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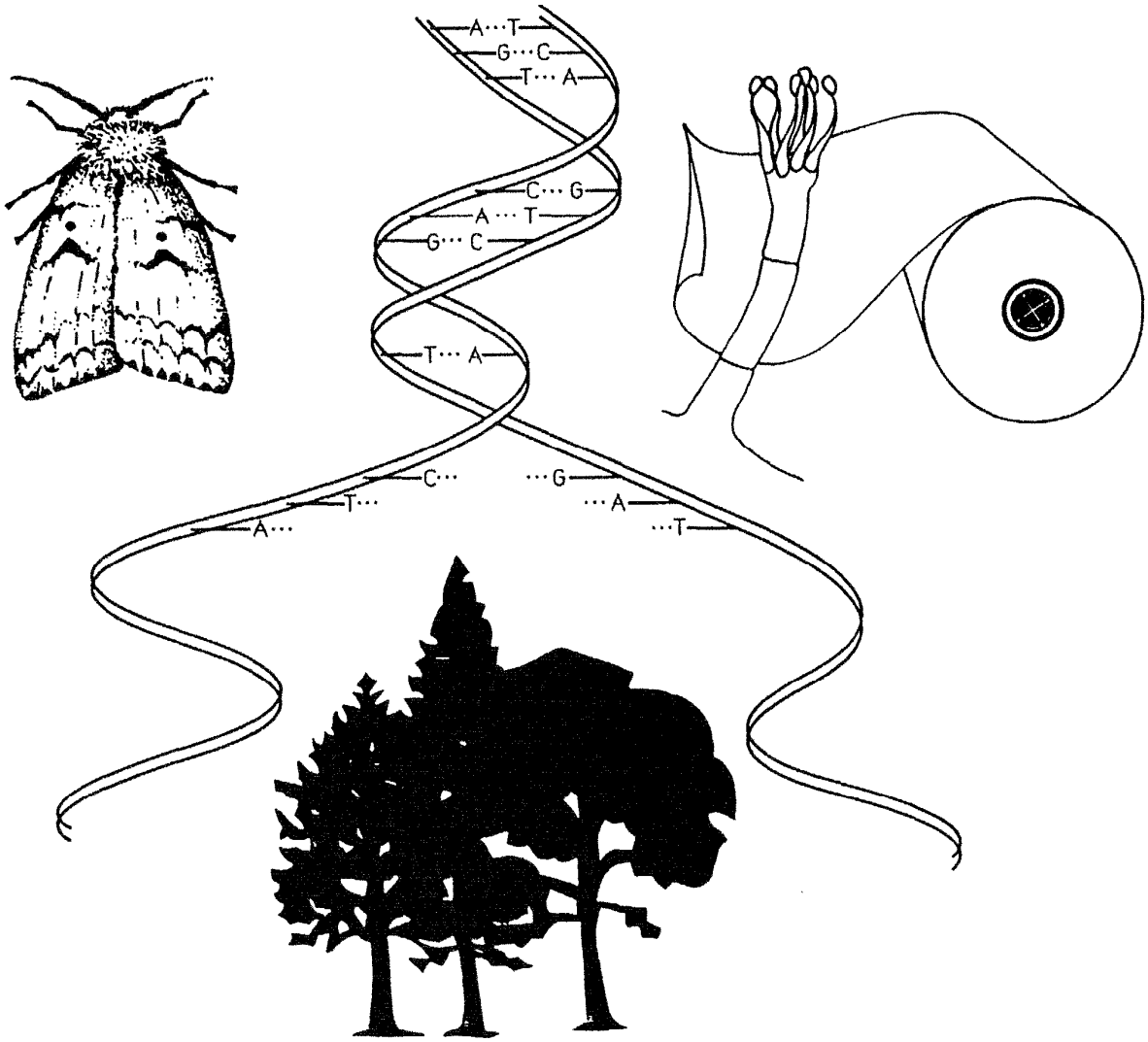
Applications of Biotechnology to Tree Culture, Protection, and Utilization



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Northeastern Forest Experiment Station
5 Radnor Corporate Center
100 Matsonford Road, Suite 200
P.O. Box 6775
Radnor, Pennsylvania 19087-4585

July 1991

Abstracts of Papers Presented

at the International Symposium on

Applications of Biotechnology to Tree Culture, Protection and Utilization

Columbus, Ohio August 5-8, 1991

Edited by:

Bruce E. Haissig
T. Kent Kirk
William L. Olsen
Kenneth F. Raffa
James M. Slavicek

Sponsored by:

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USFS Forest Insect and Disease Reseach
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Hosted by:

USDA Forest Service, Northeastern Forest Experiment Station

Executive Committee

Stanley L. Krugman
USDA Forest Service
Forest Management Research Staff
201 14th Street
P.O. Box 96090
Washington, D.C. 20090-6090

Max. W. McFadden
USDA Forest Service
Northeastern Forest Experiment Station
5 Radnor Corporate Center, Suite 200
100 Matsonford Road
Radnor, PA 19087

Richard S. Smith
USDA Forest Service
Forest Insect and Disease Research Staff
201 14th Street
P.O. Box 96090
Washington, D.C. 20090-6090

Organizing Committee

Bruce E. Haissig
USDA Forest Service
North Central Forest
Experiment Station
Box 898
Rhinelander, WI 54501

T. Kent Kirk
USDA Forest Service
Forest Products Laboratory
One Gifford Pinchot Drive
Madison, WI 53705

William L. Olsen
International Paper Company
Longmeadow Road
Tuxedo Park, NY 10987

Kenneth Raffa
University of Wisconsin
Depts. of Entomology and Forestry
345 Russell Laboratories
1630 Linden Drive
Madison, WI 53706

James M. Slavicek
USDA Forest Service
Northeastern Forest
Experiment Station
359 Main Road
Delaware, Ohio 43015

Administrative Assistant to the Organizing Committee

Rhonda Cobourn, USDA Forest Service, Northeastern Forest Experiment Station

Special Thanks to:

Edie Franson, Secretary, USDA Forest Service, North Central Forest Experiment Station

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INTERNATIONAL SYMPOSIUM ON THE APPLICATIONS OF BIOTECHNOLOGY TO TREE CULTURE, PROTECTION AND UTILIZATION

SYMPOSIUM AGENDA

Sunday, August 4, 1991

2:00 pm - 7:00 pm Registration

Monday, August 5, 1991

8:00 am - 12:00 pm Registration
1:00 pm - 4:00 pm Registration

9:00 am **Plenary Session**

Denver P. Burns US Forest Service, Director, NE Forest Experiment Station
Jerry A. Sesco US Forest Service, Deputy Chief Research

Harry C. Mussman USDA, Deputy Assistant Secretary for Science and Education

9:40 am **Symposium Keynote Address**

Ellis Cowling North Carolina State University
Forest Biotechnology: A Branch of Agriculture with a Remarkable Potential for Increasing the Productivity, Sustainablility, and Value of Forests for Humans

10:20 am Break

10:40 am **Session I Genetics/Physiology**
Moderator - Bruce Haissig

William Cheliak Forestry Canada
Impact of Biotechnology on Forest Genetics and Physiology

R.S. Nadgauda National Chemical Laboratory, India
Acceleration of Maturation, In Vitro Induction of Flowering and Its Possible Role in Bamboo Improvement

12:00 pm - 1:30 pm Lunch on your own

1:30 pm **Sara von Arnold** Swedish University of Agricultural Science
Current Status of Tissue Culture for Vegetative Regeneration of Forest Trees

Milton Gordon University of Washington
Wound-induced Genes in Hybrid Poplars

2:50 pm Break

3:10 pm **Ronald Sederoff** North Caroline State University
Gene Expression in Xylogenesis in Loblolly Pine

John Torrey Harvard University
Can Plant Productivity be Increased by Inoculation of Tree Roots with Soil Microorganisms?

Dinner on your own

7:00 pm - 9:00 pm **Mixer**

Tuesday, August 6, 1991

8:00 am - 12:00 am Registration

8:30 am **Session II Biological Control**
Moderator - James Slavicek

Lois Miller University of Georgia
Forest Stewardship and Biological Control of Insect Pests

R. Scheffer Zaadunie, Holland
Dutch Elm Disease Control: Microorganisms or Sterol Biosynthesis Inhibitors to Control a Major Vascular Wilt Disease

Ilan Chet Hebrew University of Jerusalem
Biotechnological Approaches for Plant Disease Control

10:30 am Break

11:00 am **Brent McCown** University of Wisconsin
Transgenic Trees Exhibiting Insect and Disease Resistance

Donald Dean Ohio State University
Mode of Action of B. thuringiensis Protein Toxins

12:20 - 2:00 pm Lunch on your own

2:00 pm **Session III Bioprocessing**
Moderator - T. Kent Kirk

T. Kent Kirk US Forest Service, Forest Products Laboratory
Overview of Bioprocessing

Roberta Farrell Repligen Sandoz Research Corporation
Cartapip (TM): A Biological Product for the Control of Pitch and Resin Acid Problems in Pulp Mills

Michael Sinner Voest Alpine Industrieanlagenbau, Austria
VAI - Biobleaching

4:00 pm Break

4:20 pm **Thomas Jeffries** US Forest Service, Forest Products Laboratory
Enzyme Technology for Fiber Treatment

Allan Springer Miami University of Ohio
Bioprocessing of Pulp and Paper Mill Effluents - Past, Present and Future

7:00 pm **Banquet**
Alvin Young USDA Office of Agricultural Biotechnology
Banquet Address

Wednesday, August 7, 1991 Concurrent Morning Sessions

8:00 am - 12:00 pm Registration

8:30 am **Session IV Techniques of Tree Culture and Protection**
Moderator - Bruce Haissig

David O'Malley North Carolina State University
Cloning Genes for Lignin Biosynthesis in Loblolly Pine, Pinus taeda L.

Eric Lam Rutgers University
Molecular Analysis of Factors Regulating Plant Gene Expression

Anne-Marie Stomp North Carolina State University
Transformation Vectors

10:30 am Break

11:00 am **H.G. Bradshaw Jr.** University of Washington
RFLP Mapping of Populus

Alfred Handler USDA, Agricultural Research Service
Gene-transfer Vector Analysis in Insects

12:20 pm - 2:00 pm Lunch on your own

(8/7/91 concurrent)

9:10 am **Session V Techniques in Wood Utilization**
Moderator - T. Kent Kirk

Daniel Cullen US Forest Service, Forest Products Laboratory
Recent Advances in the Organization and Regulation of Lignin Peroxidase Genes of Phanerochaete chrysosporium

Ming Tien Pennsylvania State University
Expression of Active Lignin and Mn Peroxidase in Phanerochaete chrysosporium and in a Heterologous Host

10:30 am Break

11:00 am **Peter Biely** Slovak Academy of Science
Oligosaccharides and Their Glycosides in Studies of Cellulolytic and Xylanolytic Enzymes of Microorganisms

Ronald Crawford University of Idaho
Designing Biodegradability: Lessons From Lignin

12:20 pm - 2:00 pm Lunch on your own

2:00 pm **Session VI Public Policy Issues**
Moderator - Ken Raffa

Morris Levin University of Maryland
Forestry, Biotechnology, Risk and Regulation

Robert Wachbroit University of Maryland
Ethics and Risk Analysis

William Stiles House Committee on Science, Space and Technology
Politics and Policy of Biotechnology Development

4:00 pm Break

4:20 pm **Charles Van Horn** US Department of Commerce
To be Announced

Dinner on your own

7:00 pm - 9:00 pm **Poster Session**

Thursday, August 8, 1991

8:30 am **Session VII Commercial Application**
Moderator - William L. Olsen

Patrick Trotter Weyerhaeuser Company
Biotechnology in the Pulp and Paper Industry: A Prelude to Commercialization?

Jean-Claude Pommier St. Gobain Papier-Bois Recherche, France
Liftase (TM) and Albazyme(TM): Two New Enzyme Preparations for the Pulp and Paper Industry

A.F. Mascarenhas National Chemical Laboratory, India
Biotechnology and the Paper and Pulp Industries : An Experiment

10:30 am Break

11:00 am **Chris Bornman** Hilleshog AB, Sweden
Non-Regulatory Constraints on the Application of Biotechnology in Forestry

1:00 pm **Optional Tour of the Delaware Biotechnology Facility**

International Symposium on
The Applications of Biotechnology to Tree Culture,
Protection and Utilization
August 5-8, 1991
Radisson Hotel North, Columbus, Ohio

CONTENTS

FOREST BIOTECHNOLOGY: A BRANCH OF AGRICULTURE WITH A REMARKABLE POTENTIAL FOR INCREASING THE PRODUCTIVITY, SUSTAINABILITY, AND VALUE OF FORESTS FOR HUMANS

Ellis B. Cowling

University Professor at Large
College of Forest Resources
North Carolina State University
Raleigh, North Carolina 27695-8001

Opportunities for forest biotechnology research look brighter today than at any time in history. I believe this is in part because of two recent reports by the National Research Council (NRC) -- the working arm of the National Academy of Sciences.

In the first of these reports, Investing in Research: A Proposal to Strengthen the Agricultural, Food, and Environmental System, the NRC's Board on Agriculture helped accomplish three things:

1) It provided a much broader and more appropriate definition of agricultural research (and the place of forestry research within the definition) than has ever before been embraced by the USDA;

2) It recommended a $500,000,000 increase in annual federal support for agricultural research broadly defined;

3) Since this first report was published, the federal Office of Management and Budget has approved substantial increases in funding for competitive grants (including grants for biotechnology research) in both FY 1991 and FY 1992.

In the second of these reports, Forestry Research: A Mandate for Change, a Committee on Forestry Research established jointly by the NRC's Board on Biology and Board on Agriculture, provided a ringing call for four things:

1) A new paradigm for forestry research in America -- an environmental rather than a utilitarian paradigm;

2) Greatly increased funding for research to better understand (among other things) the Molecular Genetics of Forest Organisms, Molecular Markers for Stress Response in Forests, Genetic Markers, Genetic Engineering, Cell and Tissue Culture of Forest Trees, Evaluating the Potential Risk of Genetically Modified Organisms, and Biotechnology and the Forest Environment, including biotechnology in the processing, conversion and utilization of wood;

3) Greatly improved strategies for organization, funding and providing up-to-date scientific personnel and facilities for forestry research in universities, government, and industry;

4) Establishment of a National Forestry Research Council to provide a forum for deliberations on forestry research and policy.

These two reports, coupled with the substantial progress that has been achieved in forest biotechnology research, provide substantial justification for amending the following assertion by Marion Clawson: "Forestry is the branch of agriculture with the greatest potential for increase in productivity".

Clawson, who served for many years as the chief agricultural economist for Resources For the Future, made this assertion on the basis of a long and well-researched understanding of agriculture and forestry and the importance of both for the health and welfare of people throughout the world. Indeed, Clawson's statement could now be revised to read as follows: "Forestry is the branch of agriculture with the greatest potential for increase in productivity through biotechnology".

IMPACT OF BIOTECHNOLOGY ON FOREST GENETICS AND PHYSIOLOGY

W.M. Cheliak

Forestry Canada
Forest Pest Management Institute
Sault Ste Marie, Ontario Canada

Biotechnology has created a virtual revolution in biology. It has influenced the way science is approached in fields ranging from plants to pharmaceuticals. However, impact at the operational level has, to date, been more limited. Newness of the technology, development of skilled human resources, and public acceptance are all factors influencing the implementation and impact of this technology.

Especially for plants, including forest trees, biotechnology involves tissue culture, molecular genetics, and transformation. Although each is an independent dicipline, the real impact comes from cooperation among each of these areas.

Tissue culture is essentially a vegetative propagation technique. In the past decade, excellent progress has been made in the tissue culture of trees, especially in somatic embryogenesis of conifers. In turn these advances in tissue culture have led to the development of cryopreservation techniques for the long-term storage of in vitro-cultured germplasm. There are two main impacts of tissue culture in forest genetics when integrated into a conventional, sexually-based tree improvement program. The first is essentially an economic impact, involving a saving of time. Tested, genetically improved propagules can be made available for tree improvement programs in as little as 10% of the time that it takes with conventional seed-orchard programs. Secondly, more genetic gain can be achieved by propagation of elite families or individuals. Tissue culture also provides an absolutely essential link for tree improvement programs that anticipate using advanced genetics technologies, such as transformation.

The exponential growth in molecular genetics over the past decade has created opportunities for potential impact on two fronts. The first is the unprecedented opportunities for the use of molecular markers in fingerprinting of germplasm and the potentials for using molecular markers to assist in tree

improvement programs. The second major research front is on the cloning and characterization of genes, and their promoters, that control different levels of development and function. To date, in trees, molecular genetics has had limited impact. However, the stage is set for rapid progress over the next decade.

Genetic transformation combines the unique totipotential characteristic of individual plant cells with new, or different, genetic information to produce an individual with deliberately altered characteristics. The first deliberately altered transgenic plants were regenerated in 1984. Since then over fifty different species have been successfully transformed and regenerated. These species represent major groups from the monocots and dicots, but no conifers to date. Transgenic plantlets from angiosperms, especially from the genus Populus, have been field tested for several first generation traits. Results to date have been extremely encouraging. There is every reason to expect that this technology will form part of the basis of advanced tree improvement programs.

ACCELERATION OF MATURATION, IN VITRO INDUCTION OF FLOWERING AND THEIR POSSIBLE ROLE IN BAMBOO IMPROVEMENT

Rajani S. Nadgauda, C.K. John and A.F. Mascarenhas

Division of Biochemical Sciences
National Chemical Laboratory
Pune - 411 008, India

One of the major problems in forestry is the establishment of high quality planting stock. Due to their slow growth, long vegetative phase and large size, breeding and selection of trees by conventional genetic methods are difficult. There are reports on acceleration of onset of flowering in juvenile material as a result of tissue culture (McKeand 1985; Smith et al. 1989). Due to early reproductive cycles, tissue culture raised plants can be used for seed orchards and in breeding programs, to combine the best genetic traits. The relatively smaller size of the trees makes breeding operations more convenient and less expensive. In the case of horticultural crops early onset of flowering and fruit seed set have vast potential in ensuring early returns and increased productivity. This has stimulated a great deal of interest in research on flowering.

As compared to forest trees and horticultural crops, which regularly flower in each season with the onset of maturity, the problem in bamboos is much more complex. Flowering in bamboos is cyclical, varying from 12-120 years, and gregarious in that the plants die after flowering. This flowering pattern makes improvement programs in the bamboos difficult, thereby generating interest in research on the in vitro induction of flowering.

There are few reports on in vitro induction of flowering (Tran Thanh Van et al. 1987; Nadgauda et al. 1990). In contrast, acceleration of flowering (McKeand 1985; Smith et al. 1989; Mascarenhas et al. 1988) is a phenomenon commonly observed in several tissue culture raised plants where there is a reduction in the vegetative phase preceding the flowering phase. In in vitro induction of flowering there is a sudden shift from the juvenile to mature phase.

This presentation will be divided into 2 parts. The earlier part will cover reports on in vitro induction of flowering in woody perennials. The major part of the paper will be devoted to our studies on in vitro flowering and seeding in the bamboos.

The mechanisms for control of flower initiation defy easy explanation. They appear basically different in different species and possibly evolved independently in a wide range of plant groups (Murfet 1977; Bernier 1988).

Most of the work on in vitro induction of flowering is confined to herbaceous species (Bodson 1989) with very limited work on woody perennials. In fruit crops, Patel and associates (1983) reported in vitro flowering as well as fruit set in Morus indica, and Garcia-Luis and co-workers (1989) observed in vitro acceleration of flowering in Citrus unshiu. Tran Thanh Van et al. (1987) described in vitro flowering in Sequoia sempervirens from stem explants of young sprouts. The in vitro developed cones were reported to be of similar size and shape to naturally grown ones. However, pollen grains were half the size although they were viable. An interesting observation made in this report was the retention of viability by these cones even after prolonged subcultures. In ginseng, Chang and Hsing (1980) induced in vitro flowering on basal medium supplemented with BAP and GA_3 at low temperatures from somatic embryoids developed on hypocotyl derived callus explants. The flowers were smaller with miniscule floral parts and around 90% pollen fertility. Date palm is another woody perennial where interesting observations regarding in vitro flowering have been reported (Ammar et al. 1985). The flowers were observed on in vitro grown seedlings within 5 months as compared to 8-10 years in nature. They have suggested a correlation between root development and inhibition of flowering. A similar suggestion was made by Schwabe and Al-Doori (1973), with special reference to black currant.

In three bamboo species in vitro, viz. Bambusa arundinacea, D. brandisii and D. strictus, we observed induction of flowering within 3-4 months. The inflorescence explants containing panicles of spikelets on subculture gave rise to several inflorescences. The flowers were morphologically similar to natural ones excepting for slight reduction in size (Nadgauda et al. 1990). In vitro developed pollen grains were viable and produced seed in culture. In contrast to observations of Ammar et al. (1985) and the idea propounded by Schwabe and Al-Doori (1973) that root development in herbaceous systems inhibits flowering, this was not observed in bamboos where even profusely rooted clones flowered.

Before introducing this system for breeding programs it is necessary to study in detail the different aspects of breeding behavior such as flower morphology, the timing of anthesis, stigma receptivity, mode of pollination,

effect of selfing, percent seed set, and seed viability. Since growth hormones and auxins are known to affect gametic viability, a comparison of different aspects of the breeding behavior between natural and in vitro flowering is necessary. In anemophilous bamboos even variations in the relative humidity inside the culture vessels can have a drastic effect on the onset of anthesis and the gametic viability. A detailed understanding of these aspects and others are essential for the proper planning and successful execution of a hybridization program.

We will discuss the importance of induction of in vitro flowering in bamboos, some observations on attempts at in vitro hybridization between B. arundinacea and D. strictus, and their use in bamboo improvement.

Acknowledgment: We thank National Bank for Agriculture and Rural Development (NABARD), Bombay, India, for financing a fellowship to Dr. C.K. John.

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CURRENT STATUS OF TISSUE CULTURE FOR VEGETATIVE REGENERATION OF FOREST TREES

Sara von Arnold

Department of Forest Genetics
The Swedish University of Agricultural Sciences
Box 7027, S-750 07 Uppsala, Sweden.

Forest tree breeding results in tree improvement through two main steps: (1) selection of desirable genotypes, and (2) increase of desirable genes in the breeding population. Tissue culture can contribute to the improvement of forest trees through exploitation of existing improved genotypes and through the production of new, commercially valuable genotypes (von Arnold and Wallin 1988; Cheliak and Rogers 1990). However, a prerequisite is that plants can be regenerated from cells, tissues or organs cultured in vitro. Plant regeneration in vitro can be achieved in three different ways: (1) regeneration from existing meristems, (2) regeneration from adventitious meristems, and (3) regeneration via somatic embryos.

Regeneration and propagation of forest tree species in vitro has progressed significantly during the last decade. Numerous examples of successful applications of in vitro propagation have been reported. However, the methods used vary among species as well as within species. Furthermore, the methods vary depending upon the objectives. This presentation will review several general aspects of different techniques available for tree regeneration and provide more detailed information concerning somatic embryogenesis. The latter appears to be one of the more promising regeneration methods for the future.

Regeneration from existing meristems

Propagation in vitro from existing meristems includes the following stages: establishment of explant, shoot multiplication, shoot elongation, rooting and acclimatization. For the most part, development from one state tothe next is accomplished with hormone treatments. Optimal hormone treatments (cytokinin for multiplication and auxin for rooting) varies significantly between species and among different genotypes within each species. This apparently is partially explained by the fact that the endogenous hormone levels vary among different genotypes during various developmental stages. Sometimes the weak point in the process of regenerating

the organization and the developmental stage of the somatic embryos varies. Embryogenic cell-lines can be divided into two main categories: (1) those consisting of somatic embryos with a distinct embryonic head which is clearly separated from the suspensor region, and (2) those consisting of somatic embryos where some suspensor cells are intermingled with the cells in the embryonic head. Only somatic embryos belonging to the first category undergo maturation. So far, we have been unable to convert category-2 cell-lines into category-1. Embryogenic cultures can be maintained for several years without losing their embryogenic capacity (Mo et al., 1989). One of our best cell-lines has been in culture for more than 5 years. It is also possible to establish fast-growing embryogenic suspension cultures, which have been found to be very useful for both protoplast culture and gene transfer via a particle accelerator. There is, however, a pronounced variation among different cell-lines; some cell-lines grow rapidly in liquid suspension while others do very poorly.

Somatic embryos are stimulated to mature by treatment with abscisic acid (ABA, about 15 μM). The optimal ABA treatment time varies from 1 to 3 months depending on the cell-line. ABA stimulates the accumulation of storage material including lipids, proteins and carbohydrates. Similar ABA-effects have been reported for Angiosperms. A high yield of normal, mature somatic embryos can be obtained from some cell-lines while others develop somatic embryos precociously.

Somatic embryos develop further into plants when they are isolated and cultured on a hormone-free medium. Most of the regenerated plants have a normal appearance, but some cell-lines regenerate abnormal plants. About 2000 spruce plants regenerated from somatic embryos have been transferred to a phytotron for further studies concerning growth habit and genetic stability. We, as well as several other laboratories, have transferred plants to the field (Becwar et al., 1989; Roberts et al., 1990). Their growth, compared to seedling derived plants, appears normal. However, before this technique is used for large scale propagation, it is important to perform more detailed studies and field evaluation.

Conclusions

Plants regenerated in vitro must perform in the field in a manner comparable to that of seedlings or cuttings. There is a risk that the growth of in vitro regenerated plants can be changed, owing to physiological and/or genetic aberrations. As a consequence it is very important to carefully

evaluate the performance of *in vitro* regenerated plants before the method is used for large scale propagation. Plants regenerated from preformed meristems have been tested and evaluated many times, and it seems that the risk for aberration is small. However, plants regenerated from *de novo*-formed meristems are more likely to grow differently from normal cuttings. This does not mean that the method can not be used for practical purposes. It seems to be an efficient technique for some species such as *Pinus radiata*. However, care must be taken before the technique is applied for practical purposes. Performance of plants regenerated from somatic embryos has not yet been thoroughly tested and evaluated. The results obtained so far strengthen the hope that somatic embryogenesis will become very useful.

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WOUND-INDUCED GENES IN HYBRID POPLARS

Milton P. Gordon, Harvey D. Bradshaw, Jr.,
Howard Clarke, John M. Davis, and Jay B. Hollick

Department of Biochemistry SJ-70
University of Washington
Seattle, WA 98195

Shortly (6-8 hr) after single leaves of young hybrid poplars (Populus trichocarpa x P. deltoides) are mechanically wounded, several families of wound-induced (win) mRNAs are detected in the unwounded portion of that leaf, and in specific leaves that are remote from the wounded leaf. Two of these gene families, win6 and win8, encode proteins similar to chitinases.

Chitinases are thought to be involved in defense against fungal pathogens. Nine different win6 genes have been cloned and completely or partially sequenced. Two poplar genomic clones harbor tandomly repeated win6 genes interspersed with a different chitinase gene we have designated, chitinase X (chiX). The predicted win6 and chiX proteins have a signal peptide, a cysteine-rich "hevein" domain, a hinge region, and a catalytic domain as described in Shinshi et al., (1990). The predicted win8 protein, by contrast, completely lacks a hinge region. Win6 and win8 are expected to be highly acidic (the calculated net charge is -15 to -17).

Polymerase Chain Reaction (PCR) analysis using win6-specific primers indicated that P. trichocarpa and P. deltoides transcribe alternative alleles of win6, and their interspecific hybrids express both alleles. The hybrids constitutively express a third win6 gene not expressed in either parent in response to wounding.

An additional wound-induced gene family, win3, coding for putative proteinase inhibitors contains at least 12 members as determined by genomic Southern blotting. At least 5 family members are expressed in response to mechanical wounding. Nuclear runoff transcription assays demonstrate that win3 mRNA abundance is regulated primarily at the transcriptional level.

Transgenic tobacco plants have been made that express the reporter gene, β -glucuronidase (GUS), under the control of a win3 promoter. Forty per cent of Kan^r regenerants show wound-induced GUS expression.

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GENE EXPRESSION IN XYLOGENESIS IN LOBLOLLY PINE

C.A. Loopstra, W. Bao, D. O'Malley,
A.K. Weissinger, and R. Sederoff

Department of Forestry
North Carolina State University
Raleigh, NC 27695-8008

We are studying genes and proteins active in the formation of xylem in loblolly pine. This work is part of an attempt to understand the mechanisms of gene regulation during wood formation, as a step towards the manipulation of wood properties through genetic engineering. We hope to identify specific genes and regulatory sequences that are involved in the terminal differentiation of xylem and therefore affect the properties of wood.

To carry out biochemical purification of specific components of differentiating wood, we use 12-year-old trees active in biosynthesis of wood. In the spring, trees are cut into bolts, the bark stripped from the bole, and newly forming xylem is rapidly scraped from the "bolts" into liquid nitrogen. We obtain at least 0.5 kg of differentiating xylem from individual trees. Protein and mRNA preparations can be carried out on material from an individual tree of a defined genotype. Three studies will be described: 1) Isolation and characterization of abundant xylem specific cDNAs, 2) Isolation and characterization of an extensin-like protein from differentiating xylem, and 3) Development of a transient expression system for xylem in situ, that can analyze activity of promoters using microprojectile bombardment.

I. **Abundant xylem specific cDNAs:** The objectives of this study were to determine if xylem specific or xylem enhanced cDNAs could be identified. The genes that code for such mRNAs might contain promoter sequences that could confer tissue specificity on chimeric genes. Such regulatory elements could play an important role in the genetic modification of wood properties because tissue specific modifications could be made. Many genes important in biosynthesis of wood, such as those involved in lignin biosynthesis, are known to be expressed in a number of tissues.

Differential screening of a loblolly pine xylem cDNA library was used to isolate clones from two different genes with enhanced expression in differentiating xylem. mRNA prepared from differentiating xylem was used to

prepare a cDNA library. Labelled preparations of total cDNA from megagametophyte and xylem were used to screen isolated colonies for clones containing abundant transcripts. Most of the abundant, differentially expressed sequences belonged to two genes. Northern blot analysis indicated that the genes were expressed at high levels in various samples of differentiating xylem, at much lower levels in needles, and hardly at all in megagametophytes or embryos. The low level of expression in needles may be due to the small amount of vascular elements. Nearly full length clones have been isolated for both genes. Southern blot analysis indicates that both of these genes are single copy sequences in the loblolly genome. Nucleotide sequence analysis of both genes has been carried out and putative reading frames assigned. Both genes have segments that are proline rich, and may in part code for structural extensin-like domains. Genomic clones for both genes have been isolated, mapped by restriction enzyme analysis, and subcloned for more detailed location of promoter boundaries.

II. **An extensin-like protein in xylem:** An extensin-like protein has been purified from differentiating xylem. The protein is extracted from the cell walls by $CaCl_2$ and further purified by selective solubility in TCA. The resulting protein fraction contains a single major protein component that is highly glycosylated. After deglycosylation by hydrogen fluoride, the protein has an apparent molecular weight of 68kd on SDS acrylamide gels. Amino acid composition analysis shows that the protein is rich in proline. Rabbit polyclonal antibody has been prepared against the deglycosylated protein. The specificity of the antibody was verified by Western blots using preimmune serum as the control. Antibody was then used for immunolocalizations in material from differentiating wood using alkaline phosphatase conjugated secondary antibodies. Extensin-like protein is apparent in all metabolically active differentiating cells in xylem, particularly ray parenchyma and resin duct epithelium. Antigen is apparent in cambium and phloem also. Differentiating tracheids have less staining activity than other cell types with more dense cytoplasm. Unexpectedly, we found immunolocalization in mature wood. Using either alkaline phosphatase conjugated secondary antibody or silver enhanced immunogold labelling, unambiguous activity is observed compared to preimmune serum controls. We find preferential localization of staining in early wood compared to late wood. Microscopic examination indicates that antigenicity is located in the secondary walls of early wood. Extensin-like protein may be immobilized in cell walls during formation of the secondary wall and it appears to remain there in mature wood for extended periods of time.

III. **Transient expression of introduced genes in differentiating wood using microprojectile bombardment.** We have used microprojectile bombardment for transient expression in several tissues of pines including differentiating xylem. Differentiating xylem is exposed by removal of the bark, and the xylem cells attached to the differentiating wood are bombarded by microprojectiles coated with DNA. The target tissues are assayed on the following day for histochemical activity of GUS. Detailed histological examination indicates that most foci of transient expression are due to single cells. Control plasmids without GUS or with inverted promoters show no activity. We are confident therefore, that our results are not due to bacterial contamination, endophytic bacteria, or endogenous enzyme activity. Equal numbers of control samples were bombarded in each experiment and no blue spots were ever seen in any of the controls.

Examination of hand sections of bombarded stem segments stained for GUS activity showed that at least three cell types were transiently expressing GUS: axial parenchyma, ray parenchyma and developing tracheids (Loopstra et al. submitted). Transient expression in tracheids results in thin blue streaks, usually 1 to 2 mm long, whereas ray parenchyma shows blue spots for expression and resin duct epithelium produces short blue rod shaped cells. This feature may allow identification of cell-type specific transcriptional regulatory elements in our promoters, if such elements exist.

Bombardment of developing xylem has been used to show differences in the activity of three promoters in loblolly pine xylem. The three promoters tested were CaMV 35S, nos, and the ABA-inducible promoter of the Em gene of wheat. The relatively low level of the nos promoter is of interest because it is a wound inducible promoter. The Em promoter reproducibly gave ten-fold more transient expression events in samples stained with X-gluc than did the other two promoters. This effect is hypothesized to result from a requirement for a threshold level of GUS activity necessary to cause a blue spot. Promoters of lower activity will more frequently fail to give enough transcription to form the threshold level of GUS activity.

CAN PLANT PRODUCTIVITY BE INCREASED BY INOCULATION OF TREE ROOTS WITH SOIL MICROORGANISMS?

John G. Torrey

Harvard Forest
Harvard University
Petersham, MA 01366

Over the past two decades, considerable interest has been shown by the scientific community concerning the importance of root/rhizosphere relationships and their essential benefits to the health and productivity of plants grown for agriculture and forestry. Of special interest and concern has been the development of a better understanding of symbiotic relationships that exist between soil microorganisms and the roots of the numerous host plants with which they associate. These associations focus attention on the mineral nutrient benefits afforded the host plants, especially by the provision of nitrogen and phosphorus in edaphic situations where productivity may be limited by the low availability of these elements.

Fostering symbiotic associations between appropriate soil microorganisms and their compatible hosts lies within the management capabilities of agriculturalists and foresters. Using knowledge of the fundamental scientific bases for these associations, one can facilitate the development of beneficial symbioses by inoculation of seeds, seedlings or growing plants with selected microorganisms to establish and perpetuate effective symbioses leading to increased productivity.

Four major symbiotic systems are known that demonstrably benefit plant productivity that should be the concern of foresters. Development of our knowledge of the nature of the symbioses has a scattered history. The understanding of Rhizobium-root nodulation of agricultural crops goes back a hundred years. Knowledge of important details of the symbioses between Frankia and trees of the actinorhizal group is of quite recent origin, only a decade or two. Similarly, fungal-root mycorrhizal associations have been known for many years but we are still in the process of developing an understanding of basic facts. We stand far from an effective management regime for most of these beneficial associations.

It is the purpose of this review to describe and discuss in broad terms the status of our knowledge of the basic facts in these symbioses (namely, Rhizobium/Bradyrhizobium-leguminous trees; Frankia-actinorhizal trees; ectomycorrhizae-host trees; endomycorrhizal, including vesicular-arbuscular mycorrhizae-host trees). More specifically, I plan to explore the degree to which foresters are applying this basic information by the use of inoculation technologies. To be discussed are questions relating to the isolation, characterization and culture of the microbial symbiont, the demonstrated specificity for infection and effectivity for facilitating nutrient uptake in each case, and the development of the technology for field inoculation to achieve effective symbioses in forest plantations.

The development of a useful and effective inoculation program depends upon a large number of factors. The microorganism must be readily grown in large-scale culture. It should have as wide a host range as possible. The inoculum containing the microorganism should be relatively inexpensive to produce, should have a long storage life, and should be of minimum bulk, capable of easy and inexpensive shipment. The inoculum must be free of other microorganisms or deleterious materials, must be simple to apply, and should be effective over a wide range of environmental conditions. The introduced microorganism should be capable of displacing native microbial populations in creating the effective symbiosis.

In this review it will be useful to explore a few specific examples where scientific knowledge and technology have been brought together in the most advanced form to achieve some or many of these requirements.

FOREST STEWARDSHIP AND BIOLOGICAL CONTROL OF INSECT PESTS

Lois K. Miller

Departments of Entomology and Genetics
The University of Georgia
Athens, GA 30602

With increasing public attention on global issues relating to climate change, biological diversity, air pollution, and water pollution, forests are becoming a focal point of public interest and concern. In the USA, the Forest Service will be expected to play an increasingly important and essential role in maintaining healthy, biologically diverse and sustainable forest ecosystems. These goals will be difficult to meet in the face of the demands of an enormous human population which looks to forests for wood products and recreation. Successful forest stewardship will require the acquisition of sufficient biological information to be able to manipulate, safely and specifically, the populations of single species. The need for such species manipulation is perhaps clearest in the cases of insect pest species which have been introduced into North America.

Insects have essential roles in maintaining forest equilibrium and any measures which are employed to control introduced insect pest species must preserve the delicate balance required of other insect populations. The application of chemicals which are broadly toxic to invertebrate species in forest systems does not benefit the forest in the long run. The U.S. Forest Service is acutely aware of the need to find biologically specific and environmentally safe methods of maintaining biological balance and species diversity. Because humans have had and will continue to have an enormous impact on world forests and their utilization, the Forest Service must develop new biological methods to control pest species which threaten the balance of biological diversity and forest ecosystem sustainability.

In the mid 1970's, the U.S. Forest Service took a brave and important step in the direction of developing new, biological methods of insect pest control. This step included the development of naturally occurring microorganisms for use as pest control agents. Although the very mention of "germs" can strike fear in the minds of those people (including some biologists) who are unfamiliar with the nature and behavior of such organisms, certain insect pathogens can be the most specific and useful types of biological "mechanisms"

of controlling specific biological populations. Of the microorganisms which are pathogenic to insects, the most specific are viruses, particularly baculoviruses. The baculoviruses constitute an immense group of viruses found only in arthropods. Each individual member of the group causes disease in only a limited number of insect species and some are known to cause epizootic diseases in only a single species. Over the past 15 years, the Forest Service has developed a baculovirus which can be used to control the Douglas Fir tussock moth. The Forest Service has also explored the use of different baculoviruses to control gypsy moths, spruce budworms, pine sawflies, and other tree defoliators which can emerge in massive numbers to devastate forests. Although the current dollar cost of producing and using such microbial control agents would exceed that of chemical pesticides, the environmental savings with regard to invertebrate species diversity and reduction in chemical residues in the soil and water will be enormous in the future.

In this opening symposium talk, I survey what we have learned about these viruses using molecular biological approaches and genetic engineering technology. Such techniques are essential in studying these microscopic biological entities which consist primarily of DNA with an evolutionary history. The molecular biological work on the viral DNA has been essential in identifying different viruses, identifying different variants of the same virus, following the viruses in the environment and manipulating the characteristics of the viruses to make them more effective. Genetic engineering technology is opening new avenues to developing viruses with improved efficacy and this in turn is likely to stimulate industrial interest in developing these viruses as biological control agents for forest preservation. Some of the new possibilities in viral pesticide development will be covered. The new genetic technology must, however, be used sensibly. Specific recommendations as to how to implement this technology over the next years will be provided. These recommendations include a caution to scientists too eager to insert foreign genes into baculoviruses used in forest settings and advice to government regulators to encourage industrial involvement in biological pest control development. Recommendations include: (1) limitation of chemical pesticides or taxation of such pesticides in forest pest management, (2) active encouragement of university, industrial and Forest Service interactions in developing biological control strategies, and (3) timely registration of sensibly engineered viruses with evaluation and testing based on biologically sound principles.

DUTCH ELM DISEASE CONTROL: MICROORGANISMS OR STEROL BIOSYNTHESIS INHIBITORS TO CONTROL A MAJOR VASCULAR WILT DISEASE

R.J. Scheffer

Zaadunie B.V.
P.O. Box 26, 1600 AA Enkhuizen
Willie Commelin Scholten Phytopathological Laboratory
Javalaan 20, 3742 CP Baarn, The Netherlands

Dutch elm disease, caused by the ascomycete Ophiostoma ulmi (Buisman) Nannf., can be controlled to a certain extent by various means. In this review, two recent studies carried out in the Netherlands will be discussed: a novel fungicide and a biological control method. The fungicide approach is attractive because of the low concentrations that were effective in experiments and its possibility to be used as a curative therapy. The biological control of the disease can be applied preventively only, but seems promising because of its low cost and limited (if any) environmental impact compared with more classical approaches.

Some sterol biosynthesis inhibitors can affect the morphology of dimorphic fungi. Candida albicans, for instance does not produce hyphae anymore upon exposure to some sterol biosynthesis inhibitors, probably because of an irregular deposition of chitin in the cell wall. For O. ulmi a similar phenomenon was observed: low concentrations of, for instance, fenpropimorph suppressed hyphal growth entirely.

In field trials in the city of Amsterdam and in one of the Dutch 'polders' it was shown that injection of a fenpropimorph salt (a salt because the free base is not well soluble in water) abruptly stopped symptom development in trees that already showed beginning symptoms of Dutch elm disease upon inoculation. A reasonable distribution of fenpropimorph within the tree was found and the fungicide could be isolated from new annual rings in the years after injection. For instance, from a tree injected with 7.5 g of fenpropimorph sulphate, 0.62 µg/g wood could be recovered in the third season (26 months) after injection. Comparing this 0.62 µg/g wood with the 0.05 µg/ml for in vitro inhibition of hyphal growth warrants the conclusion that the fungicide provides protection against the pathogen for at least three seasons (Scheffer et al. 1988). Compared with thiabendazole, the only fungicide currently on the market against Dutch elm disease, fenpropimorph salts have the

advantage that only two to five percent of the amount of active ingredient is needed, making application much faster and thus more affordable.

Biological control of Dutch elm disease using Pseudomonas spp. was attempted in several studies carried out in the U.S.A., the Netherlands and the United Kingdom. From these studies the following can be concluded: treating elms curatively generally failed, but preventive treatments were highly successful in several studies, depending on the methods employed and the species or clone of elm involved.

A few key experiments will be discussed here that were designed to reveal a common mechanism, if any, to explain the results of the various studies (Scheffer 1990a,b, and Elgersma, personal communication).

The selection procedures for the micro-organisms employed for biological control of the pathogen generally have been based on in vitro tests for antagonism. Therefore, explanation of a disease-controlling effect generally has been targeted on fungicidal (or fungistatic) metabolites: antibiotics or rather antimycotics, including siderophores. However, besides affecting the pathogen, a micro-organism intended to control Dutch elm disease may exert some effect on the host tree: microbial compounds probably trigger the host's defenses by acting as elicitors, or by inducing or producing them. The ultimate effect of a biological control treatment thus may be an increased resistance of the host, referred to as 'induced resistance'.

Within the species O. ulmi, a non-aggressive and an aggressive strain are distinguished. Elms with an intermediate level of resistance to Dutch elm disease, such as the clone 'Commelin' used for many of the Dutch experiments, are resistant to the non-agressive strain, but susceptible to the aggressive one. An effective suppression of Dutch elm disease symptom development was observed in such 'Commelin' elms after a preventive treatment by this non-aggressive strain and induced resistance in the host is proposed as an explanation. An equally effective isolate of Verticillum dahliae was found that suppressed symptom development even in the very susceptible field elm (Ulmus carpinifolia), in which Pseudomonas treatments failed, probably because of an insufficient induction of the trees' resistance.

Induced resistance triggered by the biocontrol agent does explain the results of the various field trials, assuming that the treatments with Pseudomonas spp. only resulted in a marginal induction of resistance. Such would explain why the methods employed became crucial in various experiments, why the treatment of a specific elm clone was not always as effective and why

the elm species or clone became important. For instance, no effect of injection with Pseudomonas spp. was observed in several experiments with the very susceptible field elms. But a preventive treatment with V. dahliae, which probably elicited a resistance response of the tree more effectively, resulted in a strong protective effect.

In one experiment injection of a large amount of dead cells of the non-aggressive O. ulmi did not have any effect on symptom development due to the subsequent inoculation of the trees with the aggressive strain suggesting that a lasting signal is needed to elicit such a resistance response. Indeed, in several experiments, V. dahliae could still be isolated by the end of the season. However, attempts to isolate the fungus in the second year failed and Dutch elm disease infections in the second year were not impeded; apparently V. dahliae did not survive and the induction of resistance was not extended to the second year.

A biocontrol agent for Dutch elm disease such as the V. dahliae discussed here would have a limited, if any, environmental impact compared with other control measures. The cost of application would be even lower than the cost of bacterial injections as an inoculation takes only seconds. In combination with a sanitation program aiming at the prompt removal of dying and dead elms (to keep vector population low), and with the fungicide treatment for trees that are affected by Dutch elm disease despite efforts to avoid it, the biocontrol would allow for an effective integrated control of the disease.

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BIOTECHNOLOGICAL APPROACHES FOR PLANT DISEASE CONTROL

I. Chet, K. Broglie*, R. Shapira and R. Broglie*

Hebrew University of Jerusalem
Otto Warburg Center for Biotechnology
Faculty of Agriculture, Rehovot, Israel
and, *E.I. DuPont de Nemours & Co. (Inc.)
Agricultural Products Department
Wilmington, DE 19880, USA

Biological and biochemical control provides an alternative means of reducing plant diseases without using hazardous pesticides. A main polymer in many fungal cell walls is chitin built of N-acetylglucosamine units. Various chitinases are capable of degrading this component and to partially lyse fungal cell walls. This is an important mechanism for controlling plant pathogenic fungi.

The mycoparasite Trichoderma harzianum is capable of parasitizing the plant pathogenic fungi Rhizoctonia solani and Sclerotium rolfsii and to reduce disease incidence under field conditions. Studying the mechanism involved in disease reduction as a result of this antagonism revealed several steps in this process. The first detectable step is a chemotropic growth of Trichoderma towards its host. It is not known, however, if this phenomenon is specific or an essential part of this interaction. Later, the antagonist recognizes and attaches to its host by sugar-lectin linkage. This "recognition" is the basis for specificity of the Trichoderma.

Rhizoctonia solani produces a L-fucose specific agglutinin which agglutinates erythrocytes type O, but not A or B and cells of Escherichia coli B. The agglutination was inhibited by L-fucose, L-galactose and their derivatives. A correlation was found between this inhibition by α-methyl L-fucoside, and the prevention of Trichoderma coiling around its host. The ability of different isolates of Trichoderma to attack S. rolfsii was correlated with the agglutination of Trichoderma conidia by S. rolfsii lectin (Chet 1987).

After attachment the mycoparasite begins to excrete extracellular lytic enzymes, especially chitinase and β-1-3 glucanase, which partially digest the cell walls of the pathogenic fungi and inhibit their growth. We have recently prepared protoplasts of several isolates of Trichoderma and fused protoplast

with different traits. This technique resulted in fusants which are more efficient as biocontrol agents in greenhouse tests (Pe'er & Chet 1990).

Serratia marcescens was found to be an efficient biocontrol agent of Sclerotium rolfsii and Rhizoctonia solani under greenhouse conditions. Populations of 10^5 or 10^6 cfu g^{-1} soil were the most effective in disease control (Ordentlich et al. 1988). The isolated Serratia was found to possess very high chitinolytic activity. The gene coding for chitinase was cloned into Escherichia coli and the enzyme was uniquely excreted from the bacterium into its growth medium. When S. rolfsii was sprayed by partially purified chitinase produced by the cloned gene, rapid and extensive bursting of the hyphal tips was observed. This chitinase preparation was found to be effective in reduction of disease incidence caused by S. rolfsii in beans and R. solani in cotton, under greenhouse conditions. A similar effect was obtained when a viable E. coli cell containing the plasmid with the chitinase gene (pLCHIA), was applied (Shipira et al. 1990).

Plants respond to attack by pathogenic fungi by activation of defense mechanisms. In part this includes the induction and accumulation of lytic enzymes such as chitinase and β-1-3-glucanase among other "PR" -proteins.

We were interested in understanding the role of chitinase in the defense response of the plant and in determining whether manipulation of chitinase gene expression can be used to generate fungal resistant plants.

In healthy, uninfected plants chitinase activity is present at low basal levels; however, treatment with oligosaccharide elicitors or infection by fungal pathogens causes a rapid induction of chitinase mRNA levels and enzyme activity. To study the expression of this gene in response to fungal infection a chimeric gene containing the flanking sequence from a bean chitinase gene fused to the reporter gene, β-glucoronidase (GUS), was introduced into tobacco plants by Argrobacterium-mediated transformation.

Histochemical analysis of the transgenic tobacco seedlings infected with R. solani and S. rolfsii indicated that activation of the bean chitinase promoter occurred locally in the tissue surrounding the infection site. The increase in GUS activity was correlated with an increase in endogenous tobacco chitinase activity produced in response to fungal infection (Roby et al. 1990).

Transgenic tobacco plants containing a modified chitinase gene in which the active promoter was replaced by a high level, constitutive promoter (35S of cauliflower mosaic virus) were tested. They were found to exhibit increased resistance to infection by R. solani. These plants exhibited less root damage and an increased rate of survival when grown in soil infested with this

phytopathogen (K. Broglie, I. Chet, H. Holliday and R. Broglie, unpublished data).

It appears that genetic engineering of the gene coding for lytic enzymes such as chitinase, which play an important role in plant disease control, may improve the efficacy of biocontrol agents as well as enhance plant resistance.

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TRANSGENIC TREES EXHIBITING INSECT AND DISEASE RESISTANCE

Brent H. McCown, David D. Ellis, Kenneth F. Raffa and Daniel J. Robison

Departments of Horticulture, Entomology and Forestry
University of Wisconsin-Madison
Madison, WI 53706

Tree crops are some of the most difficult plants to manipulate genetically and have less research and development directed to their improvement than any of the major crops. However, enough progress, including the application of biotechnological techniques, is being made to anticipate that in the next decade, a more rapid introduction of genotypes with highly desired traits will be evident. Of the traits that can be predicted to give the greatest gain in productivity and economics, improved disease and pest resistance is paramount (Stomp 1987).

The incorporation of disease and pest resistances into trees using biotechnological approaches has lagged behind such genetic improvement in the annual agronomic and horticultural crops. This has been due in large part to the concurrent lag in the development of reliable genetic engineering and somaclonal technologies applicable to tree crops. However, it appears that these technological problems are being solved and should not in themselves be the major obstacle in future research. For example, the incorporation and effectiveness of Bt-type pest resistance in a major tree species (Populus) has been recently reported (McCown et al. 1991).

Besides the continuing shortage of support for research and development on tree crops, two other major obstacles are apparent: (1) a lack of a conceptual framework and proven strategies for the deployment of biotechnologically-engineered trees, and (2) the untested response of regulatory agencies to the large scale introduction of genetically-engineered, long-term perennial crops.

This discussion will focus on our concepts of how the genetic engineering and testing of pest resistance in various tree crops should proceed in light of our current understanding of the problems and potentials of deployment of such plants in (1) large commercial stands of forest trees harvested on long-rotation bases, (2) intensively-managed, short rotation stands of forest trees, (3) intensively-managed, long-rotation orchards of fruit and nut trees and (4) long-term ornamental plantings mostly in urban settings (urban

forestry). In each situation, the feasibility of engineering a trait into the crop, the value of the trait in the production and utilization of the trees, the potential of biotype evolution, the potential impact on non-target organisms, and the compatibility with cultural and biological controls will be evaluated (Raffa 1989). Such considerations should be utilized in formulating a regulatory framework for the adoption of tree biotechnology.

An equally exciting prospect that comes with the ability to reasonably rapidly modify the genetics of trees is the opportunity to conduct experiments that heretofore were impossible or impractical. One such project involving the creation of populus genotypes that combine native insect resistances (Robison and Raffa 1990) with engineered resistances will be described. This project will include field plantings to begin the process of evaluating alternative deployment strategies for bioengineered trees.

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MODE OF ACTION OF *B. thuringiensis* PROTEIN TOXINS

Donald H. Dean

Departments of Biochemistry, Molecular Genetics and Entomology
The Ohio State University
Columbus, Ohio 43210

Bt Impact on Tree Pathogens

Bacillus thuringiensis is currently the most commonly used biological control agent. In forestry, it comprised 74% of the total pesticide used in Canada against *Choristoneura fumiferana* (spruce budworm) in 1986 (Morris *et al.* 1986) and is in even greater use today. In the United States, it surpassed other control measures for *Lymantria dispar* (gypsy moth) in 1989 (Twardus 1989). Over 2.3 million kilograms of *B. thuringiensis* are used worldwide (Rowe and Margaritis 1987).

Varieties of Bt and Types of Insecticidal Crystal Protein Genes

B. thuringiensis is a gram-positive, spore-forming bacterium which is very closely related to the common soil bacterium *B. cereus* and is virtually indistinguishable by standard biochemical tests, except that it produces an insecticidal crystal, the δ-endotoxin. By latest count, there are about 39 *B. thuringiensis* serotypes and another 2 non-flagellated biotypes. It has been known for 20 years that insecticidal specificity was unrelated to serotype, and now it is clear that this is due to the variety of insecticidal crystal protein genes which *B. thuringiensis* may contain and share by genetic exchange. Hofte and Whiteley (1989) list 14 insecticidal crystal protein genes by the mnemonic of *cry*, for crystal formation, and several more have recently been identified. Each of these genes encodes a toxic protein which has a different specificity (Milne *et al.* 1990).

Mode of Action

B. thuringiensis δ-endotoxin must be eaten by a susceptible insect. The toxin is then solubilized in the midgut and digested by trypsin-like proteases. The activated toxin then binds to specific receptors located on the epithelial brush border and forms a K^+ channel which leads to lysis of epithelial cells by osmotic shock. Insect specificity of the toxin has been suggested to be correlated to receptor-binding specificity, but in the case of gypsy moth, the correlation between toxin specificity and binding is inversely correlated; that is, the CrylA(b) is more active but binds poorly, while

CrylA(c) binds well but is weakly active. The spore enhances toxicity such that insects that are only marginally susceptible to the insecticidal crystal protein are more susceptible in the presence of the insecticidal crystal protein and the spore. Presence of the spore will also shorten the time of mortality of insects which are only marginally susceptible to the toxin alone (Milen et al. 1990).

Insecticidal Crystal Protein Structure and Function

Comparison of the derived amino acid sequences of the insecticidal crystal protein genes reveals several remarkable features which may help locate functional domains of the protein. Two major structural groups exist: Type I, the large insecticidal crystal proteins of approximately 130 kDa (cryl series, and crylVA and B); and Type II, the small insecticidal crystal proteins of approximately 65 kDa (cryll series; crylllA; crylVC and D).

In the Type I protoxins, the -COOH terminus is apparently wound in an escargot manner around the toxic core and processively exposed to digestion with trypsin and insect gut proteases. The structure of the toxic core is composed of two domains. The domain structure has been proposed on the basis of differential denaturation characteristics. The $-NH_3$ terminal half of the toxin unfolds at pH 8.0-11.0 in 4 M GuHCI, while the -COOH terminal half unfolds at pH 7.0 down to 4.0 in 4M GuHCI. The topology of the two-domain structure fits nicely with the natural division of the CrylA toxins into conserved $-NH_3$ terminal and hypervariable -COOH terminal regions. It also fits well with recent protease studies on the toxins. Several authors have found that the tryptic limit toxin may be further cleaved into two peptides of approximately equal size with select proteases; namely, papain, subtilisin, chymotrypsin, or thermolysin. These studies indicate that the two structural domains are linked by a protease-sensitive bridge. The conformational nature of the two domains is also quite different. The $-NH_3$ terminal domain is rich in α -helices as determined by circular dichromatic spectroscopy, Raman spectroscopy and infrared spectroscopy. This empirical determination matches very nicely the predictions of secondary structure. The -COOH terminal structural domain has a very low α -helical content and is predominantly β -sheets (Dean and Adang 1991).

Location of functional sites on the protoxin molecule has been a major effort in our laboratory in the last few years. We have positioned the "specificity domain" for B. mori to the central region of the CrylA(a) toxin (Ge et al. 1989), and for Trichoplusia ni in the same location on the CrylA(c)

toxin. The "specificity domain" for Heliothis virescens is over a broader region of the CrylA(c) toxin involving components of the whole -COOH terminal domain (although a construct involving the central region of CrylA(c) into a CrylA(a) gackground has 30X more activity than CrylA(c) itself) (Ge et al., in preparation).

Location of receptor binding domains for CrylA(a) on B. mori brush border membrane vesicles has recently been completed in our lab (Lee, Ge, Milne and Dean, in preparation), and the receptor binding region corresponds to the "specificity region." We are currently analyzing the location of other functional properties of insecticidal crystal proteins, such as amino acid residues associated with K^+ channel formation.

Engineering Better Insecticidal Crystal Proteins

There are several routes of providing protection to trees through the genetic engineering of B. thuringiensis insecticidal crystal protein genes. One of these approaches is the direct introduction of cry genes into trees. Certain tree cell cultures are available and may be regenerated into whole plants, as revealed in this meeting. Efforts are currently being made to introduce and express cry genes into these cells. This approach would provide relief for insect-resistant ornamentals and commercial forest trees, but would provide little relief for existing forests. Another approach is to introduce cry genes in tree endophytes or epiphytes and inject or spray these on the threatened trees. Some work in this area is currently in progress. The approach receiving the most attention in our laboratory is the direct attempt to improve cry genes by protein engineering. By locating the functional domains on the toxins, we hope to alter the active amino acid to obtain more active mutant forms of the toxins. We have had some progress in obtaining enhanced toxinsfor the agricultural insect pest, H. virescens, and we are currently working on two forest pests, Spruce budworm and gypsy moth.

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OVERVIEW OF BIOPROCESSING

T. Kent Kirk

Institute for Microbial and Biochemical Technology
Forest Products Laboratory, Forest Service, USDA
1 Gifford Pinchot Drive
Madison, WI 53705 USA

Raw materials from the forest are substrates for the enzymes of numerous microorganisms. It follows that such microorganisms and their isolated enzymes can be used to modify, convert and break down those raw materials. Numerous applications and potential applications of such bioprocessing in forest products manufacture are being explored in laboratories worldwide. Current commercial applications include waste treatment processes, fermentations of wood sugars to ethanol and single cell protein, and the latest: use of enzymes and fungi to remove pitch in connection with pulping, and enzymatic bleaching of chemical pulps. New applications being researched include biopulping, using enzymes to improve virgin and recycled fibers, using microorganisms to remediate soils contaminated with wood preservatives and other chemicals, biotransforming extractives with enzymes and microorganisms, and biodegrading solid wastes. It is to be expected that the next few years will see many new and improved applications of biotechnology in forest products manufacture. The touted advantages of biotechnological approaches to manufacture are environmental compatibility and opportunities for novel products. In many cases, cost savings, energy savings and processing simplification can also be expected.

A most interesting new commercial application of bioprocessing is the use of mixtures of xylanases and cellulases to partially bleach kraft pulps. The color in pulps is due primarily to residual lignin, which is attached to polysaccharides. Treating the pulps with enzymes that cleave exposed polysaccharide chains frees chromophoric material so it can be washed out of the pulp. The interest in such use of enzymes is that it reduces the chlorine requirement for chemical bleaching--a major current objective of the pulp industry. There is considerable room for improvement in enzymatic bleaching, through such avenues as a) developing enzymes with improved rates, selectivities, stabilities, and ease of manufacture, b) using lignin as well as

polysaccharide-degrading enzymes, and c) optimizing bleach plant design to accommodate enzymes.

The potential of enzymes to improve the properties of mechanical and chemi-mechanical pulps, and especially recycled fibers, is being explored in many laboratories. Enzymes should be able to alter surface properties, perhaps decrease brightness reversion, remove fines, assist in deinking, remove certain other contaminants, etc.

Isolated enzymes are already being used to remove the pitch from mechanical and chemi-mechanical pulps. A problem particularly with pine wood pulp, pitch sticks to the paper machines and slows them down; removing pitch allows the machines to be run at a higher rate. Pitch is also removed by another new commercial process that employs a living fungus growing in wood chips before they are converted into pulp. The fungus feeds on the pitch, and does not attack the structural components.

Processes that employ fungi that do attack the structural components of wood could eventually be of even greater significance. Biopulping is the use of lignin-degrading fungi to "soften" wood chips prior to manufacturing pulp. During the last four years considerable effort has been expended to evaluate the technical feasibility of such fungal pretreatment for refiner mechanical pulping. Results to date on a bench scale indicate that with selected fungal strains and conditions, energy savings of at least 25% in the pulping step are obtained, and that paper strength properties can be enhanced. Industrial interest in biopulping is primarily in its potential to save energy. Research now is needed primarily to evaluate biopulping on a larger scale so that engineering parameters can be defined and process economics can be worked out.

Generating an energy source through the production of ethanol from wood and wood processing wastes has been practiced for many years, but the full potential has not been approached. Interest in generating alternative liquid fuels from wood--and funding for the required research and development--comes and goes as petroleum prices fluctuate. Two of the biggest unsolved problems are how to ferment xylose commercially, and how to hydrolyze the wood polysaccharides without generating byproducts that are toxic to the fermenting strains.

Various other fermentations could be developed for transforming extractives from wood, bark, etc., into higher value products. For example, research has demonstrated that the abundant tall oil sterols in kraft pulping liquor can be transformed by bacteria to useful steroids. In a less glamorous application, attention is being paid to composting and deliberate biodegradation of solid

wastes, reflecting the increasing urgency of the concern about accumulation landfills.

A related application that is also receiving increasing attention is the use of white-rot fungi for remediating soils contaminated with wood preservatives, coal tar and other chemicals. Some of these fungi can also b used to degrade the wood preservatives found in treated wood that is no long in use.

Various other microbiological processes are being studied increasingly f improved and novel treatments for wastewaters, including those from wood-usi industries.

"CARTAPIP(TM): A BIOLOCICAL PRODUCT FOR THE CONTROL OF PITCH AND RESIN ACID PROBLEMS IN PULP MILLS

R.L. Farrell*, P.A. Wendler*, Y. Hadar#,
R.A. Blanchette^,T.S. Brush* and C.Ho@

*Repligen Sandoz Research Corporation, 128 Spring Street
Lexington, Massachusetts 02173

#The Hebrew University of Jerusalem
Rehovot 76-100, Israel

^University of Minnesota, Department of Plant Pathology
St. Paul, Minnesota 55108

@Sandoz Chemicals Corporation, Mallard Creek Center
Charlotte, North Carolina 28213

One cause of production and paper quality problems in pulp and paper mills is depositable pitch. Pitch is composed of fatty acids, resin acids, sterols, glycerol esters of fatty acids, other fats and waxes. Pitch is less than 10% of total weight of wood, but causes major problems. Composition and concentration of pitch varies within the tree itself, between wood species, geographical location and seasons of the year. Problems in the pulp and paper industry can include relatively frequent and lengthy down-time for cleaning, breakage of paper on paper machines, decreased paper strength, increased toxicity from resin acids in the effluent and other drawbacks.
Pitch problems are also dependent upon the type of pulp processing, chemical versus mechanical. Pitch is liberated from the pulp fibers at various processing timepoints and particularly when there is a change of pH and/or temperature. Pitch can be deposited alone or with fibers, fillers, defoamer components, coating binders from broke and insoluble inorganic salts. Current solutions for pitch include aging or seasoning of wood chips, use of wood species with low pitch content, control with alum, talc, dispersants or surface active agents, cationic polymers of retention aids.

Reported here is a biotechnological solution for pitch problems; it is a naturally occurring non-pathogenic fungal product, called Cartapip(TM), which removes pitch by degrading it. Cartapip(TM) is a formulation of the Ascomycete, Ophiostoma pilifera. It is marketed as a dry powder fungal product; it is a living organism; when diluted with water to a 1-3% solids solution and sprayed on wood chips it rapidly proliferates and removes the

pitch/resin from the wood chip prior to the production of pulp and subsequently paper. It has been tested and shown to be completely not harmful and safe to the environment. It is exclusively saprophytic; in harvested wood it proliferates through ray parenchymal cells, tracheids and resin ducts, as followed by scanning electron microscopy, metabolizing readily available nutrients such as simple sugars and pitch.

Cartapip(TM), which stands for Pulp Improvement Product, was discovered in collaboration with a thermomechanical pulp mill in Virginia, U.S.A., Bear Island Paper Company (B.I.P. Co.), which produces newsprint for the Washington Post and the Wall Street Journal.

Repligen Sandoz Research Corporation (RSRC) of Lexington Massachusetts began a biological screening program at B.I.P. Co. which eventually led to the identification and isolation of a number of fungal species growing in the chip pile, some capable of reducing pitch. RSRC identified a natural fungus from the chip pile which reduced pitch by 50% or more in less than 10 days from southern yellow pine wood chips. The organism is called Ophiostoma pilifera. RSRC has now isolated these Ophiostoma strains throughout the United States as well as Malaysia, Australia, Portugal and Finland.

Since Ophiostoma pilifera is a fungus of the classification called Ascomycete, it produces sexual spores called ascospores which are homokaryotic. This allowed for selection of Ophiostoma pilifera homokarotes; crosses were made of various isolated homokaryotes which allowed for the development of strains which maintained chip brightness as well as rapidly reducing pitch.

Laboratory analysis has shown that the Cartapip(TM) strains produce extracellular lipases and small amounts of hemicellulases, but no cellulytic or ligninolytic enzymes. Pitch analysis by gas chromatography showed a reduction of total fatty acids and total resin acids and identified changes in the levels of individual pitch components following Cartapip(TM) treatment of southern pine wood chips.

Field trials were initially conducted on southern yellow pine chips at B.I.P. Co. at the 6-10 wet ton chip pile size. Chips were routinely prepared from 7-60 day old logs without affecting the efficiency of the process. In the field was shown easy application of Cartapip(TM) at a dose of 1×10^{11} cells per ton chips in a volume of one liter water. Within four to ten days, chip piles had complete coverage of Cartapip(TM) with pitch being reduced by typically 50% or more.

Mill trials were then conducted at the 1400 wet ton level: chips were inoculated with a dose of 5×10^{10} cells per ton in the chipper facility immediately after chipping as the chips were tumbling on an auger screw conveyor and then blown out to the chipyard. The spray system consisted of a 50 gallon reservoir of fungal inoculum, motor driven propellor to maintain cells in suspension, and a pump delivering the inoculum through a series of nozzles three feet above the chips. Chips were stored in the chipyard for ten days prior to normal TMP processing and the resulting pulp showed reduced pitch levels, improved paper machine efficiency and paper strength, reduction of bleaching chemicals needed for seasoned chips and reduction of insolubles to waste water treatment systems.

RSRC and Sandoz IBS Paper Biotechnology Group continue to work on the development of Cartapip(TM) for worldwide introduction. To date the wood species it has been shown to effectively remove pitch from include many different pine species, aspen and mixed tropical hardwoods. The dosage of Cartapip(TM) and storage time in the chip pile situation varies depending on wood species and temperature. Cartapip(TM) is a unique solution to pitch problems before the wood enters the mill.

VAI - BIOBLEACHING

Dr. Michael Sinner, Donatusgasse 3b, A-4020 LINZ
Dr. Gunther Ditzelmuller, Schweizerstr, 8-10/5/8, A-1130 WIEN
Dipl. Ing. Wolfgang Wizani, Grillparzerstr. 3/4/11, A-4400 Steyr
Prof. Dr. Walter Steiner, Im Hoffeld I, A-8046 Graz
Prof. Dr. Hermann Esterbauer, Ziegelstr. 21 w, A-8045 Graz

The pulp industry is endeavoring to reduce the use of chlorine in pulp bleaching and to eliminate it completely in the near future. The first successes were "Extended Cooking" and oxygen delignification. New technologies using oxygen-containing bleaching agents, particularly ozone, are being developed, for instance, at the Osterreichische Zellstoff Forschungsgesellschaft m.b.H. (OZF).

VAI has developed an enzyme for a non-chemical pre-bleaching stage: Bio-bleaching is the application of enzymes - up to now, cellulase-free xylanases - for the treatment of unbleached or semi-bleached pulp in chemical bleaching stages. The R&D work of VAI focussed on finding a suitable microorganism that does not produce cellulase. The investigations yielded a fungus - without gene manipulation - which produces large quantities of stable xylanase. Based on beech xylan from viscose production, inexpensive lignocellulose-containing residues were used as carbon sources and different technical nitrogen sources and mineral substances for a simplified and economic xylanase production on an industrial scale.

On a laboratory level, the improvement of fermentation using lignocellulose as a carbon source yielded some thousands of XU/ml. Fermentation trials were conducted on a 15 m^3 fermenter reaching over 600 XU/ml. The xylanase-containing enzyme filtrate can be stored for weeks without any marked loss of activity at a temperature of 20°C. and at a temperature of 4°C for several months. By means of industrial equipment, the enzyme filtrate was successfully processed to xylanase concentrates and spray-dried xylanase powder. The original activities remain practically unchanged. A fermenter charge of approximately 10 m^3 is sufficient for bleaching approximately 500 - 1500 tons of pulp. The enzyme filtrate can be used for bleaching without any special treatment. The washed and unbleached or pre-bleached pulp is set to a pH value of 4.5 - 5.0 and a temperature of 50 - 55°C. By means of a static mixer, the VAI xylanase is dosed within 2-8 XU/g bone-dry pulp. After a retention time of approximately 2 hours, e.g. in a storage tank, the pulp is transported to the subsequent bleaching stage without intermediate washing.

Lab-scale bleaching trials resulted in a savings of chemicals, unchanged final brightness and nearly the same physical properties of pulp. Regarding softwood, the yield loss was nearly zero; with respect to hardwood, it was below 1%. The savings in chlorine gas as well as the reduction in AOX amounted to about 40%. Up to now, bleaching sequences using oxygen-containing agents have resulted in savings of such agents within a range of 20%.

ENZYME TECHNOLOGY FOR FIBER TREATMENT

Thomas W. Jeffries

Institute for Microbial and Biochemical Technology
USDA Forest Products Laboratory
Madison, Wisconsin 53705, USA

Within the past five years, microbial enzymes have seen increasingly diverse applications in bioprocessing lignocellulose. In the early 1980's virtually the only use envisioned for lignocellulose degrading enzymes was the saccharification of cellulose to glucose for ethanol production. Enzymes that would attack lignin had not yet been described, and hemicellulases were considered irrelevant. Today, we stand on the verge of a new era for lignocellulose bioprocess technology in which:

- Cellulase preparations from recombinant fungi are being supplied commercially with progressively higher yields and tailored isoenzyme profiles at significantly lower costs
- Xylanases, mannanases and pectinases are finding specialized industrial applications in pulp bleaching, fiber modification and recovery
- Lignin-degrading enzymes are being used in laboratories for pulp bleaching and fiber modification
- Lipases are being applied to pitch removal and deinking

Future technologies will see the application of these and other enzymes in organic solvents, at elevated temperatures and extreme pH. Individually, these developments can be seen as the products of highly innovative minds identifying new applications for versatile technology. Collectively, they point the way toward a revolution in lignocellulose bioprocessing in which specific enzymatic catalysts working under well-defined conditions can be used to remove individual lignocellulosic components and impart new properties to the products.

Microbial enzymes have been used to increase pulp fibrillation and water retention, and to reduce beating times in virgin pulps. With recycled fibers, enzymes restore bonding and increase freeness. Specialized applications include the reduction of vessel picking in tropical hardwood pulps, and the selective removal of xylan from dissolving pulps. The single most important application is in the removal of lignin from kraft pulps.

Microbial enzymes have relatively little effect on unmodified wood. Particle size and substrate surface area play large roles in determining enzyme accessibility. The resistance of wood to attack is attributable in part to the presence of acetyl esters. Acetyl groups are removed and other ester linkages are broken by dilute alkali. Cell-free enzymes show much greater activity against wood pulps. Relatively low enzyme dosages can be employed, and the effects are notable.

Numerous substances can interfere with enzyme activity. In addition to contaminants and heavy metals, the non-specific adsorption or inactivation of xylanases by cellulose can reduce the effect of the enzyme on xylan. For these reasons, great care must be taken in evaluating enzyme activities in the presence of heterogeneous substrates. The activity of microbial enzymes may be reduced against secondary fibers where inks, adhesives and numerous other contaminants are present.

Enhanced fibrillation was the first application of cell free enzymes to pulps. Both cellulases and hemicellulases have been employed. In addition to enhancing beating, cellulases selectively remove fines, thereby facilitating drainage. In other applications of this sort, cellulases have been used to remove fines from pits and felts in the papermaking machinery.

One of the disadvantages of using cellulases on pulps is that they will attack the fiber and can diminish pulp strength. Cellulases decrease the degree of polymerization (DP) of cellulose and thereby reduce viscosity. In order to get around this difficulty, a number of "cellulase-free" xylanase preparations have been investigated. Treatments with xylanases and cellulases have been used to improve the freeness of recycled fibers. Recycled fibers contain a larger fraction of fines than virgin fibers, and fines account in large part for the slow drainage of pulps. Rapid drainage is desirable because in order to increase the efficiency of the paper machine, the drying phase must be as short as possible.

Many different cellulases and hemicellulases can be used to improve freeness, but good results have been obtained with commercial cellulases from Trichoderma reesei. Recently, the drainability of mechanical pulp has been shown to be enhanced by the addition of hemicellulases rather than cellulases. While using xylanases can avoid degradation of the cellulose, hemicellulose is removed, and inter-fiber bonding strength is reduced. Hemicellulases are also less effective than cellulases in restoring freeness.

The use of tropical hardwoods such as eucalyptus for pulp production has increased in recent years. The vessel elements of tropical hardwoods are,

however, large and hard, and they do not fibrillate during normal beating. As a consequence, they stick up out of the surface of the paper. During printing, they are torn out leaving voids. This characteristic reduces their value. Commercial cellulases can enhance the flexibility of hardwood vessels and thereby reduce vessel picking.

Certain applications of pulps require purified cellulose having a high degree of polymerization. Rayon, for example, is a regenerated cellulose formed by extruding a cellulosic dissolving pulp through a fine nozzle. When xylan is present, bulking occurs, and the quality of the rayon fiber is diminished. Xylan can be removed by partial acid hydrolysis, but this also degrades the cellulose, leading to diminished strength properties. By using a specific xylanase to remove xylan, the polymeric properties can be preserved. Some xylanases can remove xylan from pulps without affecting other components. The purified cellulose can then be used in making dissolving pulps.

The most studied application of enzymes to pulps it to enhance bleaching. At least three enzyme-based approaches have been investigated: One is to use extracellular oxidative enzymes, another is to use hemicellulases, and a third is to use low molecular weight biomimetic agents.

Kraft pulp is commonly bleached with chlorine and chlorine dioxide. Alkali extraction then removes chlorinated aromatic derivatives of lignin. Some of these derivatives include dioxins and other toxic compounds. They are recalcitrant, and pass through conventional biological waste treatment. Moreover, residual chlorine compounds left in the paper are released during incineration. The chlorine bleaching of paper is a major concern to the industry, and it is one of the principal areas under research and development.

Chlorination is essentially an oxidative process, so a number of biological oxidative agents have been examined for their abilities to break down lignin in pulps. The principal enzymes of interest are lignin and manganese peroxidases (LiP and Mnp, respectively) from white-rot fungi.

The published scientific literature concerning the effect of ligninases on pulps is sparse. Farrell (1986) pointed out the potential applications of lignin degrading enzymes. These include the bleaching of chemical pulps, the partial delignification of coarse thermomechanical pulps, decolorization of bleach plant effluents, and enhancing the utility of kraft lignin. Viikari et al. (1990) applied crude lignin and manganese peroxidases from Myrothecuim graminaceum and Phlebia radiata to birch and pine peroxy acid pulps. M. graminaceum produced high MnP, but relatively little LiP. Initial studies

indicated that enzyme treatments would reduce the kappa numbers of peroxyacid pulps, but that the lignin contents were not affected.

There are at least two U.S. patents (Farrell 1987a,b) and one European patent application (Olsen 1989) pertaining to the use of ligninases from white-rot fungi for bleaching pulps. In addition, there is a European patent application on the production of recombinant ligninase (Farrell et al. 1987). All of these patents focus primarily on lignin peroxidase from P. chrysosporium.

Olsen et al. (1989) has presented data showing the effects of LiP and MnP on pulps. This work focuses on the development of an enzymatic bleaching procedure using partially purified MnP and LiP isoenzymes from P. chrysosporium. The enzymes were applied in three stages, with gradually decreasing amounts of enzymes at each stage. Several replicates of buffer controls and several levels of enzymes were employed with a northern hardwood and a southern pine kraft pulp. Low levels of hydrogen peroxide were supplied by adding glucose oxidase and glucose to the reaction mixtures, but in a commercial process, hydrogen peroxide would be added by an automated metering device. The amounts of LiP and MnP added were relatively high. Cumulative enzyme additions amounted to as much as 32 IU of LiP and 88 IU of MnP per oven dry g of hardwood pulp. Similar but slightly lower levels were used for the softwood pulp. Reductions in kappa were significant, and they appeared to be dose-responsive. In the case of the hardwood, the kappa of enzyme-treated pulp was less than half that of the controls, and similar reductions in kappa were achieved with the softwood. Viscosities were also reduced by the enzyme treatments, albeit to a slightly lesser extent. These findings indicated that under the conditions employed, fungal lignin-degrading enzymes can facilitate the removal of lignin from pulp.

Several reports have shown that bleaching is enhanced by various hemicellulase preparations. Microbial hemicellulases include various xylanases, acetylmethylglucuranosidases, arabinofuranosidases and glucomannanases. Xylanases generally enhance bleaching of hardwood more than softwood pulps. Purity of the xylanase appears to be important. Some preparations contain appreciable amounts of cellulase and therefore reduce the viscosity of pulps, so cellulases should not be present, but because the kraft pulping process can introduce chemical bonds or chromophoric groups into cellulose, some cellulase activity might be necessary to enhance bleaching. However, mixtures of hemicellulases with different action patterns can be more effective than single enzyme fractions.

Glucomannanases appear to be more useful with softwood pulps. The only reliable method to evaluate an enzyme is to determine its effects on pulp properties and bleachability. Pulps have been treated effectively with cloned xylanases, and it seems likely that products will eventually be developed that will contain a mixture of enzymes to enhance and affect bleaching.

Xylanase treatments can in some instances release lignin from kraft pulp, but more commonly,they reduce chemical consumption in subsequent bleaching and extraction steps while attaining greater brightness than pulps that are not enzymatically treated. The mechanical strength of fibers is not necessarily affected, however, inter-fiber bonding often decreases. Xylan does play an important role in fiber strength properties, so excess removal of hemicellulose can be detrimental. If xylanases alone are used to reduce residual lignin content, the negative effects can be significant. Typical enzyme loading rates used are in the range of 30 to 150 nkat/g of pulp (approximately 2-10IU/g). (Viikari et al. 1990) However, useful effects have been observed with some commercial enzyme treatments using as little as 0.5 to 5 IU of xylanase per g dry wt of pulp (Pedersen 1989).

Viikari et al. (1990) evaluated the capacity of five different microbial xylanases and a bacterial mannanase to enhance the alkaline peroxide delignification of a pine kraft pulp. The endo xylanases were the most effective. Only slight differences in brightness or pulp strength properties were observed with the fungal and bacterial xylanases. In keeping with other studies (e.g. Jeffries and Lins 1990; Puls et al. 1990) only a portion of the xylan was susceptible to enzyme hydrolysis following repeated enzyme treatment and lignin extraction. Relatively short treatments with low quantities of enzyme are sufficient to remove the susceptible fraction of xylan present. These findings have led to the hypothesis that the efficacy of xylanase treatments are largely limited by substrate accessibility or structure.

At least two patent applications have been filed concerning the hemicellulase-facilitated bleaching of kraft pulps. One application indicates that a mixture of cellulases, hemicelluases and esterases might be used (Salkinoja-Salonen et al. 1989). The other patent makes specific reference to bleaching sequence employing the xylanase from Aureobasisium pullulans (Farrell 1989). The usefulness of the latter enzyme derives from the fact that the wild-type strain produces the enzyme easily in very high yield without coproducing significant amounts of cellulase. Neither of these preparations nor other presently available commercial preparations have very much activity at neutral or alkaline pH.

The use of cellulases and hemicellulases in enhancing freeness of secondary fibers has already been mentioned. In addition, there have been a number of recent patents directed toward the use of enzymes for deinking. One of these employs cellulases in a pulp slurry at 3 to 10% consistency. Following enzyme treatment, a deinking agent is applied (Anon 1990a). Other approaches employ alkaline lipases which act by deesterifying vegetable lipids used as the carrier in some ink formulations (Anon 1990b; Sharyo and Sakaguchi 1990).

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BIOPROCESSING OF PULP AND PAPER MILL EFFLUENTS: PAST, PRESENT AND FUTURE

Allan Springer, Professor
Department of Paper Science and Engineering
Miami University
Oxford, Ohio 45056, USA

A brief history of biological treatment as it has evolved in the U.S. pulp and paper industry will be presented. The legislative and economic forces involved, and the way biotreatment technology has evolved to meet these challenges will be discussed.

The evolution has been from storage oxidation lagoon,to aerated stabilization basins, to high rate activated sludge systems. There are currently about 600 pulp and paper mills in the United States and over 98% have some form of biological treatment. The majority of mills have their own treatment plants, but some are tied into publicly owned treatment plants. Of those which have their own treatment plants, two thirds have aerated stabilization basins, and one third have activated sludge. The reasons for this will be discussed. Some mills have decided to use more specialized treatment systems such as oxygen activated sludge, oxidation ditches and deep tank systems. The reasoning behind these choices will be discussed. One of the main disadvantages of activated sludge biological treatment is the production of a large amount of sludge, which is difficult to dewater and costly to dispose of. A modification of the activated sludge system which overcomes this problem will be discussed.

As the mills closed their water systems, the concentration of biochemical oxygen demand (BOD) in the effluents built to the point where it became viable to consider anaerobic treatment as a pre-treatment stage before applying aerobic treatment. The evolution of anaerobic treatment in the paper industry will be traced. The success of anaerobic lagoons, anaerobic contact, upflow anaerobic sludge blanket, anaerobic biofilter and anaerobic fluidized beds will be considered. The challenge of biological treatment has always been the removal of BOD and the systems meet this challenge well. The new issue facing the industry is removal of chlorinated organics. This challenge is not being met well with biological treatment as measured by the gross parameter AOX. Biological treatment seems to remove between 13-54% of the AOX present in a bleached kraft mill effluent. Some of the research to improve this removal will be briefly discussed.

The future challenges for biological treatment will be color and toxic substance removal. Other biotech systems, currently at the research stage, which attempt to meet this challenge are Specialized Cultures and MyCoR. These systems will also be reviewed.

CLONING GENES FOR LIGNIN BIOSYNTHESIS IN LOBLOLLY PINE, PINUS TAEDA L.

David M. O'Malley, Ross W. Whetten and Ronald R. Sederoff

Department of Forestry
North Carolina State University
Raleigh, NC 27695-8008

Our overall strategy is to exploit the unique biology of conifers to explore questions of vascular development that are not easily approached in herbaceous angiosperms. Little is known about the mechanisms that regulate gene expression in the lignin biosynthetic pathway during development in vascular plants. Lignification is an important step in the differentiation of vascular elements into a functional water-conducting tissue, lending mechanical strength and impermeability to the cell walls. Environmental cues such as wounding, pathogen attack or mechanical stress can also induce lignification. The regulation of phenylpropanoid metabolism by environmental cues has been studied extensively in herbaceous plants, but the developmental regulation of lignin synthesis has attracted less attention.

Our general approach has been to progress from biochemical analysis of proteins to molecular genetic analysis of DNA and RNA, using material from individual trees in order to avoid the problems of genetic variation between individuals. We have focused our efforts on two enzymes of lignin biosynthesis. Phenylalanine ammonia-lyase (PAL, EC 4.3.1.5) catalyzes the deamination of L-phenylalanine to cinnamic acid, the first step in the phenylpropanoid pathway of plant secondary metabolism. Cinnamyl alcohol dehydrogenase (CAD, EC 1.1.1.195) catalyzes the reduction of substituted cinnamaldehydes to the corresponding alcohols, the final step in the production of lignin precursors (monolignols). PAL is involved in the production of many phenolic products in addition to lignin (e.g. flavonoids, coumarins and stilbenes), while CAD is specific to the production of monolignols, although monolignols may not be specific to lignification.

In loblolly pine, our preliminary results indicate that PAL and CAD may be encoded by single genes. Campbell and Ellis (1990) have begun work on PAL in Pinus banksiana suspension cultured cells. They find only a single PAL isozyme in elicitor-induced cultures, in contrast to results from angiosperms, where multiple isozymes encoded by different genes are found in elicitor-treated cell

cultures (e.g. Bolwell et al. 1985b; Dalkin et al. 1990). Parallel investigation into elicitor-induced PAL activation in gymnosperms thus also supports the idea that there may be only a single pal gene in pines. If single genes encode these important enzymes, the genetic control of lignin biosynthesis in conifers may be less complex and more easily analyzed than in more highly evolved angiosperm systems. In this case, it should be relatively easy to identify all of the cis acting DNA sequence elements regulating the transcription of the single genes of interest. Furthermore, these single genes must contain the cis acting sequences needed for response to both environmental and developmental control.

We exploit the advantages of differentiating xylem in pine as a developmental system for investigating the biosynthesis of lignin. We have chosen this tissue for the following reasons:

1) Immature xylem is readily obtained in quantities suitable for biochemical uses such as nucleic acid or protein purification, as well as for molecular biology techniques requiring isolated nuclei. A single rapidly-growing tree can yield more than a kilogram of immature xylem.

2) This tissue is composed largely (>90%) of a single cell type, differentiating tracheids. These cells are in the process of terminal differentiation into a highly specialized tissue, and they are likely to demonstrate unique patterns of regulation. The homogeneity of this specialized tissue is an important advantage.

3) Transient gene expression assays are possible in differentiating xylem using microprojectile bombardment. Promoter constructs can therefore be tested for activity in differentiating cells without the time required to make and characterize transgenic plants. We will be able to isolate regulatory sequences for genes known to be active in lignin biosynthesis in specific tissues, and to test their activity in a transient assay system in the tissue of interest.

4) We can carry out genetic mapping of enzymatic and nucleic acid polymorphisms, because of the unique biology of the conifer megagametophyte and recent advances in PCR-based mapping techniques.

Background: PAL is the best studied of the enzymes in all of plant secondary metabolism (Jones 1984). As the first enzyme of the phenylpropanoid pathway, PAL is active in many tissues in response to many different stimuli, including wounding, infection, hormones, light and development. It is likely that PAL is regulated in many different ways. In addition to control at the transcriptional level, there is evidence for post-translational control of

enzyme activity (Lamb 1979; Bolwell et al. 1985; Elkind et al. 1990). The enzyme is a tetrameric protein, usually present in multiple forms which differ in their physical and kinetic properties. In bean, PAL is represented by a family of at least three genes which each encode a different isozyme (Cramer et al. 1989). The different pal genes show marked differences in response to developmental programs and to environmental stimuli (Bevan et al. 1989; Liang et al. 1989). Similarly, in parsley, PAL is encoded by a small family of at least 4 genes, and at least three respond to different stress stimuli (Lois et al. 1989).

Phenylpropanoid metabolism can be divided into the "common portion" and the branch pathways. The branch of phenylpropanoid metabolism that leads to monolignols starts with the conjugation of p-coumaric, ferulic or sinapic acid by 4-coumarate: CoA ligase (4CL) to Coenzyme A. These phenolic acid-CoA thioesters are in turn converted to the corresponding aldehydes by cinnamoyl CoA oxidoreductase (CCR). The final step in precursor synthesis is the conversion of the aldehydes to alcohols by CAD. The enzymes for the reduction of substituted cinnamic acids to the monolignols may therefore be regulated differently from PAL and the other steps before 4CL in the pathway. We are far from understanding the regulation of the lignin pathway in any system. Cloning of the gene for CAD has proven to be elusive. Walter et al. (1988) reported isolation of a cDNA clone from bean. Recently, they have shown that this sequence has high similarity with malic enzyme (Walter et al. 1990). Schuch and co-workers (pers. comm.) have recently isolated putative cDNA clones for CAD from tobacco. The size of the CAD gene family in tobacco has not been established. This issue is of particular interest because tobacco is an allotetraploid.

Work carried out by our laboratory on PAL: We have purified PAL from developing wood of loblolly pine. Polyclonal antibodies were raised against the PAL subunit purified on SDS gels, and a partial cDNA clone was isolated from a xylem cDNA expression library. The nucleotide sequence of the clone shows 60% to 65% identity to homologous regions of angiosperm PAL clones. Examination of purified pine PAL by isoelectric focusing and kinetic analysis has yielded no evidence for the existence of multiple isozymes, and Southern blots of pine genomic DNA probed with the pine PAL cDNA at high stringency show bands consistent with a single-copy gene. Low stringency hybridizations identify multiple bands, but the degree of sequence similarity is so low as to raise doubts as to whether these bands represent functional genes or diverged nonfunctional sequences. One genomic clone isolated from a loblolly pine

library using a heterologous probe (Hutchison and Smith, pers. comm.) shows no more similarity to the clone we have isolated than does the bean PAL-2 pal-gene. The region of similarity to PAL in this genomic clone is interrupted by a sequence of unknown origin that is not at the site of the conserved intron in the known PAL genes (Cramer et al. 1989; Lois et al. 1989; Minami et al. 1989) and does not conform to the hallmarks of intervening sequences (Mount 1982). We conclude that there may be only a single functional PAL gene in the loblolly pine genome, in contrast to the multi-gene families found in all herbaceous angiosperms examined to date. We suggest that PAL may be regulated by different mechanisms in pine than in angiosperms, as a reflection of the differences in gene structure.

Work carried out by our laboratory on CAD: We have purified CAD enzyme from loblolly pine xylem to homogeneity using column chromatography and HPLC methods (following Sarni et al. 1984). Loblolly pine CAD enzyme is a dimer, with apparent molecular weights of 82 and 44 kd, respectively, for native and denatured forms (O'Malley et al., submitted). We determined the substrate specificity of CAD and found that the K_m for three substrates in the reduction reaction were similar to reported K_m from other plants.

The isozyme diversity and tissue specificity of CAD were analyzed by native gel electrophoresis. CAD enzyme can be distinguished from related enzyme activities by its substrate specificity: only CAD can utilize both coniferyl and cinnamyl alcohols. We found no evidence of coniferyl alcohol dehydrogenase (EC 1.1.1.194). An aromatic alcohol dehydrogenase (EC 1.1.1.91) appeared as a monomorphic isozyme band on gels stained using cinnamyl alcohol. The xylem CAD enzyme displayed a high level of electrophoretic variation, with five different allozymes of CAD in a sample of 30 individuals. Mendelian segregation of allozyme variants showed that CAD in encoded by a single functional gene. We were surprised to find CAD expressed in the haploid tissue of conifer seeds (megagametophytes) because this tissue does not lignify. Correspondence of allozyme phenotypes from xylem and megagametophyte show that the same CAD gene is expressed in both tissues. We do not detect CAD in needle tissue.

We are confident that we have identified the xylem form of CAD from loblolly pine. We have prepared monoclonal and polyclonal antibodies to CAD. In addition, we have obtained protein sequences from the N-terminus and two other peptides totaling about 50 amino acids. We have the tools and information to clone and authenticate the gene encoding of the xylem form of CAD from loblolly pine. We have carried out preliminary work to screen cDNA expression libraries with the antibodies, but have not yet identified a

putative CAD clone. Work to clone the gene by PCR using degenerate oligonucleoptide primers is in progress. In the last week, we have identified a 1400 bp DNA fragment which was amplified from xylem cDNA using a CAD N-terminal degenerate PCR primer and oligo dT. Other fragments were amplified using the 1400 bp fragment as template for degenerate primers to other CAD protein sequences. It is likely that this DNA fragment encodes CAD. We will confirm the identity of this fragment from nucleotide sequence and by immunological assay of expressed fusion protein in bacteria.

Significance: Knowledge of the regulation of lignin biosynthesis in developing wood has practical applications. Wood is the major product for which most trees are grown, and the ability to manipulate lignin quantity or quality would have a significant impact on the wood products industry. Any attempts to manipulate lignin content in wood should take into account the role of lignification in the defense response; an understanding of how these two aspects of lignin biosynthesis are integrated at the level of gene regulation would be vital to the success of such a genetic engineering experiment.

MOLECULAR ANALYSIS OF FACTORS REGULATING PLANT GENE EXPRESSION

Eric Lam

AgBiotech Center, Waksman Institute
Box 759, Rutgers State University
Piscataway, NJ 08854

The ability to express desired gene products in a spatially and temporally specific manner will play an important role in genetic engineering and biotechnology. Elucidation of the mechanisms by which gene expression can be regulated may provide the necessary information from which "designer" promoters and transcription factors can be constructed *in vitro*. These will provide new tools in the design of future plant transformation vectors. In addition, understanding of the regulatory mechanisms that govern gene expression should help to advance our knowledge on plant developmental control and may eventually allow us to alter the growth characteristics through manipulation of endogenous levels of specific transcription factors. In this presentation, I will discuss some of our recent studies concerning the cis- and trans-acting elements of the cauliflower mosaic virus (CaMV) 35S promoter, a strong enhancer element that is able to confer expression in diverse cell types.

The 35S promoter of CaMV is one of the strongest promoters known in transgenic plants. Its expression is mostly, if not completely, dependent on host transcription factors. In monocots and dicots, a 352 base-pair derivative of this promoter (-343 to +8) has been found to confer expression in diverse cell types and under different physiological conditions (Odell *et al*. 1985). This promoter sequence has also been functionally dissected into at least 6 different regions, each with a distinct pattern of expression in transgenic tobacco (Benfey and Chua 1990). To better understand the factors that contribute to this strong promoter, we have mapped protein binding sites within two of these regions (Lam *et al*. 1989; Lam and Chua 1989). In the following, I will describe our current work concerning one of these factors, *A*ctivating *S*equence *F*actor - 1 (ASF-1).

We have first detected ASF-1 as a DNA-binding activity from pea and tobacco leaf nuclei that interacts with the -75 region of the CaMV 35S promoter. Two putative CAAT motifs, known to be common enhancer elements in other systems, were previously identified in this region (Fang *et al*. 1989).

However, from methylation interference assays, we have shown that two TGACG motifs in this region are the critical sites for ASF-1 binding. Moreover, site-specific mutations of the putative CAAT motifs did not interfere with ASF-1 binding whereas mutations in the TGACG motifs decreased binding of ASF-1 dramatically (Lam et al. 1989). Functionally, the binding site of ASF-1, from -83 to -63 of the 35S promoter, is known to be required for maximal expression of the upstream sequences in tobacco leaf (Fang et al. 1989). However, a derivative of the 35S promoter, truncated at -90, is active only in root cells and not in leaf cells (Benfey and Chua 1990). In addition, the region between -90 and -46 of the 35S promoter is also known to be required for expression of heterologous enhancer elements (Benfey and Chua 1990). These observations lead us to conclude that the binding site for ASF-1 can interact with upstream elements and activate expression of these sequences in a synergistic manner. The region that ASF-1 interacts with, -83 to -63, is thus designated as Activating Sequence - 1 (as-1). Mutations of the as-1 sequence in the context of the 35S promoter severely attenuated root expression of this promoter but have only minor effects in the leaf (Lam et al. 1989). Moreover, insertion of a synthetic as-1 element into a leaf-specific promoter can confer high levels of root expression in transgenic tobacco (Lam et al. 1989). These results suggest that although the as-1 site is functional in the leaf, its expression in this tissue is apparently dependent on the presence of other upstream factors. We proposed that the cause for this behavior may be due to a limiting concentration of ASF-1 in tobacco leaves (Lam et al. 1989). Thus, a single as-1 site is not sufficient to compete with other promoters in leaf nuclei. To test this hypothesis, we have made a synthetic tetramer of the as-1 sequence and fused upstream of the minimal 35S promoter (-46 to +8). We expected that the multiple as-1 sites should now be able to compete better with other promoters in the nucleus for ASF-1 and thus overcome the limitations in factor concentration. Indeed, we found this artificial promoter element to be active in both leaf and root cells of tobacco (unpublished data).

Using the binding sites for ASF-1 as probe, we have been able to isolate a numer of tobacco cDNA clones that encode sequence-specific DNA-binding proteins (Katagiri et al. 1989). Southern blot analysis suggests that this is a small family of closely related genes with about 3 to 4 individual members. Northern analysis shows that the transcript level of this gene family is about 10 times higher in root than in leaf cells of tobacco. The deduced amino acid

sequence predicts that the encoded protein is 373 amino acid residues long and contains a leucine-zipper region found in the DNA-binding domain of a class of transcription factors (Katagiri et al. 1989; 1990). This cDNA clone has been inserted into a bacterial expression vector and the encoded protein over-produced in E. coli. The putative ASF-1 protein produced in bacteria has been shown to have similar sequence specificity in DNA binding and can function as a bona fide transcription factor in vitro (Yamazaki et al. 1990). Furthermore, the bacteria-produced protein has been found to enhance transcription initiation by increasing the number of active preinitiation complexes at the promoter (Katagiri et al. 1990). Thus, the structural and functional characteristics of our cDNA clone suggest strongly that it indeed encodes ASF-1 and its preferential expression in root tissues is consistent with our hypothesis that the concerntration of this factor is limiting in leaves but not in roots.

ASF-1 has also been shown to interact with a number of different promoters other than the as-1 site of the CaMV 35S promoter. In addition to a conserved hexameric motif (TGACGT) found upstream of many plant histone genes (Lam and Chua 1989; Katagiri et al. 1989) ASF-1 has also been found to bind to functionally critical regions of the promoters for octopine synthase (Fromm et al. 1989; Bouchez et al. 1989) and nopaline synthase (Bouchez et al. 1989; Lam et al. 1990). These genes are located on the T-DNA of Agrobacterium tumefaciens and encode the enzymes responsible for opine biosynthesis in the plant host. The promoters of a number of other plant viruses, T-DNA and R-DNA genes also apparently contain ASF-1 binding sites (Bouchez et al. 1989). However, their functional properties in vivo remain to be defined. It is interesting that ASF-1 binding has apparently been selected by these different organisms as an important part of their promoters. We have recently found that at least two of these binding sites for ASF-1 can function as auxin-responsive elements. Thus, their activity in transgenic tobacco can be substantially increased by exogenous application of auxin. Since auxin plays an important role in plant cell proliferation and differentiation, our results suggest that ASF-1 may in fact mediate some of the physiological effects of this plant hormone. In addition, some pathogens such as Agrobacterium tumefaciens are known to increase auxin concentrations in the infected plant cells, suggesting that ASF-1 can be used by these plant pathogens as a positive feed-back regulator for the high expression of certain genes. Future study of the signal transduction pathway(s) by which auxin activates ASF-1

dependent transcription may provide new insights into the mechanism of growth regulation by plant hormones.

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RFLP MAPPING OF POPULUS

H.D. Bradshaw, Jr. and R.F. Stettler

Department of Biochemistry and College of Forest Resources
University of Washington
Seattle, WA 98195

The study of forest tree genetics is complicated by the large size, long generation interval, and outcrossing mating habit of woody plants. Furthermore, the inheritance of traits unique to woody plants, such as frost hardiness, patterns of secondary growth, and wood formation, is usually quantitative and often shows low heritability. These traits cannot be investigated thoroughly in herbaceous model systems even though powerful genetic tools are available to do so.

One approach to quantitative genetics research in plants with particular promise for trees is the use of detailed genetic maps for counting and identifying quantitative trait loci (QTLs). Application of high-density linkage mapping to problems of quantitative inheritance in trees has been delayed because of the lack of suitable pedigrees with sufficient segregating genetic and phenotypic variation. To address this problem, we have produced and begun to map a three-generation inbred pedigree founded by hybridization between a female Populus trichocarpa (T) and a male P. deltoides (D). The F_1 generation (TxD) contains 23 hybrid offspring. Two F_1 hybrids were crossed to produce the F_2 (TDxTD), and each F_1 was backcrossed to one of the founding parents to produce the two possible backcrosses (TDxD; TxTD). Among the 612 advanced-generation offspring, there is striking variation in phenology, form, and growth. Genetic variation is assessed and linkage analysis performed using DNA markers, primarily restriction fragment length polymorphisms (RFLPs) and amplified sequence polymorphisms (ASPs). Linkage blocks containing several markers have been assembled, and we are searching for correlations between RFLP alleles and quantitative traits.

Peripheral to the mapping of quantitative trait loci we have found that some hybrids are not diploid, but triploid or aneuploid. This was first revealed by RFLP analysis in F_1 and F_2 offspring. Tri/aneuploidy in the F_1 has been confirmed by measurement of nuclear DNA content and by karyotype. As is typical of triploid aspen, triploid TxD hybrids have larger leaf epidermal cells and larger leaves than diploids.

Other phenotypic traits, such as leaf shape, abaxial leaf color, and stomatal physiology, are skewed in the direction of the female "T" parent. At least 10 different female P. trichocarpa clones in our breeding program have produced one or more tri/aneuploid TxD hybrids as judged by abaxial leaf color. The ramifications of tri/aneuploidy for tree growth, wood quality, and fertility are being investigated.

GENE-TRANSFER VECTOR ANALYSIS IN INSECTS

Alfred M. Handler

U.S. Department of Agriculture, Agricultural Research Service
Insect Attractants, Behavior, and Basic Biology Research Laboratory
Gainesville, FL 32608

Molecular biological methodologies provide new approaches towards achieving highly efficient genetic-sexing and sterilization for sterile insect technique programs. Genetic-sexing is possible using classical genetic manipulations, such a linkage by translocation of a selectable gene to a sex-specific chromosome, yet problems such as genetic instability after mass-rearing have impeded their wide-scale implementation. Molecular techniques should overcome such problems by the use of relatively small chimeric gene-fusions transferred into a host genome (see Handler, 1991). Sex-specific selection may be achieved when genes coding for selectable products are linked to regulatory DNA sequences that cause sex-specific expression of the gene. Regulatory sequences may include promoter elements from sex-specific genes such as yolk protein genes whose transcription is usually restricted to female fat body and/or ovarian tissue. Sex-specific expression may also be achieved by alternative intron splicing sites. Drosophila sex-determination genes have alternative male and female-specific 3' intron splice sites that result in the open-reading frame being maintained, and therefore a functional product produced, only in females. Male selection can occur when a gene resulting in lethality, by its own activity or after selection, is either linked to a female-specific promoter or has a female-specific splice acceptor site introduced into it. A more speculative approach to genetic sexing, though potentially most effective, may involve manipulation of sex-determination genes themselves which can result in breeding insect populations giving rise solely to sterile male offspring. An insect model system for this approach is the temperature-sensitive transformer-2 mutant strain in Drosophila melanogaster. At the non-permissive temperature chromosomal females develop as sterile males, while chromosomal males also become sterile. The ability to genetically sterilize males is of particular importance in lepidopterans where sterilization by X-irradiation may not be feasible for some species.

Many of the molecular techniques and some of the genetic material to achieve these goals are already available. The major obstacle to their implementation is the inability to transfer appropriate recombinant DNA molecules into insect host genomes (see Handler and O'Brochta 1991). When gene transformation techniques are available, testing and implementation of these methods should occur rapidly. Presently, gene transformation is an efficient and routine procedure in only one insect, D. melanogaster, which uses a gene vector system developed from the Drosophila P transposon. In an effort to develop gene transformation methods for nondrosophilid insects, we have developed methods to rapidly test putative vector systems for gene transfer by assaying their mobility properties in the insect embryo (O'Brochta and Handler 1989). The first assay involves the injection of two plasmids, one containing P sequences within a reporter gene and another plasmid containing a transposase gene, into preblastoderm embryos. The function of the transposase gene (and the ability of the embryonic milieu to support that function) are measured by the precise excision of P sequences resulting in restoration of the reporter gene function (β-galactosidase activity). Use of this assay in a variety of insects has indicated that P mobility occurs as a function of relatedness to D. melanogaster, with a lack of P mobility outside the Drosophilidae. A second excision indicator plasmid allows the unbiased detection and recovery of all products (imprecise as well as precise) resulting from P element excision (O'Brochta et al. 1991). The frequency and sequence analysis of excision products from the drosophilids D. melanogaster, D. virilis, and Chymomyza proncemis indicated both quantitative and qualitative differences in the activity of transposase in the three species. Consistent with earlier results, P mobility, and thus probably P gene vector function, was limited in species distantly related to D. melanogaster. A transient expression assay for transposase RNA intron splicing indicated a limitation in proper RNA processing in a tephritid fruitfly, Anastrepha suspensa. Although a transposase cDNA, having all introns deleted, was found not to facilitate P mobility in this species, sequence analysis of the cDNA revealed a base deletion in the first exon. After repair of the deletion by oligonucleotide site-directed mutagenesis, the new transposase cDNA helper plasmid was found capable of supporting P mobility in three nondrosophilid species (A. suspensa, Lucilia cuprina, and Musca domestica), for the first time, based upon excision assays. However, mobility frequencies were variable and significantly lower than in D. melanogaster, suggesting differences in requisite cofactors in nondrosophilid species. Testing the ability of the

transposase cDNA to promote P-mediated gene transfer in Anastrepha is in progress.

Another limitation on P mobility may be the action of repressors. We have found that the somatic product of a full-length P element, which results in a truncated polypeptide, represses the activity of the normal transposase product in a dose dependent fashion. Thus, inefficient transposase RNA processing in nondrosophilids might also serve to repress the activity of any normal product that is produced. We have made antibody to transposase in order to test transposase product formation in nondrosophilids.

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RECENT ADVANCES IN THE ORGANIZATION AND REGULATION OF LIGNIN PEROXIDASE GENES OF PHANEROCHAETE CHRYSOSPORIUM

Daniel Cullen

Institute for microbial and Biochemical Technology
Forest Products Laboratory
1 Gifford Pinchot Drive
Madison, WI 53705

Lignin depolymerization is catalyzed by extracellular peroxidases of white-rot basidiomycetes such as Phanerochaete chrysosporium. In submerged culture, production of multiple lignin peroxidase (LiP) isozymes is derepressed under carbon, nitrogen or sulfur limitation. The roles of the individual isozymes in lignin degradation and their genetic regulation are poorly understood.

In recent years much progress has been made toward elucidating the basic molecular genetics of P. chrysosporium. Important techniques have been developed such as genetic transformation, electrophoretic separation of chromosomes, and genetic maps based upon restriction fragment length polymorphisms (RFLP). At least seven closely-related LiP genes have been cloned and sequenced. Linkage and allelic relationships have been investigated.

The extreme sequence homology and allelic variation among LiP clones has substantially complicated investigations of transcriptional regulation. In this laboratory we have developed two techniques allowing quantitative detection of specific transcripts: 1) reverse transcriptase-mediated primer extension and 2) polymerase chain reaction amplification of transcripts. These methods will be described.

To provide pure enzyme for biochemical investigations we are attempting to overproduce recombinant enzyme in foreign hosts. In particular, we have expressed a LiP gene in another fungus, Aspergillus nidulans, and we have devised a strategy for altering expression in P. chrysosporium.

To establish the role of individual isozymes in lignin degradation and in processes such as biomechanical pulping, we are trying to disrupt certain genes and over-express others. Toward this end, we have recently developed a genetic transformation system based upon antibiotic resistance. It is hoped that these investigations will elucidate the importance of specific genes and provide recombinant strains with improved biopulping performance.

EXPRESSION OF ACTIVE LIGNIN AND Mn PEROXIDASE IN PHANEROCHAETE CHRYSOSPORIUM AND IN A HETEROLOGOUS HOST

Linda Miller, Elizabeth Pease, Ann B. Orth and Ming Tien
Department of Molecular and Cell Biology
Pennsylvania State University
University Park, PA 16802

The lignin-degrading white-rot fungus Phanerochaete chrysospoirum secretes a family of lignin-degrading enzymes under nutrient limitation. Most of these enzymes are peroxidases which can be divided into two distinct catalytic classes, the lignin peroxidases and the Mn peroxidases. The lignin peroxidases catalyze the oxidation of a large number of non-phenolic aromatic substrates. The Mn peroxidases catalyze the oxidation of Mn (II) to Mn (III); Mn (III), in turn, can oxidize a large number of phenolic substrates. Both enzymes have been shown to depolymerize lignin in vitro. One of our interests is to produce large quantities of both of these isozymes for enzymology studies and for potential applications.

We have developed a strain of P. chrysosporium, PSBL-1 which produces these isozymes in much larger quantities than the wild type under nonlimiting nutrient conditions. Lignin peroxidase, manganses peroxidase and glyoxal oxidase activities for PSBL-1 under nonlimiting conditions were from 4- to 10-fold higher than those of the wild type under nitrogen-limiting conditions. PSBL-1 was still in log phase of growth while secreting the enzymes, whereas the wild type had ceased to grow by this time. Addition of veratryl alcohol to the culture medium stimulated lignin peroxidase activity, inhibited glyoxal oxidase activity, and had little effect on manganese peroxidase activity in PSBL-1, as was found in the wild type. FPLC analysis shows production of large amounts of isozyme H2 in PSBL-1 as compared to the wild type. These properties make PSBL-1 very useful for isolation of large amounts of all ligninolytic enzymes for biochemical study, and open the possibility of scale-up production for practical use.

Large scale production of these oxidative enzymes is also being pursued through recombinant DNA technology. We have isolated the cDNAs encoding ligninases and an Mn-dependent peroxidase. These cDNAs were expressed in insect cell cultures of Spodoptera frugiperda (SF-9 cells) using Autographa californica nuclear polyhedrosis virus (AcNPV) baculovirus expression vectors. The recombinant proteins from all of the cDNAs were active without the need for in vitro protein engineering. The addition of exogenous heme to the growth

medium is an integral aspect of obtaining active enzyme. Analysis of the protein indicates that the enzyme is secreted. The leader sequence, directing secretion, is removed in the final protein. This technology is not economically feasible for large-scale production of lignin- or Mn peroxidase. It is, however, a valuable research tool. We have initiated site-directed mutagenesis studies to characterize structure/function relationships. Three mutant genes were constructed, a ligninase gene without its N-linked glycosylation site; a ligninase gene without an active-site histidine, putatively assigned a catalytic function in mechanistically similar peroxidases; and an Mn-dependent peroxidase gene without the homologous active-site histidine. The heterologously-expressed proteins, both mutant and wild-type, will be isolated and kinetically characterized.

OLIGOSACCHARIDES AND THEIR GLYCOSIDES IN STUDIES OF CELLULOLYTIC AND XYLANOLYTIC ENZYMES OF MICROORGANISMS

P. Biely, M. Vrsanska, and S. Kucar

Institute of Chemistry
Slovak Academy of Sciences
84238 Bratislava, Czechoslovakia

In spite of extensive studies of cellulolytic enzymes, the mechanism of cellulose degradation is still not fully understood. The mode of action of cellulolytic enzymes has not been sufficiently elucidated. The use of crystalline or amorphous cellulose or soluble cellulose derivatives as substrates enables division of cellulases into two groups; endo- and exo-acting 1,4-β-glucanases. Several lines of evidence indicate that there is no sharp boundary between these two groups of cellulases. The name cellobiohydrolases (CBHs) persists in the literature for the enzymes which do not attack soluble cellulose derivatives. Those which do hydrolyze them are considered to be endo-1,4-β-glucanases (EGs). Significant progress in differing cellulolytic enzymes within the groups of EGs and CBHs was achieved by means of aryl β-glycosides of cellobiose, lactose and cellotriose (van Tilbeurgh and Claeyssens 1985). The fact that the enzymes differ in the structures of their substrate binding sites allows formation of the productive enzyme-substrate complexes with different glycosides. Another useful criterion for classification of cellulases is their ability to hydrolyze xylan as an additional polysaccharide substrate (Biely 1990). A combination of low molecular mass and polysaccharide substrates facilitated identification of products of four cloned cellulase genes of the most extensively studied cellulase producer, Trichoderma reesei (Knowles et al. 1987). β-glycosides of cellooligosaccharides are also convenient substrates to follow the stereochemistry of the hydrolysis of the glycosidic bond (Claeyssens et al. 1990).

It is generally accepted now that the cellulolytic system of T. reesei consists of five enzymes, three EGs and two CBHs. EG I is a non-specific glucanase hydrolyzing also xylan and aryl β-cellobiosides and β-lactosides at the agluconic bond. EG III (also termed EG II) is a specific glucanase which does not liberate aryl aglycones from β-cellobiosides and β-lactosides and which does not attack xylan. EG III liberates aglycones from β-cellobiosides. The third EG of T. reesei (pI 7.5) is a low-molecular mass enzyme which is als

specific, but which, in contrast to EG III, is not detectable with 4-methylumbelliferyl β-cellobioside. CBH I, which has a high sequence homology with EG I (Penttila et al. 1986), shows another property common to EG I, the capability of liberating aryl aglycones from β-cellobiosides and β-lactosides. CBH II is the only T. reesei cellulase which does not liberate aryl aglycones from β-cellobiosides, β-lactosides and β-cellotriosides. The best way to identify CBH II is the immunochemical approach (Mischak et al. 1989).

The above differentiation of cellulolytic enzymes produced by one microorganism is only the first step toward elucidation of the mechanism of cellulose hydrolysis. No doubt that the way to this goal will be paved by determination of three-dimensional structures of the enzymes. The first study of this type (Rouvinen et al. 1990) suggested tunnel-shaped active sites for CBHs and open clefts or grooves for substrate binding sites of EGs. Our contribution to how the cellulose chain is attacked by individual glucanases comes from an investigation of the mode of cleavage of [1-^{3}H]-reducing end labelled cellooligosaccharides, cellotriose through cellohexaose, by purified enzymes of t. reesei (Biely et al. 1990). Determination of initial bond cleavage frequencies and relative rates of hydrolysis of the labelled substrates pointed out that 1) four investigated enzymes of t. reesei, EG I, EG III, CBH I and CBH II exhibit different patterns of the cleavage of cellooligosaccharides; 2) neither CBH I nor CBH II behaves as typical exoglucanases and they do not liberate cellobiose as the only product of hydrolysis of cellooligosaccharides; 3) CBH I shows some preference for attacking the substrates from their reducing ends, whereas CBH II attack from the non-reducing ends; 4) considerable amounts of D-glucose are generated from cellooligosaccharides larger than cellotriose by three enzymes, CBH I, EG I and EG III; 5) EG I is the only enzyme catalyzing glycosyl transfer reactions at high substrate concentration. Both cellooligosaccharides and xylooligosaccharides can serve as glycosyl donors and acceptors (Biely et al. 1991). Although these results point to some new aspects of enzymic cellulose degradation, we must keep in mind that the native cellulase substrate is initially completely insoluble in water and that adsorption of enzymes on its surface may be related to the mode of action (Henrissat et al. 1988; Klyosov 1990).

The crucial enzyme component of xylanolytic systems is endo-1,4-β-xylanase (EX). As a rule, microorganisms produce more than one EX. The reason for this is unknown, but it may be related to the branched structure of most plant xylans. One can assume that EXs produced by one microorganism may differ in

their requirements for the length of unsubstituted part of the xylan main chain which may be determined by the size of the substrate binding site. However, artificial substrates for differentiation of EXs according to such a criterion have not yet been introduced. From analogy with β-glycoside-mediated differentiation of cellulolytic glucanases, we have realized that a similar approach may be successful with EXs. For this purpose 4-methylumbelliferyl β-xylobioside and β-xylotrioside were chemically synthesized in our group by a modified Koenigs-Knorr synthesis (Helferich and Ost, 1962). 2,3,4-Tri-O-acetyl-β-xylopyranosyl bromide and 2,3,2',3',4'-penta-O-acetyl-β-xylobiosyl bromide were condensed with 4-methylumbelliferyl 2,3-di-O-acetyl-β-D-xylopyranoside to give 4-methylumbelliferyl-per-O-acetyl β-xylobioside and β-xylotrioside. On deacetylation the compounds afforded 4-methylumbelliferyl β-xylobioside and β-xylotrioside. Both glycosides were found to be specific and extremely sensitive substrates of EXs, and suitable for detection of the enzymes in gels and determination of their activity fluorometrically. Detection of EXs pure or present in crude systems resolved by IEF indicated that EXs can be differentiated more on the basis of the rate of liberation of the fluorogenic aglycone from the glycosides than on the basis of ability to hydrolyze one of them. A number of fungal EXs, including pairs produced by one strain, liberated 4-methylumbelliferone from both β-xylobioside and β-xylotrioside. This is somewhat in contrast to behaviour of cellulolytic enzymes towards aryl β-cellobiosides and β-cellotriosides.

A considerable amount of information of the mechanism of action of EXs was obtained using [1-^{3}H]-reducing end labelled xyloologosaccharides. The substrates allow determination of initial bond cleavage frequencies and V/K_m parameters which are sufficient input data to map the substrate binding sites. Three Exs have been characterized using such an approach; however, they always represented only a single enzyme species of one microorganism. The future task is to compare several EXs of one producer and to relate catalytic properties of the enzymes to their performance in various technological processes.

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DESIGNING BIODEGRADABILITY: LESSONS FROM LIGNIN

A. Paszczynski, M. Pasti, S. Goszczynski,
D. Crawford and R. Crawford

University of Idaho
Center for Hazardous Waste Remediation Research
and Department of Bacteriology and Biochemistry
Moscow, Idaho 83843

Azo dyes are excellent examples of synthetic chemicals that are extremely resistant to biodegradation once they enter the environment. Preliminary studies in our laboratory have shown, however, that these dyes may be made biodegradable by slightly altering their chemical structures. The changed chemistry involves introducing naturally occurring, lignin-like chemical substitution patterns into a dye's molecular structure. The recalcitrant dye is thereby converted to a modified molecule which retains its properties as a dye, but is degraded readily by soil microbes such as extracellular lignin-peroxidase-producing Streptomyces and white-rot fungi such as Phanerochaete chrysosporium. We hypothesize that the modified dye is more susceptible to peroxidase-initiated enzymatic destruction because 1) azo dyes are conjugated unsaturated compounds that can be subject to enzymatically catalyzed free radical degradation, 2) dye oxidation by extracellular microbial peroxidases involves a cation radical mechanism for initial attack on the molecule, and 3) the lignin-like moiety introduced into a dye's structure provides a previously unavailable access point for initial enzymatic attack on the molecule. This discovery may also be applicable to increasing the biodegradability of persistent agricultural chemicals and to plastics such as polystyrenes, provided their chemistry can be manipulated similarly.

Our initial work has been funded by the Idaho State Board of education (SBOE) in the form of a grant from the University of Idaho Hazardous Waste Remediation Research Center. In this work, three azo dyes, including commercially available acid yellow number 9 (4-amino-1, 1'-azobenzene-3, 4-disulfonic acid), and two synthetically modified dyes, azo dye 1 ([4-(3-methoxy-4 hydroxphenylazo)-azobenzene-3, 4-disulfonic acid]), and azo dye 2 (3-methoxy-4-hydroxy-azobenzene-4'-sulfonic acid), plus sulfanilic acid (4-aminobenzenesulfonic acid), and vanillic acid (3-methoxy-4-hydroxybenzoic acid), were tested in liquid cultures as substrates for degradation by twelve Streptomyces species and by the white-rot fungus P. chyrsosporium. None of the

Streptomyces degraded acid yellow 9 or sulfanilic acid. However, linkage of a guaiacol molecule (1-hydorxy-2nethoxybenzene) on the acid yellow 9 or sulfanilic acid via azo linkages to form dyes number 1 and 2 resulted in dyes that were degraded by five of the twelve streptomycetes. These were the strains that could also degrade vanillic acid, which has the same ring substitution pattern as guaiacol. While P. chrysosporium degraded both acid yellow 9 and sulfanilic acid, the two guaiacol-substituted dyes were degraded more readily by the fungus than were the unsubstituted molecules. Additional studies showed ligninase and manganese peroxidase preparations from P. chrysosporium were involved in oxidation of the dyes, and dye metabolism was associated with appearance of the ligninolytic enzyme system of the fungus.

Recently, we have also examined selected strains of the Streptomyces and P. chrysosporium for their abilities to degrade eighteen different azo dye structures (see Tables 1-3). Degradation was significantly influenced by dye substitution pattern. Table 1 shows degradation of the different dyes by P. chrysosporium, while Table 2 shows their degradation by selected Streptomyces species. Table 3 provides the chemical structure of each dye.

Table 1 shows degradation of the dyes by P. chrysosporium. Degradation of the eighteen dyes was examined at four concentrations in 50 ml cultures of low-nitrogen mineral medium (Kirk, et al. 1978, Arch. Microbiol. 117:277-285) incubated in 125-ml flasks at 200 rpm and 37°C. Specific dyes were added as sterile solutions to the cultures at the time of inoculation. The results of the degradation study show that the dyes vary in toxicity to the fungus, but on average, dye concentrations above 200 ppm inhibit degradation. Only one compound, dye 3, was completely decolorized at 300 ppm (97.6% of dye was removed from the culture). The linkage in this dye (3-methoxy-4-hydroxy) resembles a guaiacyl-type lignin substructure. Dyes 2,4,10,12 and 14 were more than 90% degraded (90-95%). After 10 days, cultures with these compounds still had a slight color. Dyes 2, 4 and 10 resemble lignin in their substructure substitution patterns. Good degradation of dye 12 indicates that a halide can substitute for a methoxyl or methyl group in a position adjacent to a hydroxyl in the second aromatic ring of the dimeric molecule. The remaining dyes fell into two groups. In the first group were those dyes largely degraded (70-90%). The second group includes those degraded to less than 50% under the experimental conditions. Interestingly, during degradation all of the dyes formed varying amounts of a transition polymer ranging in molecular weight from 6800 for dye 1 to 23,200 for dye 2 (determined by HPLC), which is equivalent to a range of 20-70 monomeric ring units per polymer molecule. The largest amount

of polymer was formed from dyes 14, 13, 10, 8, 7, 6, and 5. The first two of these compounds contain ternary amine structures, which are known to form polymers when incubated with Pseudomonas strains (Kuller et al. 1983, Arch. Microbiol. 135: 1-7). A common structural feature of the next four dyes is a hydroxyl group ortho to the azo linkage. Compound 5, containing a phenolic ring, also formed polymer. In the case of the dyes biodegraded by P. chrysosporium, it appears that the polymers are transitory, since the fungus also ultimately degrades the polymer, as shown by HPLC analyses of culture filtrates over time.

As shown in Tables 2 and 3, with the Streptomyces significant degradation of dyes occurred only when the hydroxy group was para to the azo linkage (dyes 1,2,3, and 4). Dyes 1 and 2 were degraded to a greater extent than dyes 3 or 4, and indication that the presence of two methyl groups (dye 1) or two methoxyl groups (dye 2) facilitates catabolic enzyme access to the hydroxyl group on the second ring. With these dyes we observed no polymer formation during degradation. Dye 3 was more readily degraded by the Streptomyces than dye 4, an indication that a methoxyl substituent is preferred to a methyl substituent by these actinomycetes. Their enzymatic machinery recognized both substitution patterns, but apparently with differing efficiencies. Slight degradation of dye occurred when a hydroxyl group was in the para position and a sec-butyl group was ortho to the hydroxyl. It is important to note that the Streptomyces used in this study were known to degrade vanillic acid (4-hydroxy-3-methoxy benzioc acid). Thus, their preference for this type of substitution pattern within the azo dye structure is not surprising. Other bacteria that degrade aromatic compounds with different substitution patterns might be expected to show different degradative patterns on this same group of dyes.

These preliminary data show that fungi such as P. chrysosporium and actinomycetes such as Streptomyces species have the ability to degrade azo dyes if the substitution pattern on the dye's second ring is appropriate. The catabolic mechanisms involved, however, may differ between the fungus and the actinomycetes.

Table 1. Azo dye (ppm) remaining after 10 days' cultivation (37°C) of *P. chrysosporium* in low-nitrogen mineral medium supplemented with dyes at different initial concentrations[a].

Azo Dye[b]	Initial Dye Concentration (ppm)			
	100 ppm	150 ppm	200 ppm	300 ppm
	ppm Dye Remaining after 10 Days			
1	0.4	1.2	1.9	114.8
2	1.6	3.8	4.2	16.3
3	2.5	6.4	10.1	7.3
4	6.1	9.9	14.5	22.7
5	12.9	14.0	33.3	39.3
6	62.7	41.0	10.2	246.0
7	16.1	28.4	29.7	135.7
8	81.9	109.8	32.0	153.7
9	8.1	15.3	46.6	42.9
10	6.8	14.4	30.1	21.8
11	2.4	44.2	6.2	198.2
12	7.7	20.8	28.3	20.1
13	4.4	11.8	15.5	36.8
14	1.9	10.6	17.9	17.8
15	67.8	92.7	179.3	219.1
16	29.8	58.9	93.3	136.1
17	8.4	20.3	40.9	77.1
18	ND[c]	2.9	ND	252.6

[a] Residual dye concentration was determined from standard curve prepared for each dye, using an appropriate wavelength of light (Table 2) for measurement.

[b] Structures are shown in Table 3.

[c] ND = Not done

Table 2. Degradation of different azo dyes by selected *Streptomyces*[a] strains after 14 days' incubation (37°C) in a low-nitrogen defined medium supplemented with dye (0.005% w.v.).

Azo Dye[b]	Wavelength (nm)[c]	Percent Degradation[d]			
		Strains			
		A10	A11	A12	A13
1	396	68	73	39	10
2	416	79	83	39	8
3	396[e]	77	73	43	16
4	386[f]	9	20	3	8
5	376[g]	-[j]	-	-	-
6	430	-	-	-	-
7	422[h]	-	-	-	-
8	420	-	-	-	-
9	394	11	16	7	6
10	420	4	9	-	-
11	376	-	-	-	-
12	376	2	5	-	-
13	466	-	-	-	-
14	474	2	3	-	-
15	350[i]	-	-	-	-
16	408	-	-	-	-
17	386	-	-	-	-
18	ND[k]				

[a] These are *Streptomyces* known from our previous work to degrade guaiacyl-substituted (4-hydroxy-3-methoxy) aromatic rings.

[b] Structures are shown in Table 3.

[c] Wavelengths chosen on the basis of spectral analysis of each dye in the medium being used for culture growth.

[d] Determined from standard curves prepared for each dye at the appropriate wavelength.

For *P. chrysosporium*, the following wavelengths were used for measuring dye concentration:

[e] = 400 nm

[f] = 388 nm

[g] = 380 nm

[h] = 426 nm

[i] = 416 nm

[j] No degradation observed

[k] ND = Not done

Table 3. Chemical Structures of the Modified Azo Dyes to Be Used

1. 4-[(3,5-dimethyl-4-hydroxyphenyl)-azo]-benzenesulfonic acid
2. 4-[(3,5-dimethoxy-4-hydroxyphenyl)-azo]-benzenesulfonic acid
3. 4-[(3-methoxy-4-hydroxyphenyl)-azo]-benzenesulfonic acid
4. 4-[(3-methyl-4-hydroxyphenyl)-azo]-benzenesulfonic acid
5. 4-[(4-hydroxyphenyl)-azo]-benzenesulfonic acid
6. 4-[(2-hydroxy-4,5-dimethylphenyl)-azo]-benzenesulfonic acid
7. 4-[(2-hydroxy-5-methylphenyl)-azo]-benzenesulfonic acid
8. 4-[(2-hydroxy-5-ethylphenyl)-azo]-benzenesulfonic acid
9. 4-[(3-sec-butyl-4-hydroxyphenyl)-azo]-benzenesulfonic acid
10. 4-[(2-hydroxy-3-methoxy-5-methylphenyl)-azo]-benzenesulfonic acid
11. 4-[(3,5-difluoro-4-hydroxyphenyl)-azo]-benzenesulfonic acid
12. 4-[(3-chloro-4-hydroxyphenyl)-azo]-benzenesulfonic acid
13. 4-[(4-dimethylaminophenyl)-azo]-benzenesulfonic acid
14. 4-[(4-diethylaminophenyl)-azo]-benzenesulfonic acid
15. 4-[(4-methoxyphenyl)-azo]-benzenesulfonic acid
16. 4-[(3,4-dimethoxyphenyl)-azo]-benzenesulfonic acid
17. 4-[(2-hydroxy-7-sulfo-1-naphthalenyl)]-benzenesulfonic acid
18. 9-(4-amino-1,1'-azobenzene-3,4'-disulfonic acid)

FORESTRY, BIOTECHNOLOGY, RISK AND REGULATION

Dr. M. Levin

Maryland Biotechnology Institute
Center for Public Issues in Biotechnology
Fine Arts, 519
Baltimore, MD 21228

Risk analysis has become an increasingly important component in the safe development and regulation of biotechnology. Many of the issues involved in the analysis are not well understood.

A fundamental problem regarding safety is the realization that risks cannot be reduced to zero. Hence, regulating field tests and commercial use of a product requires identifying the possible adverse and beneficial effects and making "trade-offs". Relative to the rest of the world, the United States has served as a guinea pig. The number of US field tests exceeds the total number of tests for all of the rest of the world. Not only does the US have an elaborate regulatory system, but after almost 200 field tests of biotechnology products, no adverse effect has been noted.

Risk Assessment and Management

The overall process of risk management has two components: risk assessment and risk management. Assessment is the determination of the probability of harm. The assessment process consists of the collection and analysis of the appropriate data that can lead to an estimation of likelihood of harm. Risk management focuses on the actions that should be taken. The management process begins with the risk assessment but takes into account a number of other factors including the benefits of the product and the social implications of the activity. Since many of these factors will suggest different responses, proper risk management - i.e., maximum safety with minimal impact on research and product development - involves the necessity of making tradeoffs.

Because of the involvement of life forms and interaction with the environment, biotechnology risk assessments rely more heavily on probability estimates than other technology assessments. As a result, they incorporate a greater degree of uncertainty into the analysis.

Risk Communication

The differences in the educational background of the recipients of risk analysis information and their perspective will affect evaluation of the results and the management decisions, especially where probabilistic determinations are involved. Thus, communication between assessor and those potentially affected becomes especially critical. Drawing these groups together to reach agreement also requires strong coordination efforts among those conducting risk assessments as well as attention to the means of communication.

A number of authors have emphasized the need for involving the lay public early and the need for clearly communicating risk information (Slovic 1986; Sun 1989). All risk analysis have at least five common elements which strongly affect the communication of risks to the public. These elements include (1) the path by which information reaches the audience, (2) the limited value of experts when the process becomes highly politicized, (3) the extent to which the local community believes it is affected, (4) the role of the mass media and (5) the technical or cultural background of the audience.

This paper will discuss the science base for assessing risks related to environmental applications of biotechnology products and how the findings are used.

Background

To understand the efforts to conduct and coordinate biotechnology risk management in the US, it is important to understand how the regulatory framework is structured. Three agencies (the Environmental Protection Agency [EPA], The Department of Agriculture [USDA] and the Food and Drug Administration [FDA]) are heavily involved, each of which is required to comply with a general statute for environmental protection and each of which has a specific mission. There are no statutes specifically dealing with the safety of biotechnology products. A coordinating committee has been formed to harmonize activities.

Each Agency is guided by its particular legislation. All of the relevant legislation is based on safety as viewed from a number of perspectives (e.g. workers in general, industrial emissions on the environment, public health). Each perspective provided the basis for specific statutes. As a result, legal issues such as the source of funding or purpose of the experiment become involved in determining jurisdiction.

Each of the three Agencies is also responsible for compliance with the National Environmental Policy Act (NEPA) which is binding on all US Federal Agencies. NEPA attempts to ensure that all federal actions are environmentally sound. It requires each Agency to evaluate the possible environmental outcomes of its proposed actions and look for a balance between benefits and possible adverse impacts. Agencies must conduct and document a review of all pertinent available information, including alternatives, and seek public comment. EPA is not required to specifically address NEPA issues since environmental protection is the basis for the agency's activities.

The first effort to deal with the safety of biotechnology experiments was not the result of law making but grew out of concerns voiced by the scientists involved. The protocol was developed and administered by the National Institutes of Health, resulting in its Recombinant Advisory Committee and the well-known RAC guidelines. The guidelines focused on laboratory worker safety. They function with a great deal of success by stressing containment. In the initial version of these guidelines, any type of release of genetically engineered organisms into the environment was completely prohibited. At present, field work is reviewed by the RAC only if no other agency is responsible and the experiment fits within the RAC definition of molecular biology.

Although only federally funded molecular biological research was covered, nonfederally funded researchers were expected to comply on a voluntary basis. An outcome of this procedure was the creation of Institutional Biosafety Committees (IBC's). Although they were developed to aid the RAC by providing local review of some applications for rDNA research, IBC's currently review most of rDNA research requiring review in the US.

Specific Agency Activities

With the scale up of molecular biology to industrial levels, safety emerged as an industrial issue. Worker safety, the environment and public health in general became involved. Because of their stated missions, three Agencies -- the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), and the Department of Agriculture (USDA) -- became heavily involved in biotechnology regulation. This paper will focus on the USDA and the EPA because of their involvement in environmental applications.

The EPA is the primary agency responsible for ecological and related public health issues. EPA regulates biotechnology under two statues, the Toxic Substances Control Act (TSCA) and the Federal Fungicide and Rodenticide ACT (FIFRA) in separate offices. These two acts are "gateway legislation", are

oriented towards preventive actions and are invoked before new products are released into the environment. FIFRA covers all pesticidal products. New chemicals trigger TSCA. (Recombinant DNA is considered a new chemical and the resulting life form is included in the coverage).

EPA is committed to the concept that risk is the result of combining hazard and exposure. Each office reviews products to estimate the extent of the hazard and risk components. Specific procedures vary because of the terminology of the specific act, but the assessments focus on estimating the hazard by examining data relevant to environmental or public health effects and estimating the exposure in terms of the fate of the introduced product (i.e. transport to other sites and persistence under environmental conditions). EPA risk assessment research has been reviewed (Levin 1987). The results are then combined in a risk assessment document for a regulatory decision.

USDA has the responsibility for enhancing production and assuring the safety and nutritional quality of food and fiber. The Agency's environmental concerns are the safety of crop plants and cattle. The USDA has three divisions that deal with biotechnology. The Agricultural Research Service (ARS) deals with research issues and USDA funded research. The Agency's Food Service and Inspection Service function to assure the safety and the wholesome characteristics of all food products. Through the Animal and Plant Health Inspection Service (APHIS) the Agency meets its responsibilities for licensing veterinary biological material and for issuing permits for the transport of biological material. ARS has produced guidelines and APHIS has published regulations describing the procedure for assessing risks associated with biotechnology products which fall in their respective jurisdiction.

All of the above agencies and their offices have produced general documents describing the type and depth of information required to meet their decision making needs.

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ETHICS AND RISK ANALYSIS

Robert Wachbroit

Institute for Philosophy and Public Policy
University of Maryland
College Park, MD 20742

Risk analysis has become an important tool in the regulation and development of biotechnology. Government agencies require it, science and industry tries to provide it, and the public reacts to it. Risk analysis is crucial in determining when a genetically engineered organism can be tested in the field and when it can be commercialized. Because risk analysis is such an imperfect science, and yet so much hangs on it, it is therefore important that we be aware of the values implicit in a risk analysis. The aim of this paper is to identify some of the values and ethical controversies that are part of assessing risk.

A good place to begin is by defining risk. A simple characterization of "risk" is probability of harm. There are other characterizations of risk, including risk as the magnitude of harm and risk as the expectation value of the harm (i.e., the product of the probability of harm and the magnitude of harm) (Vlek 1987). We can find support in everyday speech for these various characterizations of "risk". Nevertheless, insofar as an understanding of risk builds on a prior understanding of probability and of harm, the choice of definition is not important for this discussion (Rayner and Cantor 1987). This understanding of risk suggests that we should proceed first by examining issues raised in identifying harms and then by considering the probability claims involved in risk judgements.

Differences in risk analysis can often be traced to differences in what harms are to be recognized. We can distinguish thin from thick conceptions of harm. On a thin conception, harms are physical harms, understood in terms of mortality and morbidity. Thus, the thin harms that could occur in an intentional release of a genetically engineered organism consist of the possible physical harms -- that is to say, death or disease. On a thicker conception of harm, we look at a much broader range of losses and damages. Many of these losses and damages might be considered "social harms". They might include economic losses, social disruption, the abandonment of certain conventions and values, and the undermining of political and social

institutions. The paper will discuss some of the problems and controversies over the identification of thick harms and over whether thick harms should inform risk analysis.

The concept of probability employed in risk analysis also raises a number of important questions. There are two standard interpretations of probability -- probability as relative frequencies and probability as subjective (Bayesian) degrees of belief. Which interpretation is the more appropriate for risk analysis? The paper will discuss this issue and its implications for risk communication.

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POLITICS AND POLICY OF BIOTECHNOLOGY DEVELOPMENT

William A. Stiles, Jr.

Legislative Director
House Committee on Science, Space, and Technology
2320 Rayburn House Office Building
Washington, D.C. 20515

As modern biology leaves the laboratory and enters commercial application, it is encountering a broad set of social, economic, and ethical issues. Some of these issues are unique to, and prompted by, the scientific advances in modern biotechnology. However, some are unresolved issues which have been attached to biotechnology as the next step in an ongoing debate. In either case, the level of public discussion surrounding the commercialization of this technology is higher than the limited debates accompanying the commercialization of physics or chemistry.

The heightened level of the debate is partly the result of a skeptical, non-scientific public, who blindly trusted physicists and chemists and were not prepared for some of the risks of nuclear power and toxic chemical wastes. The debate is also a result of the unique tradition of open discussion which has marked the development of biotechnology since the Gordon and Asilomar Conferences. The debate, properly focused, should be viewed as a positive development and a chance to avoid the disruptions being experiences in the nuclear power and chemical industries.

Governmental activity at all levels has increased in response to these issues and the attendant public discussion. However, there has been little resolution of the proper role of government in biotechnology after over five years of intensive examination. This lack of resolution, while useful in an evolving field such as biotechnology, can also cause uncertainty and confusion in the commercial sector. It also leaves the federal and state governmental systems vulnerable to pressures for inappropriate levels of regulation in response to problems or emergencies which might develop during the course of commercialization. A predictable level of regulation would help avoid that, and should be a goal of government at present.

The most intractable issues involved are the ethical issues which biotechnology raises. Ownership of life forms, the narrow boundary between genetic therapies and eugenics, the shattering of species barriers to genetic

transfers, and a host of other real or imagined developments have sparked a roiling ethical debate. These discussions are the most passionate and are the most alien to the scientific community. But these issues must be settled before biotechnology commercialization can proceed.

In the end, the current debate should be encouraged and mechanisms and forums for resolution of these issues should be established. This final "fitting" of a technology into society is the most difficult part of a proper technology assessment, but can be the most critical to a technology's success.

BIOTECHNOLOGY IN THE PULP AND PAPER INDUSTRY: A PRELUDE TO COMMERCIALIZATION?

Patrick C. Trotter

Private Consultant

Considerable research effort has been devoted in the last several years to biotechnology applications in areas closely identified with the pulp and paper industry. These include tree improvement, primary pulp manufacture, pulp modification (i.e., bleaching and upgrading pulp properties), effluent treatment and remediation, and by-product conversion. In tree improvement, researchers have been concentrating on genetic manipulation of lignin content and composition with a view to easier pulping with less expenditure of chemicals and energy, but recent advances in cellulose biosynthesis could eventually lead to ways to control fiber structure and properties as well. Progress in tree improvement via genetic engineering is still impeded by lack of a system for regenerating transgenic plantlings of important pulpwood species. Biopulping research is active in many places around the world, and appears to be following two tracks: the use of white-rot organisms in biomechanical pulping processes, and the production of ligninase enzymes for possible direct use as pulping reagents. Biobleaching research focuses on hemicellulase enzymes, with several supplier companies now offering quantities of these enzymes for experimental evaluation. Hemicellulase enzymes have also been used in "enzymatic beating" experiments to improve pulp strength properties, and mixtures of cellulase and hemicellulase enzymes have been used to increase the freeness and paper machine runnability of recycled fibers. Xylanase enzymes are also being studied for use in preparing high quality dissolving pulps. Effluent treatment has been a stronghold of biotechnology within the industry for years, and continues to be the only significant commercial use of biotechnology today. Interesting new approaches may emerge for bleach plant effluent decolorization and for AOX removal via ongoing research on bioremediation of contaminated soils and groundwaters. Finally, research on by-product conversion has waned in recent years.

LIFTASE(TM) AND ALBAZYME(TM): TWO NEW ENZYME PREPARATIONS FOR THE PULP AND PAPER INDUSTRY

Jean Claude Pommier

Saint Gobain Papier-Bois Recherche
Biopulp International
351 Cours de la Liberation
33405 Talence, France

We had already demonstrated in previous publications that certain cellulases preparations (LIFTASE[TM]) added to the stock preparation produce a substantial increase in freeness which can be used in different ways on the paper machine, i.e. increased machine speed, decreased head box concentration, or changes in the furnish composition. Since the basic laboratory work was carried out in the middle of the eighties, mill scale trials were performed on various kind of papers. Today, the commercial development of this enzyme application is still in the start up phase because, if the effect on drainage is observed each time, the effect on the paper machine cannot be predicted and each mill case must be thoroughly studied before a regular use of LIFTASE(TM).

Work carried out in Finland in 1985 showed that an hemicellulase pretreatment of kraft pulp would help to decrease the amount of active chlorine needed in the bleaching sequence. Typical laboratory experiments have yielded and average reduction of 25 to 35% in active chlorine consumption in the first chlorination stage. Nowadays, a large number of mill scale trials involving hemicellulase pretreatment have been completed with success in Northern and Central Europe. Commercial success of this new technology is now mainly dependent on the price of this enzyme prebleaching.

The technical aspects and the first commercial developments of these two applications will be presented.

BIOTECHNOLOGY AND THE PAPER AND PULP INDUSTRIES - AN EXPERIMENT

A.S. Mascarenhas and S.S. Khuspe

Division of Biochemical Sciences
National Chemical Laboratory
Pune - 411 008, India

The pulp industry depends solely on cellulosic raw materials, the three main sources of which are the grasses (including bamboos and sugarcane) conifers, eucalypts and a few non-conventional trees. In India it is expensive to utilize conifers which are confined to the inaccessible regions of the Himalayas. Bagasse and other agricultural residues are now finding application as commercially viable alternatives for specific grades of paper. Bamboos and the eucalypts have a wider application for production of quality paper, boards and rayon grade pulp.

The total demand of forest raw materials for pulp wood is estimated to increase dramatically in India and the world. To meet this demand, scientific tree improvement programs have been introduced so that productivity of the forests in India can be increased from the present average low yield of 0.5 M^3/Ha/Year.

This paper will be restricted mainly to the results of various projects undertaken at our laboratory (NCL) on different tree species. The final goal is to produce sufficient quality planting stock required to replenish the rapidly depleting forest cover in the country. The species covered in this paper will include the eucalypts and bamboos, the two major sources of wood for the pulp industries.

Eucalyptus species

Experimental procedures for culture of explants from mature plus trees of E. tereticornis and E. torelliana, their transfer to field and the methods for determining specific gravity, pulp, yield, biomass, etc. have been described earlier (Mascarenhas et al. 1988: 1990).

The plantlets produced from selected elite trees were field grown in Pune, in 1983 as described by Khuspe et al. (1987) and a comparison made with seedlings of the same trees.

Biomass yields of tissue culture grown plants of E. tereticornis and E. torelliana at 12, 34 and 52 months were 200, 34 and 16%, and 700, 100, and 100%

higher than controls, respectively. Thereafter the growth rates levelled out. A high degree of uniformity is observed in tissue culture raised plantlets.

D. strictus

There are now several reports on micropropagation of bamboos (Nadgauda et al. 1991). The method developed in our laboratory using seedling explants (Mascarenhas et al. 1990) has now been extended to mature clones and also to B. arundinacea and D. brandisii (unpublished) which are some of the major species used in the pulp industry. The results obtained (Mascarenhas et al. 1989) regarding early culm formation have now been confirmed in field trials conducted by Misra et al. (1990). Besides obtaining increased culm heights and rate of culm production in tissue culture raised plants, they also observed a reduction of the gestation period from 2 years to 6-7 months.

Experiment I

The main objectives for this project, financed by the National Bank for Agriculture and Rural Development, Bombay (NABARD), have been to develop the tissue culture technologies, advise forestry agencies on setting up of laboratories, impart intensive training of their personnel and design trials for field evaluation. As an outcome of the results of the field trials, which indicated increased yields and higher growth rates of tissue culture plants over controls, the experiment was extended to include two forestry agencies situated in Karnataka and Tamil Nadu, two states in the South of India. This project covers setting up of tissue culture laboratories at these agencies, each with an annual production capacity of around 1.0 million plants for direct transfer to plantation sites. Several candidate trees identified on the basis of phenotypic traits serve as parent material for plant production and field evaluation in comparative trials.

Experiment II

In a novel and unique experiment, and in continuation of the NABARD project, the Department of Biotechnology (DBT) Delhi, India sponsored a more daring project at two centers in India, NCL, Pune, and Tata Energy Research Institute (TERI), Delhi, for the pilot scale production of plantlets from different tree species and their field testing at different locations. At NCL, the trees selected are teak, eucalypts and bamboos. The objectives of this project are: (a) to confirm the earlier results regarding increased productivity of tissue culture raised plants in field verification trials, (b) to refine the procedures for reducing costs of plants, and (c) if successful, to mass produce plants. A simplified protocol eliminating the in vitro rooting stage, thereby resulting in reduction of plantlet costs, has been developed.

This also reduces the time that would be involved if *in vitro* rooting were adhered to.

Conclusion

Following the pioneering experiments of the team from Ara Cruz, Brazil, propagation of *Eucalyptus* by rooting of cuttings using juvenile plant material is now being successfully employed for many species. The growth rates and the yields are significantly higher in clonal than in seed raised plantations (Brando 1984). Several commercial companies in India are now following this procedure.

With eucalypts, growth is very rapid in plantlets raised by tissue culture in the first 48 months. If the tissue culture process developed for the eucalypts is to find wider application, the increased productivity will have to be significantly higher than from rooted cuttings, because of the high cost factors in production of plants. Evaluation in a comparative trial between seed raised, rooted cuttings and tissue culture plants is in progress.

Techniques have recently been developed for vegetative propagation of *D. strictus* through macro proliferation (Kumar 1990) using culm cuttings. However, tissue culture assures a steady, unlimited supply of high quality planting material eliminating dependence on the unpredictable flowering behavior in the bamboos. Moreover, plantlets being small in size make handling and transport easy. The present process needs refinement for cost reduction although trials conducted at two locations indicate significant benefits. Our more recent success in inducing precocious flowering *in vitro* with different bamboo species makes breeding programs for improvement of the species a possibility and also offers a new approach, if refined, for obtaining an alternative continuous source of seed (Nadgauda *et al*. 1990).

Plantlets produced from selected material can be raised in clonal banks and seed orchards, for production of genetically improved plants. The results of experiments and field trials conducted at NCL and other laboratories point to the fact that planting of forests with quality planting stock raised via tissue culture will increase productivity and also reduce the normal rotation cycles. The interest in the use of tissue culture as an alternative method of clonal propagation is increasing. Even minor gains per plant in a uniform population will result in major benefits in large scale afforestation programmes involving millions of plants. The cost factors are gradually being made more attractive and the benefits could justify the costs.

Acknowledgements

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NON-REGULATORY CONSTRAINTS ON THE APPLICATION OF BIOTECHNOLOGY IN FORESTRY

Chris H. Bornman and William Cheliak

Research Division, Hilleshog AB, Box 302, S-26123
Landskrona, Sweden and Science Directorate, Forestry Canada
351 St. Joseph Blvd, Hull, Quebec K1A 1G5 Canada

Applications of biotechnology in forestry are primarily, but not exclusively, focussed on regeneration. Among the more restrictive non-legal constraints are expected to be factors relating to the integration of biotechnology into existing programmes of improvement, biodiversity, the environment, positional effects and temporal stability of introduced genes, costs, and ultimately, time.

Advances in tissue culture, especially the recent success with somatic embryogenesis in many different coniferous species, has created a potential constraint by conjuring up visions of vast mono-genotypic stands. At the stand level, the developmental homeostasis and biodiversity represented by a single genotype is clearly inadequate and completely unrealistic. Perhaps, rather than constraints, the advances in tissue culture should be viewed as having created new options for the regeneration of forests and the conservation of germplasm. At the same time, operational experience with more conventional rooted cuttings programmes (for example in Picea abies) has demonstrated that problems and constraints of maturation are less serious than formerly believed.

The unique characteristic of long regeneration and rotation times for trees poses real biological and ecological constraints for application of advanced gene technology. Some of these include maturation of genotypes and methylation of introduced genes, evolution of pest tolerance in trees transformed with biocide-coding genes, and the potentials for transfer of introduced genes to wild gene pools, that is, to natural, non-domesticated populations. When integrating this technology, work will need to progress simultaneously on many different genotypes with many transformation events per genotype so as to minimize possible penalties in yield or performance. Confirmation of these, and other constraints, will take years of field testing.

Finally, production of selected genotypes for use in reforestation on a large scale demands high reproducibility, as this directly affects cost and competitive pricing.

Energy savings and improvements in paper strength properties during biomechanical pulping of loblolly pine chips with selected white-rot fungi.

Masood Akhtar and Michael C. Attridge, University of Wisconsin Biotechnology Center, Madison, Wisconsin-53705.

Current mechanical pulping methods have increased in popularity because they produce high pulp yields, and because mechanical pulp mills have a much lower capital investment than chemical pulp mills. Mechanical methods also enhance stiffness, bulkiness and opacity of the pulp fibers, making them particularly desirable for some products. The main disadvantages of mechanical methods are the production of lower strength paper, brightness reversion, and high energy requirement for the pulping. Chemical pretreatments are currently being used in conjunction with mechanical methods to circumvent some of these problems; chemical pretreatments of wood chips are used to enhance the strength properties. However, chemical pretreatments lower pulp yield and produce troublesome waste products. The overall energy requirement during such chemimechanical processes is still high.

The disadvantages of mechanical pulping processes are the primary reasons for evaluating the potential of using fungal treatments prior to mechanical pulping (biomechanical pulping). Biomechanical pulping has the potential to ameliorate many of the problems associated with mechanical and chemical pulping processes. We have achieved promising results with a nonoptimized bench-scale process that uses selected white-rot fungi to alter the wood cell wall and thus "soften" wood chips prior to refiner mechanical pulping. With aspen chips, the process reduces energy consumption, and improves strength properties of handsheets made from biomechanical pulps.

In the work described here, loblolly pine chips were treated with several white-rot fungi in two different bioreactor configurations for four weeks prior to refiner mechanical pulping. Irrespective of the bioreactor configuration, all fungal treatments caused some chip weight loss and saved electrical energy during fiberization and refining as compared to the untreated control. Some of the fungal treatments improved strength properties of paper handsheets, whereas brightness and light scattering coefficient of the handsheets were decreased by all of the fungal treatments. Opacity of the handsheets after the fungal treatments remained unchanged. Based on energy savings and improvements in the strength properties, regardless of bioreactor type, the white-rot fungus *Ceriporiopsis subvermispora* was superior to the other white-rot fungi tested. When incubated in stationary tray bioreactor, *C. subvermispora* caused only 6% weight loss, saved 42%energy during fiberization and refining, improved burst index by 32% and tear index by 67%, as compared to the control.

Acknowledgements. This work was supported by a Biopulping Consortium involving 20 pulp and paper and related companies, the University of Wisconsin Biotechnology Center and the USDA Forest Products Laboratory.

MICROPROPAGATION OF *ACACIA MEARNSII* FROM SHOOT TIP EXPLANTS

Jameel M. Al-Khayri, Feng H. Huang, and Zheng Gong

Department of Horticulture and Forestry,
University of Arkansas, Fayetteville, AR 72701 USA

Black wattle (*Acacia mearnsii* Wild) is a tropical fast growing tree species with important economical value and multiple use. Tissue culture methods could be useful for the rapid propagation of elite individual trees of this species but a system for its micropropagation has not been reported. Because culture requirements are species specific, this study was conducted to establish a system for propagating this species using shoot tip explants.

Seeds, obtained from University of Stellenbosch, South Africa, were soaked overnight in water, then surface sterilized in 70% ethanol for 5 min and 2.6 w/v sodium hypochlorite (50% v/v Clorox) for 20 min, rinsed 5 times in sterile distilled water and germinated on Murashige and Skoog (MS) medium solidified with 0.6% agar. After 2 weeks, shoot tips 10 to 15 mm in length were excised and cultured on MS medium supplemented with 200 mg L^{-1} casein hydrolysate, 3% sucrose, and benzyladenine (BA) at 0.5, 1, or 2 mg L^{-1} combined with naphthaleneacetic acid (NAA) at 0, 0.05, or 0.1 mg L^{-1}. Media were adjusted to pH 5.8 and solidified with 0.6% agar. Cultures were maintained at 26±3°C exposed to 16 h photoperiod (70 μmol $s^{-1}m^{-2}$).

Four weeks after culture initiation, observations were made on shoot length, number of axillary bud, rooting, and callus formation. The experiment was designed as a 3x3 factorial with 18 replications and data were subjected to analysis of variance. Shoot growth was not significantly affected by the growth regulators tested but axillary bud formation was significant influenced by BA concentration; 2 mg L^{-1} gave maximum number of buds (2.8 bud/explant). Rooting and callus formation at the base of the explants were also affected by the level of BA. As the concentration of BA increased callus formation frequency was promoted but the root formation was inhibited. Callus formation ranged from 8 to 83% and root formation ranged from 10 to 92% depending upon BA concentration. At 2 mg L^{-1} BA callus formation was not influenced by the level of NAA, but at lower levels (1 and 0.5 mg L^{-1}) callus production was directly proportional to NAA level. The concentration of NAA played a minor role in the root development. Axillary buds were isolated and transferred to multiplication medium, containing BA at 1, 2, 5, 10, 15, or 20 mg L^{-1} combined with NAA at 0, 0.01, or 0.1 mg L^{-1}, for further production of buds; and the tips were transferred to rooting medium containing 0.1 mg L^{-1} BA and IAA, IBA, or NAA at 0, 0.25, 0.5, or 1 mg L^{-1}.

This method provides a means by which continuous source of buds is maintained for propagation purpose. This system utilizes buds multiplications rather than callus culture; thereby, minimizing somaclonal variations associated with callus cultures, an important consideration in clonal propagation.

Poster Abstract # I

Title

Plant regeneration from anther culture of natural rubber tree (*Hevea brasiliensis*).

M.P. Asokan, P. Kumari Jayasree, S. Sushama Kumari and P. Sobhana., Rubber Research Institute of India, Kottayam - 686 009, Kerala, India.

Summary

Plant regeneration from anther culture of rubber tree (*Hevea brasiliensis*) was carried out in three steps, namely (I) callus generation (II) embryo induction and (III) plantlet formation. The growth regulator requirements for this steps were the following:

Step I - Benzyladenine (BA) - 1.5 mg l^{-1}
2,4-Dichlorophenoxyacetic Acid (2,4-D) - 0.5 mg l^{-1}

Step II - Kinetin 1.0 mg l^{-1} - 2.5 mg l^{-1}
Gibberellic Acid (GA) - 0.5 mg l^{-1}

Step III - GA - 1.0 mg l^{-1}

The regenerated plants were successfully hardened and planted in the field.

Poster Abstract # 2

Title

In vitro propagation of rubber tree (*Hevea brasiliensis*): a commercial prospective.

M.P. Asokan, S. Sushama Kumari, P. Sobhana and P. Kumari Jayasree and S. Sobha, Rubber Research Institute of India, Kottayam - 686 009, Kerala, India.

Summary

An *in vitro* propagation system for the natural rubber (*Hevea brasiliensis*) clone-311 was developed. The optimum growth regulator range for shoot and root development was 1.0 mg l^{-1} —3.0 mg l^{-1} Kinetin with 0.5 mg l^{-1} - 1.0 mg l^{-1} Indoleacetic Acid (IAA). Rooted plants were successfully transplanted in the field.

IMPROVEMENT OF EXTRACELLULAR PEROXIDASES EXCRETION USING FREE OR IMMOBILIZED CELLS OF PHANEROCHAETE CHRYSOSPORIUM BKM-F-1767 and INA-12

Pascal BONNARME, Michel DELATTRE, Georges CORRIEU and Marcel ASTHER

Laboratoire de Génie des Procédés Biotechnologiques Agro-Alimentaires,
CBAI, INRA, 78850 THIVERVAL-GRIGNON, FRANCE.

Phanerochaete chrysosporium secretes two extracellular heme peroxidases, Mn(II) dependent peroxidases (MnPs) and lignin peroxidases (LiPs), which are apparently the major components of its lignin degrading system.

In order to produce peroxidases in a low-shear environment, immobilization on various supports such as polyurethane foam (1,2) or nylon (3) was achieved with success.

This study reports on the comparison of extracellular peroxidases secretion (i.e. LiPs and MnPs) by free and immobilized cells of two strains of P. chrysosporium wild type BKM-F-1767 and the mutant INA-12 (4).

For both strains, in presence of polyurethane, LiPs and MnPs synthesis was significantly improved and fermentation time for maximum activity was reduced as compared to free pellets. Best results were obtained with strain INA-12 as compared to BKM-F-1767 ; 2580 and 1296 $U.l^{-1}$ were produced respectively. In addition to peroxidase synthesis, exoprotein secretion was also enhanced.

In parallel, the organelle content of P. chrysosporium was investigated. Several intracellular enzymes were estimated as biochemical markers for cellular compartment determination (5,6) namely, cytochrome-c-oxydoreductase a typical marker of endoplasmic reticulum and succinate dehydrogenase a mitochondria marker.

The results demonstrated that in immobilized cells of P. chrysosporium the endoplasmic reticulum as well as the mitochondria syntheses accompanied peroxidases production.

Peroxidase activity has recently been demonstrated to be involved in the loss of LiP activity after the active production phase (7). Our results show that in immobilized cells cultures, protease activities were reduced as compared to free cells cultures.

Production of LiPs in an air-lift type biorector (8) by both strains has also been achieved with success.

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LIGNIN PEROXIDASE OF *Phanerochaete chrysosporium*: EVIDENCE FOR AN ACIDIC IONIZATION CONTROLLING ACTIVITY

Danying Cai and Ming Tien

Department of Molecular and Cell Biology
The Pennsylvania State University, University Park, PA 16802

The active site amino acid residues of lignin peroxidase are homologous to those of other peroxidases; however, in contrast to other peroxidases, no pH dependency is observed for the reaction of ferric lignin peroxidase with H_2O_2 to form compound I. Chloride binding is used in the present study to further investigate this reaction. Chloride binds to lignin peroxidase at the same site as cyanide and H_2O_2. Chloride binding is pH dependent: it binds only to the protonated form of lignin peroxidase. Transient-state kinetic studies demonstrate that chloride inhibits lignin peroxidase compound I formation in a pH-dependent manner with maximum inhibition at low pH. An apparent pK_a was calculated at each chloride concentration; the pK_a increased as chloride concentration increased. Extrapolation to zero chloride concentration allowed us to estimate the intrinsic pK_a for the ionization in the lignin peroxidase active site. The results reported here provide evidence that an acidic ionizable group ($pK_a \approx 1$) at the active site controls both lignin peroxidase compound I formation and chloride binding. We propose that the mechanism for lignin peroxidase compound I formation is similar to that of other peroxidases in that it requires the deprotonated form of an ionizable group near the active site.

MONOCLONAL ANTIBODIES TO PROBE LIGNIN COMPOSITION AND STRUCTURE

Jeffrey F.D. Dean & Karl-Erik L. Eriksson

Department of Biochemistry, CBRR
University of Georgia
Athens, GA 30602

Water-insoluble preparations of lignin are immunogenic. We have obtained polyclonal antisera from mice immunized with birch and loblolly pine ball-milled wood lignins, as well as dehydrogenative polymers (DHPs) synthesized from highly-purified preparations of each of the three monolignol precursors of lignin, *p*-coumaryl, coniferyl and sinapyl alcohol. Hybridoma cell lines developed from these immunized mice were screened with enzyme-linked immunosorbent assays (ELISA) for the production of monoclonal antibodies (mAbs). The mAbs from different cell lines recognize different lignins and DHPs to varying degrees. Some mAbs appear to recognize structures containing multiple phenolic rings, while others can bind to single phenolic rings. In some cases, these latter antibodies appear able to discriminate between rings bearing methoxyl groups at different positions. Lignin-specific monoclonal antibodies will improve our ability to detect lignin deposition in plant cells at very early stages of differentiation, and mAbs which can discriminate between the three different ring structures of lignin monomers will be useful for mapping variations in lignin composition in different portions in the plant cell wall.

Factors Affecting Host-Pathogen Interaction between Elm Callus Cultures and Ophiostoma ulmi.

Subhash C. Domir, Lawrence R. Schreiber, and Jann M. Ichida, USDA-ARS,U.S. National Arboretum, Ohio Research Site, 359 Main Road; Delaware, OH 43015

ABSTRACT

We examined the effects of temperature, inoculum concentration, elm selection, explant source, and media composition on growth of the Dutch elm disease (DED) fungus, Ophiostoma ulmi, on callus cultures. Calli were generated from young leaf and stem tissue of an American elm (Ulmus americana L.) seedling,A, susceptible to the disease; an American elm selection, 8630, resistant to the disease; and a Siberian elm (U. pumila L.) seedling, resistant to DED. Calli were generated on modified Murashige-Skoog (MMS) medium either with (MMSC) or without coconut milk. Calli generated on MMS medium were incubated at 16,22, and 28 C for 3 days, inoculated with 15 x 10^6, 2 x 10^6, or 0.3 x 10^6 O. ulmi conidia/ml and returned to treatment temperatures. After 72 hr, the rates of fungal growth for all treatments were most rapid on calli from 8630 followed by A and Siberian. Maximum growth of fungus occurred at 22 C and was directly proportional to the inoculum concentration. A temperature of 22 C and inoculum concentration at 2 x 10^6 conidia/ml were considered optimum for studies of explant source and media composition. Explant source did not affect the fungal growth rate on the callus. Rate of O. ulmi growth on A callus was similar on both media; on Siberian and 8630, fungal growth rate was most rapid on callus cultured on MMS than on MMSC. However, in absence of callus tissue, O. ulmi growth on MMSC medium was more than five times as rapid as it was on MMS. Significant interaction was observed between explant source and selection and medium and selection. Fungal growth was fastest on 8630 followed by A and Siberian. While fungal growth was more rapid on 8630, it was more dense on A. Scanning electron microscopy revealed heavy fungal sporulation on A, slight on Siberian, but none on 8630.

TRANSFORMATION OF WHITE SPRUCE BY ELECTRICAL DISCHARGE PARTICLE ACCELERATION

D. Ellis[1], D. McCabe[2], S. McInnis[3], B. Martinell[2], D. Roberts[3] and McCown[1]. [1]University of Wisconsin-Madison, Madison, WI; [2]Agracetus Inc., Middleton, WI; [3]Forest Biotechnology Centre Vancouver, B.C.

Electrical discharge particle acceleration has been used to deliver DNA into several different tissues of white spruce which are capabl of regeneration *in vitro*. These tissues include mature and immatur zygotic embryos, seedlings, embryogenic callus and somatic embryos. Based on transient ß-glucuronidase (GUS) gene expression assays, ea individual tissue requires a different set of physical parameters t maximize delivery of DNA into the various cell types. With the use of particle acceleration, the delivery of the DNA into the cells is no longer a major obstacle. The regeneration of organized transformed tissues from these individual cells which are expressir and contain the introduced DNA is however still a limitation. Somatic embryos have been used to define biological parameters important for high levels of transient GUS expression. Our researc has focused on developmental stages of the somatic embryos, as well as pre- and post-particle acceleration treatments of the embryos. Under optimal conditions, cell division of GUS expressing cells car be identified as early as three weeks following particle accelerat and well-formed proembryos expressing GUS in the suspensors and proembryonal head cells can be identified at six weeks. In our wor it is clear that the developmental stage of the explant is crucial for the regeneration of organized transformed structures.

PSEUDOMONAS GLADIOLI AS AN ANTAGONIST AGAINST VASCULAR WILT FUNGAL PATHOGENS

Steven M. Eshita and Nick H. Roberto, USDA Forest Service, Forestry Sciences Laboratory, 359 Main Road, Delaware, OH USA 43015

Pseudomonas gladioli is being investigated as a potential biocontrol agent against the following vascular wilt fungal pathogens: Ophiostoma ulmi, Cryphonectria parasitica, Ceratocystis fagacearum, and Verticillium dahliae. The bacterium inhibited fungal growth on potato dextrose agar and cleared mycelial mats of the fungi on cellulose films. Light microscopy of the degraded fungi revealed extensive hyphal damage and leakage of cytoplasm. Protection experiments using bacteria-treated greenhouse elms challenged with O. ulmi are in progress. The bacterium produced antifungal compounds on agar and in several culture broths; the active compounds of the latter are being isolated for structure determination. The activity passes through a 10,000 MW filter, is destroyed by autoclaving, and is not due to pyrrolnitrin, suggesting production of a novel antifungal compound by this species.

In vitro multiplication of conifers

(Fourré J-L, Misson J-P, Van Schendel D, Giot P, André P)

Université Catholique de Louvain
Laboratoire des Eaux et Forêts-Gragef
Place Croix du Sud 2, 1348 LLN, BELGIUM
Tél : 32.10473698 - Fax : 32.10473697

The two clonal propagation biotechnologies studied in the laboratory are "classical micropropagation" and "somatic embryogenesis".

Classical micropropagation :

The method of multiplication used here is the axillary bud formation, i.e. the activation of preexisting meristematic zones. It is believed to preserve genetic stability.

After organ initiation, several additional steps, including shoot elongation, rooting, and acclimatization, are required before propagules suitable for planting are produced.

For most of the coniferous species studied, response to *in vitro* culture decreases dramatically with age.

Generally, it has not been possible to propagate *in vitro* sexually mature individuals.

But for some species it is sometimes possible to bypass this problem by using "rejuvenation" techniques.

Using these "time expensive" methods we have get interesting results for some old clones of *Thuya plicata*.

Gymnosperms generally, and northern temperate conifers in particular, have been difficult to propagate through this propagation system.

It is labor intensive, and consequently, the investment per propagule is high.

Nevertheless it could be a good technique to multiply at a small scale a selected superior genotype in order to establish seed or hedge orchards.

Somatic embryogenesis :

Somatic embryogenesis refers to a developmental process where somatic cells can be cultured to produce an organized bipolar structure displaying shoot and root poles connected by a functional vascular tissue.

Somatic embryogenesis proceeds after the exposure of a responsive explant (a zygotic embryo in this case) to critical concentrations of exogenously supplied hormones during the initial culture phase.

So, we can obtain an "embryogenic callus" (also called proembryogenic mass) consisting in proembryos (i.e. immature embryos).

Subsequent development of immature somatic embryos into mature embryos proceeds after transfer of the embryogenic callus to another culture medium, which contains abscisic acid (ABA).

Finally after *in vitro* "germination" of the naked somatic embryos in a last medium, the plantlets obtained must be acclimatated.

Although successful for the propagation of different angiosperms since about 30 years, somatic embryogenesis is a recent development for conifers.

For many species like *Pseudotsuga menziesii*, conversion of immature to mature embryos remains one of the major challenges.

For *Picea abies* we are more advanced, but field and genetic testing will be necessary to determine performance and genetic stability.

So, it is premature to assume that somatic embryogenesis will necessarily be a viable option for the efficient production of clonal propagules for use in reforestation programs.

One objective of research on somatic embryogenesis is the production of dormant embryos capable of being encapsulated to form artificial seeds.

Experience from other plants suggests that it might be possible to store these embryos for future use, similary to sexually derived seeds.

Because the rates of production are potentially high and the time spent on any single propagule is small (automation can be done in bioreactors) somatic embryogenesis may provide an attractive method of producing commercial quantities of clonal propagules for use in forest regeneration programs.

It is also possible to use cryopreservation techniques to store these types of cultures, providing a convenient way to store clones during the long field testing phase.

Coupled with methods of gene transfer, somatic embryogenesis offers potential for an efficient system to transform and screen cultures and regenerate transgenic conifers.

ACACIA MEARNSII TISSUE CULTURE: CALLUS INDUCTION FROM HYPOCOTYL

Zheng Gong, Jameel M. Al-Khayri, and Feng H. Huang

Department of Horticulture and Forestry,
University of Arkansas, Fayetteville, AR 72701 USA

Tissue culture research with black wattle (*Acacia mearnsii* Wild) has been limited. Development of a tissue culture method for the regeneration of black wattle from callus is a prerequisite for the application of various biotechnological techniques. This report describes a method for the production of callus from hypocotyl explants of seedlings.

Seedlings were established from seeds obtained from University of Stellenbosch, South Africa. The seeds were soaked overnight in water, then surface sterilized in 70% ethanol for 5 min and 2.6 w/v sodium hypochlorite (50% v/v Clorox) for 20 min, rinsed 5 times in sterile distilled water after which they were cultured on Murashige and Skoog (MS) basal medium. Hypocotyl sections 2 mm in length were isolated from 2-week-old seedlings and cultured on MS medium supplemented with 1 g L^{-1} casein hydrolysate, 3% sucrose, and benzyladenine (BA) at 0.5, 1, or 2 mg L^{-1} combined with either 2,4-dichlorophenoxyacetic acid (2,4-D) at 0.5, 1, or 2 mg L^{-1}, or naphthaleneacetic acid (NAA) at 1, 2, or 3 mg L^{-1}. Media were adjusted to pH 5.8 and solidified with 0.6% agar. Cultures were maintained at 26±3°C exposed to 16 h photoperiods (70 μmol $s^{-1}m^{-2}$).

Four weeks after culture initiation, callus growth was measured in millimeters. The experiment was designed as a 3x2x2 factorial with 24 replications and data were subjected to analysis of variance.

Callus production commenced within 2 weeks after the start of the cultures. All treatment tested resulted in callus production. No significant difference of percentage callus formation was observed. Up to 100% callus formation was achieved with 3 mg L^{-1} NAA combined with 1 mg L^{-1} BA. Callus size, however, was influenced by both auxin and BA concentrations. Maximum callus growth of 4.8 mm was obtained on 2 NAA mg L^{-1} combined with 1 mg L^{-1} BA. When NAA was included in the callus induction medium, the callus produced appeared whitish and friable as compared to the yellowish more compacted callus grown on 2,4-D-containing medium. Occasionally roots differentiated directly form the explants or from the callus on the NAA-containing medium.

Our study has led to defining a suitable medium for the production of callus from hypocotyl of black wattle. The next phase of this study involved testing the regenerative capacity of calli. The regeneration medium contained BA at 0.5 or 1 mg L^{-1} combined with either 0.05 mg L^{-1} 2,4-D or 0.2 mg L^{-1} NAA. Plant regeneration has not been observed yet. Once plantletes are obtained, this system could be useful for the rapid multiplication and potential genetic transformation of this species.

THE ROLE OF IRON AND IRON-CHELATING COMPOUNDS ISOLATED FROM DECAY FUNGI IN BIOLOGICAL DEGRADATION. Barry Goodell, Wood Science and Technology, Jody Jellison, Plant Biology and Pathology, Vikas Chandhoke, Forest Biology, and Frank Fekete, Microbiology, University of Maine, Orono, Maine 04469.

The degradation of woody plant cell walls, particularly by brown-rot fungi cannot occur by enzymatic action alone. We are currently investigating the action of low molecular weight chelators produced by wood degrading fungi and believe that these compounds may play a direct role in the oxidative degradation of cellulose, and potentially, to lignin. Their mode of action may be similar to that of biomimitic chelating compounds whose oxidative potential has been recently investigated by other researchers.

The brown-rot fungus Gloeophyllum trabeum produces multiple chemically and serologically distinct metal chelators. HPLC purification has resulted in the identification of several iron-binding peaks. Chemical analysis (IR, NMR and MS) of one of these compounds suggests the presence of a tri-substituted phenolate structure similar to the structure of phenolate siderophores previously isolated from bacteria.

Antibodies produced to chelators purified from low-iron liquid cultures of G. trabeum reacted quantitatively with the inject antigen and were used in TEM immunolocalization studies to allow the in situ visualization of these compounds during the degradative process. In addition, numerous assays have indicated the ability of our isolated compounds to participate in lignocellulose degradation. Iron-chelating compounds are active in cellulose-azure cleavage and preliminary x-ray diffraction data suggest that siderophores, in the presence of 10^{-4}M iron, may be able to reduce the crystallinity of treated poplar wood. Isolated chelators have also shown limited but repeatable activity in cellulose depolymerization, as monitored by viscosity assays, and have shown oxidative activity in the KTBA assay for one-electron oxidation.

Our data suggest that biological chelators produced by brown-rot fungi can potentially penetrate the wood cell wall and oxidize ligno-cellulose in a manner which is consistent with previous reports documenting cell wall degradation. Although all functional aspects of chelator action have not been elucidated, experimental results suggest a potentially significant role for these compounds in the fungal degradation of wood.

EFFECT OF IBA, NAA AND GELLING AGENT ON ADVENTITIOUS ROOT FORMATION OF *IN VITRO* SHOOTS OF HYBRID POPLAR

Feng H. Huang, Jameel M. Al-Khayri, and Zheng Gong

Department of Horticulture and Forestry,
University of Arkansas, Fayetteville, AR 72701 USA

Poplar is a deciduous hard wood tree cultivated primarily for pulp production. *In vitro* methods has been successfully applied to vegetatively propagate a number of species of *Populus*. Our objective is to optimize the rooting of *in vitro* regenerated shoots of hybrid poplar (*Populus nigra* L. var. betulifolia Torr x *P. trichocarpa* Torr and Gray) strain NC5331. This strain, obtained from USDA Forest Service, North Central Forest Experiment Station, Forest Sciences Laboratory, Rhinelander, WI 54501, was previously selected via tissue culture methods for herbicide glyphosate tolerance. The effects of indolebutyric acid (IBA) and naphthaleneacetic acid (NAA) concentrations as well as agar and phytagel as a medium solidifying agents were tested.

Adventitious buds maintained on Murashige and Skoog (MS) medium supplemented with 0.1 mg L^{-1} benzyladenine, 200 mg L^{-1} casein hydrolysate, 0.25% w/v sucrose, and 6 g L^{-1} agar. Shoots of 15 to 20 mm in length were isolated and transferred to rooting media individually in 25x150 mm tubes containing 15 ml medium per tube. The rooting media consisted of MS medium supplemented with NAA or IBA at 0, 0.1, 0.25, 0.5, or 1 mg L^{-1} and solidified with either agar or phytagel at 2 or 6 g L^{-1}. The cultures were maintained at 20± 3°C exposed to 10 h photoperiod (70 μmol $s^{-1}m^{-2}$). Data recorded 5 weeks later included number of shoots rooted, number of roots produced, length of roots, and shoot length. The experiment was setup as 2x2x2x4 factorial with 18 replications and the data were subjected to analysis of variance.

Within 2 weeks on rooting media, root initiation commenced. The percentage of shoots that exhibited rooting was significantly influenced by time passed on rooting medium, gelling agents, auxin type, and auxin concentration. In the end of 5 weeks, maximum rooting percentage (89%) was observed on a medium containing 1 or 0.5 mg L^{-1} IBA and solidified with 6 g L^{-1} agar; these treatments gave rise to 8.3 and 7.9 roots per shoot, respectively. Most numerous roots (8.5 roots/shoot) was obtained with 0.25 mg L^{-1} NAA. Root length was affected by auxin type and auxin concentration. The length of root was inversely related to the level of auxin. Although auxin-free medium resulted in the longest roots, root length was enhanced by IBA (10.3 mm) but was inhibited by NAA (6.8 mm). Shoot growth was not affected by IBA concentration but NAA reduced shoot growth at a concentration higher than 0.25 mg L^{-1}.

This study has identified an optimum medium composition for root induction and growth of tissue culture produced poplar shoots; thus, a maximum potential of poplar micropropagation can be realized by obtaining a high level of rooting of regenerated shoots.

TRANSFER AND INTEGRATION OF *BACILLUS THURINGIENSIS* INSECTICIDAL GENE INTO *LARIX DECIDUA*

Yinghua Huang and David F. Karnosky

School of Forestry and Wood Products
Michigan Technological University
1400 Townsend Drive
Houghton, Michigan 49931, USA

Abstract

Larch (*Larix* spp.) is an economically important conifer species which has shown rapid growth and good wood and pulp quality. Therefore, larch species are becoming an alternative choice for reforestation in northern climates. There is a need to develop insect resistant larches as these trees are susceptible to serious insect pests. About thirty years ago, it was known that the bacterial species *Bacillus thuringiensis* (*Bt*) is toxic to insects. The toxic factors of *Bt* strains have been recently identified as a group of insecticidal crystal proteins, and subsequently the insecticidal protein gene (*Bt* toxin gene) of *Bacillus thuringiensis* has been cloned. Our research approach has demonstrated the transfer of the insect resistance gene to larch trees via an *Agrobacterium*-mediated gene transfer system.

The insecticidal protein gene was inserted into a plant expression vector plasmid pWB139 as follow: The T-DNA of plasmid pWB139 contains a single gene, which codes for a translational fusion of the first half of the insecticidal crystal protein of *Bt* HD-73, fused to kanamycin resistance gene (*npt II*) from Tn5. The gene is under control of the CaMV 35S promoter and the 3' region of tomato protease inhibitor I gene. It is flanked by the nopaline promoter and the nopaline 3' end. This engineered vector was then transferred into a host *Agrobacterium rhizogenes* strain 11325, which was used to transform hypocotyls of seven-day-old larch seedlings. Transformed tissues (e.g., root clusters and/or adventitious buds) developed from the inoculated sites were first tested for expression of kanamycin resistance gene by subculturing on the selection medium containing antibiotic kanamycin. For DNA analysis, genomic DNAs were extracted from the positive lines on the kanamycin selection. Southern blot analysis showed that the *Bt* toxin gene was physically transferred and stably integrated into larch genome. The *Bt* gene transgenic regenerants were also recovered via adventitious bud initiation from transformed seedling tissues.

We report that the insect resistance gene has been successfully transferred to European larch. We believe that genetically engineered insect resistant forest crops by the biorational biopesticide biotechnology should contribute great value to both tree improvement and pest management programs.

IN VITRO REGENERATION OF SHOREA ROBUSTA GARTEN.F.

Madhu Jain and H.C. Chaturvedi*
Tissue Culture Laboratory, National Botanical Research Institute, Lucknow-226001, India

Shorea robusta (Sal), one of the most important commercial timber trees, the tissue culture regeneration of which has been found intractable so far, could successfully be multiplied in vitro. The axillary buds of single-node stem segments, taken from aseptically-grown seedlings, were induced to proliferate in a greatly modified Murashige and Skoog's (1962) medium supplemented with 1 mg/l 6-benzylaminopurine and 0.25 mg/l indole-3-acetic acid (IAA). Cultures were incubated under 3 klx fluorescent light for 14 h a day at 27±1°C. Necrosis of explants as well as of regenerated shoots, which ensued after 3rd subculture was controlled in the liquid state of the medium by certain nutritional changes effected in the medium in respect of inorganic salts and the amino acid supplement. The use of antioxidants, like, polyvinylpyrrolidone (insoluble) and glutathione was not as effective as ascorbic acid in checking necrosis. A single axillary bud proliferated to give rise ca. 10 off shoots in 25 days. Subculture of explants along with the regenerated off shoots resulted in further proliferation of shoots with development of normal leaves on grown up shoots. Cultures of regenerated shoots were kept proliferating by repeated subculture of small groups of shoots, after every 30 days, in the same medium for the past two years without decline in their regenerative potentiality. The isolated developed shoots of ca. 3 cm in length were rooted on filter paper bridges, particularly under the influence of IAA (1 mg/l) using the same liquid basal medium. The initial slight vitrification of shoots was alleviated by further growth of rooted shoots on filter paper bridges. According to a moderate estimate, ca.700000 shoots could be produced within a year from one explant.

*To whom any correspondence should be addressed.

BIODEGRADATION OF LIGNOCELLULOSE BY THREE STRAINS OF STREPTOMYCES

V.M. Kaluskar*, S.J. Pathak and S.M. Pandya, Department of Biosciences, Saurashtra University, Rajkot 360 005, India.

The lignocellulose decomposing ability of the three strains of **Streptomyces** spp.(Actinomycetes) viz., VMK2, VMK3 and VMK4, isolated from rotted stem of **Ficus religiosa** Linn, were studied in relation to their potential utilization for the bioconversion of wheatstraw **(Triticum aestivum** Linn.)as lignocellulose substrate to useful chemical products. Lignin from the substrate was found to be degraded by the three strains of **Streptomyces** spp. The bioassay of the substrate after its decomposition by the three strains included weight loss, lignin loss and total APPL formation. Both the inoculated and the control culture supernatants were found to be composed of solvent extractable simple phenolic lignin-derived compounds which were analysed on HPLC to compare the products qualitatively and their existence during each week of the incubation. These lignin fragments that were identified qualitatively included 10 identified and 21 non-identified products. The identified products included dihydrocinnamic acid, vanillin, veratraldehyde, ferulic acid and other benzoic acid derivatives. The studies supported suitability of wheatstraw for the bioconversion.

GENE PRODUCTS ACTIVATED AT FERTILIZATION IN WHITE PINE MEGAGAMETOPHYTES. Joe C. Kamalay, Stokes Baker, Clayton Rugh and Bill Whitmore, Agronomy Dept. and Forestry Division,Ohio State Univ., Columbus, OH 43210

To study molecular events which may be significant in early embryo development, we constructed and screened a cDNA library from post-fertilization ovule mRNA. Messenger RNA levels of these sequences indicate that the initiation of their expression in ovule tissues of Pinus strobus L. (eastern white pine) appears to coincide with fertilization. (1) Two distantly related multigene families code for 18-20 kd peptides; sequence homology searches have revealed no relationship to any known sequences in the GENBANK or EMBL databases. (2) The largest group of sequences encodes a family of legumin-like proteins, showing 30-40% identity to the known angiosperm legumins. We have constructed a phylogentic tree based on the conservation of the globulins in gymnosperms and angiosperms. Cloned cDNA sequences hybridized to 8-10 distinct genomic DNA restriction fragments under moderate stringency conditions and strongly hybridized with 3 bands. (3) A partial cDNA clone encodes a peptide homologous to the COOH terminus of rat and rabbit NADPH:cytochrome P-450 reductase. Hybridization to genomic DNA revealed homology to one or two restriction fragments in the Pinus strobus L. genome, indicating that the gene is probably present as a single locus. The expression of this mRNA sequence appears to be modulated in ovule tissues following fertilization. To date, clones from (2) and (3) were used to examine tissues within the pine ovule by in situ hybridization and mRNA blots of dissected tissues. Each of these sequences was detected in the haploid prothallium.

Molecular cloning and genomic organization of glyoxal oxidase from *Phanerochaete chrysosporium*

Philip J. Kersten[1,2] Chris Witek[3] and Daniel Cullen[1]

Institute for Microbial and Biochemical Technology[1], Forest Products Laboratory, U.S. Department of Agriculture, Madison WI; Biotechnology Center[2], and Department of Bacteriology[3], University of Wisconsin-Madison, Madison WI.

An important component of the ligninolytic system of *Phanerochaete chrysosporium* is the H_2O_2 required as oxidant in the catalytic cycles of the extracellular peroxidases. Glyoxal oxidase is an extracellular enzyme of *P. chrysoporium* and catalyzes the oxidation of simple aldehydes and α-hydroxycarbonyl compounds with the production of H_2O_2. The appearances of glyoxal oxidase, oxidase substrates, and ligninolytic peroxidases (lignin peroxidase and manganese peroxidase) in cultures starved for nitrogen suggest a close physiological connection between these components.

We have cloned and sequenced two cDNAs corresponding to glyoxal oxidase, *glx-1* and *glx-2*, that can be distinguished by their polymorphism with *Kpn I*. The predicted N-terminal sequences of both show 100% identity with the experimentally determined sequence of the purified enzyme. The cDNA sequences also indicate that the mature enzymes are preceded by a leader sequence of 22 amino acids. The molecular weight of 57 kDa (537 amino acids) for the predicted mature peptides is in reasonable agreement with the experimentally determined molecular weight of 68 kDa. The difference possibly could represent the carbohydrate content of the glycoprotein; five potential N-glycosylation sites (Asn X Ser/Thr) are indicated from the DNA sequence. Nucleotide homology of *glx-1*and *glx-2* is > 99% in the coding region for the mature peptides. However, a predicted translational difference (Thr ↔ Lys) is observed. Northern analysis indicates that the *glx* transcript is abundant in ligninolytic cultures, expressed only during secondary metabolism and coordinant with lignin peroxidase in nitrogen-starved cultures.

We have also cloned the genomic equivalents of *glx-1* and *glx-2*. The sequences indicate that the coding region is interrupted by four introns ranging in size from 53-61 bp. Importantly, Southern analyses of parental and single-basidiospore derivatives of *P. chrysoporium*, show that these are allelic forms of the gene. CHEF analysis also indicates that the gene resides on a single dimorphic chromosome distinct from the lignin peroxidase and cellulase genes.

Effect of Glyoxylate on the Enzymatic Activity of Mn peroxidases from *Phanerochaete chrysosporium*

I-Ching Kuan and Ming Tien
Department of Molecular and Cell Biology
Pennsylvania State University, University Park, PA 16802

Mn peroxidases (MnP) are a family of hemeproteins secreted by ligninolytic cultures of *Phanerochaete chrysosporium*, a wood-degrading fungus. The oxidation reactions catalyzed by these enzymes are dependent on H_2O_2. However, we discovered that glyoxylate is capable of supporting Mn peroxidase activity even without added H_2O_2. This Mn peroxidase activity is oxygen-dependent and the oxygen consumption is actually observed. The involvement of active oxygen species in this process is suggested by inhibition of Mn peroxidase activity by $O_2^{\cdot-}$-scavenging agents, superoxide dismutase or tetranitromethane. Moreover, the addition of catalase caused a release of O_2, indicating the production of H_2O_2 during this process. Formate is detected by the formate dehydrogenase assay as one of the oxidation products of glyoxylate. The stoichiometry of the reaction between glyoxylate and Mn^{3+} approaches 1:1. Mechanisms on how glyoxylate supports Mn peroxidase activity are proposed.

Upgrading of Empty Fruit Bunch of Oil Palm to Animal Feeds by Radiation-Fermentation Treatment

T. Kume, H. H. Mutaat*, M. R. Awang*, H. Ito and S. Hashimoto

Takasaki Radiation Chemistry Research Establishment, Japan Atomic Energy Research Institute, Takasaki, Gunma 370-12, Japan.
* Nuclear Energy Unit, Ministry of Science, Technology and the Environment, Kompleks PUSPATI, Bangi, 43000 Kajang, Malaysia.

Empty fruit bunch (EFB) is a major cellulosic waste of the palm oil industry. The current availability of EFB in Malaysia is estimated to be more than 2 million tones (dry weight basis) per year. EFB is mainly burned and emits considerable amount of smokes and pollutants thus affecting surrounding areas. Upgrading of these fibers into useful end-products can be expected not only to reduce pollution but also to increase their values.

Gamma irradiation is presently being utilized for sterilization of medical products and preservation of foods in bulks by virtue of its high penetration ability. In relation to the similar application, we have been investigating the upgrading of oil palm wastes to animal feeds by fermentation. The process is as follows; decontamination of microorganisms in fermentation media using EFB by irradiation, inoculation of useful microorganisms, and subsequent microbial digestion of cellulosic materials as well as production of proteins.

The initial contamination of microorganisms in EFB was very high. Total aerobic bacteria ranged from 10^8 to 10^{10} cells/g, and fungi ranged from 10^4 to 10^8 cells/g. The dose of 25 kGy was required to sterilize the contaminated bacteria whereas the dose of 5 - 10 kGy was enough to eliminate the fungi. The contents of alpha-cellulose, hemicellulose and lignin in EFB were ca 40%, 20% and 22% respectively, and the change in components by irradiation was small.

The EFB media was irradiated at 10 kGy and then inoculated with various fungi such as Coprinus, Pleuorotus, Aspergillus, Vertisillium and Trichoderma. Among these fungi, C. cinereus was selected as the most suitable seed microorganism for the fermentation of EFB. The protein content increased to 13% and the crude fiber content decreased to 20% after 30 days incubation with C. cinereus at 30°C in solid state fermentation. The optimum temperature and pH for growth of C. cinereus were 30 - 40°C and 7 - 9 respectively, and EFB suspension shows the alkaline pH of 8 - 9 because it has a high potassium content. It is, therefore, considered that the fermentation of EFB using C. cinereus is appropriate to produce animal feeds in Malaysia without pH adjustment and incubation temperature control.

IN VIVO ROOTING OF MICROPROPAGATED SHOOTS OF JUGLANS SINENSIS (ORIENTAL WALNUT)

Y.J. Kwon, Y. Youn and S.K. Lee

Institute of Forest Genetics
P.O. Box 24, Suwon 441-350
Republic of Korea

Shoots produced by bud culture of 1 - and 2 - year - old seedlings were rooted in vitro and in vivo. In vitro rooting was not successful and only callus was formed at the lower part of the shoot.

Shoot rootability (76%) was obtained from in vivo rooting with pretreatment of DKW liquid media with IBA 0.15 mg/l in cutting media of 1 perlite: 1 peat moss: 1 vermiculite mixture.

Shoots were irrigated with diluted liquid fertilizer (11N-3P-5K) before and after rooting.

Rooted shoots went through hardening process and out-planted before June.

EMBRYOGENIC SUSPENSION CULTURES FOR GENETIC TRANSFORMATION AND MASS PROPAGATION OF HARDWOOD FOREST TREES

S.A. Merkle, H.D. Wilde, A.T. Groover, D.T. Carraway and B.A. Watson-Pauley

School of Forest Resources, University of Georgia, Athens, GA 30602

Embryogenic suspension cultures of the hardwood forest trees yellow-poplar (*Liriodendron tulipifera*) and black locust (*Robinia pseudoacacia*) offer the potential for large scale propagation and gene transfer via microprojectile bombardment. Embryogenic cultures of yellow-poplar were established from immature zygotic embryos derived from controlled pollinations of genetically superior parents in a Tennessee seed orchard. Suspension cultures were maintained in a medium containing 2 mg/l 2,4-D, which promoted proliferation of proembryogenic masses (PEMs). Populations of synchronous somatic embryos were created by size fractionation of PEMs, followed by culture of the desired fraction on a layer of filter paper overlayed on semisolid hormone-free (basal) medium. Application of this fractionation/plating technique resulted in up to 70% conversion of embryos to plantlets, depending on clonal line. Size-fractionated PEMs were transformed by microprojectile bombardment using DNA which encoded ß-glucuronidase and neomycin phosphotransferase. The resulting kanamycin-resistant microcalli were employed to initiate suspension cultures, from which transgenic yellow-poplar plants were regenerated.

Black locust embryogenic cultures were initiated from immature seeds pulsed for as little as 3 days on a 2,4-D-containing medium prior to transfer to basal medium. Initially, somatic embryos were formed by direct, repetitive embryogenesis, a system which could not be successfully maintained in suspension. However, by manipulation of the osmotic environment of the somatic embryos, the cultures were modified to proliferate as PEMs. PEMs could be maintained on a new medium containing 2,4-D, and readily formed embryogenic suspension cultures when inoculated into a liquid version of this medium. A transformation strategy similar to the protocol used for yellow-poplar is being applied to black locust.

Effects of embryo explant type and developmental maturity on Eastern white pine (Pinus strobus L.) embryogenic callus initiation. Charles H. Michler, Therese M. Voelker, and Rebecca Moioffer, USDA Forest Service, North Central Forest Experiment Station, Forestry Sciences Laboratory, P.O. Box 898, Rhinelander, WI 54501.

Eastern white pine is an important forest tree species with value in recreation, wildlife, and timber. Attempts at reforestation over much of the species natural range has been limited because of few pest-resistant genotypes for use as breeding or planting stock. Biotechnological methods, such as direct gene insertion and somaclonal selection, could be used to enhance resistance of white pine to damaging stress agents. Currently, there are two major limitations to tree improvement using biotechnology. First, there is a lack of knowledge of how tree genes control or modify stress from damaging agents. Second, tissue culture methods with white pine, such as somatic embryogenesis, are inadequate for regeneration of genetically modified cells and tissues.

The initiation of embryogenic callus, which may be both genotypically and developmentally dependent, is an important step in conifer somatic embryogenesis. This study examined half-sib seed from four white pine genotypes that originated from three upper-midwest states (WI,MN,MI). Seed collection began on 7/25/90 and was extended over four dates which included developmental stages ranging from precotyledonary to maturity. For each clone and collection date, embryos and gametophytes (with imbedded embryo) were explanted (100 each) on Gupta and Durzan basal media (DCR) with 3 mg/l 2,4-D, 0.5 mg/l benzyladenine, 0.2 g/l myo-inositol, 0.5 g/l casein hydrolysate, 30 g/l sucrose, 0.25 g/l glutamine and solidified with 2 g/l gelrite. Explants were cultured in darkness and transferred at 14-day intervals to fresh medium. At eight weeks, qualitative and quantitative measurements were made to determine callus initiation frequency and callus types. Three callus types were produced: non-embryogenic, slow-growing embryogenic, and fast-growing embryogenic. Production of embryogenic callus was greater from embryo explants, and the highest percentage of embryogenic callus initiation from embryo explants occurred at the first, two collection dates (7/25/90 and 8/1/90) which corresponded to pre- and early cotyledonary stage embryos. There was also a clonal difference in the ability to produce embryogenic callus. Only one fast-growing embryogenic callus line, that was initiated from a precotyledonary embryo, was produced for future developmental studies.

FORMATION OF LIGNIN-LESS PLANTS WITH ANTISENSE RNA METHOD

Faculty of Agriculture, Tokyo University of
Agriculture and Technology
Noriyuki Morohoshi, Keiko Yamada, Shinya Kajita, Keishi Osakabe,
Shinya Kawai, and Yoshihiro Katayama

Jujo Paper Co., Ltd.
Kunio Hata

The molecular breeding of wood is one of the important method solving the environmental problems and keeping the resources and energy to live us on the earth. As an application of the molecular breeding techniques, we tried to form a lignin-less tree which is brought about by controlling the production of lignin biosynthesis enzymes by the antisense RNA method. These results will give an useful raw material to pulp and paper industry, biomass conversion companies, etc.

Populus kitakamiensis which is a crossed tree between Canadian poplar (*Populus grandidentata* Michx.) and Japanese poplar (*Populus sieboldii* Miq.) was used. The callus of it was formed from the leaves in Murashige-Skoog (MS) medium containing 2,4-D, the adventitious bud in a MS medium containing benzyladenine and zeatin for two months, and the root in the MS medium containing naphtyl-acetic acid for two weeks. We succeeded in the differentiation and regeneration of *P. kitakamiensis* from the callus to seedlings in a high frequency, above 90 percent.

An acidic peroxidase isozyme fraction of *P. kitakamiensis* was isolated and its monoclonal antibody was prepared. It was concluded that the acidic peroxidase fraction is involved in the lignin biosynthesis *in vivo*, judging from staining of wood thin sections treated with the monoclonal antibody by use of immunogold-silver staining.

A part of the genomic gene of the acidic peroxidase was amplified by the polymerase chain reaction method and its nucleotide seqnence was determined.

In order to establish a stable transformation system of *P. kitakamiensis*, we tried to use the binary vector system with Ti-plasmid in *Agrobacterium tumefaciens* and to transduce a foreign gene by the leaf disk method. Used vector and *A. tumefaciens* are a binary vector, pBI121 and non-oncogenic *A. tumefaciens* LBA4404, respectively. The transformed callus was screened on MS medium containing kanamycin and carbenicillin, and the introduction of pBI121 was recongnized with the detection of β-glucuronidase expressed by the transformants. We concluded that we have a certain transduction system in *P. kitakamiensis*.

On the other hand, we had a preceding experiment on the control of peroxidase enzyme by antisense RNA method in tabacco. A flipped DNA (45 nucleotides, 15 amino acids) constructed from the acidic peroxidase gene in *Nicotiana tabacum* samson NN was synthesized and was inserted into the understream of the califlower mosaic virous 35s-promoter of pBI121. Transformed tobaccos were regenerated. The patterns of acidic peroxidase in the leaf, pith and root of them were analyzed by isoelectric focusing. Obtained results show that some species of transformed tobaccos lost the acidic peroxidase isozymes. We concluded that the antisense RNA method can be used to control the lignification enzymes in plants.

The Influence of Guaiacol and Syringyl Groups in Azo Dyes on Their Degradation by Lignocellulolytic *Streptomyces* spp.*

M. B. Pasti, S. R. Hagen, S. Goszczynski, A. Paszczynski,
R. L. Crawford, and D. L. Crawford

Department of Bacteriology and Biochemistry, College of Agriculture, and Center for Hazardous Waste Remediation Research, University of Idaho, Moscow, Idaho 83843, USA

Azo dyes are the largest class of commercially produced dyes. Their structural diversity is great, and they are not readily degradable by microorganisms (1).

Efforts to isolate microorganisms able to transform azo dyes have been somewhat successful. Various anaerobic bacteria have been shown to decolorize azo dyes via reduction of the azo linkage (1, 2), but they produce toxic aromatic amines (3). Under aerobic conditions azo dyes have been considered to be essentially non-biodegradable (3, 4).

Recently researchers have reported azo dye degradation by cultures of the white-rot fungus *Phanerochaete chrysosporium* (5, 6) and the actinomycetes *Streptomyces rochei* A10, *S. chromofuscus* A11, *S. diastaticus* A12, *S. diastaticus* A13 and *S. rochei* A14 (6). Lignin-ase and manganese peroxidase preparations from the *P. chrysosporium* culture were involved in the degradation. The streptomycete strains were unable to degrade the commercial azo dye Yellow #9 (4-amino-1,1'-azobenzene-3,4'-sulfonic acid), but were able to utilize it when its structure was modified by linkage of a guaiacol molecule in the dye via an azo linkage (6).

In the present research twelve new modified azo dyes were synthesized (Table 1) to study the influence of aromatic substituents on azo dye degradation by actinomycetes, and to explore the possibility of enhancing the biodegradability of azo dyes by linking selected readily degradable substituents into the dye's chemical structure.

Significant degradation of the dye by *Streptomyces* spp. occurred only when a hydroxy group was in *para* position to the azo linkage and at least one methoxy group and/or one alkyl group were *ortho* to the hydroxy group (Table 1).

A correlation was also found between the degradation by *Streptomyces* spp. and the rate of dye oxidation by a commercial preparation of horseradish peroxidase (type II, Sigma), an enzyme that shows some similarity in substrate specificity to the peroxidases produced by selected lignocellulolytic streptomycetes (7, 8). The possible involvement of peroxidases produced by the *Streptomyces* spp. in azo dye degradation and the products of such biodegradation will be discussed.

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Table 1. Degradability of azo dyes by *Streptomyces* spp. during a growth period of 14 days and its correlation with dye oxidation by horseradish peroxidase. Growth conditions were as previously described (6).

Compound	Strain A10	Strain A11	Strain A12	Strain A13	Peroxidase Enzyme
Yellow #9	--	--	--	--	--
4-hydroxy-*	--	--	--	--	--
3-methyl-4-hydroxy-*	+	+	+	+	+
3,5-dimethyl-4-hydroxy-*	+	+	+	+	+
2-hydroxy-5-methyl-*	--	--	--	--	--
2-hydroxy-4,5,-dimethyl-*	--	--	--	--	--
3-methoxy-4-hydroxy-*	+	+	+	+	+
3,5,-dimethoxy-4-hydroxy-*	+	+	+	+	+
2-hydroxy-3-methoxy-5-methyl-*	--	--	--	--	--
4-methoxy-*	--	--	--	--	--
3,4,-dimethoxy-*	--	--	--	--	--
3-ethyl-2-hydroxy-*	--	--	--	--	--
3-sec-butyl-4-hydroxy-*	+	+	+	+	+

* -azobenzene-4'-sulfonic acid

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Degradation of azo compounds by lignin peroxidase from *Phanerochaete chrysosporium*: Role of veratryl alcohol*

A. Paszczynski, R. L. Crawford, S. Goszczynski, M. B. Pasti, and D. L. Crawford

Department of Bacteriology and Biochemistry, College of Agriculture, and Center for Hazardous Waste Remediation Research, University of Idaho, Moscow, Idaho 83843, USA

Two functions for veratryl alcohol (VA) in the catalytic reaction of lignin peroxidases (LiP) from *P. chrysosporium* have been suggested. One suggestion is that VA may act as a cation radical mediator between the enzyme and the lignin substrate (1). However, it has been observed that free cation radicals derived from VA are not released from the enzymes during catalysis (2). Recently, VA and 1,4-dimethoxybenzene were found to be ineffective mediators for the oxidation of anisyl alcohol by a lignin peroxidase model, iron *meso*-tetra (2,6-dichloro-3-sulfonatophenyl)porphyrin chloride (3). Thus, the mediator role of VA appears unlikely.

Subsequent studies with whole cultures of *P. chrysosporium* indicated an alternative function for VA might be to protect ligninase against inactivation by hydrogen peroxide (4). This appeared to be confirmed by experiments where high concentrations of hydrogen peroxide were added to non-protein-synthesizing cultures (5). The concentration of VA necessary for protection of ligninase activity was in direct proportion to the rate of hydrogen peroxide synthesis by the cultures.

Gold et al. further investigated the protective mechanism of VA *in vitro* in the catalytic cycle of lignin peroxidase (6, 7). They concluded that VA protects lignin peroxidase against inactivation, by reducing LiPII (lignin peroxidase compound II) or LiPIII back to the native enzyme. Unfortunately, they were not able to measure oxidations of *p*-anisyl alcohol and VA simultaneously in LiP reaction mixtures because the spectra of the two compounds were similar. A recent report by Hammel and Moen (8) clearly showed a requirement for VA during depolymerization of ^{14}C-labeled synthetic lignin *in vitro* by crude LiP, but the role of VA was not determined.

We investigated degradation of several azo dyes by *P. chrysosporium* using pure ligninase preparations. Oxidations of VA and azo compounds could be monitored simultaneously because they have considerably different absorption maxima (Figure 1). Our results suggested that LiP was oxidized by H_2O_2 to LiPI. LiPI then oxidized polyaromatic azo dyes, with formation of LiPII. The LiPII was then reduced back to the native enzyme by VA.

Decolorization of some azo dyes by LiP was almost totally dependent on the presence of VA. The VA significantly increased the oxidation rates of other azo dyes. A simultaneous inhibition of VA oxidation by azo compounds was observed. When azo dye oxidation was terminated, the rate of VA oxidation increased again. These results suggest that LiPI is able to oxidize these dyes, but the LiPII formed requires VA to recycle to the native state. Similar enzymatic oxidation interactions could be involved during degradation of lignin and other recalcitrant compounds.

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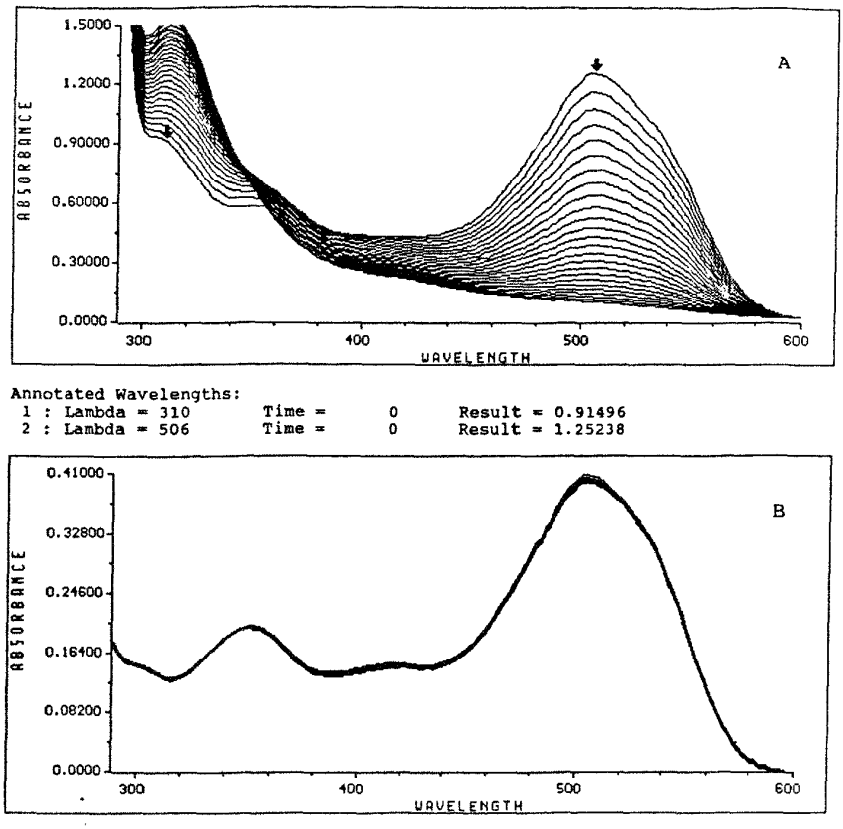

Figure 1. Oxidation of veratryl alcohol and Biebrich Scarlet [international dye no. 4196-99-0] by lignin peroxidase. The reaction mixture (1 ml) contained 0.2 U of enzyme, 0.2 mM hydrogen peroxide, 15 μg of azo dye, 1 mM veratryl alcohol, and 50 mM sodium tartrate; pH 3. Reaction time = 2 min; cycle time = 5 sec. **A.** A_{310} ↑ = oxidation of veratryl alcohol; A_{506} ↓ = oxidation of dye; ↓ = time 0. **B.** Dye oxidation by LiP in the absence of veratryl alcohol.

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DIFFERENTIAL EXPRESSION OF MANGANESE PEROXIDASE ISOZYMES OF *PHANEROCHAETE CHRYSOSPORIUM* IN RESPONSE TO NUTRIENT LIMITATION

Elizabeth A. Pease and Ming Tien
Department of Molecular and Cell Biology
The Pennsylvania State University,University Park, PA 16802.

Three manganese peroxidase isozymes are secreted by *Phanerochaete chrysosporium* in response to nutrient carbon or nitrogen limitation. The pattern of isozyme expression, however, is different depending on the nutrient limitation imposed on the fungus. Under carbon-limiting conditions, the increase in manganese peroxidase levels did not correlate with the RNA levels. In contrast, previous studies showed that in nitrogen-limited cultures, the activity, protein levels and RNA levels all correlated and appeared early in idiophase and decreased over time. This result is explained, in part, by enzyme stability. The addition of the protein synthesis inhibitor cycloheximide to cultures showed that manganese peroxidase enzyme activity is stable for over 72 hours in carbon-limited cultures and for less than 24 hours in nitrogen-limited cultures. The isozymes in the extracellular fluid of the nutrient-limited cultures were analyzed by analytical isoelectric focussing. Silver staining, western blotting and manganese peroxidase activity staining showed that in carbon limited cultures the levels of manganese peroxidase isozyme H4 remained constant at a high level from day 3-6, however the level of manganese peroxidase isozyme H3 increased on days 5 and 6, and no detectable H5 was expressed. Since the Northern blots indicated a decrease in message, this data implies differences in H3 and H4 nucleotide sequences so that they do not cross hybridize. N-terminal amino acid sequencing confirmed a sequence difference between H3 and H4.

CRYOPRESERVATION FOR GERMPLASM STORAGE OF RECALCITRANT AND SUB-ORTHODOX TREE SPECIES

Pence, VC. Center for Reproduction of Endangered Wildlife, Cincinnati Zoo and Botanical Garden, Cincinnati, OH 45220; and Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221

Cryopreservation, or the storage of tissues in liquid nitrogen (LN), should be adaptable for the storage of germplasm of both economically important and wild endangered tree species. Seeds which can be dried to very low moisture levels (less than approximately 15%), generally survive LN exposure. A number of tropical and large-seeded temperate tree species, however, have seeds which are recalcitrant (desiccation sensitive) and must remain hydrated. Others have seeds which can be dried, but which do not remain viable for long in that condition (sub-orthodox). Various protocols, focusing on embryo freezing, have been adapted for use with these different types of seeds.

For large, temperate sub-orthodox and recalcitrant seeds, desiccated storage of the excised embryo axis has allowed survival through cryostorage of tissues of species of Aesculus, Carya, Castanea, Corylus, Juglans, and Quercus. In several cases it has been found that there is an optimal point of desiccation for freezing, as determined by time-course experiments. Differences in the sensitivity of species to this technique have also been observed. For example, Juglans species are very tolerant and germinate after thawing and rehydration, while species of Quercus are more sensitive, producing only callus after freezing. Sensitivity may also differ within the embryo tissue, as with Aesculus in which the root axis is more sensitive to desiccation than the shoot, and Castanea, in which the shoot is more sensitive than the root.

For recalcitrant tropical species, such as Theobroma cacao, cryostorage has been obtained using hydrated freezing of immature embryos, since desiccation and freezing of mature embryo axes have not been successful. Pre-maturation embryos are cultured on a pre-freezing medium for several days and are cryoprotected before slow freezing. Although the zygotic embryo does not survive freezing intact, somatic embryos, which can be germinated into plants, are produced on recovery medium containing 1.5-3.0 mg/liter naphthaleneacetic acid.

Other approaches, such as cell suspension freezing, can also be used with species which can be regenerated from cell cultures, such as the endangered tropical Brunfelsia densifolia. The development of these and other techniques should help broaden the application of cryopreservation to the ex situ preservation of commercially important and endangered tree species.

IN VITRO PROPAGATION OF ROCK ELM (ULMUS THOMASII)

Pence, VC and Biederman, EL Center for Reproduction of Endangered Wildlife, Cincinnati Zoo and Botanical Garden, 3400 Vine Street, Cincinnati, OH 45220; and Department of Biological Sciences, University of Cincinnati, Cincinnati, OH 45221

Rock elm, Ulmus thomasii, is listed as an endangered species by the State of Ohio, with only a few specimens located in the State. Explants for tissue culture were taken from several trees in Mount Storm Park, Cincinnati, which were approximately 100 years of age and were scheduled to be removed. Cuttings were made in May of 1989 from shoots which had been etiolated by covering the branches with black plastic before growth began.

Tissues were disinfested with a 1:10 dilution of commercial sodium hypochlorite for 10 minutes, followed by 2 rinses in sterile distilled water. Shoots approximately 2 cm in length were cultured, with lateral leaves and any large apical leaves removed. Some leaf tissue was also placed on media. Three basal media, Woody Plant (WP), Murashige and Skoog (MS) and Driver and Kuniyuki (DKW), were tested. The following growth regulators were tested, in mg/liter: With WP and MS, kinetin (K) and benzylaminopurine (BAP) at 0.2 or 1.0; K+BAP each at 0.2; DKW media with either 1.0 BAP plus 0.1 naphthaleneacetic acid (NAA) or the same plus 0.5 K. Seven or 8 explants were placed on each medium, in 25x150 mm culture tubes, 10 ml medium/tube. After 1 week 47% of the cultures had become contaminated, from an endogenous source of bacteria and fungus. Of those remaining clean, 67% had lateral buds which were swelling and appeared capable of growth. Only 16% had produced callus, and these were all on BAP-containing media. Shoots were subcultured weekly for the first 8 weeks.

With further subcultures, lateral bud growth did not occur from these explants, although they did produce a light brown, friable callus from shoot bases as well as from leaf explants. Best callus growth occurred on MS medium and subcultures were routinely made onto this medium containing 0.5 mg/liter BAP. Although ranging from light to dark brown, Rock elm callus continually produced green shoots, which in some cases elongated. At approximately 1 cm in length, the shoots were excised and rooting was attempted. In vitro rooting attempts were not successful, and the shoots were then placed directly into soil in $2^1/2$" plastic boxes where rooting occurred. Plants obtained in this manner have been acclimated and over-wintered out-of-doors.

Rock elm, like other elm species which have been cultured (e.g. Fink et al, 1986), appears to regenerate well in culture. The most important limiting factor in the recovery of plants from Rock elm cultures appears to be limitations in the control of successfully stimulating elongation of the shoots which develop from Rock elm callus.

Acknowledgements: The authors wish to acknowledge Mr. David Gamstedder, City of Cincinnati Department of Urban Forestry, for inviting our participation in the propagation of these trees.

Reference: Fink et al., Plant Cell Tissue and Organ Culture 7:237 (1986)

EFFECTS OF EMBRYO EXPLANT ORIENTATION, THIDIAZURON, AND AGAR ON EASTERN WHITE PINE (*PINUS STROBUS* L.) ADVENTITIOUS SHOOT INITIATION.

Paula M. Pijut, Charles H. Michler, and Therese M. Voelker, USDA Forest Service, (PP) North Central Forest Experiment Station, 1992 Folwell Ave., St. Paul, MN 55108, and (CM, TV) Forestry Sciences Laboratory, P. O. Box 898, Rhinelander, WI 54501

Eastern white pine remains an economically important conifer species in the northeastern and midwestern United States, despite its population decline as a result of overlogging, conversion of forest land to agriculture, and pests such as white pine blister rust, white pine weevil, and deer. Interest in planting white pine is increasing in the northeast and the Lake States. Reforestation with white pine has been limited because of few pest resistant genotypes and unreliable vegetative propagation methods. Development of *in vitro* propagation methods would begin to alleviate both of these limitations through improved vegetative propagation and use of biotechnological applications such as, somaclonal selection and direct gene insertion for enhanced tolerance to damaging agents.

Adventitious shoot production induced on embryo explants was examined as influenced by explant orientation, thidiazuron, and agar type and concentration. Mature seeds were surface sterilized in 25% Clorox for 25 minutes, rinsed three times in sterile, distilled water, and imbibed for 72 hours in 1% hydrogen peroxide at 26°C in darkness. Seeds were re-sterilized following imbibition. Zygotic embryos were excised from the surrounding gametophytic tissue and cultured on a modified Schenk and Hildebrandt (SH) medium supplemented with 30 g/l sucrose, growth regulator treatments (thidiazuron, 0-100 μM), and agar treatments (Difco Bacto vs. Gibco Phytagar, 0-1.0%). The embryos were also oriented in seven different positions, including variations of vertical and horizontal placement either on the surface or submerged into the medium. Cultures were incubated under an 18/6 hour photoperiod (125 $\mu E/m^2/sec^1$) in a growth chamber at 26°C.

Agar concentration at 0.8%, regardless of brand, had the greatest effect on adventitious shoot initiation on SH medium containing 4.4 μM 6-benzylaminopurine. Thidiazuron increased shoot production with increasing concentrations, although higher concentrations reduced shoot elongation. In orientation experiments, vertically placed embryos with only the cotyledons submerged into the medium produced the greatest number of shoots per explant. Completely submerged embryos failed to produce adventitious shoots.

Propagation of Interior Spruce through Somatic Embryogenesis

D.R. Roberts, F.W. Webster, B.S. Flinn, W.R. Lazaroff, D.R. Cyr. Forest Biotechnology Centre, B.C. Research Corp., 3650 Wesbrook Mall, Vancouver, British Columbia V6R 2L2

To apply somatic embryogenesis to clonal forestry, the technique must be applicable to a broad range of genotypes and allow efficient regeneration of phenotypically normal plants. The abscisic acid (ABA) dependent developmental profile (the number of rooty, shooty, precociously germinating and late cotyledonary embryos at different concentrations of ABA) differed among genotypes. Only late cotyledonary embryos accumulated storage proteins and, for most genotypes, the best concentration of ABA for production of late cotyledonary embryos was 40 and 60 μM. Somatic and zygotic embryos contained similar levels of storage proteins and showed the same developmentally-specific accumulation during maturation. A high relative humidity (HRH) treatment promoted high frequency and rapid germination of somatic embryos that was similar to seed germination. Comparison between temporal changes in embryo water content during the HRH treatment and its subsequent effects on germination revealed that although the majority of water was lost during the first week, gradual improvements in germination occurred over a five week treatment period. No changes in major proteins were observed in embryos during the HRH treatment. However, decreases in storage protein m-RNA were temporally related to changes in germination, suggesting that metabolic events occur after the biophysical changes are complete which are required to achieve normal germination. Survival of emblings following transfer to soil, acclimatization and first seasons growth in the nursery was 80% or greater for most genotypes while growth rate, final height, shoot and root morphology were similar for emblings and seedlings.

RESPONSES OF FOREST TENT CATERPILLAR AND GYPSY MOTH TO POPLAR GENETICALLY ENGINEERED WITH THE B.t. d-ENDOTOXIN

Daniel J. Robison, Kenneth F. Raffa and B.H. McCown

Behavioral, developmental and consumptive trials of larval forest tent caterpillars, Malacosoma disstria, and gypsy moths, Lymantria dispar, were conducted on genetically engineered Populus clone NC5339, P. alba x P. grandidentata, "Crandon". The engineered transformants contained the Bacillus thuringiensis K. d-endotoxin gene. Results indicate that these insects behaviorally discriminate against the d-endotoxin expressing plants, develop more slowly and consume very small amounts of the engineered leaf tissue, and that survival and development are dependent upon the insect species and the duration of feeding. The gypsy moth was generally less sensitive to the d-endotoxin than the forest tent caterpillar. These results suggest the utility of B.t. engineered trees for plant protection; and provide insight into the challenges of resistance management, secondary pest development, nontarget species effects, deployment strategies within plants (i.e., controlling natural resistance), and deployment strategies among plants (individuals and/or clones) in plantations.

EFFECTS OF UV-B RADIATION ON YOUNG PLANTS OF *QUERCUS ROBUR* L.

Isabel Santos, J.M. Almeida & R. Salema - Experimental Cytology Center (INIC) & Institute of Botany, University of Porto - Portugal

The increase of the solar UV-B radiation reaching the Earth's surface, as a consequence of depletion of the ozone layer is known to be harmfull to life since it is readily absorbed by nucleic acids and protein chromophores affecting important physiological functions. Following previous studies concerning the effects of UV-B radiation on corn plants it was deemed of interest to subject a woody plant to this type of treatment and follow its effects at ultrastructural and biochemical level.

Young plants of *Quercus Robur* L. were irradiated with UV-B radiation provided by 2 Philips TL 40/12 lamps (2.5 W m^{-2}) together with white light (700 $\mu E\ m^{-2}\ s^{-1}$) under a photoperiod of 12h /12 h light/darkness. Control plants were kept under white light only. Leaf samples were collected after 1,3,8,16 and 25 days and some prepared for TEM and others used for total protein, chlorophyll and flavonoid quantification and for SDS-PAGE.

At the end of the treatment the most conspicuous alterations in UV-B irradiated plants were the smaller surface area and reddish-brown color of their young leaves as well as easily perceived symptons of dryness in the foliage; indeed treated leaves had about 16% less water than control material. Our results showed that under the conditions used, UV-B radiation did not destroyed the chlorophylls since from the outset of the treatment the values found for these pigments were very close in control and treated material; at the end of the experiment the values found were 5.92 and 5.39 mg / g dry weight (d. w.) respectively, however the a/b ratio increased in the latter ones. Contrary to that, the spectrophotometric scan of extracts in methanol + ammonia showed a considerable increase of UV-screening pigments (240-340 nm),with a well marked peak around 275 nm (generally considered flavonoid glycosides) in treated leaves which was already appreciable after 1 day treatment. The level of total flavonoids was about 35% higher than in control material.

The total protein level was reduced by radiation and at the end of the treatment the values of 519.64 mg protein / g d. w. and 417.60 mg protein /g d. w. were found respectively in control and treated mature leaves. These results are in accordance to densitograms of the SDS-PAGE gels which showed that 2 protein bands did not appeared in irradiated material and all the other but one were thinner and took less silver stain.

The ultrastructural study of mature leaves revealed that chloroplasts and mitochondria appeared affected after 1 day of treatment. The former displayed swelling of the thylakoids which however reverted to normal with ongoing treatments. After 3 days the most striking difference was the high amount of starch in plastids of the treated leaves,deposits that were even more preeminent at the end of treatment. Mitochondria, that initially appeared swollen, with large electron translucent areas and even odd-shaped, also recovered displaying the current ultrastructure. Another difference was the increase of the number of cells that accumulate electron dense material in their vacuoles observed in treated leaves.

Young leaves were more severely affected and no recovery was observed either on chloroplasts or mitochondria. The leaves developed a thicker cuticule in the adaxial side very early in the treatment (3 days); the stomata, even if located only on the abaxial side, were somewhat affected (as also observed in corn) and the leaves loose water to the extend of becoming curled at the edges. Some meristematic cells in the shoot apex were affected as could be seen in plants that were irradiated and later transferred to normal light conditions which developed abnormally shaped leaves.

This study showed that the effects on plant leaves depend on the developmental stage at which they began to be irradiated, that the plant respond both metabolic and morfologically and further studies are needed to better understand the total effects of the UV-B radiation.

In vitro culture of Taxus embryos: A developmental approach to pathogenicity.
STANLEY SCHER* and NASSER HAGHIGHAT-KASHANI, Department of Biology, Sonoma State University, Rohnert Park, CA 94928.

Pacific yew (Taxus brevifolia), other native yew species, ornamental Taxus cultivars and their hybrids are the principal sources of taxol--a promising experimental anticancer agent in clinical trials. The National Cancer Institute, in cooperation with the USDA Forest Service recently initiated a study of T. brevifolia populations in the Pacific northwest, to identify genotypes with a high taxol content, determine if these genotypes maintain their high taxol content when cultivated in nursery environments, and ensure that these genotypes are available for future propagation in nurseries and plantations (Miller, personal communication). National Forests from five states contributed Taxus samples for analysis and studies of vegetative propagation in Forest Service nurseries. In these settings, the potential for transmission of root diseases by soil-borne fungi is markedly increased. Phytophthora lateralis, a cause of a destructive root disease on Port-Orford Cedar, also attacks Pacific yew (DeNitto and Kliejunas, in press). Accordingly, we initiated a study of Phytophthora pathogenicity to identify possible disease-resistant strains. Approaches to this investigation include: Growing seedlings from seed; propagating stem cuttings vegetatively; culturing Taxus tissues in the laboratory. But, germination of Taxus seed requires stratification and a long--one to two year--dormancy period; root production on cuttings is also a slow process requiring several months; limited data on callus and nodule cultures with Taxus tissues suggests extremely slow growth rates. When excised from immature arils and cultivated in synthetic media, embryos of T. brevifolia and other Taxus species germinate within two weeks (Flores and Sgrignoli, in press). Using precocious germination, embryos can be obtained over a range of aril maturation stages. Embryos and seedlings exhibit similar responses to infection by Phytophthora (Sharma and Bedi, 1990). This approach facilitates developmental studies with Phytophthora or, in principle, other infectious agents.

DeNitto, G.A. and Kliejunas, J.T. 1991, First report of Phytophthora lateralis on Pacific yew. Plant Disease 75: (in press).

Flores, H.E. and Sgrignoli, P.A. 1991, Immature embryos of Taxus species display precocious germination in vitro. In vitro Cellular and Developmental Biology-PLANT (in press).

Sharma, N.K. and Bedi, S. 1990, Effects of Phytophthora palmivora on zygotic embryos of Papaya in vitro. Annals of Botany 66: 597-603.

*Mailing Address: P.O. Box 9456, Berkeley, CA 94709

ANALYSIS OF VIRAL GENOMIC HETEROGENEITY IN THE LYMANTRIA DISPAR NUCLEAR POLYHEDROSIS VIRUS FORMULATION GYPCHEK

James M. Slavicek
USDA Forest Service, Northeastern Forest Experiment Station
359 Main Road, Delaware, Ohio

The Lymantria dispar nuclear polyhedrosis virus (LdNPV) was developed as a microbial insecticide for use against the gypsy moth and registered by the US Forest Service with the EPA. This product, Gypchek, is a mixture of LdNPV genotypes. We initiated an analysis of Gypchek to assess the degree of genomic heterogeneity present, and have generated several purified viral isolates for use in studies designed to identify the basis for genomic heterogeneities. In addition, isolates of differing potencies were identified for use in studies directed at elucidation of the molecular basis for potency differences, and for development of a more efficacious LdNPV product to replace Gypchek.

LdNPV isolates were generated and purified in vivo by infecting 4th instar gypsy moth larvae per os with Gypchek polyhedra at a dose that resulted in approximately 2-6% mortality. Polyhedra were collected from individual insects that died from 11-13, 14-17, and 18-21 days after infection. These isolates were repurified through one or two additional passages in larvae at a dose that resulted in 2-6% mortality. The purity of eleven viral isolates chosen for analysis was assessed by the lack of submolar DNA genomic fragments after restriction endonuclease digestion.

Genomic heterogeneity of the viral isolates was assessed through the presence of restriction endonuclease fragment length polymorphisms after digestion with restriction endonucleases. Heterogeneities in viral isolate DNA fragment lengths were found using several different restriction enzymes. Digestion with Bgl II provided a range of DNA fragment lengths that facilitated comparisons between the isolates, and was therefore used for this analysis. Of the eleven viral isolates examined, digestion with Bgl II resulted in 9 unique and two duplicate DNA fragment profiles. Of the unique DNA digestion profiles, eight exhibited 17 fragments ranging in length from approximately 3.2 - 25 kb, and the remaining isolate contained 16 fragments. Thirteen Bgl II fragments, ranging in length from approximately 3.5 to 25 kb, were common to all nine isolates. The sum length of the remaining 3 or 4 fragments ranged from approximately 15.6 - 17.3 kb, accounting for the differences in genomic size between the isolates.

The nine LdNPV isolates with distinct genomes were plaque purified on a 652Y cell line maintained in cell culture. During plaque purification four additional unique isolates were detected. These unique viral genotypes may have been present in the original isolate as an undetected minor component, or may have arisen through mutation during plaque purification.

This analysis has shown that Gypchek is a heterogeneous mixture of at least nine different viral genotypes. Since nine of eleven isolates generated were unique, it is probable that many more unique LdNPV genotypes exist within Gypchek. Studies are in progress to identify the regions of the viral genome that contain the heterologous Bgl II sites, and to determine if the LdNPV genome contains preferential sites for insertion of foreign DNA sequences.

LACCASE AND PEROXIDASES, EXTRACELLULAR ENZYMES INVOLVED IN LIGNIN BIOSYNTHESIS IN TREE CELL SUSPENSION CULTURES

Raja Sterjiades, Jeffrey F.D. Dean & Karl-Erik L. Eriksson

Department of Biochemistry, CBRR
University of Georgia
Athens, GA 30602

Our current understanding of lignin biosynthesis in trees and other higher plants is limited with respect to what enzymes are involved and how they are regulated. Using cell suspension cultures of sycamore and eastern cottonwood, we have undertaken an in-depth study of peroxidase isoenzymes and laccase, the oxidative enzymes most likely responsible for catalyzing the dehydrogenative polymerization of monolignols in the extracellular matrix. We have determined the time course for secretion of these enzymes into culture media with respect to age and mass of the cells. We have separated the enzymes using column chromatography and documented some of their characteristics using different types of gel electrophoresis (IEF, native, and SDS-PAGE). We will discuss the activities of these two enzymes on various common substrates, including those which may represent their *in vivo* substrates, as well as how the *p*-diphenol oxidase activity of laccase may be differentiated from *o*-diphenol oxidase activities. We will also present initial studies of the *in vitro* synthesis of lignin (DHPs) using laccase and peroxidases together and separately with each of the three monolignols.

Conifer molecular genetics: Applications in population genetics and developmental studies.

B.C.S. Sutton, C.H. Newton, J.R. Gawley, S.C. Pritchard, B.S. Flinn. Forest Biotechnology Centre, B.C. Research Corp., 3650 Wesbrook Mall, Vancouver, British Columbia, V6R 2L2

In British Columbia (B.C.) introgression occurs between three spruce species, white (*Picea glauca*), Sitka (*P. sitchensis*) and Engelmann spruce (*P. engelmannii*). Two main groups of hybrids occur naturally; interior hybrids which comprise mixes of white and Engelmann spruce and interior/coastal hybrids which comprise mixes of the interior species with Sitka spruce. Distinguishing these hybrids based on morphological traits is difficult yet important for tree improvement programs. Our efforts have been focussed on the development of diagnostic probes to identify and quantify the occurrence of natural hybrids of the latter group. Chloroplast, mitochondrial and nuclear DNA sequences have been used to define restriction fragment length polymorphisms which distinguish the white, Engelmann or Sitka spruce populations. Analysis of progeny from crosses between the species revealed that mitochondrial DNA was maternally inherited in all cases (32 progeny from 5 independent crosses). Chloroplast DNA exhibited paternal inheritance (27 progeny from 4 crosses); however, when naturally occurring hybrids were analyzed, individuals exhibiting chloroplast polymorphisms specific to both coastal and interior spruce could be found. In most cases mitochondria and chloroplasts within individuals from zones of introgression were contributed by different species. Use of an rRNA gene probe to distinguish the nuclear genomes of coastal and interior spruce allowed preliminary estimation of the degree of hybridization occurring in one geographic area. A cDNA library was also constructed from somatic embryos of white spruce. Analysis of these clones with mRNA from somatic embryos during various stages of maturation, partial drying and germination allowed us to define developmental stage specific mRNA's. DNA sequencing of both cDNA and genomic clones, and Southern hybridization experiments, allowed us to identify two embryo storage protein sequences encoded by multigene families. The cDNA sequences are being used for both RFLP studies and to refine the spruce somatic embryogenesis.

ROLE OF EXTRACELLULAR ENZYME RATIO FROM *Trichoderma harzianum* ON PROTOPLASTING OF *Trichoderma reesei* MYCELIUM

K.Theodore and T.Panda
Biochemical Engineering Division
Department of Chemical Engineering
IIT, Madras - 600 036
INDIA

Abstract

Trichoderma harzianum NCIM 1185 was studied for its extracellular cellulolytic and other hydrolytic enzymes viz., laminarinase, cellobiase, and endoglucanase. Both surface- and submerged-culture studies were performed using the conditions : different slant age (between 2 and 10 days) of *T.harzianum*, temperature - 30^{o}C, pH (initial) - 5.0, inoculum level - 30 mg dry wt./l, and rpm - 160 (for submerged culture). A 100 ml glucose-nutrient medium was dispensed in 500 ml Erlenmeyer flask. It was observed that the proportion of laminarinase, cellobiase, and endoglucanase varied during the entire fermentation cycle. This phenomenon was also found using different slant age. Further analyses have shown that the ratio of laminarinase, cellobiase, and endoglucanase has influenced the protoplasting efficiency in *Trichoderma reesei* mycelium. The ratio of laminarinase : endoglucanase : cellobiase :: 6.98 : 1 : 1.61 was found to be more suitable for protoplasting in *T.reesei* mycelium (yield of protoplast - 72 X 10^{6} /(g dry wt. of mycelium) (ml of lytic enzyme)). This will be critically analysed in the communication.

AFFINITY PURIFICATION OF GYPSY MOTH JUVENILE HORMONE ESTERASE

Algimantas P. Valaitis
USDA Forest Service
359 Main Road
Delaware, Ohio 43015

During the final larval instar of the gypsy moth the expression of juvenile hormone esterase (JHE) activity contributes to the elimination of juvenile hormone from circulation prior to pupation. Since the decline in the titer of juvenile hormone is a prerequisite for pupation it has been suggested that JHE may be exploited for biocontrol. In order to clone the gene for JHE we began to purify and study the properties of the gypsy moth hormone esterase. Earlier we reported the isolation and characterization of JHE from the hemolymph of gypsy moth larvae using classical biochemical procedures. Subsequently, we demonstrated that polyclonal antibodies raised against the gypsy moth enzyme cross-reacted with JHE purified from Trichoplusia ni but not with JHE from Manduca sexta. Amino acid sequence data revealed that there are distinct differences in the primary structures of JHEs present in the hemolymph of different Lepidoptera.

A new and improved method has been developed for the purification of juvenile hormone esterase from the gypsy moth. We have successfully applied the ability of Sepharose-coupled concanavalin A to bind to glycoproteins for the purification of JHE by affinity chromatography. Gypsy moth JHE was bound strongly by Sepharose-concanavalin A and could be displaced by 0.2 M methyl a,D mannopyranoside in 0.1 KCl. JHE has been purified both from the fat body and the hemolymph using concanavalin-A chromatography. Experiments have shown that the fat body and the hemolymph enzymes may be purified up to 160-fold by this affinity column step. These results are consistent with JHE being a glycoprotein. Characterization of the carbohydrate moiety remains to be determined.

PURIFICATION OF TREHALOSE-HYDROLYZING ENZYME FROM THE MUSCULATURE OF THE GYPSY MOTH LARVAE.

Algimantas P. Valaitis
USDA Forest Service
359 Main Road
Delaware, Ohio 43015

In gypsy moth the major source of energy for muscle metabolism is the non-reducing disaccharide trehalose which is stored in the larval hemolymph. The hydrolysis of trehalose into two glucose molecules is catalyzed by the enzyme trehalase. In the gypsy moth larvae, trehalase is present in the muscle cells and is absent both in the hemolymph and in the fat body of the immature insect. There appears to be no inhibitor of trehalase present in the hemolymph. Therefore, using gypsy moth virus as the delivery system for trehalase, the disruption of the normal trehalose levels may result in a effective biological control agent. A possible scheme in relating the effect of an engineered virus expressing trehalase has been developed.

The gypsy moth trehalase was purified from the gypsy moth larvae by a method that includes concanavalin-A lectin chromatography and FPLC. The molecular weight of the enzyme (60,000 daltons) was determined by molecular sieve chromatography and SDS-polyacrylamide gel electrophoresis. The gypsy moth larval trehalase is a soluble enzyme that is highly specific for trehalose. The relationship between trehalose concentration and the rate of hydrolysis has been examined for the determination of Km for trehalose.

In vivo experiments were carried out to evaluate the effect of injection of porcine kidney trehalase into the hemocoel of gypsy moth larvae. Preliminary results showed a dramatic increase in the concentration of glucose in the hemolymph of trehalase-treated larvae and an impact on the normal growth and development of the larvae which may be related to the hyperglycemic effect of the administered enzyme.

Virulence factors of C. parasitica , the chestnut blight fungus.

Varley, D., Podila, G.K. and Hiremath, S.T.[1] Department of Biological Sciences, Michigan Technological University, Houghton, MI 49931 and [1] Northeastern Forest Experiment Station, Delaware, OH 43015.

The filamentous fungus *Cryphonectria parasitica* (Murr.) is the causal agent of chestnut blight, which has virtually eliminated the American chestnut, *Castanea dentata*, from the eastern United States. Although hypovirulent strains of *C. parasitica* exist, their inability to disseminate spontaneously in nature has made it necessary to search for alternate approaches to control the American virulent strain. While chestnut blight is a vexing problem, the factors that determine the virulence of the fungus have not been studied. In order to infect and colonize the chestnut tree, *C. parasitica* may use various genes. Genomic DNA of both virulent and hypovirulent strains was probed with heterologous gene probes for cutinase, pectin-degrading enzymes, cellulases, and lignin-degrading enzymes. Results from these analyses showed the presence of homologous genes for cutinase and lignin peroxidase in all the strains. Enzyme assays indicated that cutinase and lignin peroxidase activities in the virulent strain were severalfold higher than those in the hypovirulent strains. To determine whether the decreased activities in the hypovirulent analyses is due to a decrease in the synthesis of these enzymes, northern analyses (using DNA probes) and western analyses (using antibodies to the enzymes) are being performed. Preliminary evidence suggests that several of these genes are involved in the pathogenesis of the chestnut by *C. parasitica*.

HETEROLOGOUS EXPRESSION OF AND MUTAGENESIS STUDIES ON THE LIGNIN-DEGRADING ENZYMES OF *Phanerochaete chrysosporium*

Ross E. Whitwam, Linda Miller, Elizabeth A. Pease, and Ming Tien*
Dept. of Molecular and Cell Biology, Pennsylvania State University, University Park, Pa 16802

Genes for a ligninase (H8) and a Mn-dependent peroxidase (H4) from the white rot fungus *Phanerochaete chrysosporium* were expressed in insect cell cultures of *Spodoptera frugiperda* (SF-9 cells) using *Autographa californica* nuclear polyhedrosis virus (AcNPV) baculovirus expression vectors. Site-directed mutagenesis was performed on the same enzymes and the mutant variants were expressed in the same system. Three mutant genes were constructed -- a ligninase gene without its N-linked glycosylation site; a ligninase gene without an active-site histidine, putatively assigned a catalytic function in mechanistically-similar peroxidases; and a Mn-dependent peroxidase gene without the homologous active-site histidine. The heterologously-expressed protein products -- both mutant and wild-type -- were isolated and kinetically characterised for comparison with their native counterparts.

BIOCONTROL OF CHESTNUT BLIGHT

Eva Wilhelm and Harald Zipko

Austrian Research Centre
Seibersdorf G.m.b.h.
A-2444 Seibersdorf, Austria

Chestnust blight is a severe disease of European (Castanea sativa) and American (Castanea dentata) chestnuts. The causal agent is a fungus Cryphoneciria parasitica, an ascomycete of the family Diaporthaceae. Two strategies to control this disease are under investigation.

1. Hypovirulence

   We have isolated several fungal strains from different regions in Austria which show different morphological symptoms (growth rate, color of the mycelium and ability to produce asexual spores). Hypovirulence means a debilitation of the fungus and these isolates can convert aggressive fungal strains via hyphal fusions or spores into less aggressive presence of dsRNA which has been located from Newhouse et al. (1990) in virus-like particles of a size from 50-100nm. The transmission of hypovirulence is not possible between different compatibility groups. Preliminary investigations to detect the amount of different compatibility groups in Austria have been made.

2. Antagonistic bacteria

   Selected bacterial strains from the xylem sap of a healthy chestnut, leaves of lilac and roses show an inhibitory effect <u>in vitro</u> on the growth of the fungus. Inoculations of the plant with bacteria could prevent chestnut blight.

Investigations into the feasibility of these methods under field conditions will be demonstrated.

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