36839 PDF

FY 97 FINAL REPORT

1. PROJECT TITLE: Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expressions Studies (PP97-FS-8)

2. PRINCIPAL INVESTIGATOR: Dr. Gerald S. Pullman

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3. EXECUTIVE SUMMARY OF WORK COMPLETED

- We have developed a new rapid solid phase method of performing differential display. This allows us to perform differential display with very small amounts of starting material, in many cases with as little as a single embryo.
- We have monitored the activity of hundreds of genes over the course of development of loblolly pine somatic embryos. This is the first time this technique has been used to follow plant embryogenesis.
- We have identified potential marker bands for many stages of development.
- We have cloned 96 cDNAs which are differentially expressed during somatic embryogenesis, 81 cDNAs have been sequenced and similarities to 18 previously characterized genes have been recognized. The remaining cDNAs may be derived from novel genes, previously unidentified or may represent regions which are poorly conserved between species. (see Attachments)
- We have monitored the activity of hundreds of genes over the course of development of loblolly pine zygotic embryos.
- We have identified potential marker bands for many stages of development. Some of these appear similar to those in somatic embryos however the timing of expression can differ. Some bands are present in somatic but absent in zygotic or vice versa.
- We have cloned 98 cDNAs which are differentially expressed during zygotic embryogenesis, all 98 cDNAs have been sequenced and similarities to 27 previously characterized genes have been recognized. The remaining cDNAs may be derived from novel genes, previously unidentified or may represent regions of genes which are poorly conserved between species (see Attachments).
- Protein synthesis in somatic and zygotic embryos has been assessed by 2D gel analysis
- Three Research Papers are in preparation, one Poster Abstract has been Published (see attached)
- Two Seminar and Three Poster Presentations will be made at International Meetings within the next three months
- A Post-Doctoral Scientist with several year experience in International Research laboratories has been recruited to work on the project and a Ph.D. student has been supported by IPST

Introduction

This study was initiated to gather information on the physiology of natural embryo development which in turn will be used to assess and improve the quality of somatic embryos. Zygotic and somatic embryo development has been divided into specific stages and specific proteins, m-RNAs, and amino acids have been analyzed in order to identify stage specific nutritional requirements and markers. This work will generate fundamental knowledge and from this develop protocols which can be quickly applied in industrial settings; in tissue culture operation and forestry programs in Georgia. These techniques have the potential to provide a new and fundamental tool which will find widespread application and greatly accelerate the process of improving somatic embryo production.

Summary of Work Completed in FY 97

- Using Differential Display The activity of approximately hundreds genes has been followed over the
- **A new rapid solid phase method of performing differential display has been developed by our laboratory**. With Somatic Embryos, the continual supply of embryos of different stages allows us to increase quantities of starting material and thus overcome the problem, however, where embryos of a specific stage are limiting, such as when working with zygotic embryos, this approach cannot be employed. To overcome these problem, we used coated magnetic beads with attached oligo(dT) to isolated poly(A) RNA in a method modified from Rosok et al 1996. (Biotechniques 21:114-121)
- Compared to the traditional approach using total RNA, this method has offered several advantages:
- **Easy and fast**. Bypasses the laborious and capricious total RNA isolation. All the procedures, from poly(A) RNA isolation, reverse transcription to PCR, can be performed on a single day. Many samples can be processed at the same time.
- **Dramatically increases the reliability of DD in loblolly pine**. The number of steps in RNA isolation and handling are greatly reduced compared to other methods, this allowed us to generate repeatable and clear band patterns on the differential display gels.
- **Very small numbers of embryos are required**. This new procedure eliminated the RNA precipitation step, and thus avoids loss of RNA. This is important when the amount of embryos available for RNA isolation is small. Using the beads-oligo(dT) approach, the poly(A) RNA is captured by the oligo(dT) on the beads, no matter how small the amount of starting material. So far, we are able to generate clear DD band pattern of a single stage 7 embryo. This pattern is almost identical to those generated when 2, 5 or 10 embryos at the same stage are used as starting material.

- **Many sets of DD have been performed which generated clear and informative band pattern**. We have performed solid-phase RNA differential display on embryos at suspension stage (stages 1 and 2), stage 3, 4, 5, 6, 7 and 9. Eight sets of primer combinations were used for each stage. In a initial step, more than 70 bands on the DD gels were identified that either increase or decrease their abundance from early to late stages. 115 of these bands were cut out the DD gels and successfully re-amplified. We have cloned 96 of these amplified sequences into a plasmid vector and screened 200 clones using PCR. More than 100 clones contained a plasmid that carries insert sequence of the expected size. Characterization of the PCR products of these clones using restriction enzymes have identified was used as a preliminary screen of the number of cDNAs cloned. We are in the process of re-examining their abundance at different stage of somatic embryo development, and sequencing and have developed new techniques to do so.

- **Differential Display with Zygotic Embryos**. DD has been successfully carried out using as little as 1 mid- to late-stage embryo using the oligo-dT-bead primed method

- DD performed for embryos of 18 stages, 216 band patterns obtained, many stage-specific bands have been identified.
- The DD patterns of zygotic embryos were compared to those of somatic embryos. Differences and similarities have been identified. These are currently being analyzed in more detail and will be discussed during PAC presentations.

- Reproducible, high-resolution 2-dimensional gel patterns have been obtained for late- stage (stage 8 and stage 9) zygotic and somatic embryos of loblolly pine. The somatic embryos, which appear to have the most difficulty proceeding past these stages, showed markedly different patterns of protein accumulation from zygotic embryos. In particular, protein spots previously identified as belonging to the high molecular weight heat shock proteins and heat shock cognate proteins, such as the hsp70 family, accumulated to high levels in zygotic embryos but at far lower levels in somatic embryos. Somatic embryos accumulated other, unidentified proteins not expressed in zygotic embryos. These patterns suggest that stress protein accumulation should be investigated as markers for maturation of zygotic embryos of loblolly pine, and may be important for successful maturation of somatic embryos.

4. DELIVERABLES

Major Milestones & Dates	Original Proposal	Actual
1. Begin isolation and staging of somatic embryos	Months 1-24	ONGOING
2. Begin isolation and staging of zygotic embryos	Mo. 1-3 &12-14	Mo. 1-3 COMPLETED Mo. 12-14 UNDERWAY
3. Begin Differential Display experiments and 2-D gel profiles with somatic and zygotic embryo as they become available	Mo. 1-24	ONGOING
4. Begin Amino acid analysis with somatic and zygotic embryos as they become available	Mo. 3-5	ONGOING
5. Begin NMR studies of somatic and zygotic embryos	Mo. 1-3, 12-15, 17-19	COMPLETED
6. Complete 'first order' evaluation of somatic embryos (simple, reproducible). Commence detailed, multi primer'anaylsis and protein profile	Mo. 6-14	ONGOING
7. Complete 'first order' evaluation of zygotic embryos (simple, reproducible). Commence detailed, multi-primer'anaylsis and protein profile	Mo. 9-18	ONGOING
8. Based on NMR and preliminary Differential Display and amino acid analysis, design protocol changes for later evaluation	Mo. 3-9	ONGOING

5. BUDGET

State Funds	Total FY 97 $NA	6-Month Expended $ NA
Matching Funds	Original Proposal $ NA	Actual $ NA

6. Additional Information/Results/Graphics can be attached if available

Time Table for the Tasks Proposed for FY 1997 and Potential for Future Work

Title: Ensuring Fiber Supply Through Biotechnology: New Tissue Culture Tools from Gene Expressions Studies (PP97-FS-8)

Authors (Affiliation): Gerald S. Pullman (IPST), John Cairney (IPST), Jung H. Choi (GIT), Art Ragauskas (IPST)

1. Begin isolation and staging of somatic embryos
2. Begin isolation and staging of zygotic embryos
3. Begin Differential Display experiments and 2-D gel profiles with somatic and zygotic embryos as they become available
4. Begin Amino acid analysis with somatic and zygotic embryos as they become available
5. Begin NMR studies of somatic and zygotic embryos.
6. Complete 'first order' evaluation of somatic embryos (simple, reproducible). Commence detailed, multi primer'anaylsis and protein profile
7. Complete 'first order' evaluation of zygotic embryos (simple, reproducible). Commence detailed, multi-primer'anaylsis and protein profile
8. Based on NMR and preliminary Differential Display and amino acid analysis, design protocol changes for later evaluation.
9. Continue analyses, evaluatilng different genotypes of differeing embryogenic potential and integrate results with tissue culture program.
10. Analyse the data to produce a detailed molecular description of events during somatic and zygotic embryogenesis of Loblolly Pine..

Months	1	3	6	9	12	15	18	21	24	Estimated Cost in $
Tasks										
1	xxxxx				xxxxx					
2	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx									NA
3	xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx									NA
4		xxxxx		xxxxx			xxxxx			NA
5	xxxxx			xxxxxx		xxxxx				NA
6			xxxxxxxxxxxxxxxxxxxxx							NA
7				xxxxxxxxxxxxxxxxxxxxxxx						NA
8		xxxxxxxxxxxxxxxx								NA
9				xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx						NA
10				xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx						

Potential for Future Work:
Design a continually upgraded tissue culture program which is continually monitored by molecular methods.
Streamline methods for easy 'export' to industrial and academic forest biology laboratories

Clones from Somatic Embryos

LPS clones, arranged according to stage of expression.

Clone name	Appearance at stages								GeneBank match (accession number)
	S	3	4	5	6	7	8	9	
LPS075	*								tXET-b1 mRNA^{H}, *L. esculentum*, X82685
LPS076	*								
LPS045	*	*							
LPS046	*	*							
LPS047	*	*							
LPS048	*	*							
LPS006, 007, 008, 009	*	*	*						
LPS028	*	*	*						
LPS029	*	*	*						-
LPS031	*	*	*						
LPS010	*	*	*	*	*				T-cell receptor^{L} J, human, S80858
LPS011	*	*	*	*	*				
LPS042	*	*	*	*	*				
LPS044	*	*	*	*	*				
LPS052	*	*	*	*	*				
LPS026	*	*	*	*	*	*			
LPS030	*	*	*	*	*	*			
LPS056	*	*	*	*	*	*			
LPS058	*	*	*	*	*	*			
LPS059	*	*	*	*	*	*			cDNA clone 2897s^{L}, *Pinus taeda*, H75243
LPS062	*	*	*	*	*	*			
LPS063	*	*	*	*	*	*			
LPS064	*	*	*	*	*	*			
LPS082	*	*	*	*	*	*			
LPS024	*	*	*	*	*	*	*		
LPS025	*	*	*	*	*	*	*		
LPS060	*	*	*	*	*	*	*	*	XET1^{H}, *Tropaeolum majus*, L43094
LPS061	*	*	*	*	*	*	*	*	
LPS084	*	*	*						
LPS085	*	*	*						mRNA for α-tubulin^{H}, *P. amygdalus*, X67162
LPS078		*							60S ribosomal protein L35^{H}, rat, P17078
LPS027		*	*	*					
LPS057		*	*	*					
LPS083		*	*	*					
LPS012		*	*	*	*				

LPS013			*	*	*	*			
LPS014			*	*	*	*			
LPS077			*	*	*	*	*		
LPS053		*	*	*	*	*			rab7b mRNA[M], *N. tabacum*, L29275
LPS054		*	*	*	*	*			
LPS066		*	*	*	*	*	*	*	*
LPS079			*	*					
LPS089			*	*	*				
LPS090			*	*	*				
LPS065			*	*	*	*			Embryonic ectoderm development protein mRNA[L], *Mus musculus*, U97675
LPS041			*	*	*	*			-
LPS088				*					
LPS093				*					Serine kinase mRNA[H], human, U09564
LPS095, 096				*					
LPS069				*	*	*			
LPS070				*	*	*			
LPS086				*	*	*			
LPS087				*	*	*			
LPS091				*	*	*	*	*	*
LPS092				*	*	*	*	*	*
LPS055				*	*	*	*	*	*
LPS094					*				
LPS037				*	*	*	*		
LPS049					*	*	*		
LPS050					*	*	*		
LPS038, 039					*	*	*		
LPS040					*	*	*		
LPS080						*			
LPS067			*	*	*	*	*	*	
LPS020					*	*	*	*	*
LPS081							*		
LPS036			*	*	*	*	*	*	
LPS071						*	*	*	*
LPS072						*	*	*	*
LPS032		*	*	*	*	*	*	*	*
LPS043				*	*	*	*	*	*
LPS068				*	*	*	*	*	*

LPS073	*	*	*	*	*	*	Expressed tag[H], *A. thaliana*, ATEST958
LPS074	*	*	*	*	*	*	
LPS015, 016, 017, 033		*	*	*	*	*	
LPS021		*	*	*	*	*	
LPS022		*	*	*	*	*	
LPS023		*	*	*	*	*	
LPS018			*	*	*	*	
LPS019			*	*	*	*	
LPS034			*	*	*	*	
LPS035			*	*	*	*	
LPS051			*	*	*	*	
LPS001, 002				*	*	*	Cyt B6-F Fe-S subunit[M], *Cyanobacterium nostoc*, P14698
LPS004				*	*	*	
LPS005				*	*	*	
LPS003					*	*	

*. Match is labelled H if BLAST score is >400 for DNA and >200 for protein, M if score is 300-399 for DNA and 150-199 for protein, and L if score is 200-299 for DNA and 80-149 for protein.

Clones from Zygotic Embryos

Clone name	Unique batch #	PCR # (06/02/97)	Band name (05/22/97)	Appearance at stages(*, DD; +, Southern)																	
				1	2	3	4	5	6	7	8	9.1	9.2	9.3	9.4	9.5	9.6	9.7	9.8	9.9	9.10	
LPZ001	1	12 (4/21)	Z1C4-2	*	*	*	*															
LPZ002	4	22 (4/21)	Z2C4-1	*	*	*	*	*	*	*	*	*	*	*								
LPZ003	7	4-3 (4/28)	Z3C4-1			*	*															
LPZ004	9	14 (4/20)	Z3C4-4				*	*	*	*	*											
LPZ005	15	21 (4/20)	Z4C4-2				*															
LPZ006	17	10 (Ken)	Z9.2C4-1										*									
LPZ007	19	49 (4/25)	Z9.3C4-1											*	*	*	*	*	*	*	*	ERECTA receptor protein kinase[L], (1345132)
LPZ008	22	52 (4/25)	Z9.3C4-2											*	*	*	*	*	*	*	*	Lily mRNA[H] in flowering buds, (D21823)
LPZ009	26	13-4 (4/28)	Z9.3C4-3											*	*	*	*	*	*	*	*	
LPZ010	31	15-1 (4/28)	Z9.5C4-1													*						Human aorta cDNA[L], (C16240)
LPZ011	34	99 (4/25)	Z9.5C4-2													*						
LPZ012	37	18-4 (4/28)	Z9.5C4-4															*	*	*	*	
LPZ013	1	1	Z2G3-1	*	*	*	*	*	*	*	*	*	*									
LPZ014	2	2	Z2G3-1	*	*	*	*	*	*	*	*	*	*									
LPZ015	3	7	Z3G3-1		*	*	*	*														
LPZ016	5	15	Z3G3-2		*	*																Cyclic P-diesterase[M], *A. thaliana*, (e311727)
LPZ017	6	16	Z3G3-3		*	*	*	*	*													
LPZ018	7	24	Z3G3-4			*	*	*														
LPZ019	8	25	Z3G3-4			*	*	*														Ribosomal protein L5[H], rice, (P49625)
LPZ020	9	29	Z3G3-5		*	*	*	*	*	*	*	*	*									
LPZ021	10	30	Z3G3-5		*	*	*	*	*	*	*	*	*									
LPZ022	11	32	Z5G3-1		*	*	*	*														Human EST sequence[L], (F18185)
LPZ023	12	33	Z5G3-1		*	*	*	*														Similar to insulin 1[H], (C06946)
LPZ024	13	38	Z5G3-2				*	*	*													
LPZ025	14	39	Z5G3-2				*	*	*													*A. thaliana* cDNA clone[H], (T43932)
LPZ026	15	41	Z6G3-1						*	*	*	*										
LPZ027	16	42	Z6G3-1						*	*	*	*										
LPZ028	17	48	Z8G3-1	*	*	*	*	*	*	*	*											

LPZ029	19	52	Z8G3-2			*	*	*	*	*	*	*	*	*									Photosynthetic OEC[L], *S. oleracea*, (X05512)
LPZ030	20	59	Z9.5G3-1												*	*	*						
LPZ031	22	63	Z9.5G3-2												*	*	*						
LPZ032	23	65	Z9.5G3-2												*	*	*						2S stroage protein[H], *P. strobus*, (X62433)
LPZ033	24	66	Z9.6G3-1													*	*	*					
LPZ034	25	72	Z9.6G3-2												*	*	*	*	*				
LPZ035	26	77	Z9.7G3-1														*	*	*	*			
LPZ036	27	78	Z9.7G3-1														*	*	*	*			
LPZ037	28	81	Z9.9G3-1														*	*	*	*			OEC 17kD protein, *Zea. mays*, (M87435)
LPZ038	29	83	Z9.9G3-1														*	*	*	*			
LPZ039	30	84	Z9.9G3-1														*	*	*	*			Actin[L], *S. lycopersicum*, (U60481)
LPZ040	31	86	Z9.9G3-2														*	*	*	*			
LPZ041	32	87	Z9.9G3-2														*	*	*	*			
LPZ042	33	88	Z9.9G3-2														*	*	*	*			
LPZ043	35	91	Z9.10G3-1			*	*	*	*	*	*	*	*	*	*	*	*	*	*	*			
LPZ044	36	94	Z9.10G3-2											*	*	*	*	*	*	*			
LPZ045	37	96	Z9.10G3-2											*	*	*	*	*	*	*			Rac-like protein, *A. thaliana*, (S79308)
LPZ046	1	21 (5/24/97)	Z1C3-1	*	*																		
LPZ047	2	22 (5/24/97)	Z1C3-1	*	*																		
LPZ048	3	62 (5/24/97)	Z1C3-2	*	*																		ZK792.1[L], *C. elegans*, (e219740)
LPZ049	4	3	Z1C3-3	*	*	*	*	*	*	*	*	*											Starch synthase[H], *M. esculenta*, (X74160)
LPZ050	5	5	Z1C3-3	*	*	*	*	*	*	*	*	*											Starch synthase[H], *M. esculenta*, (X74160)
LPZ051	6	8	Z1C3-4	*	*																		
LPZ052	7	9	Z1C3-4	*	*																		Dynamin[H], soybean, (S63667)
LPZ053	8	11	Z5C3-1		*	*	*	*															Rice cDNA[H], (D41438)
LPZ054	9	14	Z5C3-1		*	*	*	*															
LPZ055	10	17	Z5C3-2		*	*	*	*	*	*	*	*	*	*	*	*	*	*					
LPZ056	11	20	Z5C3-2		*	*	*	*	*	*	*	*	*	*	*	*	*	*					
LPZ057	12	21	Z6C3-1					*	*	*	*												ZK792.1[L], *C. elegans*, (e219740)
LPZ058	13	24	Z6C3-1					*	*	*	*												
LPZ059	14	27	Z7C3-1						*	*													Transposon[L], *Lilium henryli*, (X13886)
LPZ060	15	30	Z7C3-1						*	*													
LPZ061	16	31	Z7C3-2		*	*	*	*	*	*	*	*	*	*	*								

LPZ062	17	32	Z7C3-2	*	*	*	*	*	*	*	*	*	*	*							
LPZ063	18	36	Z7C3-3				*	*	*	*	*										
LPZ064	19	38	Z7C3-3				*	*	*	*	*										
LPZ065	20	42	Z7C3-4					*	*	*											
LPZ066	21	44	Z7C3-4					*	*	*											Acyl-CoA-binding protein^{L}, (e286172)
LPZ067	22	48	Z7C3-5			*	*	*	*	*	*										
LPZ068	23	50	Z7C3-5			*	*	*	*	*	*										
LPZ069	24	53	Z8C3-1				*	*	*	*											
LPZ070	25	54	Z8C3-1				*	*	*	*											
LPZ071	26	56	Z9.2C3-1			*	*	*	*	*	*	*	*	*							
LPZ072	27	60	Z9.2C3-1			*	*	*	*	*	*	*	*	*							
LPZ073	28	62	Z9.2C3-2			*	*	*	*	*	*	*									
LPZ074	29	67	Z9.2C3-3									*									
LPZ075	30	69	Z9.2C3-3									*									
LPZ076	31	74	Z9.2C3-4									*									
LPZ077	32	75	Z9.2C3-4									*									
LPZ078	33	78	Z9.3C3-1										*								
LPZ079	34	79	Z9.3C3-1										*								
LPZ080	35	81	Z9.3C3-2								*	*	*	*	*	*					Acyl-CoA-binding protein^{M}, (e286172)
LPZ081	36	82	Z9.3C3-2								*	*	*	*	*	*					
LPZ082	37	86	Z9.4C3-1										*	*	*	*	*				
LPZ083	38	87	Z9.4C3-1										*	*	*	*	*				
LPZ084	39	91	Z9.4C3-2										*	*	*						Acyl-CoA-binding protein^{M}, (e286172)
LPZ085	40	92	Z9.4C3-2										*	*	*						
LPZ086	41	99	Z9.4C3-3										*	*	*	*	*	*			Acyl-CoA-binding protein^{M}, (e286172)
LPZ087	42	100	Z9.4C3-3										*	*	*	*	*	*			
LPZ088	43	101	Z9.5C3-1												*						
LPZ089	44	102	Z9.5C3-1												*						
LPZ090	45	106	Z9.5C3-2												*	*	*	*			Acyl-CoA-binding protein^{M}, (e286172)
LPZ091	46	114	Z9.5C3-3										*	*	*	*	*	*			LMW hsp^{L}, *P. menziessii*, (X92983)
LPZ092	47	116	Z9.6C3-1													*					
LPZ093	48	118	Z9.6C3-1													*					
LPZ094	49	122	Z9.7C3-1												*	*	*	*			

LPZ095	50	123	Z9.7C3-1										*	*	*	*
LPZ096	51	126	Z9.8C3-1								*	*	*	*	*	*
LPZ097	52	131	Z9.8C3-2									*	*	*	*	*
LPZ098	53	136	Z9.8C3-3	*	*	*	*	*	*	*	*	*	*	*	*	*

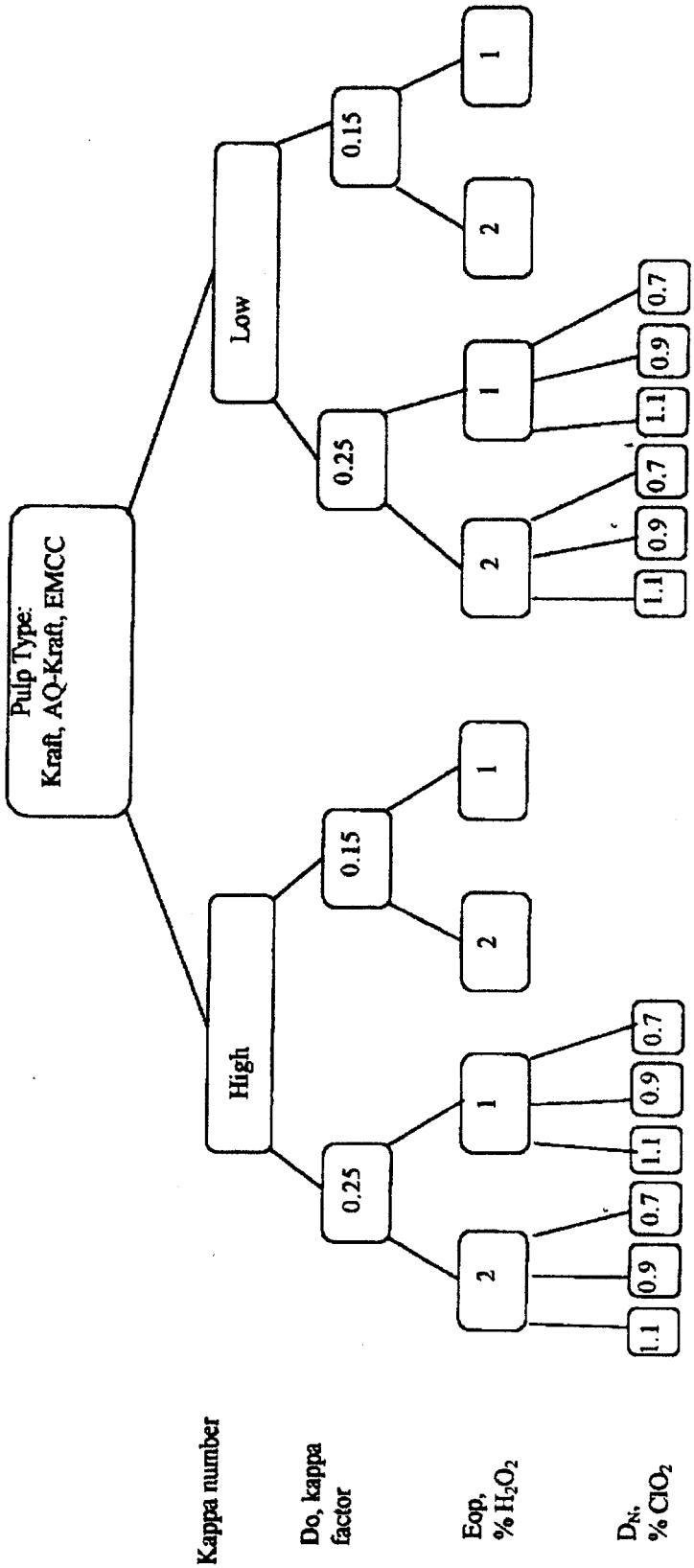

Figure 1: Conditions for D(PO)D_ND

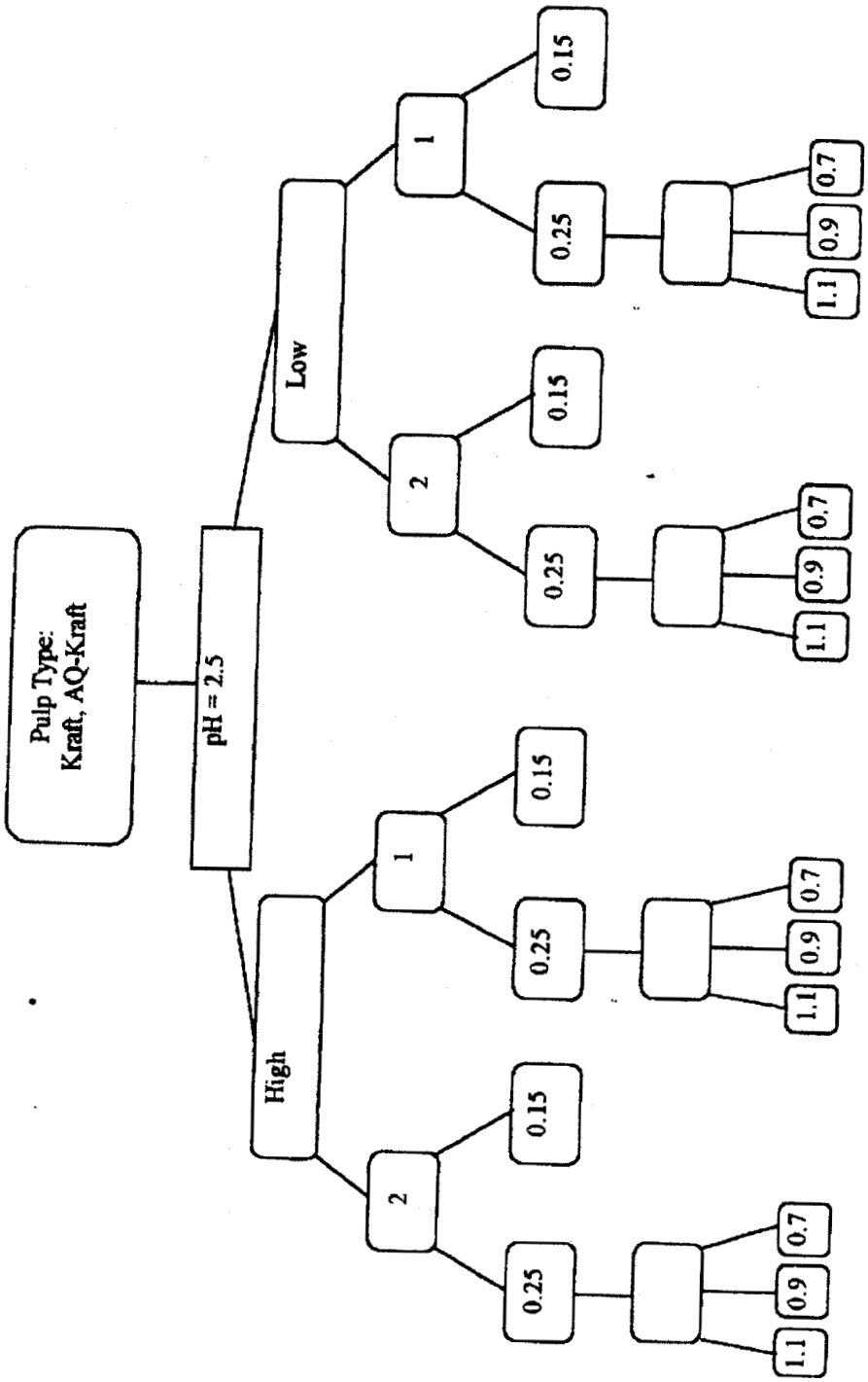

Figure 2: Conditions for A(PO)DED_N

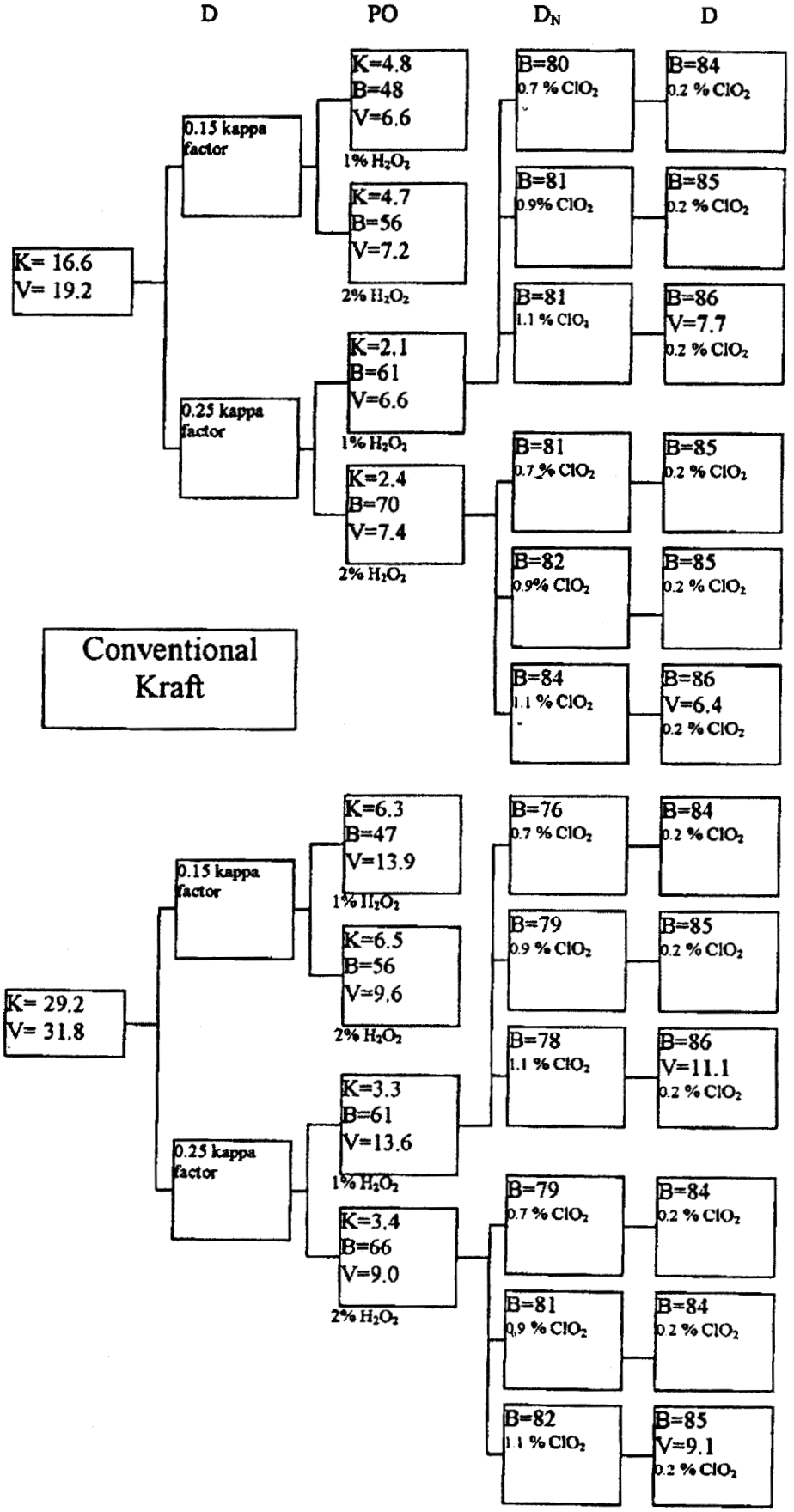

Figure 3: Results from D(PO)D_ND for conventional kraft pulp

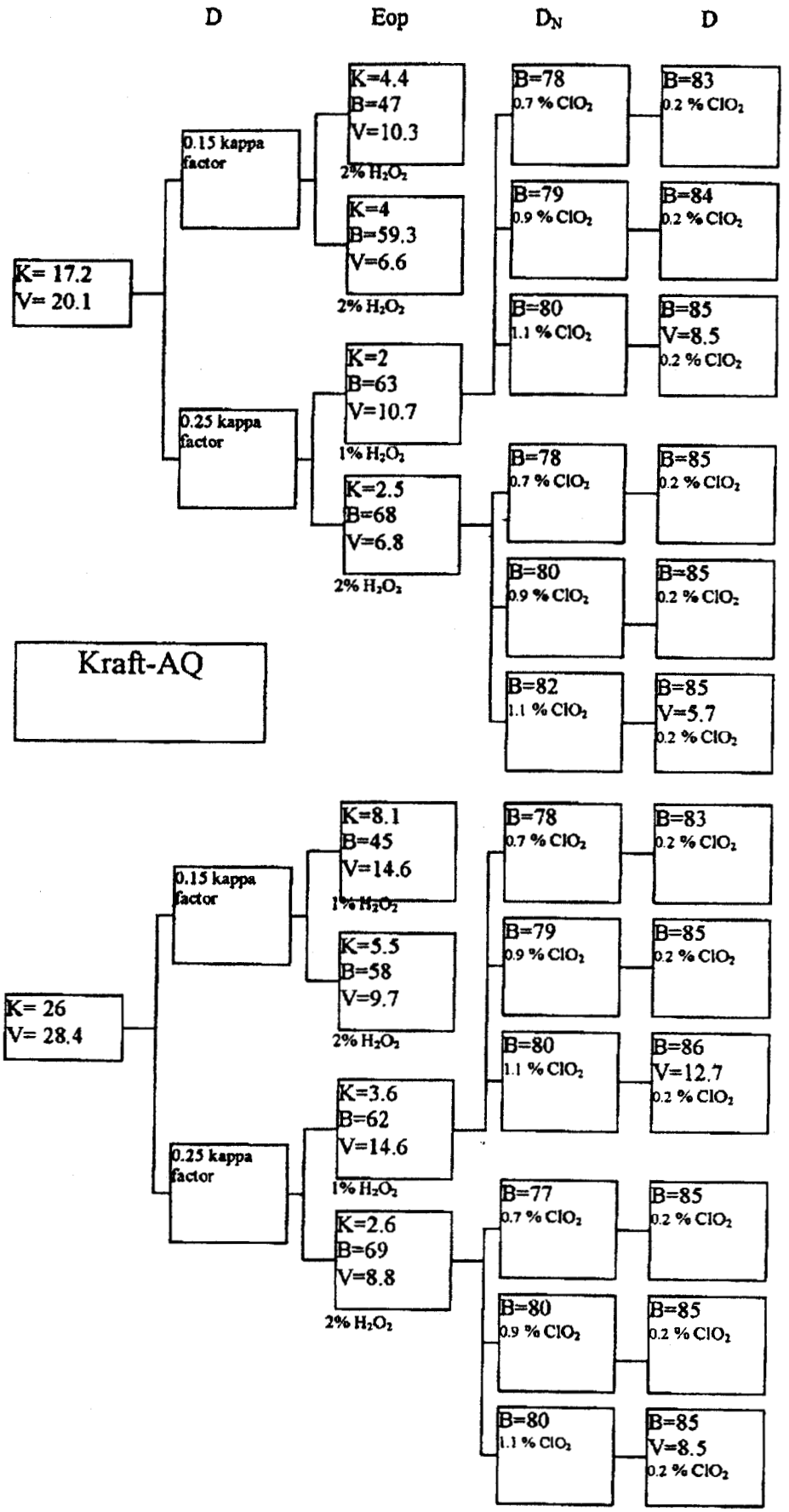

Figure 4: Results from D(PO)D_ND for kraft-AQ pulp

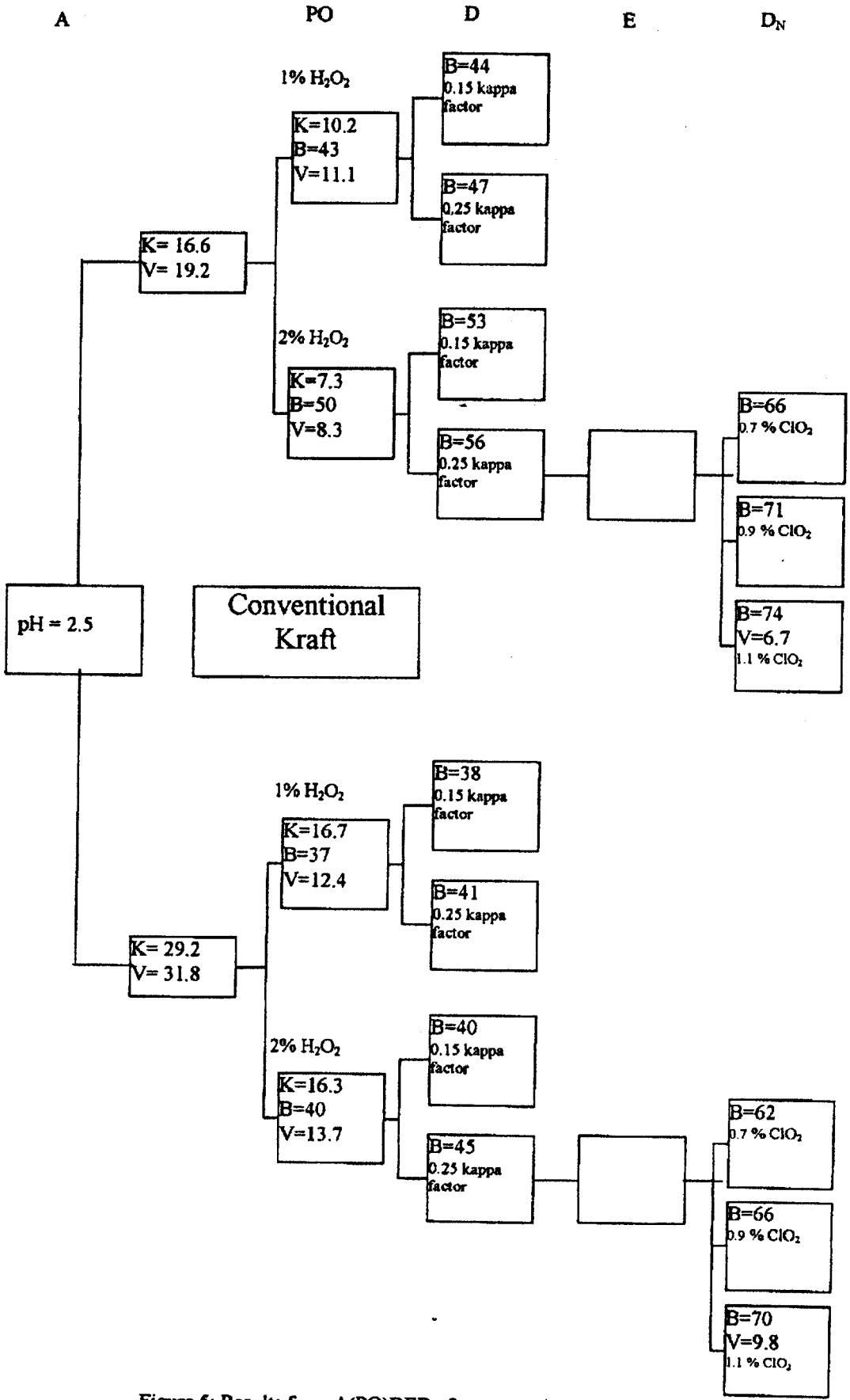

Figure 5: Results from A(PO)DED_N for conventional kraft pulp

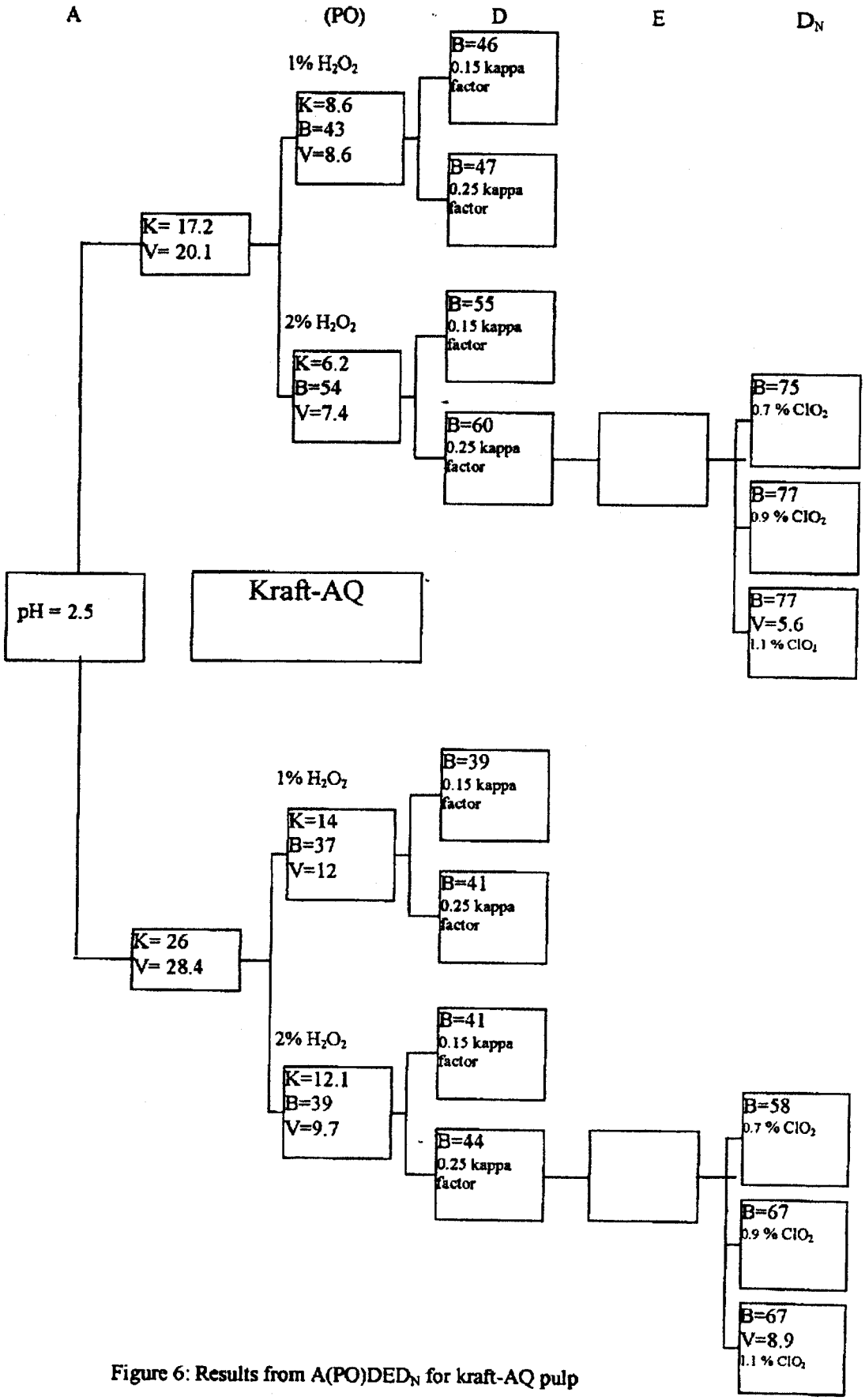

Figure 6: Results from A(PO)DED_N for kraft-AQ pulp

1511 Session 61, Seed Physiology

Enhanced gene transcription of thiol protease during solid matrix priming and germination of loblolly pine seeds. Wu, Luguang *Forestry Department, Oklahoma State University* Huang, Yinghua *Forestry Department, Oklahoma State University* Hallgren, Stephen W. *Forestry Department, Oklahoma State University* Ferris, David M. *Forestry Department, Oklahoma State University*

Thiol protease gene expression can be either developmentally regulated during seed germination or induced by environmental stress in vegetative tissues. Solid matrix priming (SMP), a controlled water stress treatment, can improve seed germination performance, but thiol protease inhibitors can negate SMP effects. The objectives of this study were to determine whether SMP can affect gene expression of thiol protease at the transcription level during SMP and subsequent germination and to determine effects of thiol protease inhibitors and of RNA/protein synthesis inhibitors on transcript accumulation of thiol protease genes in loblolly pine seeds. Northern blot showed a thiol protease gene transcript (1.5 Kb) in embryo peaked at day 2 in a period of 6 d priming. The transcript accumulated more rapidly in primed compared with unprimed seeds once the seed was placed under conditions favorable to germination. The transcript was not detected in the megagametophyte during priming and subsequent germination. When treated with thiol protease inhibitors such as E64, $CuCl_2$and $HgCl_2$before priming, the seeds had the same level of transcript accumulation as the control. Either the RNA synthesis inhibitor (alpha-amanitin) or the protein synthesis inhibitor (cycloheximide) suppressed the gene transcript accumulation of thiol protease. The results imply: 1) SMP promotes thoil protease gene expression at the transcription level, 2) this regulation probably occurs in the embryo rather than megagametophyte, 3) thiol protease inhibitors have no negative effects on the transcript accumulation though they negate SMP effects on germination, and 4) newly synthesized protein during SMP might be involved gene transcription of thiol protease.

1512 Session 61, Seed Physiology

Commercial-scale desiccation and storage of spruce somatic embryos. Eastman, P. Ann K. *BC Research Inc., Vancouver, BC* Stodola, Tom F. *Silvagen Inc., Vancouver, BC* Comeau, Mamta *Silvagen Inc., Vancouver, BC* Percy, Robin E. L. *BC Research Inc., Vancouver, BC*

Maintenance of somatic embryo viability and quality during extended storage is an essential requirement for commercial nursery production of somatic conifer seedlings. A patented somatic embryo drying treatment (Canadian Patent # 2,064,697; US Patent # 5,183,757) yields high-quality spruce (*Picea glauca* x *engelmannii* complex) somatic seedlings; however, this process is not adequate for long-term embryo storage, nor is it readily amenable to automation. This patented treatment is being refined to develop a commercial-scale process for conditioning the somatic embryos to survive long-term storage without loss of quality. Current research indicates that embryos desiccated to low relative water contents, 0.15 -0.20, (0.34- 0.40 g H_2O g^{-1}fm) are capable of germinating to yield high-quality somatic seedlings. Preliminary assessments of embryos subjected to drying treatments and then stored for extended periods at ambient or low temperatures indicate that this system has significant potential for commercial-scale conifer somatic seedling production. (Research funded by the British Columbia Ministry of Forests, IRAP-NRC, Silvagen Inc., British Columbia Research Inc.)

1513 Session 61, Seed Physiology

Desiccation and Cryopreservation of Spruce Somatic Embryos. Percy, Robin E.L. *BC Research Inc., Vancouver, BC* Livingston, N.J. *University of Victoria, Victoria, BC* von Aderkas, P. *University of Victoria, Victoria, BC*

The effects of drying and cryopreservation on the survival of spruce (*Picea glauca* and *Picea glauca* x *engelmannii*) somatic embryos were investigated. This work was undertaken to develop a simple, quantifiable technique for drying and storage of mature conifer somatic embryos. The specific research objectives were to: (i) characterize water release from somatic embryos by drying over salt solutions of known water potential (psi), (ii) determine whether embryos could be desiccated in dry air to low relative water contents (RWC) without loss of viability, and (iii) determine whether embryos dried to defined RWC could be frozen directly in liquid nitrogen (LN_2), without cryoprotectants, and remain viable after thawing and rehydration. Our results show that mature somatic embryos survive drying to bound water, and subsequent freezing in LN_2. No cryoprotectant additives or preculture steps were required. For mature conifer somatic embryos, this is the first report of survival following exposure to LN_2. Somatic embryos survived drying to a RWC of approximately 0.13 (or 0.28 g H_2O g^{-1}fm), similar to the water content of spruce zygotic embryos from dry seed (0.325 to 0.365 g H_2O g^{-1}fm). This suggests that given the appropriate storage conditions, somatic embryos dried to bound water might survive extended dry storage. Highest survival after freezing in $LN_2$was in those embryos pre-dried to psi of -15 to -20 MPa, which yielded RWC close to bound water values. Minimal or no survival after freezing was observed in embryos pretreated at higher psi or at very low psi (silica gel). This work provides a basis for development of reliable commercial-scale storage protocols. (Graduate research partially funded by NSERC)

1514 Session 61, Seed Physiology

Treatments effective in terminating the dormancy of yellow-cedar seeds. Schmitz, Nancy *Simon Fraser University* Xia, Jian-Hua *Simon Fraser University* Kermode, Allison R. *Simon Fraser University*

Following dispersal from the parent tree, seeds of yellow-cedar (*Chamaecyparis nootkatensis*) exhibit a low capacity for germination as a result of a deep dormancy mechanism. Only a low percentage of seeds will germinate the first year after seed dispersal; the remainder require an additional year to meet their stratification requirements. Effects of seed coat and megagametophyte removal on germination of immature yellow-cedar embryos indicate that the former may be more important in supplying factors which block germination. Germination of the mature (intact) seed requires several months of cold stratification; however, the requirement for cold stratification can be reduced considerably (to 60 days), if preceeded by a 2-d GA_3treatment. We have initiated studies to further shorten the requirement for cold stratification and to investigate the underlying basis of dormancy maintenance in yellow-cedar seeds. The dormancy breaking protocols incorporate treatments that target either a decline in ABA levels or embryo sensitivity to ABA (re-drying and fluridone), manipulate water uptake (rapid imbibition and PEG), or enhance the GA_3-promotive effect (GA_{4+7}, cytokinin and red light). The protocols will be discussed in relation to their effectiveness in inducing germination and post-germinative growth. Northern and Western blot analyses will be presented to analyse the fate of several markers of development and post-germinative growth. Ultimately, we wish to address the question: Is dormancy of yellow-cedar seeds maintained by a heightened sensitivity of the embryo to ABA? Further, are there other factors that contribute to dormancy maintenance, such as low levels of endogenous GA?

1515 Session 61, Seed Physiology

Is dormancy inception in yellow cedar seeds a consequence of embryo immaturity? Xia, Jian-Hua *Department of Biological Sciences, Simon Fraser University, Burnaby, B.C., V5A 1S6, Canada* Kermode, Allison R. *Department of Biological Sciences, Simon Fraser University, Burnaby, B.C., V5A 1S6, Canada*

Development of yellow cedar seeds is completed by about 17 to 21 months after pollination. Following dispersal from the parent plant, the seeds exhibit a low capacity for germination as a result of deep dormancy. Only a low percentage of seeds will germinate the first year after seed dispersal. The remainder require an additional year to meet their stratification requirement. We have initiated studies to investigate the underlying basis of dormancy inception in yellow cedar seeds. These studies address whether a state of dormancy is imposed upon the seeds because the embryo is immature at the time of seed shedding and hence requires a period of time to complete critical developmental events. We examined the accumulation of major soluble and insoluble storage protein reserves in the embryo and megagametophyte at different times during development. Major protein reserves of yellow-cedar seeds are the buffer-insoluble crystalloid storage proteins and the water-soluble albumin proteins. SDS-PAGE and quantitative analysis of the insoluble and soluble protein fractions showed that the greatest increase in both fractions occurred between the first and the second years of development, and that deposition was largely completed in seeds of second year cones by August, 2-3 months prior to seed dispersal. Fluorographs of soluble proteins synthesized in vivo showed a continued synthesis of developmental proteins at the time of seed shedding. Northern blot analyses to detect changes in storage protein mRNAs during development will also be presented. Two ER-resident proteins associated with maturation of storage proteins (PDI and BiP) were most abundant in seeds of first year cones. Accumulation of proteins implicated in the acquisition of desiccation tolerance was also examined.

1516 Session 61, Seed Physiology

Differential Display as a Tool to Monitor Embryo Development in Loblolly Pine. Xu, Nanfei *Institute of Paper Science and Technology* Ciavatta, Vincent *Institute of Paper Science and Technology* Johns, Babara *Institute of Paper Science and Technology* Pullman, Gerald *Institute of Paper Science and Technology* Cairney, John *Institute of Paper Science and Technology*

Differential Display as a Tool to Monitor Embryo Development in Loblolly PineNanfei Xu, Vincent Ciavatta, Babara Johns, Gerald Pullman and John CairneyInstitute of Paper Science and Technology, Forest Biology Group, 500 10th Street NW, Atlanta, GA 30318Loblolly pine is an important fiber source for paper industry. High quality and fast growing trees are needed to sustain our growing demands on paper. However, the propagation of a desirable tree to commercial scale through seeds is slow and many years are required. A promising alternative is to propagate a tree through somatic embryos. Although somatic embryos can now be produced through plant tissue culture, the efficiency is not high enough for commercial use. This low efficiency is likely due to the different developmental conditions of the somatic embryos, which do not totally mimic the ovule of the zygotic embryos. Currently, no molecular markers are available to follow embryo development. We have employed differential display to follow gene expression during the development of somatic and zygotic embryos. Using a solid-phase RNA differential display technique, we

were able to generate a clear band pattern using just a few embryos, and have identified many short sequences that change in abundance at different stages of somatic and zygotic embryo development. We have cloned and sequenced a number of these cDNAs and compared their expression in different genotypes. Here we report the characterization of these sequences and the isolation of their corresponding genes.

1517 Session 61, Seed Physiology

Triacylglycerol and carbohydrate metabolism in loblolly pine (*Pinus taeda* L.) seeds during germination and early-seedling growth. Stone, Sandra L. *University of Alberta, Department of Biological Sciences, Edmonton, AB, T6G 2E9* Gifford, David J. *University of Alberta, Department of Biological Sciences, Edmonton, AB, T6G 2E9*

The main reserves in mature, desiccated loblolly pine (*Pinus taeda* L.) seeds are triacylglycerols (TAGs), which are stored in lipid bodies. During germination and early-seedling growth, TAGs are broken down and converted into carbohydrates that the seedling uses as carbon and energy sources for growth. Like other conifer seeds, loblolly pine embryos are enveloped by the megagametophyte, a tissue that stores the majority of the seed's reserves. Over 80% of the mature, desiccated seed's TAGs are stored in the megagametophyte. Following seed imbibition, the initiation of TAG breakdown is approximately the same in the megagametophyte and seedling. However, the most rapid rate of TAG breakdown occurs in the radicle and hypocotyl of the seedling. Soluble carbohydrate levels in the seedling decrease during germination, then begin to rise after radicle emergence when TAG breakdown is occurring. In the megagametophyte, the soluble carbohydrate level decreases until two days after radicle emergence, after which the level increases. Our data shows that soluble carbohydrates are exported from the megagametophyte to the seedling during germination and early-seedling growth. The timing of TAG breakdown and carbohydrate appearance in the different seed parts of loblolly pine will be discussed and compared to other germinating Pinaceae systems.

1518 Session 61, Seed Physiology

Utilization of arginine, a key amino acid in loblolly pine during germination and early seedling growth. King, Janice E. *Department of Biological Sciences, University of Alberta, Edmonton AB T6G 2E9* Gifford, David J. *Department of Biological Sciences, University of Alberta, Edmonton AB T6G 2E9*

The major seed storage proteins in the megagametophyte of loblolly pine (*Pinus taeda* L.) account for 70% of the total protein in the mature seed. Arginine makes up approximately 45% of the nitrogen in these storage proteins, and is a major amino acid in the free amino acid pools of both the megagametophyte and the seedling during early seedling growth. One route by which free arginine is integrated into metabolic and biosynthetic pathways in the loblolly pine seedling during this stage of development is via arginase, an enzyme of the urea cycle that catalyzes the conversion of arginine to ornithine and urea. Arginase activity is located mainly in the cotyledons and shoot apex, and increases with the increase in free arginine levels in the seedling. The megagametophyte is required for both the induction and maintenance of arginase activity in the seedling. Arginase activity in seedlings cultured in the absence of megagametophytes can be partly induced by incubation with 100 micromolar arginine. Blocking the metabolism of urea - one of the products of arginase action on arginine - with the urease inhibitor phenylphosphorodiamidate results on a marked decrease in arginase activity. These data suggest that arginase is regulated by both feed forward activation and feedback inhibition. To allow us to further examine the regulation of arginase expression, the enzyme is currently being purified from loblolly pine seedlings.

1519 Session 61, Seed Physiology

Mineral nutrition in seeds and somatic embryos of white spruce (*Picea glauca*). Reid, Daryl A. *Department of Biology, McMaster University, Hamilton, Ontario, Canada, L8S 4K1* Lott, John N.A. *Department of Biology, McMaster University, Hamilton, Ontario, Canada, L8S 4K1* Attree, Stephen M. *Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 5E2* Fowke, Larry C. *Department of Biology, University of Saskatchewan, Saskatoon, Saskatchewan, Canada, S7N 5E2*

Somatic embryos produced in tissue culture are larger but morphologically very similar to spruce zygotic embryos. Energy dispersive x-ray analysis was performed on globoids (phytate deposits in seed protein bodies) and Fe-rich particles (in plastids destined to become chloroplasts) in several regions of seeds and somatic embryos. Globoids in all regions were very similar, containing P, K, Mg and traces of Ca, Mn, Fe, and Zn. However, globoids in the zygotic embryo cotyledon procambium had significantly higher Fe levels than did globoids in the same tissue of somatic embryos. Fe-rich particles contained higher Fe and lower P, K, Mg and Zn than globoids. Fe-rich particles had similar composition regardless of location. Total P and phytic-acid P were determined using a molybdenum blue colorimetric reaction and anion exchange resins. Somatic embyros contained less P (mg per g tissue) than either zygotic embyros or combined zygotic embryo and female gametophyte. Each somatic embryo had slightly more ug P than a zygotic embyro but had 7.4 times less P than a combined zygotic embryo and female gametophyte, which are the normal nutrient storage tissues of a seed. Each somatic embryo contained half as much phytic-acid P as a zygotic embryo and had 57.4 times less phytic-acid P than a combined zygotic embryo and female gametophyte. Even though somatic and zygotic embryos of spruce are structurally similar, they certainly differ in their mineral nutrient stores. This research was funded by a grant from the Natural Sciences and Engineering Research Council of Canada.

1520 Session 61, Seed Physiology

Mobilization of soybean seed reserve protein is by a subtilisin-like protease. Liu, Xiaowen *State University of New York at Binghamton* Tian, Zhen *State University of New York at Binghamton* Wilson, Karl *State University of New York at Binghamton* Gal, Susannah *State University of New York at Binghamton* Tan-Wilson, Anna *State University of New York at Binghamton*

The mobilization of the beta-conglycinin storage protein reserves in soybean seeds during germination and early growth begins with the proteolysis of its alpha and alpha', but not its beta subunits. The enzyme responsible for initiating this proteolysis is protease C1, a 70 kD serine protease that cleaves only at sites that are characterized by the presence of multiple Glu and Asp residues. Starting from RNAs isolated from developing seed and seedling cotyledons, and PCR using degenerate primers, we have found a 1.15 kb partial cDNA clone that bears three of the six peptide sequences that we had obtained from fragments of purified enzyme. With an estimated 60% of the coding region sequenced, the closest match was to cucumisin, a subtilisin-like enzyme from the fruit of the muskmelon. Aligning the sequences, we were able to identify the active site serine and histidine residues typical of serine proteases. Unlike protease C1, cucumisin exhibits a very broad cleavage specificity. Despite the fact that the subtilisins comprise the second largest family of proteolytic enzymes, there are only seven representatives from the plant kingdom, from *Arabidopsis*, Norway spruce, alder, tomato, and lily. The sequence of the cDNA clone of protease C1 matched up to all of these and to those of the bacterial subtilisins. We will be using the partial clone as a probe to study protease C1 gene expression in the cotyledons of the developing seed and seedling.

1521 Session 61, Seed Physiology

Role of calcium in regulation of proteolysis during early mung bean growth. Singh, Sushmita *State University of New York at Binghamton* Perzova, Vera *State University of New York at Binghamton* Wilson, Karl *State University of New York at Binghamton* Tan-Wilson, Anna *State University of New York at Binghamton*

Carboxypeptidase1 (Cpase1), a serine protease that is present in dry mung beans seeds, declines in activity during the first eight days of germination and early growth. Removal of the embryonic axis at 24 h after the start of imbibition results in the retention of high Cpase1 activity. Treatment of detached cotyledons with 10mM $CaCl_2$results in a nearly normal decline of Cpase1 activity. Calcium chelating agents like TMB-8 and EGTA overcome the effect of exogenously added calcium, as does 10 mM lanthanum. Treatment with 1 mM nicardipine, verapamil and diltiazem, blockers of L-type voltage-gated calcium channels, also block the restoration of normal patterns due to calcium supplementation. Restoration of Cpase1 decline is closest to normal when calcium is supplied starting from day 1 or even during seed imbibition with water. Supplementation with calcium for only one or two days has an effect but does not fully restore the normal pattern. Rather, a continuous supply for the eight days resulted in maximum restoration of the normal pattern of Cpase1 activity. Western blot studies indicate that the loss of Cpase1 activity in intact and calcium treated samples is mainly due to protein degradation. Preliminary results show the existence of a Cpase1-degrading activity with an acidic pH optimum.

1522 Session 61, Seed Physiology

Subcellular Fractionation and Characterization of the Proteolytic Activity in Douglas-fir Seeds Following Stratification and Germination. Forward, Benjamin S. *Department of Biochemistry and Microbiology, University of Victoria* Tranbarger, Timothy J. *Department of Biochemistry and Microbiology, University of Victoria* Misra, Santosh *Department of Biochemistry and Microbiology, University of Victoria*

To identify proteinases involved in the mobilization of storage protein reserves during Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) seed germination and early seedling development, proteolytic activity in protein extracts from mature seeds, stratified seeds, 2 day and 7 day old seedlings was examined using a gelatin-SDS-PAGE assay. Following stratification, four distinct bands of proteolytic activity, with approximate molecular masses of 47.5, 83, 175 and 175 kDa, were detected in seed extracts. In 2 and 7 day old seedlings the 47.5 kDa proteolytic band was not detected whereas the 83, 175 and 175 kDa proteolytic bands were detected at much lower levels. These observations suggest that