

UNIVERSIDADE ESTADUAL DE CAMPINAS



Henrique Pessoa dos Santos

IMPORTÂNCIA ECOFISIOLÓGICA DA RESERVA DE XILOGLUCANO E O CONTROLE DE SUA MOBILIZAÇÃO EM COTILÉDONES DE *Hymenaea courbaril* L.

Este exemplar corresponde à redação final
da tese defendida pelo(a) candidato (a)
HENRIQUE PESSOA DOS SANTOS
e aprovada pela Comissão Julgadora.

Tese apresentada ao Instituto de
Biologia da Universidade Estadual de
Campinas, para obtenção do Título de
Doutor em Biologia Vegetal.

Orientador: Marcos Silveira Buckeridge

2002

UNICAMP
BIBLIOTECA CENTRAL

UNICAMP
BIBLIOTECA CENTRAL
SEÇÃO CIRCULANTE

UNIDADE DE
 Nº CHAMADA T/UNICAMP
Sa 59i
 V _____ EX _____
 TOMBO BCI 50568
 PROC 16-837/02
 C _____ DX _____
 PREÇO R\$ 11,00
 DATA _____
 Nº CPD _____

CM00172312-8

BIB 10 252436

**FICHA CATALOGRÁFICA ELABORADA PELA
 BIBLIOTECA DO INSTITUTO DE BIOLOGIA – UNICAMP**

Sa59i

Santos, Henrique Pessoa dos

Importância ecofisiológica da reserva de xiloglucano e o controle de sua mobilização em cotilédones de *Hymenaea courbaril* L./

Henrique Pessoa dos Santos. –

Campinas, SP: [s.n.], 2002.

Orientador: Marcos Silveira Buckeridge

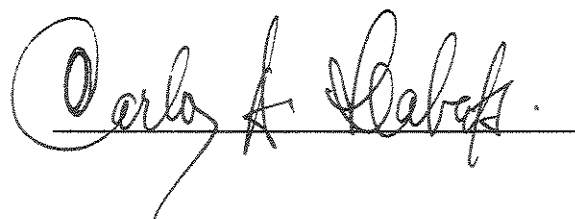
Tese(Doutorado) – Universidade Estadual de Campinas . Instituto de Biologia.

1. Xiloglucano. 2. *Hymenaea*. 3. Ecofisiologia. 4. Auxina. 5. Parede celular. I.Buckeridge, Marcos Silveira. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

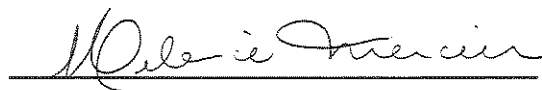
Data da defesa: 03 / 06 / 2002

Banca Examinadora:

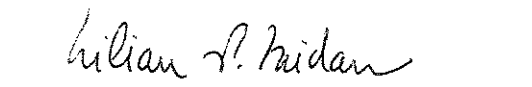
Prof. Dr. Carlos Alberto Labate



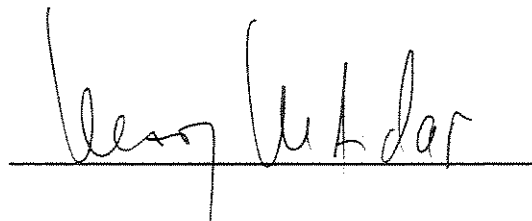
Prof. Dra. Helenice Mercier



Prof. Dra. Lilian Beatriz Zaidan



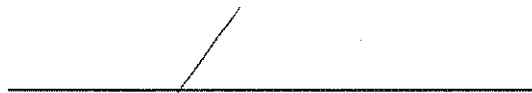
Dr. Marcos Pereira Marinho Aida



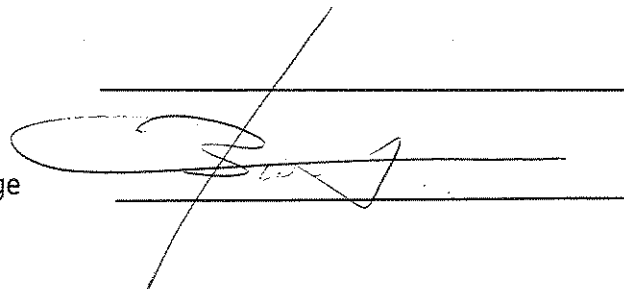
Prof. Dr. Lázaro Eustáquio Pereira Peres



Prof. Dr. Ivany F. M. Válio



Prof. Dr. Marcos Silveira Buckeridge



00239430

AGRADECIMENTOS

Ao Marcos Silveira Buckeridge pela amizade, orientação, dedicação e ensino, que sempre serão lembradas ao longo de minha vida profissional.

À Juliana pelo amor, carinho, compreensão e companheirismo, que me incentivam a romper limites, como a realização deste trabalho.

À minha família que, apesar de distante, sempre esteve comigo me incentivando na conquista dos meus ideais.

Ao Instituto de Botânica (IBt), em especial aos pesquisadores e o pessoal de apoio da Seção de Fisiologia e Bioquímica de Plantas, por permitirem a realização deste trabalho e o apoio oferecido ao longo desta jornada.

Aos meus colegas da Seção de Fisiologia e Bioquímica de Plantas (IBt), pelo apoio, críticas e amizade, em especial ao Denis, Clóvis (CI), Clóvis (CII), Cláudia, Tiné, Marília, Ana Russa, Liliana, Gustavo, Rodrigo, Marcelo, Minhoto e Paulo de Alcântara.

Ao Dr. Carlos Martinez pelos ensinamentos em avaliações fotossintéticas.

Ao pessoal do Dep. de Fisiologia (USP) e o Dep. de Farmácia de Alimentos (USP), pelo apoio e a oportunidade concedidos nas avaliações de auxina.

Aos professores do Departamento de Fisiologia Vegetal (UNICAMP), pelos conhecimentos teóricos e práticos transmitidos, os quais me auxiliaram muito na realização deste trabalho.

À Josênia, pelo apoio administrativo ao longo desta jornada.

À FAPESP, pelo suporte financeiro que tornou possível a realização deste trabalho.

ÍNDICE GERAL

ÍNDICE DE FIGURAS.....	viii
ÍNDICE DE TABELAS.....	xiv
RESUMO	xv
ABSTRACT	xvi
1.INTRODUÇÃO	1
2.REVISÃO BIBLIOGRÁFICA.....	4
2.1.Ecofisiologia do estabelecimento de plântulas.....	4
2.2.Polissacarídeos de reserva em sementes	7
2.3.Xiloglucano.....	11
2.3.1.Ocorrência, função e estrutura.....	11
2.3.2.Catabolismo de xiloglucano em paredes de reserva	15
2.3.3.Controle da mobilização de xiloglucano.....	18
3.OBJETIVOS	20
4.CAPÍTULO 1 - Cell wall polysaccharides and morphological features associated with water imbibition of seeds of <i>Hymenaea courbaril</i> L. (Leguminosae-Caesalpinioideae)	21
RESUMO	23
ABSTRACT	24
INTRODUCTION	25
MATERIAL AND METHODS	27
Plant material	27
Imbibition	27
Proportional mass and imbibition of seed parts.....	28
Extraction of water-soluble carbohydrates.....	28
Fractionation of seed coat cell walls.....	28
Separated analyses of seed coat layers	29
Neutral-monosaccharide composition of the cell wall.....	29

Linkage analysis of cell wall of seed coat.....	29
<i>In vitro</i> imbibition of water by polysaccharide composites	30
Scanning Electron Microscopy (SEM) analysis	31
RESULTS AND DISCUSSION	32
Seed imbibition.....	32
Seed morphology	33
Cell wall composition of the seed parts	34
Water imbibition by cellulose/polysaccharide composites.....	36
Physiological importance of polysaccharides in the uptake of water by seeds of <i>H. courbaril</i>	37
ACKNOWLEDGEMENTS	38
REFERENCES.....	39
5. CAPÍTULO 2 - The role of cotyledons during establishment of seedlings of <i>Hymenaea courbaril</i> L. growing under different environmental conditions	53
RESUMO	55
ABSTRACT	56
INTRODUCTION	57
MATERIAL AND METHODS	59
RESULTS.....	63
Seedling development.....	63
Dry mass	64
Root:shoot ratio	65
Seedling height	66
Leaf area	67
Photosynthesis	68
DISCUSSION	69
ACKNOWLEDGEMENTS	72
REFERENCES.....	72
6. CAPÍTULO 3 - The control of storage xyloglucan degradation in cotyledons of <i>Hymenaea courbaril</i> L. resembles the auxin-induced growth of primary cell wall. 85	

RESUMO	87
ABSTRACT	88
INTRODUCTION	89
MATERIAL AND METHODS	92
Material and experimental conditions	92
Experiments and treatment descriptions.....	92
Dry mass and leaf area of seedlings.....	94
Dry mass of cotyledons and xyloglucan determination	94
Starch, sucrose and monosaccharide analyses.....	95
Determination of enzyme activities	96
Measurements of endogenous IAA.....	97
RESULTS.....	99
Storage mobilisation in the cotyledons and early seedling growth	99
Starch, soluble sugars and xyloglucan metabolism.....	100
Xyloglucan hydrolases.....	101
Endogenous auxin	101
DISCUSSION	102
Sink strength and xyloglucan mobilisation	102
Auxin and xyloglucan mobilisation	104
Synchronism and ecophysiological function	106
ACKNOWLEDGEMENTS	107
REFERENCES.....	107
7. DISCUSSÃO GERAL	119
8. BIBLIOGRAFIA (Revisão Bibliográfica & Discussão Geral)	130

ÍNDICE DE FIGURAS

Revisão Bibliográfica e Discussão Geral

- Fig. 1. Semente (*) e plântulas de *Hymenaea courbaril* var. *stilbocarpa* durante o período de mobilização das reservas cotiledonares (A), apresentado a expansão dos eófilos (a) e dos primeiros metáfilos (b) (Flores & Benavides, 1990). Plântula crescendo sob o dossel de uma mata ciliar (B), na Reserva Biológica e Estação Experimental de Moji-Guaçu (Moji-Guaçu, SP), sob radiações de 1 a 3% da radiação plena. Barra em B corresponde a uma escala de 4 cm. 7
- Fig. 2. Representação esquemática da parede celular primária (A) e das paredes de reserva com pectina (C) ou hemicelulose (D), em corte transversal. As microfibrilas de celulose estão cobertas por hemiceluloses (xiloglucanos, arabinoxilanos ou mananos), as quais podem estar forte ou fracamente ligadas à celulose (ver B). O domínio celulose-hemicelulose está embebido em um domínio péctico (A). Em C está representada a parede de reserva em que o polímero acumulado é derivado das pectinas (arabinogalactanos em sementes de *Lupinus* e de café). Em D estão representadas as paredes cujo polímero de reserva é o galactomanano ou o xiloglucano. Este modelo foi baseado no desenho apresentado por Cosgrove (1999). O domínio protéico, que normalmente representa menos do que 10% da parede celular, foi omitido. 10
- Fig. 3. Oligossacarídeos de xiloglucano de reserva obtidos a partir da digestão com celulase de *Trichoderma reesei*. A fucose, ligada à galactose, foi omitida por não estar presente em xiloglucano de reserva. Glucose (hexágono azul), xilose (hexágono vermelho), galactose (hexágono verde). Os oligossacarídeos com 4 glucoses na cadeia principal são comuns a várias espécies, mas a série com 5 glucoses (XXXXG) só foi encontrada, até então, em xiloglucano de reserva de cotilédones de *Hymenaea courbaril* (Buckeridge *et al.*, 1997). ... 13
- Fig. 4. Representação esquemática do modelo de degradação de xiloglucano em cotilédones de *Hymenaea courbaril* (A), proposto por Tiné *et al.* (2000). Neste modelo estão descritas a provável ordem de atuação das enzimas xiloglucano endo-transglicosilase (XET), β -galactosidase (β -gal), α -xilosidase (α -xil) e β -glucosidase (β -glc). Após a degradação os monossacarídeos são translocados para o meio citoplasmático, onde irão resultar em sacarose e/ou amido transitório (visto em microscopia óptica, Tiné *et al.*, 2000). A sacarose é então direcionada para os tecidos drenos do eixo em crescimento. Em B e C estão cortes transversais (420x) dos cotilédones de *Hymenaea courbaril*,

mostrando o detalhe das células antes (**B**) e depois (**C**) da mobilização de xiloglucano, corado em azul por Iodo/Iodeto de Potássio (Tiné, 1997)..... 17

Fig. 5. Categorias de plântulas baseadas no tipo de cotilédone (papiráceo, coreáceo e globóide), persistência da casca (fanerocotiledonar ou criptocotiledonar) e a posição (epígea ou hipógea). Considerando a provável combinação destes parâmetros foram estabelecidos cinco tipos de plântulas: criptocotiledonar, hipógea e reserva (CHR); criptocotiledonar, epígea e reserva (CER); fanerocotiledonar, epígea e fotossintetizante (PEF); fanerocotiledonar, epígea e reserva (PER); fanerocotiledonar, hipógea e reserva (PHR). Adaptado de Kitajima (1996), Garwood (1996) e Ibarra-Manríquez *et al.* (2001)..... 120

Fig. 6. Filtros histórico, fisiológico e biótico que determinam a composição de espécies vegetais em um determinado local. O filtro histórico se refere à distribuição, considerando as barreiras que uma determinada espécie enfrenta para atingir um determinado local. Os filtros fisiológico e biótico estão relacionados com a capacidade de aclimação e adaptação que uma espécie apresenta para enfrentar os fatores abióticos e bióticos, respectivamente, de um determinado local. Adaptado de Lambers *et al.* (1998)..... 125

Fig. 7. Comparação fenológica de plântulas crescidas, em casa de vegetação (descrição Capítulo 2), sem (A) e com (B) os cotilédones durante o período de mobilização das reservas de xiloglucano. Plântulas com 32 dias de idade, em referência ao início da embebição das sementes. A retirada dos cotilédones (seta) foi realizada quando os eófilos (a) tornaram-se visíveis (22 dias após a embebição). (b) primeiro metáfilo 127

Capítulo 1

Fig. 1. Imbibition of seeds of *H. courbaril* at different temperatures and scarification positions in the seed. Seeds were scarified at the hilum end (●, 30 °C) and at a lateral position (□, 20 °C; ■, 30 °C). The bars represent the standard deviation among 10 seeds in each point..... 43

Fig. 2. Transversal (A) and longitudinal (B) sections of seeds at different times of imbibition. The arrows represent the scarified point, which was in the lateral (A) and in the hilum position, respectively. wl = water level..... 44

- Fig. 3. Relative increase in fresh mass of seed parts of *H. courbaril* during imbibition (20°C). Seed coat (□), cotyledon (○) and embryonic axis (▲). Bars represent the standard deviation among 15 seed parts in each point. 45
- Fig. 4. Scanning electron micrographs showing details of a cross section of one seed of *H. courbaril* L.. (A) lower magnification showing the seed coat (SC) and part of cotyledon (CT); (B) detail of seed coat double layer palisade (pal), 'hourglass' cells layer (hg) and parenchymatic layer (par); (C) parenchymatic layer of the seed coat; (D) detail of cotyledon cells, which shows one out of many pit fields through which cells are linked together (pt) and the arrows show the many intercellular spaces; (E) higher magnification of cotyledon storage cell walls showing the intercellular space (a) and deposition of pectin on the microfibrils in a pit field zone between linked cells(b) and detail of a pit field (pt)..... 46
- Fig. 5. Scanning electron micrographs showing differences among composites artificially constructed of commercial cellulose and xyloglucan extracted from cotyledons of *H. courbaril*. (A) 100 % of cellulose; (B) 100% of xyloglucan; (C) 20% of xyloglucan in cellulose; (D) 40% of xyloglucan in cellulose. 47
- Fig. 6. Time course of *in vitro* imbibition of cellulose (○), 100% xyloglucan (■), and composites of pectin:cellulose (30:70%, ●) and xyloglucan:cellulose (20:80%, ▲; 40:60%, △). The pectin and xyloglucan were both extracted from seed coat and cotyledons, respectively, of seeds of *H. courbaril*. Bars represent the standard deviation among five measurements. 48
- Fig. 7. A model for water uptake by seeds of *H. courbaril*. After scarification (SC), the water follows route A, which moves faster in the coat than enters the cotyledons, driven by pectin-rich walls (p) in the seed coat (parenchymatic layer). This behaviour promotes the expansion of seed coat (double-head arrows) and consequently induces ruptures (R) of the palisade layer, thus offering new openings to water intake. After the seed coat is saturated with water, water flows by route B, driven both through intercellular channels (c) and xyloglucan containing storage walls (x). 49

Capítulo 2

- Fig. 1. Schematic representation of development of seedlings of *Hymenaea courbaril* growing in greenhouse, growth-room and forest environments. Data represent the emergence dates of seedlings (a), eophylls (b) and first (c), second (d) and third (e) metaphylls of seedlings with and without cotyledons. The cotyledons excision was performed at 22, 24 and 27 days after the

- beginning of imbibition (black arrows). In intact seedlings fifty percent of cotyledons felt at 34 ± 2.0 , 38 ± 2.5 and 43 ± 3.4 days, respectively in greenhouse, growth-room and forest (asterisks) 75
- Fig. 2. Changes in dry mass of cotyledons (A) and total seedlings (B) of *Hymenaea courbaril*. The seedlings were growing in greenhouse, growth-room and forest with or without cotyledons, in which the excision was performed 22, 24 and 27 days after the beginning of imbibition, respectively (GH, GR and F arrows in B). The germination was 15 days after imbibition in all environments (black arrow). The cotyledons felt with an average residue mass of 278 ± 48 mg at 34 ± 2.0 , 38 ± 2.5 and 43 ± 3.4 days, respectively in greenhouse, growth-room and forest (horizontal bars in A). Vertical bars represent $\text{LSD}_{5\%}$ Tukey test ($n=5$) among treatments..... 76
- Fig. 3. Root:shoot ratio (A-C) and height (D-F) of seedlings of *Hymenaea courbaril* growing with (full symbols) or without (empty symbols) cotyledons in forest (A,D), growth-room (B,E) and greenhouse (C,F), respectively. The excision of cotyledons was performed 22, 24 and 27 days after the beginning of imbibition, in greenhouse, growth-room and forest, respectively (arrows). Vertical bars represent $\text{LSD}_{5\%}$ Tukey test ($n=5$) among treatments.. 77
- Fig. 4. Changes in leaf area of seedlings of *Hymenaea courbaril* growing with (full symbols) or without (empty symbols) cotyledons in forest (A,D), growth-room (B,E) and greenhouse (C,F), respectively. A,B and C represent the area of eophylls (circles) and of first metaphyll (triangles), in the different treatments. D, E and F represent the summation in area of all leaves present per seedlings in each treatment. The excision of cotyledons was performed 22, 24 and 27 days after the beginning of imbibition, in greenhouse, growth-room and forest, respectively (arrows). Vertical bars represent $\text{LSD}_{5\%}$ Tukey test ($n=5$) among treatments..... 78
- Fig. 5. Leaf area ratio (LAR) of seedlings of *Hymenaea courbaril* growing with (full symbols) or without (empty symbols) cotyledons in forest (A), growth-room (B) and greenhouse (C), respectively. The excision of cotyledons was performed 22, 24 and 27 days after the beginning of imbibition, in greenhouse, growth-room and forest, respectively (arrows). Vertical bars represent $\text{LSD}_{5\%}$ Tukey test ($n=5$) among treatments..... 79
- Fig. 6. Response of net photosynthesis to (A) photosynthetic photon flux density (PPFD) and to (B) internal concentration of CO_2 (C_i) by 54 days old eophylls of seedlings of *Hymenaea courbaril* growing with (full symbols) or without (empty symbols) cotyledons in growth-room. Vertical bars represent the standard deviation ($n=20$). 80

- Fig. 7. Changes in light compensation point (squares) and maximum net photosynthesis (Amax, circles) (A) and fluorescence (B) of 32, 40, 46 and 54 days old eophylls of seedlings of *Hymenaea courbaril* growing with (full symbols) or without (empty symbols) cotyledons in growth-room. The maximum net photosynthesis was measured using $200 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of PPFD and $350 \mu\text{mol.mol}^{-1}$ of CO_2 . Vertical bars represent the standard deviation ($n=20$)..... 81

Capítulo 3

- Fig. 1. Dry mass of detached and attached cotyledons (A), of whole seedlings (B) or only the shoot above the cotyledons insertions (C) and the total leaf area of seedlings of *Hymenaea courbaril*. Seedlings with cotyledons grown under control conditions (control), in the darkness or with shoot excised above the cotyledons insertions (excised). Cotyledons were detached at 19, 34 and 41 days and maintained in water. The letters at plot B refer to germination (G) and emergence (E) of seedlings and fall of cotyledons (F). Bars represent standard deviation of the mean of five replicates..... 112
- Fig. 2. Contents of xyloglucan and starch in detached cotyledons (A, B) and in cotyledons attached to the seedlings (C, D) of *Hymenaea courbaril*. The attached (legends at C) were from seedlings grown under control conditions (control), in the darkness or with shoot excised above the cotyledons insertions (excised). Cotyledons were detached at 19, 34 and 41 days and maintained in water or 10^{-6} M 2,4-D (41 days only). Bars represent standard deviation of the mean of three composed replicates 113
- Fig. 3. Specific activities of xyloglucan hydrolases in attached (A, C and E) and detached cotyledons (B, D and F) of *Hymenaea courbaril* during seedling development. The attached (legends at E) were from seedlings grown under control conditions (control), in the darkness or with shoot excised above the cotyledons insertions (excised). The detached (legends at F) were from developing seedlings at 19, 34 and 41 days and maintained in water or 10^{-6} M 2,4-D (41 days only). A, B = xyloglucan endo- β -transglycosylase; C, D = β -glucosidase; E, F = β -galactosidase. In all plots, the activities observed in attached cotyledons (control) were added as a reference. Bars represent standard deviation of the mean of three composed replicates 114
- Fig. 4. Specific activities of xyloglucan hydrolases in attached cotyledons of *Hymenaea courbaril* seedlings grown at control conditions without excision treatment (intact) and intact seedlings treated with *N*-1-naphthylphthalamic acid at $200 \mu\text{M}$ (NPA $200 \mu\text{M}$); and in shoot excised plants (excised), shoot

excised plants with light protected cotyledons (Shoot excised LPC) and shoot apex excised plants (Top shoot excised). A = xyloglucan endo- β -transglycosylase; B = α -xylosidase; C = β -glucosidase; D = β -galactosidase. In all plots, the activities observed in intact seedlings were added as a reference. Bars represent standard deviation of the mean of three composed replicates..

..... 115

Fig. 5. Concentration of endogenous indol-3-acetic acid (IAA) observed by (A) ELISA technique (Peres *et al.*, 1997) and (B) by GC-SIM-MS technique (Chen *et al.*, 1988). In A, the IAA was measured in the attached cotyledons from intact seedlings of *Hymenaea courbaril* grown in light, darkness or with excised shoot above the cotyledons insertions (excised) at 34 days. The only isolated cotyledons employed in this analysis as a reference were the ones detached at 34 days. In B, the IAA was also measured in attached cotyledons of intact seedlings of *Hymenaea courbaril* grown in light and in intact seedlings treated with *N*-1-naphthylphthalamic acid at 200 μ M (NPA 200 μ M). In the last technique, the IAA was also evaluated in shoot excised seedlings at 34 days (excised), in shoot excised seedlings with light protected cotyledons (Shoot excised LPC) and in seedlings with the shoot apex excised (Top shoot excised). Bars represent standard deviation of the mean of three composed replicates. 116

Fig. 6. Schematic representation of synchronism between xyloglucan mobilisation and shoot development in seedlings of *Hymenaea courbaril*. Auxin (IAA) is produced in expanding leaves and transported to cotyledons by polar transport, which can be stimulated by red light (1) and inhibited by *N*-1-naphthylphthalamic acid (NPA). In the cotyledons the IAA may act on the modulation of expression of the gene related to cell wall hydrolases (2); on ^+H -pump activity reducing the apoplastic pH (3) or on the establishment of vascular system (4). Following the xyloglucan (Xg) degradation (5) in storage cell walls of cotyledons by the concerted action of the hydrolases, monossacharides (Ms) are transported to the cytoplasm, where they are metabolised (6) to sucrose (Sc) and starch (St). The sucrose produced is driven mainly to growing shoot (7). In the shoot, light stimulates (8) growth (Grt), using sucrose as a carbon backbone, which results in IAA disponibility to the cotyledons and consequently stimulate the xyloglucan mobilisation..... 117

ÍNDICE DE TABELAS

Capítulo 1

Table 1 - Dry weight of seed parts in <i>Hymenaea courbaril</i> L.....	50
Table 2 - Composition of water soluble polysaccharides from cotyledons and seed coat of <i>Hymenaea courbaril</i>	51
Table 3 - Linkage analysis of Oxalate / Alkali cell wall fraction of the seed coat of <i>H. courbaril</i>	52

Capítulo 2

Table 1 - Characteristics of three different environmental conditions where the seedlings of <i>Hymenaea courbaril</i> were grown.....	82
Table 2 - Relative mobilisation rate (RMR) of the cotyledons reserves and relative growth rate (RGR) of seedlings of <i>Hymenaea courbaril</i> growing in the forest, growth-room and greenhouse conditions with and without attached cotyledons. Data are give as $\text{mg.g}^{-1}.\text{day}^{-1}$	83
Table 3 - Relative distribution of [^{14}C]-sucrose among different tissues from 35 days old seedlings of <i>Hymenaea courbaril</i> grown in growth-room. The radioactive sucrose was injected ($5\mu\text{l}$, $0.5\mu\text{Ci}$) in one cotyledon (treated) and the ^{14}C -scintillation was read after 72h. Dry tissues of root, hypocotyl and first metaphyll were submitted to an alcohol extraction (ethanol 80%, 80°C) and the ^{14}C -scintillation of insoluble material was read. The numbers represent the average of nine replicates (seedlings) \pm standard deviation.	84

Capítulo 3

Table 1 - Contents of glucose, fructose and sucrose ($\mu\text{g.mg}^{-1}$ cot) detected by HPAEC in alcohol extracts of detached and attached cotyledons of seedlings of <i>Hymenaea courbaril</i> . The attached cotyledons were from seedlings grown under control conditions (control), in the darkness or with shoot excised above the cotyledons insertions (excised) at 34 days. The detached cotyledons were isolated at 19, 34 and 41 days after beginning of seed imbibition and maintained in water or 2,4-D (10^{-6}M). All data represent the analyses of the same three samples of cotyledons used to starch determination.....	118
---	-----

RESUMO

Hymenaea courbaril é uma espécie leguminosa arbórea clímax, considerada tolerante à sombra e com uma ampla distribuição geográfica neotropical. As sementes são ricas em xiloglucano (XG), a principal reserva de carbono nos cotilédones. Esta reserva é um polissacarídeo de parede celular e o seu mecanismo de mobilização tem demonstrado ser complexo, utilizando a ação conjunta e coordenada de quatro enzimas. Os objetivos deste trabalho foram caracterizar a importância ecofisiológica de XG para o estabelecimento de plântulas de *Hymenaea courbaril* var. *stilbocarpa* e descrever o mecanismo de controle no processo de mobilização desta reserva. Os resultados demonstraram que a importância da reserva de XG vai desde a embebição das sementes até o estabelecimento de uma superfície foliar que seja capaz de manter o crescimento autotrófico no interior de uma floresta tropical. A concentração de XG apresentou uma relação inversa com a velocidade de embebição das sementes. Considerando o tamanho da semente de *H. courbaril* (aprox. 5 g), este controle evita que períodos curtos de disponibilidade de água estimulem a germinação, assim como estabelece uma proteção à dessecação após a embebição das sementes. Como fonte de carbono, o XG torna-se essencial apenas após a germinação e emergência das plântulas de *H. courbaril* (de 30 a 50 dias após o início da embebição das sementes). Os produtos de sua degradação são direcionados principalmente para a expansão dos eófilos e do primeiro metáfilo. Ao longo desse período, estas folhas estabeleceram uma atividade fotossintética capaz de tolerar radiações de até 1% da radiação solar plena (ponto de compensação de luz = $12 \mu\text{mol.m}^{-2}.\text{s}^{-1}$). A ausência de XG (com a retirada dos cotilédones antes da sua mobilização) torna-se crítica, principalmente, nas plântulas crescidas em floresta, devido à redução de 77% na área foliar total. O sincronismo entre degradação de XG e expansão foliar foi melhor caracterizado através da análise do conteúdo endógeno de auxina (ácido indol-3-acético, AIA) nos cotilédones, o qual apresentou um incremento exponencial (aprox. 8 vezes) e relacionado com a atividade das hidrolases de XG. Nessa análise, foi comprovado que o AIA vem principalmente dos eófilos em expansão, através do transporte polar de auxina. Este tipo de controle hormonal provavelmente está relacionado com o sincronismo observado entre mobilização de reservas, expansão foliar e disponibilidade de luz, o que proporciona a esta espécie um maior sucesso no estabelecimento de plântulas em ambientes sombreados de floresta. Com estes resultados levantaram-se evidências de que, em cotilédones de *H. courbaril*, tanto a reserva de XG como o seu metabolismo, são análogos ao processo de expansão celular de tecido em crescimento, o que sugere que, do ponto de vista evolutivo, este sistema seja derivado da parede primária.

ABSTRACT

Hymenaea courbaril is a climax leguminous tree species considered to be shade tolerant with wide neotropical geographic distribution. Seed cotyledons are rich in xyloglucan (XG), which is the main carbon reserve for the seedling growth. This polymer is a cell wall polysaccharide and its mobilisation mechanism, based on the co-ordinated action of four enzymes, has been shown to be rather complex. The objectives of this work were to characterise the ecophysiological importance of XG for the establishment of seedlings of *Hymenaea courbaril* var. *stilbocarpa* and to describe the control mechanism of the mobilisation process of this reserve. The results demonstrated that the importance of XG reserves range from seed imbibition up to the establishment of leaves that would be able to maintain autotrophic growth in understorey conditions. XG concentration presented an inverse relationship with the speed of seed imbibition. Considering the relatively large size of seeds of *H. courbaril* (ca. 5g), this control avoids premature initiation of germination during short periods of water availability and protect to desiccation after imbibition. As a source of carbon, XG is essential only after germination and emergence of the seedlings. (30-50 days after the start of imbibition). During this period, the products of XG degradation are directed mainly to expanding eophylls and first metaphyll. During this same period, photosynthetic activity is established and was capable to fix carbon in low light such as 1% of full sun light (light compensation point = 12 $\mu\text{mol. m}^{-2}.\text{s}^{-1}$). The absence of this reserve by excision of cotyledons before xyloglucan mobilisation becomes a critical point, mainly for seedlings growing in the forest, since in these conditions, a reduction of 77% of total leaf area was observed. This synchronism between XG degradation and leaf expansion was characterised through the analysis of the endogenous concentrations of 3-indol acetic acid (IAA) in the cotyledons, which presented an exponential increase (ca. 8 fold) in step with the rise in XG hydrolase activities. We demonstrated that, during this period, IAA is transported mainly from the expanding eophylls to the cotyledons. This type of hormonal control is likely to be related with the synchronism observed among storage mobilisation, leaf expansion and light availability, therefore providing *H. courbaril* with a powerful set of tools for establishment of its seedlings in the shaded understorey of the rain forest. These results also permit to speculate that, from the evolutionary point of view, storage XG degradation might have derived from the metabolism of primary cell walls.

1. INTRODUÇÃO

Germinação têm sido estudada extensivamente nos níveis biológico, fisiológico e bioquímico (Bewley & Black, 1994). De modo geral, esta etapa heterotrófica é crucial para o estabelecimento e sobrevivência das plantas nos diferentes ambientes. Entretanto, poucos estudos têm sido conduzidos sobre o comportamento inicial de plântulas em relação ao metabolismo e alocação de carbono (transição heterotrofia-autotrofia), principalmente enfocando a importância ecofisiológica destes processos. Nesta etapa, o suprimento de carbono para o crescimento inicial está relacionado com uma transição entre as reservas da semente e a atividade fotossintética das primeiras folhas (Maillard *et al.*, 1994). Deste modo, os tecidos fonte e os tecidos dreno de carbono mudam continuamente na plântula, mediadas pelo crescimento de novos órgãos e pelas interações com o meio ambiente, o que aumenta a complexidade nos processos de mobilização e alocação das reservas em espécies crescidas na mata.

Em plântulas de *Hymenaea courbaril*, a transição heterotrofia-autotrofia aparenta ser modulada pela reserva cotiledonar de xiloglucano, que é a principal fonte de carbono até que se estabeleça a fotossíntese das primeiras folhas. Ao contrário das reservas citoplasmáticas de amido, o xiloglucano é um polissacarídeo da parede celular que, conforme estudos efetuados até o momento, corresponde a aproximadamente 40% da massa seca da semente desta espécie e é mobilizado somente após a germinação e a emergência das plântulas (Buckeridge *et al.*, 1997; Tiné *et al.*, 2000). Até o momento, a única função atribuída aos xiloglucanos de sementes foi a de reserva. Entretanto, estudos sobre o mecanismo de hidrólise deste polissacarídeo vêm demonstrando um alto grau de complexidade (Buckeridge *et al.*, 2000a; Tiné *et al.*, 2000). Além disto, as propriedades hidrodinâmicas do xiloglucano têm sido consideradas similares às dos galactomananos (Buckeridge *et al.*, 1992), o que sugere uma função no controle da quantidade de água nas sementes durante o período de germinação. Deste modo, os xiloglucanos de sementes podem estar

desempenhando outras funções, além de reserva, conforme salientado pela teoria de transferência de funções (Buckeridge *et al.*, 2000a).

Buscando descrever a importância ecofisiológica desta reserva de xiloglucano para o estabelecimento de plântulas de *H. courbaril*, bem como salientar alguns pontos de controle nos processos de mobilização desta reserva, este trabalho está subdividido em partes distintas, porém interdependentes: a) revisão bibliográfica; b) capítulos 1, 2 e 3, escritos na forma de artigo; e c) discussão geral. No primeiro capítulo foi abordada a hipótese de que o xiloglucano exerce um controle sobre o processo de embebição da semente de *H. courbaril* pelas propriedades hidrodinâmicas que este polissacarídeo apresenta. Para testar esta hipótese foram realizados experimentos *in vivo* e *in vitro*, os quais demonstraram uma influência inversa deste polissacarídeo sobre a velocidade de embebição. Considerando estes resultados, foi elaborada uma discussão abordando a importância deste efeito para o estabelecimento inicial de plântulas de *H. courbaril*. O segundo capítulo está relacionado com a importância ecofisiológica das reservas de xiloglucano, abordando aspectos de desenvolvimento, alocação de carbono, taxa de crescimento e estabelecimento fotossintético de plântulas crescidas com ou sem a reserva de xiloglucano e em diferentes condições de temperatura e luz. Neste trabalho destacou-se uma interdependência entre o crescimento da parte-aérea e a velocidade de mobilização de xiloglucano, levando-nos a formular a hipótese de que a parte-aérea poderia estar exercendo um controle sobre a mobilização de xiloglucano através de um sinal hormonal (auxina), como descrito primeiramente por Hensel *et al.* (1991). Esta idéia da auxina estar exercendo algum efeito (direto ou indireto) também é corroborada pelo fato do modelo de degradação do xiloglucano de reserva apresentar enzimas análogas às que atacam o xiloglucano de parede primária (Buckeridge *et al.*, 2000a). Estas enzimas necessitam de um pH apoplástico ácido (<4,5), o que supõe uma ativação da bomba de prótons na membrana plasmática pela auxina, durante o período de mobilização, como descrito na teoria do crescimento ácido (Taiz & Zeiger, 1998). Deste modo, foi realizado um trabalho, no qual buscou-se testar a hipótese de que a auxina poderia estar exercendo um controle na mobilização de xiloglucano de

reserva em *H. courbaril* (Capítulo 3). Para isto foi acompanhado todo o processo de mobilização das reservas de xiloglucano, focalizando as atividades enzimáticas e a concentração de carboidratos (xiloglucano, amido e açúcares livres) e relacionando-os à concentração endógena de ácido indol-3-acético (AIA).

Na discussão geral foram abordados os aspectos mais relevantes de cada capítulo, salientando a importância ecofisiológica da reserva de xiloglucano para o estabelecimento inicial de *Hymenaea courbaril*. Nesta etapa foram também discutidos os mecanismos de controle de degradação da reserva de xiloglucano e a razão das plântulas de *Hymenaea courbaril* utilizarem uma reserva de carbono cujo mecanismo de mobilização é extremamente complexo, em comparação a outras fontes de carbono (p.ex. amido).

2. REVISÃO BIBLIOGRÁFICA

2.1. *Ecofisiologia do estabelecimento de plântulas*

A maioria da vida na terra depende das plantas para o fornecimento de alimento, combustível, madeira, forragem, oxigênio, entre outros usos. Deste modo, todos os esforços no sentido de conhecer melhor o estabelecimento das plantas são de grande importância. Em estudos de ecofisiologia, normalmente abordam-se questionamentos relacionados à sobrevivência, distribuição, abundância e a interação entre plantas ou entre plantas e outros indivíduos. Dentro desses aspectos, qual a estratégia de uma espécie para se estabelecer em um determinado local? Conforme descrito por Lambers *et al.* (1998), existem filtros que distribuem as espécies vegetais sobre as várias condições ambientais da crosta terrestre. Muitas espécies são ausentes de determinados locais por razões históricas, ou seja, nunca foram dispersadas para um determinado local. Entretanto, das espécies que chegam a um determinado local, muitas não são capazes de se estabelecerem por não apresentarem características fisiológicas apropriadas para certas condições físicas (p.ex. baixa radiação). Além disto, muitas espécies são suprimidas por fatores bióticos, tais como efeitos alelopáticos e ataque de pragas, que também delimitam o crescimento de espécies vegetais em uma determinada região. Estes filtros histórico, fisiológico e biótico, estão freqüentemente mudando e interagindo entre si, o que resulta em diversas condições de estresse para o estabelecimento de uma espécie em um determinado local.

Quando uma planta consegue se estabelecer sob uma condição de estresse, esta deve apresentar algum mecanismo de resistência. Os mecanismos variam de fuga até tolerância ao estresse, e sob três escalas distintas de resposta (Lambers *et al.*, 1998). Quando a planta recebe um estresse ela reduz o seu desempenho rapidamente (escala 1: responde em segundos ou dias, dependendo do estresse e do

metabolismo considerado), podendo ou não compensar esta perda através de ajustes morfológicos e fisiológicos individuais (escala 2: aclimatação) ou populacionais (escala 3: adaptação), o qual envolve um grande número de gerações para ser atingido.

Quando pensamos em um ambiente de floresta, estas escalas de resposta estão constantemente ocorrendo, principalmente pela grande diversidade de espécies e microambientes que lá ocorrem (Swaine, 1996). Dentre os fatores abióticos, a luz é o recurso mais limitante na distribuição demográfica de espécies em uma floresta tropical. De acordo com Whitmore (1996), na dinâmica de sucessão de uma floresta existe um contínuo de resposta à disponibilidade de luz, o qual abrange desde espécies que requerem alta radiação para germinar e se estabelecer (pioneiras), espécies que toleram uma média disponibilidade de luz (secundárias iniciais), até espécies que se estabelecem sob o dossel da floresta (clímax ou secundárias tardias), onde ocorre entre 1 a 2% da radiação solar (Canham, 1989).

De acordo com a literatura, a espécie *Hymenaea courbaril* pertence a um extremo do contínuo de respostas à luz em florestas tropicais, ou seja às espécies clímax (Paulilo & Felipe, 1998; Souza & Válio, 1999). As espécies clímax apresentam plantas que germinam, crescem, desenvolvem e sobrevivem, com menos dependência de luz, apresentando uma maior ocorrência, abundância e permanência no sub-bosque (Gandolfi *et al.*, 1995). Dentre as espécies clímax, ainda podem-se observar dois subgrupos, espécies que podem permanecer toda a sua vida no sub-bosque (típicas de sub-bosque), ou as espécies que podem crescer e se desenvolver no sub-bosque, mas que podem alcançar e compor o dossel florestal ou a condição emergente (típicas de dossel) (Gandolfi *et al.*, 1995), como é observado para a espécie *Hymenaea courbaril*.

Na última revisão do gênero *Hymenaea* (Lee & Langenheim, 1975), são descritas 14 espécies, a maior parte delas com distribuição Neotropical, estendendo-se desde o centro do México, Antilhas, até o norte da Argentina. Acredita-se que o centro de origem do grupo tenha sido na África, e o centro de diversidade na região Amazônica (Lee & Langenheim, 1975). A maioria das espécies está distribuída em

formações florestais, mas algumas ocorrem em áreas de caatinga do nordeste do Brasil e em áreas de cerrado do Brasil Central (Gibbs *et al.*, 1999). A espécie *Hymenaea courbaril* apresenta seis variedades (*courbaril*, *altissima*, *longifolia*, *villosa*, *stilbocarpa* e *subsessilis*), as quais abrangem os mais diversos tipos de ecossistemas (Lee & Langenheim, 1975), porém predominam em regiões de florestas tropicais. A variedade *stilbocarpa*, que predomina nas florestas da Mata Atlântica (Lee & Langenheim, 1975), será o objeto de estudo neste trabalho, sendo a partir de agora referida apenas como *Hymenaea courbaril*.

As espécies pioneiras normalmente apresentam plantas pequenas com cotilédones epígeos e foliáceos e com altas taxas de crescimento, porém as espécies clímax apresentam plantas maiores e com cotilédones de reserva epígeos ou hipógeos, durante a germinação (Garwood, 1996; Kitajima, 1996). De acordo com Kitajima (1994), este tipo de plântula (considerando o tipo funcional de cotilédones) apresenta também uma baixa taxa de crescimento relativo, baixos pontos de compensação à luz e elevada capacidade de sobrevivência em ambientes de floresta. Com exceção dos parâmetros fisiológicos, Flores & Benavides (1990) já descreveram as plântulas de *Hymenaea courbaril* de acordo com o último tipo funcional, ou seja, de grande porte, com cotilédones epígeos de reserva e bem adaptadas à ambientes sombreados, como pode ser observado na figura 1.

Atualmente existem vários estudos de classificação de plântulas considerando a posição (hipógea ou epígea) e o tipo (foliáceo ou reserva) de cotilédones (Kitajima, 1996; Garwood, 1996; Ibarra-Manríquez, 2001), os quais procuram caracterizar fenologicamente a posição das espécies na sucessão de florestas tropicais. Entretanto, de acordo com o nosso conhecimento, nenhum destes estudos relaciona o tipo e o metabolismo de mobilização das reservas presentes nos cotilédones com a estratégia de adaptação nos diferentes estratos de sucessão em uma floresta. A abordagem mais próxima, neste aspecto, é a associação entre tamanho de sementes e cotilédones de reservas e que, conseqüentemente, está relacionada a espécies tolerantes à sombra (Kitajima, 1994).



Figura 1 - Semente (*) e plântulas de *Hymenaea courbaril* var. *stilbocarpa* durante o período de mobilização das reservas cotiledonares (A), apresentado a expansão dos eófilos (a) e dos primeiros metáfilos (b) (Flores & Benavides, 1990). Plântula crescendo sob o dossel de uma mata ciliar (B), na Reserva Biológica e Estação Experimental de Moji-Guaçu (Moji-Guaçu, SP), sob radiações de 1 a 3% da radiação plena. Barra em B corresponde a uma escala de 4 cm.

2.2. Polissacarídeos de reserva em sementes

As Angiospermas apresentam diferentes estratégias de adaptação aos seus respectivos ambientes, entre as quais encontra-se o acúmulo de certos compostos de reserva em suas sementes. Estas substâncias são mobilizadas durante a germinação e o desenvolvimento inicial das plântulas, e seus produtos de degradação são usados

para diferentes propósitos, tais como a geração de energia e a produção de matéria prima (proteínas, ácidos nucleicos, carboidratos e lipídeos) para a construção de novas células e tecidos (Mayer & Poljakoff-Mayber, 1975).

Dentre os polissacarídeos de reserva de sementes destacam-se o amido, presente em sementes de ervilha (*Pisum sativum*) e lentilha (*Lens esculenta*) (Bewley & Black, 1994), os arabinogalactanos, presentes em sementes de *Lupinus angustifolius* (Crawshaw & Reid, 1984; Buckeridge & Reid, 1994), o xiloglucano, comum em sementes de tamarindo (*Tamarindus indica*), jatobá (*Hymenaea courbaril*) e copaíba (*Copaifera langsdorffii*) e o galactomanano em sementes de guar (*Cyamopsis tetragonolobus*), sesbania (*Sesbania virgata*, previamente chamada de *Sesbania marginata*), falso barbatimão (*Dimorphandra mollis*) e guapuruvú (*Schyzolobium parahybum*) (Reid, 1985; Buckeridge, 1988; Buckeridge & Dietrich, 1990, Buckeridge *et al.*, 1992, Buckeridge *et al.*, 2000b).

Devido ao grande número de estudos realizados com polissacarídeos de reserva de sementes principalmente durante a segunda metade do século XX, já é possível separá-los em grupos distintos (Buckeridge *et al.*, 2000a). O critério de classificação é baseado na localização desses polímeros. O amido compreende uma classe isolada de polissacarídeos de reserva que ocorre em amiloplastos, no parênquima de reserva, formando grânulos bastante característicos. Os demais polímeros citados ocorrem nas paredes celulares, em tecidos das sementes, e constituem possivelmente uma adaptação evolutiva das paredes celulares primárias que desenvolveram a capacidade de armazenar grandes quantidades de carboidratos de reserva. Esta classe de polissacarídeos é denominada Polissacarídeos de Reserva de Parede Celular (PRPC).

Os PRPC são relativamente inertes no que concerne à sua reatividade química e apresentam diferentes graus de solubilidade em água. Essas características conferem vantagens que são similares às do amido (alta compactação e baixa reatividade) e tornam possível a existência de um "compartimento celular" (a parede celular) que permite o fluxo de água com um grau de liberdade considerável, como acontece com os frutanos nos vacúolos (Buckeridge *et al.*, 2000a). Por outro lado, o

custo metabólico para produzir os PRPC é alto, pois esses compostos necessitam de um complexo sistema de biossíntese, secreção e montagem no meio extracelular (Buckeridge *et al.*, 2000a).

Enquanto o amido desempenha a função exclusiva de reserva (Bewley & Black, 1994), os polissacarídeos de reserva de parede celular têm outras funções paralelas (Buckeridge *et al.*, 2000a). Os PRPC estão associados à dureza (mananos em endospermas de sementes de palmeiras, tomate e alface), relações hídricas (xiloglucanos em cotilédones e galactomananos em endospermas de sementes de leguminosas) e no controle da expansão celular (galactanos nos cotilédones de lupino e, em menor proporção, em sementes de feijão e soja). Estas funções secundárias seriam relevantes em processos fisiológicos como amadurecimento, crescimento, desenvolvimento e senescência de frutos (Rose & Bennett, 1999). Buckeridge & Reid (1996) e Buckeridge *et al.* (2000a) propuseram que estas funções secundárias foram importantes no mecanismo evolutivo, o que conduziu as plantas a utilizarem polissacarídeos da parede celular como reservas de carbono.

Os PRPC são classificados em três grupos distintos: os mananos, os xiloglucanos e os arabinogalactanos. Esta classificação é baseada na estrutura química desses polímeros, sendo os mananos subdivididos em: mananos puros, glucomananos e galactomananos (Buckeridge *et al.*, 2000a).

Os modelos mais recentes de parede celular (McCann & Roberts, 1991; Carpita & Gibeaut, 1993) propõem que esta seja formada de três domínios independentes (celulose-hemicelulose, pectinas e proteínas). Os PRPC podem ser vistos como variações desses modelos, em que um domínio, ou um de seus polissacarídeos, tenha sido depositado em maior quantidade em relação aos demais. Os arabinogalactanos correspondem aos polímeros derivados das pectinas enquanto os mananos e xiloglucanos são depósitos de polímeros de hemicelulose (Figura 2, C e D).

Como foi descrito por Buckeridge *et al.* (2000b), em muitos casos o processo de deposição dos PRPC parece ser derivado do metabolismo de biossíntese da parede

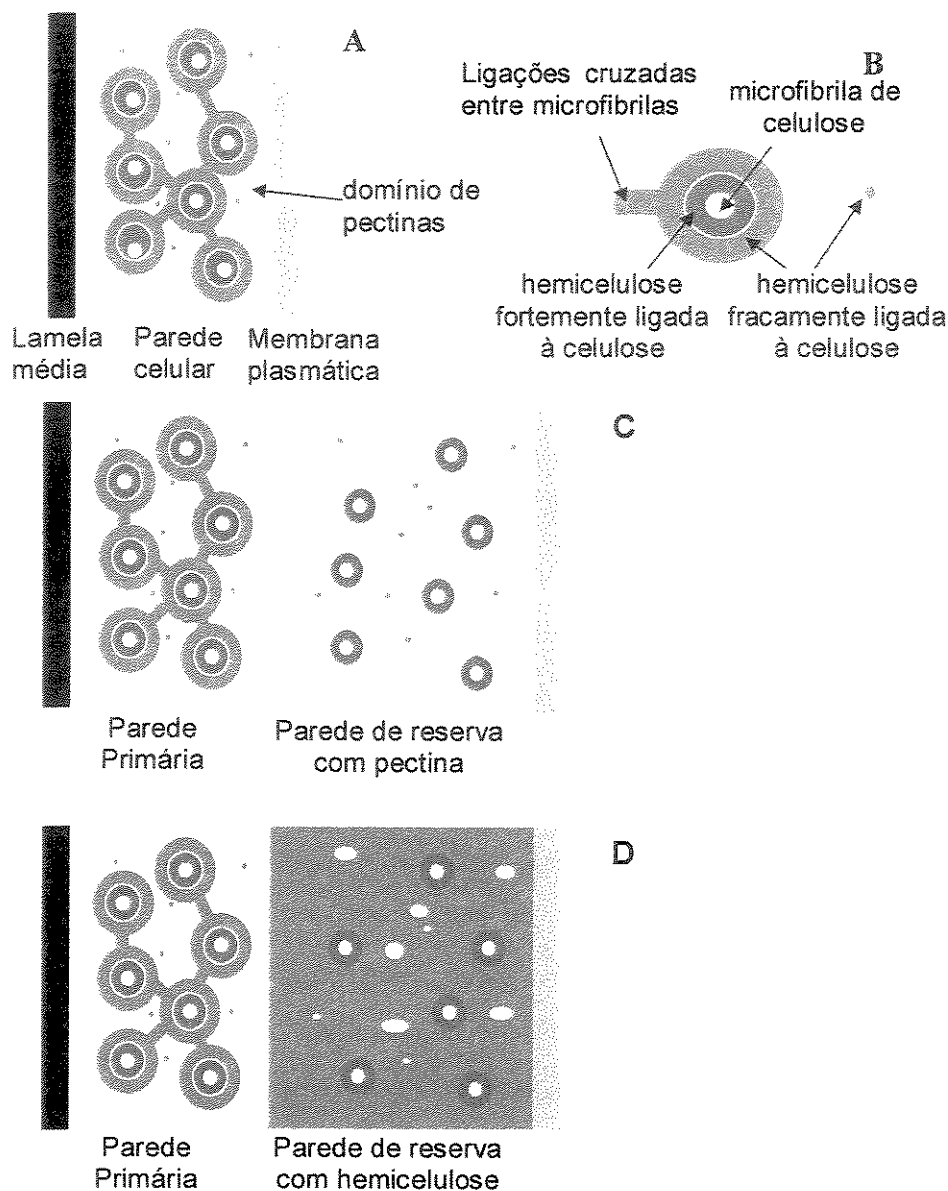


Figura 2 - Representação esquemática da parede celular primária (A) e das paredes de reserva com pectina (C) ou hemicelulose (D), em corte transversal. As microfibrilas de celulose estão cobertas por hemiceluloses (xiloglucanos, arabinoxilanos ou mananos), as quais podem estar forte ou fracamente ligadas à celulose (ver B). O domínio celulose-hemicelulose está embebido em um domínio péctico (A). Em C está representada a parede de reserva em que o polímero acumulado é derivado das pectinas (arabinogalactanos em sementes de *Lupinus* e de café). Em D estão representadas as paredes cujo polímero de reserva é o galactomanano ou o xiloglucano. Este modelo foi baseado no desenho apresentado por Cosgrove (1999). O domínio protéico, que normalmente representa menos do que 10% da parede celular, foi omitido.

celular primária. Com base nisso, estes autores propuseram que eles tenham surgido ou por diminuição na síntese de celulose ou por síntese de hemiceluloses em maior intensidade do que os polissacarídeos dos demais domínios. Entretanto, ainda não está claro se a celulose permanece nos depósitos de reserva da parede celular, mas há fortes indícios de que isso ocorra, pois em *Hymenaea courbaril* já foi observado que materiais fibrosos tornaram-se aparentes após a mobilização do xiloglucano (Tiné *et al.*, 2000).

Uma vez que este trabalho trata principalmente do xiloglucano de reserva da semente de *Hymenaea courbaril*, será apresentado o estado de conhecimento apenas sobre esse polissacarídeo.

2.3. Xiloglucano

2.3.1. Ocorrência, função e estrutura

Os xiloglucanos são polissacarídeos que ocorrem principalmente em paredes celulares de plantas da classe das Dicotiledôneas. Estes constituem a principal hemicelulose deste grupo de plantas e além disso podem ser acumulados em grandes quantidades em sementes de algumas espécies de Leguminosae-Caesalpinioideae.

Os xiloglucanos são também denominados amilóides por interagirem com I_2/KI (como o amido), fazendo com que o tecido apresente forte coloração azul. Com base nesta propriedade, a distribuição botânica de xiloglucano foi revisada por Kooiman (1960), o primeiro a observar a presença deste amilóide em cotilédones de *Hymenaea courbaril*. Buckeridge & Dietrich (1990) efetuaram extrações aquosas de sementes dormentes de *Hymenaea courbaril* e obtiveram grandes quantidades de um polissacarídeo (40% do peso seco da semente), cuja composição em monossacarídeos apresentou glucose, xilose e galactose em proporções tais que, em conjunto com a reação positiva com iodo, permitiram concluir que tratava-se de um xiloglucano. Lima *et al.* (1993) também analisaram o xiloglucano de *Hymenaea courbaril* e reforçaram as observações acima. Além disso, esses autores efetuaram

análises por metilação e mostraram que as ligações glicosídicas do xiloglucano de jatobá são idênticas aos demais xiloglucanos conhecidos.

Em paredes de reserva, até o presente momento, só tem sido descrita a função de reserva para o xiloglucano, apesar de haver hipóteses de outras funções como sugerido por Buckeridge *et al.* (2000a, 2000b). Entretanto, em paredes primárias, acredita-se que uma das funções principais do xiloglucano seja coordenar a organização das microfibrilas de celulose. São evidências em favor dessa hipótese os seguintes fatos: 1) os xiloglucanos interagem especificamente com celulose *in vitro* (Hayashi *et al.*, 1987); 2) quando extraídos de paredes intactas deixam uma rede desorganizada de microfibrilas (McCann & Roberts, 1991) e 3) quando adicionados a suspensões de celulose promovem o alinhamento das microfibrilas *in vitro* (Whitney *et al.*, 1998). Como consequência, o controle da forma celular pode estar em grande parte associado com o domínio celulose-xiloglucano em paredes celulares de Dicotiledôneas.

Estruturalmente, os xiloglucanos são polímeros compostos por uma cadeia principal celulósica em que resíduos de glucose estão ligados entre si por ligações glicosídicas do tipo $\beta(1,4)$. A cadeia principal celulósica pode apresentar três tipos de oligossacarídeos ramificadores: 1) resíduos de xilose (Xil), ligados ao carbono 6 da glucose (Glc) através de ligação $\alpha-1,6$; 2) o dissacarídeo xilose($\beta-1,2$)galactose ligado ao carbono 6 da glucose através de ligação $\alpha-1,6$ e 3) o oligossacarídeo xilose($\beta-1,2$)galactosil($\alpha-1,2$) fucopiranosil ligado ao carbono 6 da glucose através de ligação $\alpha-1,6$ (White & Rao, 1953). Exceto pela ausência de terminais fucosil ligados [α -L-(1 \rightarrow 2)] nos grupos β -D-galactosídeos, existe uma grande semelhança entre xiloglucanos de reserva (em sementes) e xiloglucanos estruturais de paredes primárias, em tecidos vegetativos de dicotiledôneas (Hayashi, 1989).

Utilizando uma celulase microbiana, Kooiman (1961) determinou que xiloglucanos de sementes de tamarindo são formados por unidades de um heptassacarídeo $\text{Glc}_4\text{:Xil}_3$, com variações nas substituições dos resíduos de galactose. Entre os anos 1980 e 1990, esta hidrólise por celulase microbiana seguida por análises por metilação, espectrometria de massa e ressonância magnética nuclear

(para uma revisão ver Hayashi, 1989) possibilitou estudos mais aprofundados sobre a estrutura fina dos xiloglucanos e hoje se sabe que a maioria dos xiloglucanos conhecidos é formada por blocos contendo 4 moléculas de glucose, 3 moléculas de xilose, 1, 2 ou nenhuma molécula de galactose e 1 molécula de fucose. Os extratos de xiloglucano normalmente apresentam uma pequena proporção de arabinose e já foi demonstrado que este monossacarídeo liga-se ao resíduo de galactose (Eda & Kato, 1978; Gidley *et al.*, 1991). Para facilitar o entendimento e a troca de informação sobre xiloglucano, em 1993 um grupo de pesquisadores da área se reuniu e propôs uma nomenclatura para os blocos estruturais do xiloglucano com base na cadeia principal (Fry *et al.*, 1993). Nesta nomenclatura, glucoses não substituídas são denominadas G; glucoses ramificadas com xilose são denominadas X e, se a galactose está ligada à xilose, o trissacarídeo é denominado L (Figura 3).

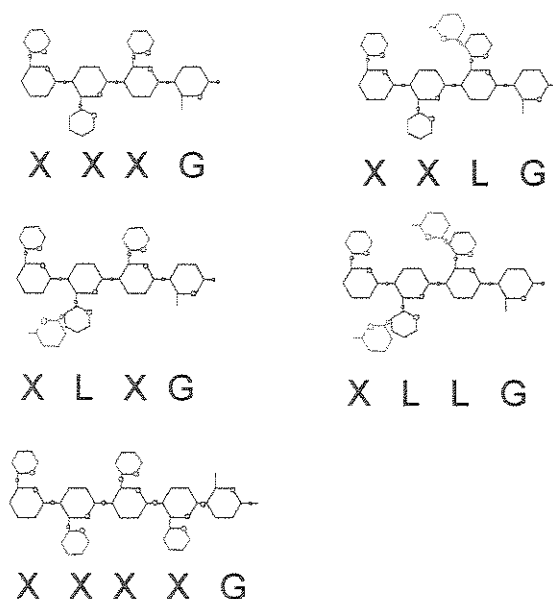


Figura 3 – Oligossacarídeos de xiloglucano de reserva obtidos a partir da digestão com celulase de *Trichoderma reesei*. A fucose, ligada à galactose, foi omitida por não estar presente em xiloglucano de reserva. Glucose (hexágono azul), xilose (hexágono vermelho), galactose (hexágono verde). Os oligossacarídeos com 4 glucoses na cadeia principal são comuns a várias espécies, mas a série com 5 glucoses (XXXXG) só foi encontrada, até então, em xiloglucano de reserva de cotilédones de *Hymenaea courbaril* (Buckeridge *et al.*, 1997).

Um estudo comparativo em xiloglucanos de sementes de *Tropaeolum majus*, *Tamarindus indica* e *Copaifera langsdorffii*, utilizando uma celulase fúngica do gênero *Trichoderma* altamente purificada, apresentou as mesmas quatro unidades estruturais básicas XXXG, XLXG, XXLG e XLLG nas três espécies (Buckeridge *et al.*, 1992). As proporções entre estas unidades demonstraram a existência de estruturas finas (distribuição das ramificações com galactose) específicas entre as diferentes espécies e entre populações de mesma espécie crescidas em diferentes ambientes. Uma diferença nas ramificações de xilose foi encontrada recentemente em xiloglucanos de sementes de *Hymenaea courbaril*, sendo este polímero formado por uma mistura de unidades de XXXG e XXXXG que podem ser ramificadas com galactose nas diferentes posições (Buckeridge *et al.*, 1997) (Figura 3). Apesar das diferenças em estrutura fina, todos os xiloglucanos de sementes examinados apresentam proporção de monossacarídeos muito próxima, preservando, desse modo, o total de ramificações com galactose de forma independente da sua distribuição.

Apesar das diferenças em estrutura fina não estarem completamente entendidas, a distribuição de galactoses ao longo do polímero de xiloglucano pode estar relacionada com propriedades hidrodinâmicas. Como consequência, pode desempenhar uma função no controle de água durante a etapa de embebição das sementes como foi demonstrado para galactomanano em sementes de *Trigonella foenum-graecum* (Reid & Bewley, 1979), *Dimorphandra mollis* e *Sesbania virgata* (Buckeridge *et al.*, 1995; Buckeridge & Dietrich, 1996). Ramificações de galactose, em galactomanano, são consideradas os principais fatores no controle de velocidade de entrada de água em espécies que armazenam este polissacarídeo no endosperma (Meier & Reid, 1982). Desse modo, a hipótese de que o xiloglucano de sementes de *Hymenaea courbaril* poderia exercer a mesma função não pode ser desprezada.

2.3.2. Catabolismo de xiloglucano em paredes de reserva

A presença e a mobilização de xiloglucano durante a germinação foi primeiramente reportada em sementes de *Impatiens balsamina*, *Tropaeolum majus* e *Cyclamen europaeum* (Heinricher, 1888; Reiss, 1889). Recentemente, a função reserva dos xiloglucanos em cotilédones tem sido demonstrada de forma circunstancial em sementes de várias espécies, como *Tropaeolum majus* (Edwards *et al.*, 1985), *Tamarindus indica* (Reis *et al.*, 1987), *Copaifera langsdorffii* (Buckeridge *et al.*, 1992) e *Hymenaea courbaril* (Tiné *et al.*, 2000), nas quais a mobilização de xiloglucano *in vivo* foi acompanhada pelo incremento e queda da atividade de quatro hidrolases: xiloglucano endo-transglicosilase (XET) ou endo- β -(1 \rightarrow 4)-glucanase; β -galactosidase; α -xilosidase e β -glucosidase.

Já foram isoladas as quatro principais enzimas responsáveis pela degradação de xiloglucano em *Tropaeolum majus*. Elas são: 1) uma endo- β -(1 \rightarrow 4)-glucanase específica para xiloglucano (ou XET) (Edwards *et al.*, 1986; Fanutti *et al.*, 1993); 2) uma β -galactosidase com alta especificidade para xiloglucano (Edwards *et al.*, 1988); 3) uma α -xilosidase ou oligoxiloglucano exo-xilohidrolase específica para oligossacarídeos de xiloglucanos (Fanutti *et al.*, 1991); e 4) uma β -glucosidase (Crombie *et al.*, 1998).

Com base nestes resultados e em outros estudos sobre o modo de ação da XET (Edwards *et al.*, 1986; Fanutti *et al.*, 1993), Crombie *et al.* (1998) propuseram um modelo aproximado para a mobilização de xiloglucano em *Tropaeolum majus*. Neste modelo, as quatro enzimas atacam o polímero de um modo sincronizado, produzindo galactose, glucose e xilose livres. Em *Tropaeolum majus*, XET e β -galactosidase são as únicas enzimas capazes de atacar o polímero. Sob baixas concentrações de oligossacarídeos de xiloglucano (receptores), a atividade hidrolítica da XET predomina (Fanutti *et al.*, 1993). Assim, quando em contato com xiloglucano de alto peso molecular, esta atividade hidrolítica produz oligossacarídeos que são prontamente atacados pelas exo-glicosidades (α -xilosidase e β -glucosidase), reduzindo o polímero aos seus monossacarídeos constituintes.

Tiné *et al.* (2000), estudando a mobilização do xiloglucano de *Hymenaea courbaril*, verificaram que o polímero foi mobilizado após a germinação e, ao mesmo tempo, observaram a produção de frutose, glucose e sacarose. As mesmas quatro enzimas encontradas por Reid e colaboradores em *Tropaeolum majus* foram detectadas em *Hymenaea courbaril* (Crombie *et al.*, 1998; Fanutti *et al.*, 1993; Fanutti *et al.*, 1991; Edwards *et al.*, 1988; Edwards *et al.*, 1986). Tiné *et al.* (2000) também encontraram evidência para a presença da atividade de transglicosilação (XET) nesta espécie, dependendo da concentração de oligossacarídeos. Como em *Copaifera* (Alcântara *et al.*, 1999), toda atividade de β -galactosidase que pôde ser detectada utilizando substrato sintético teve um pH ótimo em 3,2, enquanto as demais hidrolases foram ativas em pH 4,5. Os resultados obtidos para as β -galactosidases de *Copaifera* e *Hymenaea* sugerem que estas enzimas são um importante ponto de controle do metabolismo de xiloglucanos em leguminosas, pois em todos estudos efetuados, nenhuma β -galactosidase livre de atividade de endoglucanase apresentou atividade sobre o polímero, hidrolisando apenas os oligossacarídeos. Com base nestes resultados, Tiné *et al.* (2000) propuseram um modelo de degradação de xiloglucano em cotilédones de *Hymenaea courbaril*, como pode ser visto na figura 4.

Em função do pH ótimo das enzimas, a desmontagem do xiloglucano *in muro* pode estar sendo controlada por mudanças no pH da parede, com base na diferença de pH ótimo das enzimas. Neste enfoque a enzima β -galactosidase tem sido considerada um ponto de controle, tanto em *Copaifera langsdorffii* (Alcântara *et al.*, 1999) como em *Hymenaea courbaril* (Tiné *et al.*, 2000) devido ao seu pH ótimo estar em torno de 3,2, enquanto para as demais enzimas o pH ótimo está próximo de 4,5. Deste modo, um controle hormonal (p.ex. a auxina) poderia desempenhar um papel importante na mobilização e conseqüentemente sincronizando esta fase da germinação com o desenvolvimento da plântula de *Hymenaea courbaril*.

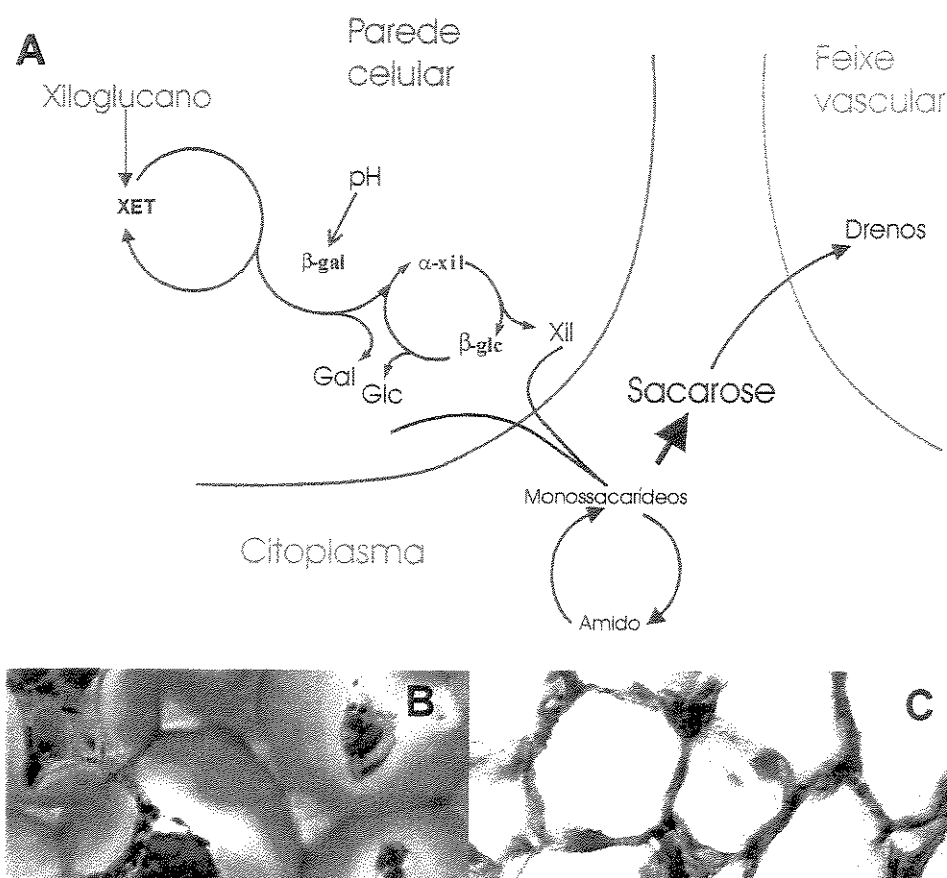


Figura 4 - Representação esquemática do modelo de degradação de xiloglucano em cotilédones de *Hymenaea courbaril* (A), proposto por Tiné *et al.* (2000). Neste modelo estão descritas a provável ordem de atuação das enzimas xiloglucano endo-transglicosilase (XET), β -galactosidase (β -gal), α -xilosidase (α -xil) e β -glucosidase (β -glc). Após a degradação os monossacarídeos são translocados para o meio citoplasmático, onde irão resultar em sacarose e/ou amido transitório (visto em microscopia óptica, Tiné *et al.*, 2000). A sacarose é então direcionada para os tecidos drenos do eixo em crescimento. Em B e C estão cortes transversais (420x) dos cotilédones de *Hymenaea courbaril*, mostrando o detalhe das células antes (B) e depois (C) da mobilização de xiloglucano, corado em azul por Iodo/Iodeto de Potássio (Tiné, 1997).

2.3.3. Controle da mobilização de xiloglucano

As auxinas, assim como as citocininas, são substâncias vitais para o metabolismo dos vegetais. Isto tem sido comprovado com plantas mutantes, as quais são metabolicamente inviáveis quando não produzem esses hormônios (Taiz & Zeiger, 1998). Dentre os vários pontos de atuação das auxinas, destaca-se o papel destas sobre a extensão celular. Em estudos com paredes celulares primárias de tecidos em crescimento, concluiu-se que as auxinas podem atuar de dois modos sobre a extensão da parede: A) através da ativação do transporte de íons H^+ para o meio extracelular (possivelmente relacionado ao aumento na expressão e na atividade de ATPases de membrana), o qual resulta em queda de pH (crescimento ácido) favorecendo a ação de glicosidases e de expansinas (Taguchi *et al.*, 1999; Cosgrove, 2000); B) ativando ou alterando a expressão gênica de glicosidases. Tais enzimas atuam sobre os polímeros de parede celular, resultando em relaxamento da parede e permitindo a extensão da mesma.

Apesar do modo de ação das auxinas na extensão da parede celular ainda não estar completamente esclarecido, o que se sabe até o momento está voltado principalmente para as alterações que são observadas nas atividades enzimáticas e na estrutura dos polissacarídeos nas paredes celulares. Dentre os polissacarídeos que são alterados nesse processo estão os xiloglucanos, que nas plantas superiores são considerados importantes no alinhamento das microfibrilas de celulose na parede (Hayashi, 1989). Acredita-se, portanto, que eles tenham um papel importante no controle das forças de tensão e coesão da parede celular como um todo (McCann & Roberts, 1991).

As sementes de *Hymenaea courbaril* e *Copaifera langsdorffii* acumulam grandes quantidades de xiloglucanos como reserva. Já se sabe, inclusive, que eles são degradados por um complexo enzimático similar ao encontrado em paredes celulares de tecidos em crescimento (Buckeridge *et al.*, 1992; Tiné *et al.*, 2000), mas pouco se sabe sobre os mecanismos de controle da degradação. As únicas evidências existentes no controle da degradação das paredes celulares de reserva contendo

xiloglucano foram obtidas por Hensel *et al.* (1991), com uma planta herbácea de ambiente temperado (*Tropaeolum majus*). Nesse sistema, a degradação apresentou-se análoga aos mecanismos de ação de auxinas que já foram demonstrados sobre paredes primárias de tecidos em crescimento, pela adição de auxina exógena (Terry *et al.*, 1981; Hayashi *et al.*, 1984).

Em estudos preliminares com *Hymenaea courbaril* (Tiné *et al.*, 2000), foi observado que a mobilização de xiloglucano nos cotilédones só ocorre quando a parte aérea já está se desenvolvendo, sugerindo que algum estímulo para degradação esteja vindo da plântula em crescimento. De acordo com Bewley & Black (1994), existem duas possibilidades para explicar a influência do eixo embrionário sobre o controle da mobilização das reservas: A) movimento de substâncias regulatórias específicas do eixo para os órgãos de reserva ou tecidos onde ocorre a produção das enzimas, por exemplo, um mecanismo hormonal; B) o eixo é um dreno, retirando os produtos finais da mobilização das reservas o que em muitos casos podem inibir competitivamente as enzimas de hidrólise (como a β -galactosidase de *Hymenaea courbaril*, Alcântara, 2000). Assim, o dreno evita o chamado mecanismo de inibição por "feedback".

Acreditamos que o conhecimento dos mecanismos de controle da mobilização irá ajudar a compreender melhor a função de reserva do xiloglucano e conseqüentemente entender quais foram os fatores que levaram à seleção desse sistema ao invés de amido ou lipídeos, como ocorre em outras espécies da família Leguminosae. Além disto, esta caracterização poderá confirmar a hipótese de que a reserva deste polissacarídeo de parede celular parece ser resultante de um mecanismo de transferência de funções durante a evolução (Buckeridge & Reid, 1996). Nesse processo, não somente o polissacarídeo, mas todo o sistema metabólico a ele relacionado (incluindo os genes que codificam para sua biossíntese e degradação), seriam provenientes de alterações nos mecanismos relacionados às paredes celulares primárias (ver Buckeridge & Reid, 1996 e Buckeridge *et al.*, 2000a e 2000b, para revisões). A eventual comprovação desta hipótese pode nos levar a sugerir que os mecanismos de extensão das paredes primárias foram mantidos nas

paredes secundárias de reserva durante a evolução o que caracteriza um mecanismo irreduzivelmente complexo (Behe, 1997), ou seja, impossível de evoluir de forma completamente distinta, em relação às paredes primárias.

3. OBJETIVOS

O presente trabalho teve como objetivos caracterizar a importância ecofisiológica das reservas de xiloglucano nos cotilédones de plântulas de *Hymenaea courbaril*, bem como salientar os principais pontos de controle sobre o metabolismo de degradação desta reserva. Dentro deste último enfoque, este trabalho teve como meta definir a relação entre auxina e o metabolismo de degradação do xiloglucano de reserva para esta espécie.

4. CAPÍTULO 1

Artigo 1 - Cell wall polysaccharides and morphological features associated with water imbibition of seeds of *Hymenaea courbaril* L. (Leguminosae-Caesalpinioideae)

Cell wall polysaccharides and morphological features associated with water imbibition of seeds of *Hymenaea courbaril* L. (Leguminosae-Caesalpinioideae)

Henrique P. Santos¹, Nicholas C. Carpita² & Marcos S. Buckeridge^{3,4}

¹ Departamento de Biologia Vegetal – UNICAMP / Seção de Fisiologia e Bioquímica de Plantas – IBt. Actual address: Rua Livramento, 515, Caixa Postal 130, CEP 95700-000, Bento Gonçalves, RS, Brazil. (henrique@cnpuv.embrapa.br)

² Department of Botany and Plant Pathology, Purdue University, West Lafayette, Indiana 47907-1155

³ Instituto de Botânica, Seção de Fisiologia e Bioquímica de Plantas, Caixa Postal 4005, CEP 01061-970, São Paulo, Brazil. (msbuck@usp.br)

⁴ To whom correspondence should be addressed.

RESUMO

A relação entre características morfológicas e químicas com a velocidade de embebição foi estudada em sementes de *Hymenaea courbaril* L., as quais apresentam geralmente 18 dias para completar a embebição e germinarem. Análises ultraestruturais revelaram que a casca da semente desta espécie é composta de três camadas onde a camada de células parenquimatosas, localizada mais internamente, corresponde à maior proporção da casca. Sob estas camadas da casca está localizado o tecido cotilédonar composto de células ovóides com espessas paredes celulares, ricas em xiloglucano. A extração seqüencial dos polissacarídeos da casca com oxalato de amônia e hidróxido de sódio revelaram que este tecido é composto principalmente de homogalacturonano, galactoglucomanano e arabinanos, com pequenas quantidades de xilanos 4-ligados. Misturas *in vitro* de celulose e xiloglucano de reserva apresentaram uma lenta embebição de água, equivalente às taxas de embebição observadas nas sementes. Entretanto, misturas preparadas com polissacarídeos da fração de oxalato de amônia da casca (pectinas) embeberam rapidamente. Isto indica que, na semente, quando a água passa pela camada palissádica, que é impermeável, ela é rapidamente absorvida pela camada parenquimática que é rica em pectina, a qual atua na distribuição da água ao redor dos cotilédones. Nos tecidos cotilédonares, o avanço da água é reduzido drasticamente devido à presença de xiloglucano nas paredes celulares. Este controle de água provavelmente está relacionado com o sincronismo entre a embebição dos tecidos e o início dos processo de mobilização das reservas. Estes resultados salientam que diferentes polissacarídeos de parede celular desempenham importantes funções ecofisiológicas na germinação das sementes.

ABSTRACT

The relationship between morpho-chemical characteristics and imbibition speed was studied in seeds of *Hymenaea courbaril* L., which usually takes approximately 18 days to be completely imbibed and germinate. Ultrastructural analyses showed that the seed coat of *H. courbaril* is composed by three layers, where the inside parenchymatous cell layer corresponded to the principal fraction of seed coat. Beneath the seed coat layers, a pair of massive cotyledons is composed of thick-walled, egg-shaped cells, rich in xyloglucan. Cell wall polysaccharides from seed coat extracted sequentially with ammonium oxalate and sodium hydroxide revealed that the seed coat is composed of homogalacturonans, galactoglucomannan and arabinans, with small amounts of 4-linked xylans. *In vitro* composites of cellulose and storage xyloglucan imbibed water very slowly, equivalent to rates observed in seeds, whereas composites prepared with polysaccharides from the ammonium oxalate fraction (pectins) imbibed faster. These results indicated that when the water crosses the palisade layer of the coat, which is impermeable, it is quickly absorbed by a pectin rich parenchymatic layer and as it enters the cotyledons, the rate of uptake is drastically lowered because the occurrence of xyloglucan in the walls. This control of water imbibition is likely to be related to a synchronisation between imbibition of tissues and the onset of storage mobilisation process. Our results emphasise that cell wall polysaccharide composition in specific cell layers plays an important ecophysiological role in the seed germination.

Key words: imbibition, xyloglucan, pectin, seed, Leguminosae.

INTRODUCTION

Imbibition of water is the first step in seed germination. In general, it includes an initial wetting of seed tissues and the establishment of a sharp wetting front, followed by a continued swelling of the seed (Vertucci & Leopold, 1983, 1987). Concomitantly, there is an associated increase in seed water potential as seed water content increases (McDonald *et al.*, 1988a). Because water uptake occurs at the same rate in both dead and living seeds, the rate of entry into the seed is interpreted to be a purely physical process (Leopold, 1980; Vertucci & Leopold, 1983). This process is not limited by the reforming membranes and is considered as a function of the diffusion of water and the hydration of polyelectrolytes in the tissue (Leopold, 1980). Thus, knowing the composition and structure of the seed parts is an important way for understanding the physical process that drives the water uptake by seeds.

Few studies have been conducted to determine how individual tissues affect water movement within the seed. Traditionally, the role of the seed coat in Leguminosae and Malvaceae has been discussed in terms of a barrier to water penetration establishing a dormancy to seeds. Usually the seed-coat become impermeable to water during seed development and is a result of waxy material and/or insoluble lignin polymers present in palisade layer of the coat (Egley *et al.*, 1983; Melo-Pinna *et al.*, 1999). On the other hand McDonald *et al.* (1988b) observed a promotive effect of osteosclereids layer (hourglass cells) of seed coat upon water uptake in seeds of soybean. They suggested that seed coat might aid in the tangential and radial movement of water into the embryo. Thus, the seed coat may alter the movement of water that surrounds the seed so that both cotyledons hydrate evenly, leading to more uniform storage mobilisation.

Hymenaea courbaril is a late successional leguminous tree native from the tropical forests of Central and South America. The seeds possess water-impermeable coat (hard seeds) and its cotyledons are rich in xyloglucan (40% of the dry mass), which is completely mobilised after germination (Tin   *et al.*, 2000a). Storage xyloglucans have a cellulose-like (1→4)β-D-glucan backbone to which single (1→6)

α -D-xylopyranoside substituents are attached (Buckeridge *et al.*, 2000). In general, some xylosyl residues are further substituted at O-2 by β -D-galactopyranosyl residues (Hayashi, 1989). The pattern of xylosyl substitution of xyloglucans from *H. courbaril* is remarkably unique when compared to other seed xyloglucans. Whereas nasturtium (*Tropaeolum majus*), tamarind (*Tamarindus indica*) and copaiba (*Copaifera langsdorffii*) xyloglucans are composed of repetitive units of glucose₄:xylose₃ with variable galactosyl substitution (York *et al.*, 1990; Buckeridge *et al.*, 1992), *H. courbaril* xyloglucan is composed of a mixture of glucose₄:xylose₃ and glucose₅:xylose₄ repetitive units with variable galactosyl substitutions (Buckeridge *et al.*, 1997). Although the importance of these differences in fine structure is not clear yet, it is a factor regarding in seed water imbibition as well as in the control of enzymatic degradation during germination (Buckeridge *et al.*, 2000). Seed storage xyloglucans have high molecular weight (Franco *et al.*, 1996) and low interaction with cellulose (Lima & Buckeridge, 2001). Each of these features may provide xyloglucans with unique hydrodynamic properties that impact water uptake by leguminous seeds (Reid & Bewley, 1979; Buckeridge *et al.*, 1992, 2000).

Depending on the species, leguminous plants may accumulate xyloglucan or galactomannan. Xyloglucan displays similar physico-chemical properties as galactomannans, where the latter have been directly related to the influence of water uptake by endosperm-containing seeds such as in *Trigonella foenum-graecum* (Reid & Bewley, 1979), *Dimorphandra mollis* and *Sesbania marginata* (Buckeridge *et al.*, 1995; Buckeridge & Dietrich, 1996). Galactosyl substitution, and probably acetylation (Meier & Reid, 1982), in galactomannan is thought to be the principal factor that controls how much and how fast water is gained or lost by the endosperm tissue.

In this work, we report that seeds of *H. courbaril* imbibe water slowly compared to those with different storage glycans. Despite of morphological, biochemical and physical analysis of the seeds, we made artificial polymer composites to better understand the basis of the low rates of water uptake. Analysis of polysaccharides from the seed coat cell walls revealed significant amounts of pectin (polygalacturonic acid), which was extracted and compared with storage xyloglucan

regarding their capacity to imbibe water *in vitro*. Whereas seed coat pectins imbibed water quickly, xyloglucans of cotyledons strongly decreased the rate of water imbibition, and this feature is suggested to be related to the control of seed imbibition. Thus, our results indicate that xyloglucan present in cotyledons and usually considered as storage polysaccharides is also an important factor in the control of water entrance and distribution throughout the different tissues and organs of seed.

MATERIAL AND METHODS

Plant material

Seeds of *H. courbaril* L. (Leguminosae-Caesalpinioideae) were obtained from two trees growing in a gallery forest in São João da Boa Vista county (22° 00' S; 47°18' W), São Paulo, Brazil. These seeds were stored for four years under dry and cool conditions (RH 35%, 8 °C). Dry seeds (6.48% moisture content) were classified according to their mass after randomly weighing 160 seeds and obtaining the frequency distribution of masses, which ranged from 2 to 7 g. Within this mass distribution, seeds weighing between 4.6 and 5.3 g were chosen for our experiments.

Imbibition

Seeds were scarified with sandpaper on the hilum or lateral position (in relation to the embryo). Seeds were placed to imbibe water so that only the scarified regions were in contact with water. For most experiments, seeds imbibed water at 30 °C, but to evaluate the effect of temperature, seeds with lateral scarification were also soaked at 20 °C. At intervals of 24 h, ten seeds of each scarification position were collected, surface dried by paper blotting, weighed and cut longitudinally (hilum scarified) or transversely (laterally scarified). The water front could be clearly seen, and the surface of the dissected seeds were copied with the help of a scanner (Genius Color-Page HR) to show seed features during imbibition.

Proportional mass and imbibition of seed parts

In order to determine the proportional mass among seed parts, 30 dormant seeds were mechanically broken and individually separated into cotyledons, seed coat and embryonic axis. These parts were weighed after drying at 70 °C for 72 h. Fifteen intact axes (0.02 ± 0.01 g) and pieces of seed coat with similar shapes (0.1 ± 0.03 g) and cotyledons (0.26 ± 0.03 g) were selected, distributed in Petri dish with filter paper moistened with water. Imbibition was followed at 20°C until completed. Every 2 h the pieces of seed parts were surface dried by paper blotting, weighed and returned to the Petri dishes under the same conditions until they reached constant mass.

Extraction of water-soluble carbohydrates

Seed coat and cotyledons from three dormant seeds were mechanically separated, dried (60 °C for 24 h) and powdered separately (three replicates). The powders of the cotyledons and seed coat (100 mg) were extracted in 30 ml of water at 80 °C for 8 h. After filtration through a nylon cloth, centrifugation was performed (10 000 *g*, 30 min, 5 °C) followed by precipitation with 3 volumes of ethanol. The precipitate was stored overnight at 5 °C, collected by centrifugation, freeze-dried and weighed. The water soluble polysaccharides obtained, when originated from cotyledons, produced a freeze dried fluffy material that was mostly (more than 90%) xyloglucan (Buckeridge *et al.*, 1992).

Fractionation of seed coat cell walls

The powdered seed coat from dormant seeds (three replicates of 100 mg each) were extracted twice for 1 h in 10 ml of aqueous 0.5% ammonium oxalate (pH 7.0) at 90 °C. The insoluble material was pelleted by centrifugation at 10 000 *g* for 15 min. The supernatants were combined and dialysed for at least 48 h against deionised water and freeze-dried. The precipitated materials were extracted twice, sequentially, in 20 ml each of 0.1, 1 and 4 M NaOH, each supplemented with 3 mg

ml⁻¹ NaBH₄ under a nitrogen atmosphere to prevent reducing end-elimination. The suspensions were stirred at room temperature for 1 h. The supernatants of the NaOH extractions were chilled, neutralised with glacial acetic acid, and dialysed for 48 h against deionised water. Samples of oxalacetate and NaOH fractions were freeze-dried, weighed and colorimetrically assayed for uronic acids (Filisetti-Cozzi & Carpita, 1991).

Separated analyses of seed coat layers

Pieces of seed coat were carefully divided into palisade and parenchymatic layers using a scalpel and a loupe (30x). Samples (5 mg) of each layer were dried for 24 h at 60°C, powdered, acid hydrolysed (as described below) and submitted to uronic acid (Filisetti-Cozzi & Carpita, 1991) and monosaccharide analyses.

Neutral-monosaccharide composition of the cell wall

The monosaccharide composition of the cotyledons and seed coat extracts were determined by acid hydrolysis of samples (4 to 5 mg) in 72% (w/w) H₂SO₄ at 30 °C for 45 min, followed by 4% (w/w) H₂SO₄ for 1 h at 120 °C (Saeman *et al.*, 1945). After acid hydrolysis the samples were neutralised with NaOH (50%) and desalted in anionic (Dowex 1x 8-200) and cationic (Dowex 50x 8-200) ion exchange columns. The resulting monosaccharides were analysed by high performance anion exchange chromatography (HPAEC) on a CarboPak PA-10 column (250 x 4 mm; Dionex Corporation, Sunnyvale, Ca, USA) by isocratic elution with 20 mM NaOH. Sugars were detected by a pulsed amperometric detector (PAD; Dionex). Detector responses were determined with the appropriate standards used to calculate molar response factors.

Linkage analysis of cell wall of seed coat

Methylation analysis was carried out on materials of the oxalate, 0.1, 1 and 4 M NaOH fractions. The samples (10 mg) were delignified with 1 ml of 340 mM NaClO₂ in acetic acid pH 5.0 for 1 h at 65 °C. The delignified materials were dialysed against

distilled water, freeze-dried and stored over P_2O_5 in a vacuum desiccator for 24 h. Samples were methylated with *n*-butyllithium and methyl iodide as described in Gibeaut & Carpita (1993). Partly methylated alditol acetates were separated by gas-liquid chromatography and identified by electron-impact mass spectrometry as described by Carpita & Shea (1989).

In vitro imbibition of water by polysaccharide composites

In order to evaluate the effect of water soluble storage xyloglucan in seed imbibition, composites were prepared with 20:80% and 40:60% xyloglucan:cellulose, and compared to cellulose and xyloglucan matrices alone. The cellulose fibres were kindly provided by "Aracruz Papel e Celulose" (Espírito Santo, Brazil), which analysis by acid hydrolysis followed by HPAEC-PAD showed 99% glucose and 1% xylose. The xyloglucan was hot water-extracted from powdered cotyledons of *H. courbaril*, as described above. Imbibition rates were also determined with 30:70% *H. courbaril* seed coat pectin:cellulose and with cellulose alone. The pectin used in the composite was from oxalate fraction of seed coat (described above), which was dialysed against distilled water (15 changes over five days) and freeze-dried. Dry samples of each polysaccharide were weighed separately and mixed with cellulose to fill one-half of the height of a 1-ml pipette tip (250 mg of composite or pure polymer). Each mixture was suspended in 500 μ l of distilled water, freeze-dried and mounted in 1-ml pipette tips (five tips per sample). This procedure was designed to avoid damage or loss of parts of the composites due to manipulation and also to simulate a single water-entrance point, i.e. analogous to the experiments with the seeds of *H. courbaril*. The tips were kept at 25°C on a tip-holding tray partially filled with distilled water with 0.025% sodium azide to avoid microbial degradation. Periodically, the tips were surface dried by paper blotting, weighed and returned to the same imbibition conditions until constant fresh mass was attained.

Scanning Electron Microscopy (SEM) analysis

For the examination of the seed tissues/parts and composites of xyloglucan/cellulose and cellulose and xyloglucan alone, samples were carefully mounted on stubs, freeze-dried, coated with gold (Baltec SCD 050 coater), examined, and photographed in a scanning electron microscope (Philips XL20).

RESULTS AND DISCUSSION

Seed imbibition

Under optimal conditions of supply, the uptake of water by seeds has a triphasic behaviour, where the first and fastest step (phase I) is a consequence of the matric forces, followed by a longer period (phase II or lag phase) of metabolic preparation for radicle emergence when growth begins (phase III) (Bewley & Black, 1994). In the present work we followed only the first two steps, where a sigmoidal curve was apparent (Fig. 1). The lag phase in *H. courbaril*, which is normally longer in other species, was nearly as long as phase I, since radicle protrusion at 20 °C occurs about 15 d after imbibition (not shown). Phase II perhaps means the rehydration of the cellular gel and reinflation of active membranes in preparation to cell expansion associated with radicle emergence and growth (phase III). This pattern of water uptake observed in seeds of *H. courbaril* suggest a delay in all phases in relation to found in pea (Waggoner & Parlange, 1976) and soybean seeds (Leopold, 1980; Vertucci & Leopold, 1983; McDonald *et al.*, 1988a, 1988b). In these seeds, the first moments of water intake (phase I) is so rapid that the imbibition curves tend to show a hyperbolic-shape. As it is well accepted that phase I is related mainly to the matric potential (Bewley & Black, 1994), the delay observed in this first phase during imbibition of seeds of *H. courbaril* might be related to chemical, physical and/or morphological properties that impact the path of water uptake and the chemical nature of the individual tissues.

Temperature strongly influences the rate of imbibition. A decrease of 10 °C was sufficient to delay maximum water uptake by 3 d (Fig. 1). Temperature may influence the rate of conversion of the dehydrated polymers during wetting when they acquire their solution conformation (Lüscher-Mattli & Rüegg, 1982). In this context, two common storage polysaccharides of thick-walled cells, galactomannan and xyloglucan, can be compared with respect to their hydrodynamic properties. Xyloglucan forms highly viscous solutions at ambient temperature, and an increase in temperature markedly reduces this viscosity, as observed with galactomannan

(Carlson *et al.*, 1962; Chudzikowski, 1971). Thus, water uptake into the cotyledons containing xyloglucan would be expected to be enhanced by increase in temperature. According to Leopold (1980) the rate of water entry in the seed is dependent on the rates of both water diffusion and the hydration of polyelectrolytes in the tissue, and both of these physical parameters are temperature-dependent.

Scarification was another factor influencing water uptake. Imbibition was slower when the hilum rather than when a lateral point was scarified (Fig. 1). This delay is probably related to the greater surface of contact with water in the laterally scarified seeds (Fig. 2). As water entered the seeds, ruptures were increasingly noted in the seed coat, therefore opening new ways for water entrance. This behaviour is more evident in the laterally scarified as opposed to the hilum scarified seeds (Fig. 2).

Our experiments demonstrated that different seed parts imbibe water with different speeds. Whereas the seed coat and the embryonic axis imbibed water quickly, the cotyledon absorbed water rather slowly (Fig. 3). As will be shown below, this phenomenon appears to be directly related to the chemical composition of the cell walls in different tissues of the seed.

Seed morphology

Dormant seeds of *H. courbaril* weigh about 5 g. Seed mass is mainly related with the cotyledons and seed coat (Table 1), suggesting that, at least quantitatively, their chemical compositions are important for imbibition. The seed coat is composed by three different layers (Fig. 4), typical of seeds of the Caesalpinioideae (Flores & Benavides, 1990). The outer layer is the palisade, which comprises a double cell layer with their larger axis pointing inwards (Figs 4A, B). This layer is often regarded as a barrier to water entrance into hard-coated seed mainly due to the presence of waxy material and/or insoluble lignin polymers (Egley *et al.*, 1983; Melo-Pinna *et al.*, 1999). Underlying the palisade is the osteosclereid, or 'hourglass', cell layer (Fig. 4B), which is normally found in relatively high proportions in the seed coat of legume species of Faboideae, such as *Phaseolus* (Esau, 1977), *Sesbania* (Bevilacqua *et al.*, 1987) and

Glycine (McDonald, 1988b). Normally, this layer is thought to be a vector of radial water distribution for the whole seed coat (McDonald, 1988b). The relatively reduced proportion of "hourglass" cells in *H. courbaril* (~ 4% of the coat) is balanced by an increase in thickness of the underlying parenchymatic layer (third layer) (Fig. 4B). The parenchymatic layer contains a greater number of cell layers, corresponding to ca. 70% of the entire thickness of the seed coat (Figs. 4A, C). This layer is composed of large and anisodiametric cells with thin walls (Fig. 4C). Following imbibition, this layer expands to approximately twice the thickness as the same layer in dormant seeds, from 370 to 780 μm (not shown). As a consequence of cell expansion, the palisade layer ruptures, introducing new openings in the seed coat to allow enhanced water intake (Fig. 2). These results suggest that the greater thickness of seed coat, mainly the parenchymatic layer, might be one of the factors involved in the control of the rate of water uptake by seeds of *H. courbaril* during the first phase of imbibition.

After entering the seed coat tissues, water enters the cotyledons, which contain egg-shaped cells with their larger axes pointing inwards (Figs. 4A, D). Cotyledon cells are less densely packed than parenchymatous cells of seed coat. The formers are interlinked by large pit fields (Fig. 4D), and further magnification shows that "channels" appear to be present in the intercellular spaces (Figs. 4D, E). The primary cell walls show striations, which are pectin-rich as seen with toluidine blue under light microscopy (Tin   *et al.*, 2000b). Underlying the primary wall, the very thick storage cell wall containing xyloglucan is present (Tin   *et al.*, 2000a).

Cell wall composition of the seed parts

Polysaccharide extraction of the seed parts with hot water showed marked differences between cotyledons and seed coat (Table 2). In the cotyledons, this extraction yielded 54% of the dry mass while in the seed coat it was only 13%. On the other hand, the content of uronic acids was higher in seed coat than in cotyledons (43 and 2%, respectively). The monosaccharide composition of water soluble polymers from the cotyledons confirms the previous evidence of that xyloglucan is the principal storage polysaccharide in the seeds (Buckeridge *et al.*,

1992, 1997; Tiné *et al.*, 2000a) and add new information about the distribution of other type of polysaccharides, such as pectins.

A study of the cell wall components of the seed coat showed that uronic acids, which represent the major fraction of cell wall pectins, corresponded to the largest proportion of cell wall material and is associated mainly with ammonium oxalate fraction (Table 2). Although analysis of cell wall fractions by Fourier Transformed Infrared Spectroscopy (FTIR) has shown that some of the seed coat pectins are methyl esterified (unpublished results), the unesterified pectins, which are negatively charged (Carpita & Gibeaut, 1993), are the principal component. It is therefore likely that pectins might be a control point of the rate of imbibition in the parenchymatic layer of seed coat, before the cotyledons hydration. This hypothesis is corroborated by a faster imbibition of the seed coat in relation to cotyledons (Fig. 3) and also by *in vitro* experiments with composites (see below). Methylation analysis of the more abundant ammonium oxalate fraction revealed that the main components of this fraction are polygalacturonic acid, a highly branched 5-linked arabinan and galactoglucomannan (Table 3).

Matrix glycans found from 0.1 to 4 M NaOH fractions, are minor components of the seed coat walls (ca. 12%) and are composed primarily of a xylan with limited branching with arabinose, as determined by *t*-Ara, 4-Xyl, and 2,4-Xyl (Table 3). However, as no xyloglucan oligosaccharides could be detected in HPAEC after extensive hydrolysis with *Trichoderma* cellulase (not shown), it is possible that 4-Glc, along with 4- and 4,6-Man, are from galactoglucomannan.

Altogether, our data are consistent with the hypothesis that the high rate of water uptake into the seed coat is related to the high proportions of pectin. Furthermore, separate analyses of uronic acids of the palisade and parenchymatic layers showed that the parenchymatic layer has twice as much uronic acid (18.7%), as well as a higher amount of rhamnose, arabinose and galactose. We conclude that the parenchymatous cell layer is primarily responsible for water conduction during early stages of imbibition.

Water imbibition by cellulose/polysaccharide composites

Beyond higher rates of imbibition by the seed coat further water uptake resides primarily in the hydrodynamic properties of the cotyledons. As mentioned before, the route for water uptake by the cotyledons, is possibly through capillary channels in the intercellular spaces (Fig. 4E). However, once in the channel, the water can be absorbed by the thick cell wall, which is rich in xyloglucan (Table 2). Storage xyloglucan has been regarded as a hydrocolloid, with similar properties to galactomannan (Buckeridge & Reid, 1996). The latter is well known as a water interactive polymer of high importance for seed imbibition and of some utility in the industry (Chudzikowski, 1971, Reid & Bewley, 1979).

In order to understand the importance of the storage xyloglucan for water imbibition in *H. courbaril*, we constructed composites of xyloglucan:cellulose of 20:80% and 40:60% and compared their pattern of water imbibition with the ones from cellulose and xyloglucan matrices alone. The “naked” fibres are clearly seen in the controls of cellulose alone (Fig. 5A). Their monosaccharide analysis after acid hydrolysis yielded 99% glucose (not shown). When xyloglucan was added to the fibre at a proportion of 40%, the fibres became barely distinguishable, presenting characteristics partly similar to a matrix made of pure xyloglucan (compare Figs 5B, D). These results agree with the observations by Lima & Buckeridge (2001), who found that the storage xyloglucan from *H. courbaril* binds to cellulose, whereas galactomannan forms independent complexes (see also Whitney *et al.*, 1995, 1998).

To test the effects of types of cellulose composites on imbibition rate empirically, an *in vitro* system was designed to mimic the micropylar route of the seeds. The proportion of xyloglucan in the mixture strongly influenced the imbibition of water by the complexes by changing both rate of water entrance and total amount of water imbibed (Fig. 6). In all instances, there was a relatively rapid first phase of imbibition, which is clearly associated with water movement within the capillaries. After this period, longer log phases were observed in both 20 and 40% xyloglucan composites (Fig. 6). Furthermore, the total amount of water imbibed by the composite after the lag phase was much higher than the amount of water taken by

cellulose alone. Matrices made with pure xyloglucan did not show the first rapid phase and imbibed water rather slowly. This confirms that the fast first phase of imbibition observed in other composites are related with the capillaries, since they are not present in pure xyloglucan matrices (Fig. 5B). Controls with two starch concentrations (not shown) revealed a pattern very similar to the cellulose alone, and, like xyloglucan, galactomannan strongly decreased water imbibition (not shown). Our results suggest that, regarding xyloglucan, the first rapid phase is related with water movement into the capillaries present among the cellulose microfibrils, whereas the second, slower phase is related to the water taken by the xyloglucan that covers most the cellulose surface (Figs. 5C, D). Composites containing 30:70% *H. courbaril* seed coat pectin:cellulose (Fig. 6) displayed an imbibition pattern which was almost indistinguishable from the cellulose fibres (Fig. 6), confirming the results on the imbibition of seed parts (Fig. 3) and reinforcing the hypothesis that the large decrease in imbibition rate of seeds is due to xyloglucan and not to pectin.

Physiological importance of polysaccharides in the uptake of water by seeds of H. courbaril

On the basis of the results of rate of imbibition, seed coat polysaccharide composition, hydrodynamic properties of xyloglucan, and ultrastructural observations, the following tentative model for water uptake by seeds of *H. courbaril* can be proposed.

In seeds of *H. courbaril* the coat can be considered as a bifunctional tissue playing a role in water retention and acceleration at the same time. On one hand, its palisade layer, which is richer in cellulose and matrix structural glycans such as arabinoxylan, xyloglucan and mannan, serves to severely restrict water entry to the micropyle or any scarified region. These polymers might contribute also to render a higher degree of brittleness to the cell layer. After this barrier is crossed, water has access to the parenchymatic layer, which is rich in water soluble pectin and whose function appears to be the rapid distribution of water around the cotyledons surface

(represented by route A, Fig. 7). As this pectin-rich layer takes up water, it expands to twice its original size (not shown), promoting ruptures in several regions of the palisade layer and leading to the opening of several new water entry locations (Fig. 2). These ruptures appear to be related directly to the progression of water front following route A (Fig. 7) and tend to be faster than the water front within the cotyledons. This agrees with our observation that a proportion of the seed coat is composed of a relatively more water-soluble pectin whereas in cotyledons the main imbibing substance is xyloglucan, which takes up water slowly (Fig. 6). This reflects the differences found in water absorption by every seed tissue (Fig. 3).

In the cotyledons, route B (Fig. 7) is possibly subdivided into two further routes, one accounting for the channels [B(c)] present in intercellular spaces (Fig. 4E), whereas route B(x) is associated with the absorption of water mainly by xyloglucan. Because of the similarity of the hydrodynamic features of galactomannans and storage xyloglucans (Reid, 1985; Buckeridge *et al.*, 2000), it can be speculated that the xyloglucan-water interaction produces a highly viscous medium, which restricts the advance of water into the matrix.

Although seed xyloglucans are very well known as storage polymers, being mobilised following germination of several legume species (Reid, 1985; Buckeridge *et al.*, 1992; Tiné *et al.*, 2000a; Buckeridge *et al.*, 2000), our results strongly indicate that they also play a role in the control of water imbibition and distribution before root protrusion. The delay in water imbibition by the cotyledons could be related to a protection against the start of germination with lower water availability or once the seeds are imbibed against desiccation. This could be also related to synchronisation of the processes of water imbibition and the activation of the biochemical processes that take place during germination.

ACKNOWLEDGEMENTS

This study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo-FAPESP (BIOTA Programme, Grant Nº 98/05124-8). The authors thank Mrs

RT Shirasuna for technical assistance with the SEM and MSc. MAS Tiné for helping with HPAEC analyses. We thank Dr. MR Braga and Dr. SMC Dietrich for the reviews of the text and suggestions.

REFERENCES

- Bevilacqua, L.R.; Fossati, F. & Dondero, G. 1987. 'Callose' in the impermeable seed coat of *Sesbania punicea*. *Annals of Botany* 59: 335-341.
- Bewley, J.D. & Black, M. 1994. Seeds - Physiology of Development and Germination. Second edition. New York: Plenum Press
- Buckeridge, M.S. & Dietrich, S.M.C. 1996. Mobilisation of the raffinose family oligosaccharides and galactomannan in germinating seeds of *Sesbania marginata* Benth. (Leguminosae-Faboideae). *Plant Science* 117: 33-43.
- Buckeridge, M.S. & Reid, J.S.G. 1996. Major cell wall storage polysaccharides in legume seeds: Structure, catabolism and biological functions. *Ciência e Cultura Journal of Brazilian Association for the Advancement of Science* 48: 153-162.
- Buckeridge, M.S.; Crombie, H.J.; Mendes, C.J.M.; Reid, J.S.G.; Gidley, M.J. & Vieira, C.C.J. 1997. A new family of oligosaccharides from the xyloglucan of *Hymenaea courbaril* L. (Leguminosae) cotyledons. *Carbohydrate Research* 303: 233-237.
- Buckeridge, M.S.; Panegassi, V.R. & Dietrich, S.M.C. 1995. Storage carbohydrate mobilisation in seeds of *Dimorphandra mollis* Benth. (Leguminosae) following germination. *Revista Brasileira de Botânica* 18: 171-175.
- Buckeridge, M.S.; Rocha, D.C.; Reid, J.S.G. & Dietrich, S.M.C. 1992. Xyloglucan structure and post-germinative metabolism in seeds of *Copaifera langsdorffii* from savannah and forest populations. *Physiologia Plantarum* 86: 145-151.
- Buckeridge, M.S.; Santos, H.P. & Tiné, M.A.S. 2000. Mobilisation of storage cell wall polysaccharides in seeds. *Plant Physiology and Biochemistry* 38: 141-156.
- Carlson, W.A.; Ziegenfuss, E.M. & Overton, J.D. 1962. Compatibility and manipulation of guar gum. *Food Technology* 16: 50-54.

- Carpita, N.C. & Gibeaut, D.M. 1993. Structural models of the primary cell walls of flowering plants. Consistency of structure with the physical and biochemical changes during growth. *Plant Journal* 3: 1-30.
- Carpita, N.C. & Shea, E.M. 1989. Linkage structure by gas chromatography-mass spectrometry of partially-methylated alditol acetates. In Biermann CJ, McGinns CD. *Analysis of Carbohydrates by GLC and MS*. CRC, Boca Raton, FL, 155-216.
- Chudzikowski, R.J. 1971. Guar gum and its applications. *Journal of the Society of Cosmetic Chemists* 22: 43-60.
- Esau, K. 1977. Anatomy of Plant Seeds. Second edition. New York: John Wiley & Sons.
- Filisetti-Cozzi, T.M.C.C. & Carpita, N.C. 1991. Measurement of uronic acids without interference from neutral sugar. *Analytical Biochemistry* 197: 157-162.
- Flores, E.M. & Benavides, C.E. 1990. Germinación y morfología de la plántula de *Hymenaea courbaril* L. (Caesalpinaceae). *Revista de Biología Tropical* 38: 91-98.
- Franco, T.T.; Rodrigues, N.R.; Serra, G.E.; Panegassi, V.R. & Buckeridge, M.S. 1996. Characterization of storage call wall polysaccharides from Brazilian legume seeds and the formation of aqueous two-phase systems. *Journal of Chromatography B Biomedical Applications* 680: 255-261
- Gibeaut, D.M. & Carpita, N.C. 1993. Synthesis of (1→3),(1→4)β-D-glucan in the Golgi apparatus of maize coleoptiles. *Proceedings of the National Academy of Sciences U. S. A.* 90: 3850-3854.
- Hayashi, T. 1989. Xyloglucan in the primary cell wall. *Annual Review of Plant Physiology and Plant Molecular Biology* 40: 139-168.
- Leopold, A.C. 1980. Temperature effects on soybean imbibition and leakage. *Plant Physiology* 65: 1096-1098.
- Lima, D.U. & Buckeridge, M.S. 2001. Interaction between cellulose and storage xyloglucans: the influence of degree of galactosylation. *Carbohydrate Polymers* 46: 157-163.

- Lüscher-Mattli, M. & Rüegg, M. 1982. Thermodynamic functions of biopolymer hydration. I. Their determination by vapor pressure studies, discussed in an analysis of the primary hydration process. *Biopolymers* 21: 403-418.
- McDonald, M.B.Jr.; Vertucci, C.W. & Roos, E.E. 1988a. Soybean seed imbibition: Water absorption by seed parts. *Crop Science* 28: 993-997.
- McDonald, M.B.Jr.; Vertucci, C.W. & Roos, E.E. 1988b. Seed coat regulation of soybean seed imbibition. *Crop Science* 28: 987-992.
- Meier, H. & Reid, J.S.G. 1982. Reserve polysaccharide other than starch in higher plants. In: Loewus FA and Tanner W, eds. *Plant carbohydrates*. New York: Springer, 418-471.
- Reid, J.S.G. 1985. Structure and function of legume-seed polysaccharides. In: Brett and Hillman, eds. *Biochemistry of Plant Cell Walls*. Cambridge University Press. 259-268.
- Reid, J.S.G. & Bewley, J.D. 1979. A dual role for the endosperm and its galactomannan reserves in the germinative physiology of fenugreek (*Trigonella foenum-graecum* L.), an endospermic leguminous seed. *Planta* 147: 145-150.
- Saeman, J.F.; Buhl, J.L. & Harris, E.E. 1945. Quantitative saccharification of wood and cellulose. *Industrial Engineer and Chemical Analyses Edition* 17: 35-37.
- Tiné, M.A.S.; Cortelazzo, A.L. & Buckeridge, M.S. 2000a. Xyloglucan mobilisation in cotyledons of developing plantlets of *Hymenaea courbaril* L. (Leguminosae-Caesalpinoideae). *Plant Science* 154: 117-126.
- Tiné, M.A.S.; Cortelazzo, A.L. & Buckeridge, M.S. 2000b. Occurrence of xyloglucan containing protuberances in the storage cell walls of cotyledons of *Hymenaea courbaril* L. *Revista Brasileira de Botânica* 23: 413-417.
- Vertucci, C.W. & Leopold, A.C. 1983. Dynamics of imbibition of soybean embryos. *Plant Physiology* 72: 190-193.
- Vertucci, C.W. & Leopold, A.C. 1987. Water binding in legume seeds. *Plant Physiology* 85: 224-231.
- Waggoner, P.E. & Parlange, J. 1976. Water uptake and water diffusivity of seeds. *Plant Physiology* 57: 153-6.

- Whitney, S.E.C.; Brigham, J.E.; Darke, A.H.; Reid, J.S.G. & Gidley, M.J. 1995. *In vitro* assembly of cellulose/xyloglucan networks - Ultrastructural and molecular aspects. *Plant Journal* 8: 491-504.
- Whitney, S.E.C.; Brigham, J.E.; Darke, A.H.; Reid, J.S.G. & Gidley, M.J. 1998. Structural aspects of the interaction of mannan-based polysaccharides with bacterial cellulose. *Carbohydrate Research* 307: 299-309.
- York, W.S.; van Halbeek, H.; Darvill, A.G. & Albersheim, P. 1990. Structural analysis of xyloglucan oligosaccharides by ^1H -NMR spectroscopy and fast-atom-bombardment mass spectrometry. *Carbohydrate Research* 200: 9-31.

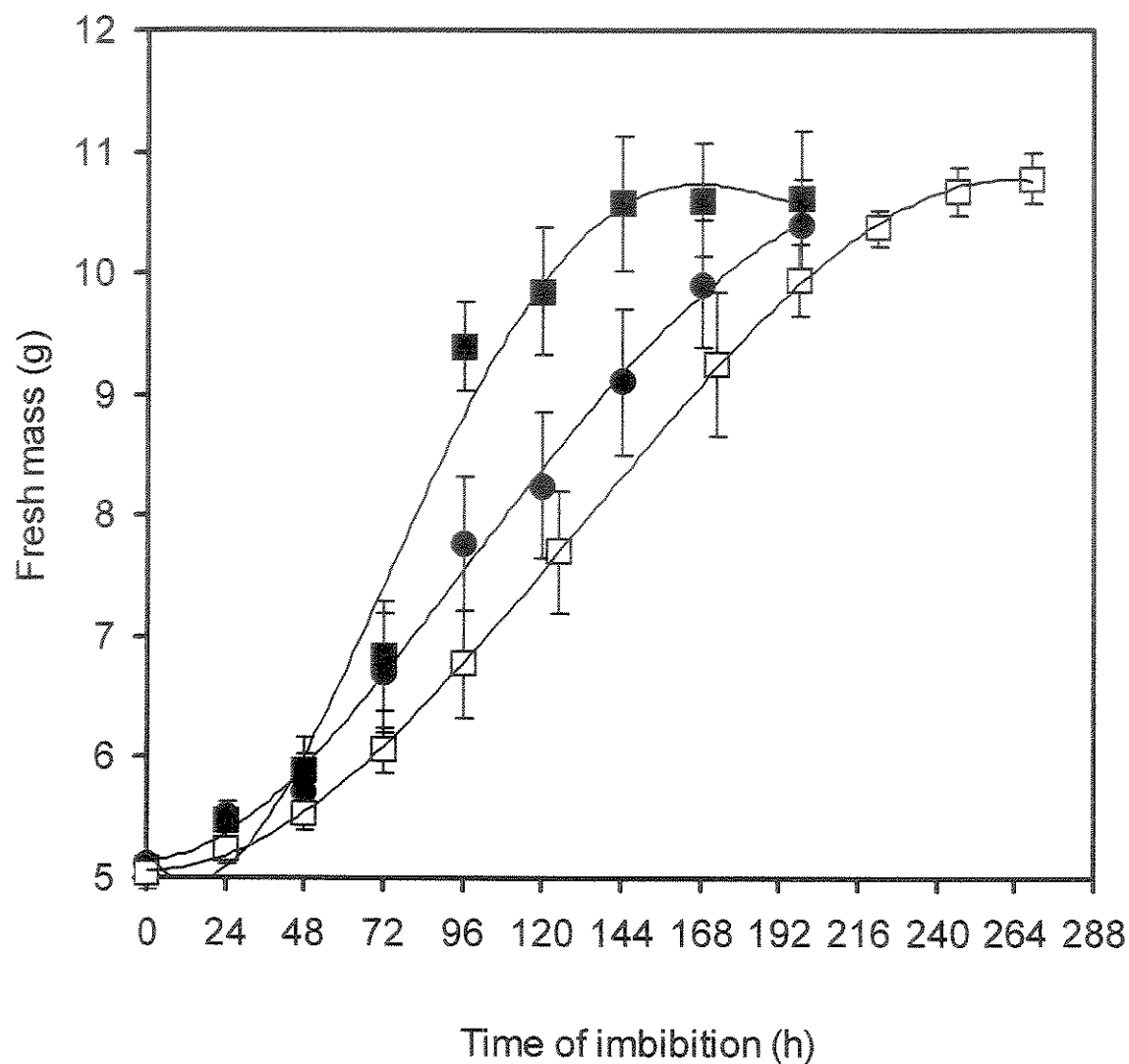


Fig. 1. Imbibition of seeds of *H. courbaril* at different temperatures and scarification positions in the seed. Seeds were scarified at the hilum end (●, 30 °C) and at a lateral position (□, 20 °C; ■, 30 °C). The bars represent the standard deviation among 10 seeds in each point.

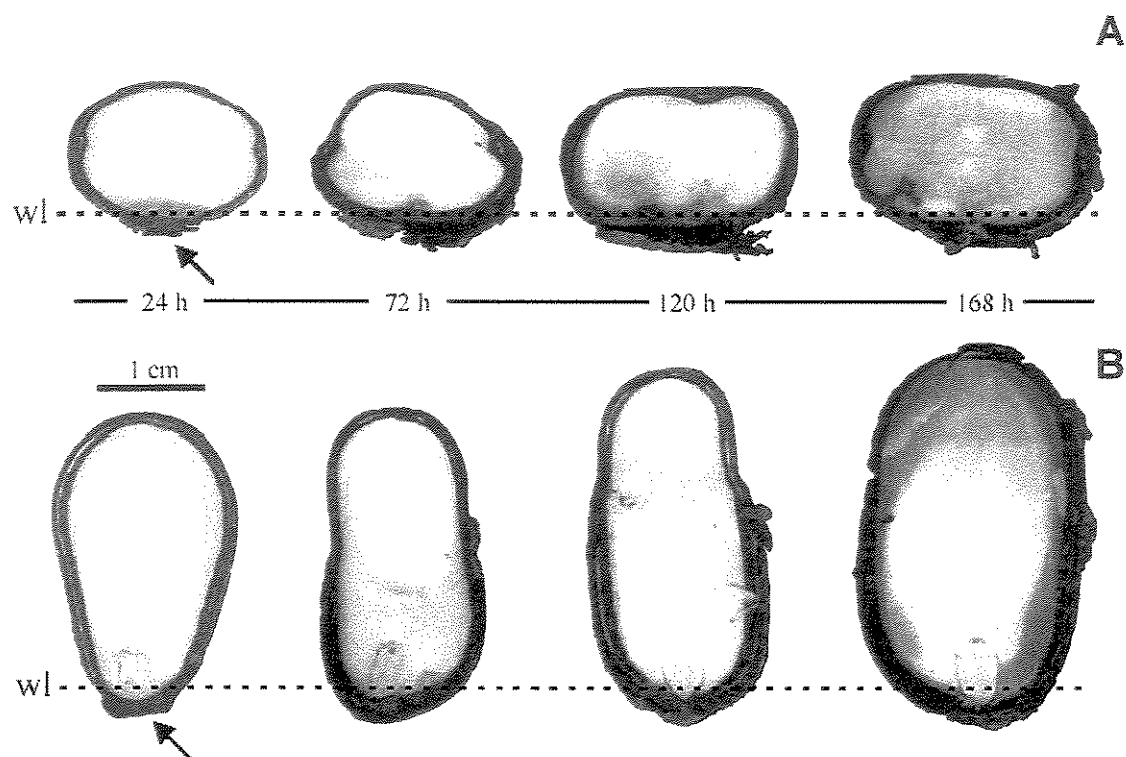


Fig. 2. Transversal (**A**) and longitudinal (**B**) sections of seeds at different times of imbibition. The arrows represent the scarified point, which was in the lateral (A) and in the hilum position, respectively. wl = water level.

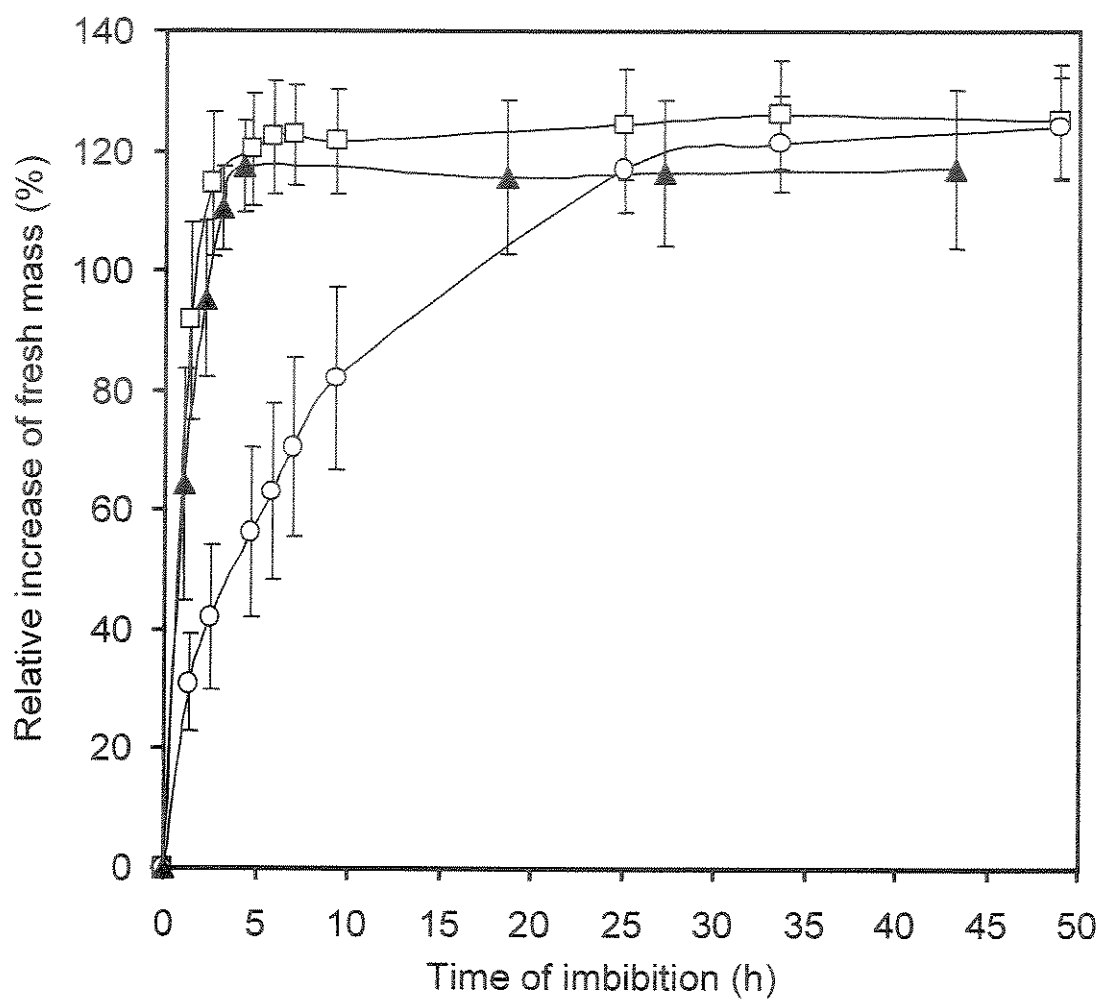


Fig. 3. Relative increase in fresh mass of seed parts of *H. courbaril* during imbibition (20°C). Seed coat (□), cotyledon (○) and embryonic axis (▲). Bars represent the standard deviation among 15 seed parts in each point.

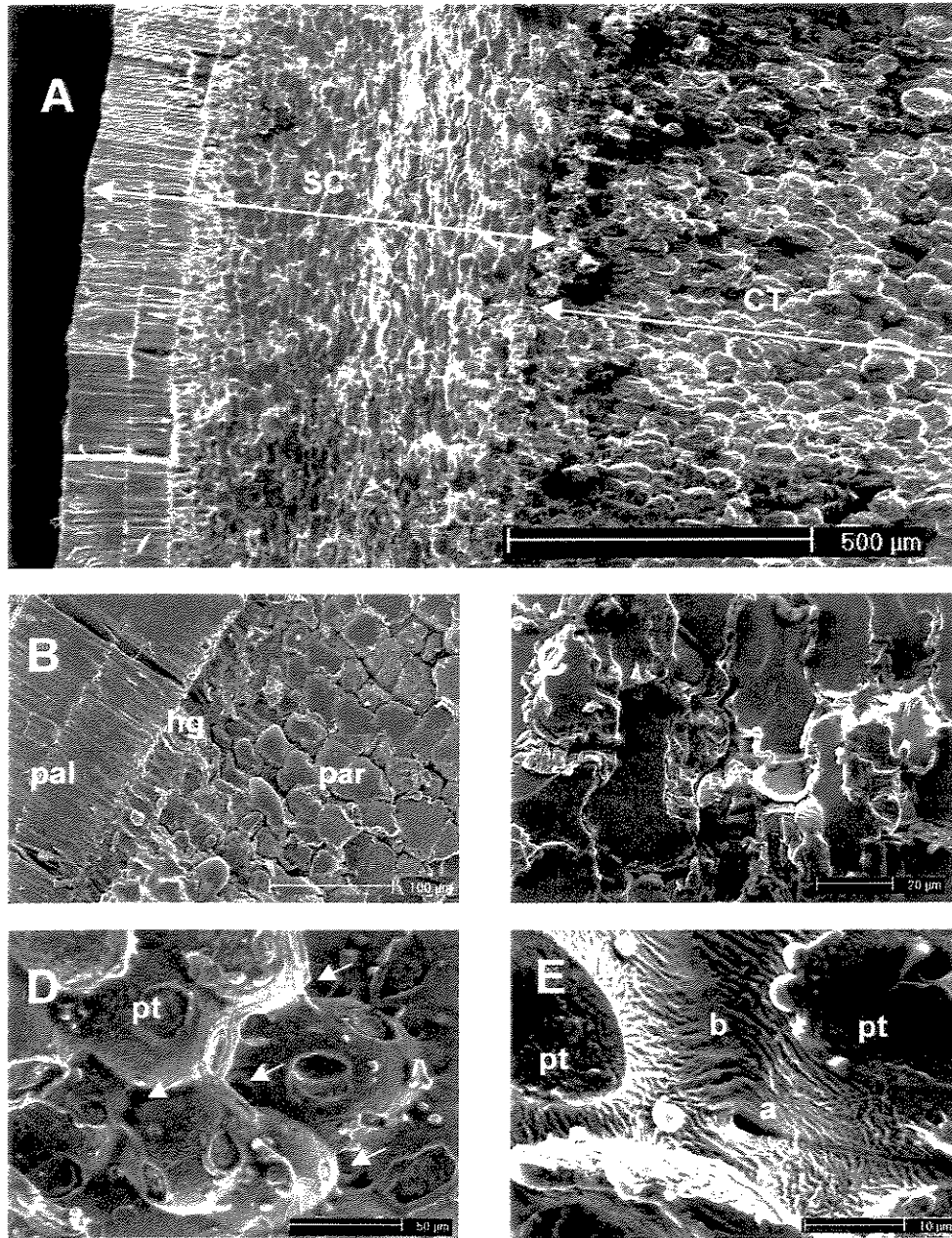


Fig. 4. Scanning electron micrographs showing details of a cross section of one seed of *H. courbaril* L.. (A) lower magnification showing the seed coat (SC) and part of cotyledon (CT); (B) detail of seed coat double layer palisade (pal), 'hourglass' cells layer (hg) and parenchymatic layer (par); (C) parenchymatic layer of the seed coat; (D) detail of cotyledon cells, which shows one out of many pit fields through which cells are linked together (pt) and the arrows show the many intercellular spaces; (E) higher magnification of cotyledon storage cell walls showing the intercellular space (a) and deposition of pectin on the microfibrils in a pit field zone between linked cells (b) and detail of a pit field (pt).

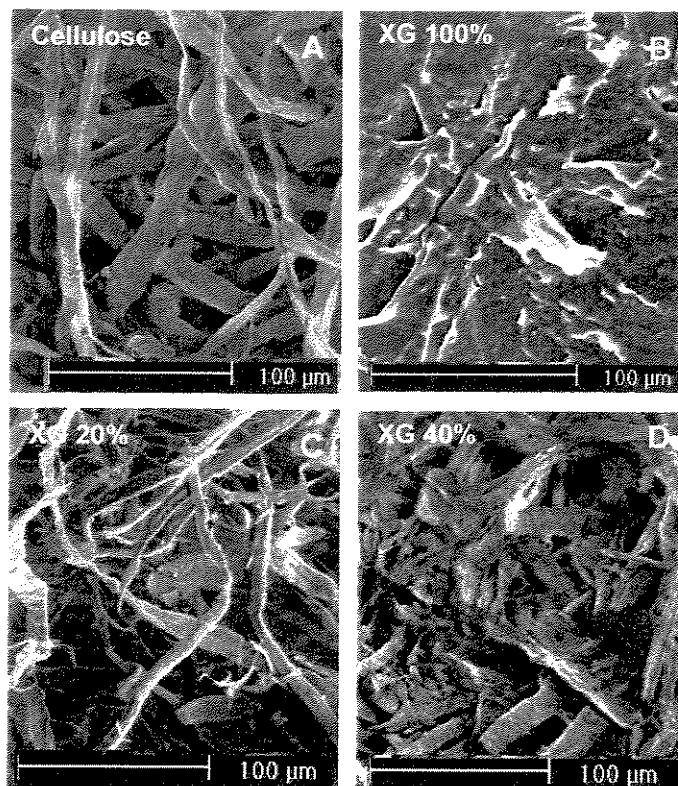


Fig. 5. Scanning electron micrographs showing differences among composites artificially constructed of commercial cellulose and xyloglucan extracted from cotyledons of *H. courbaril*. (A) 100 % of cellulose; (B) 100% of xyloglucan; (C) 20% of xyloglucan in cellulose; (D) 40% of xyloglucan in cellulose.

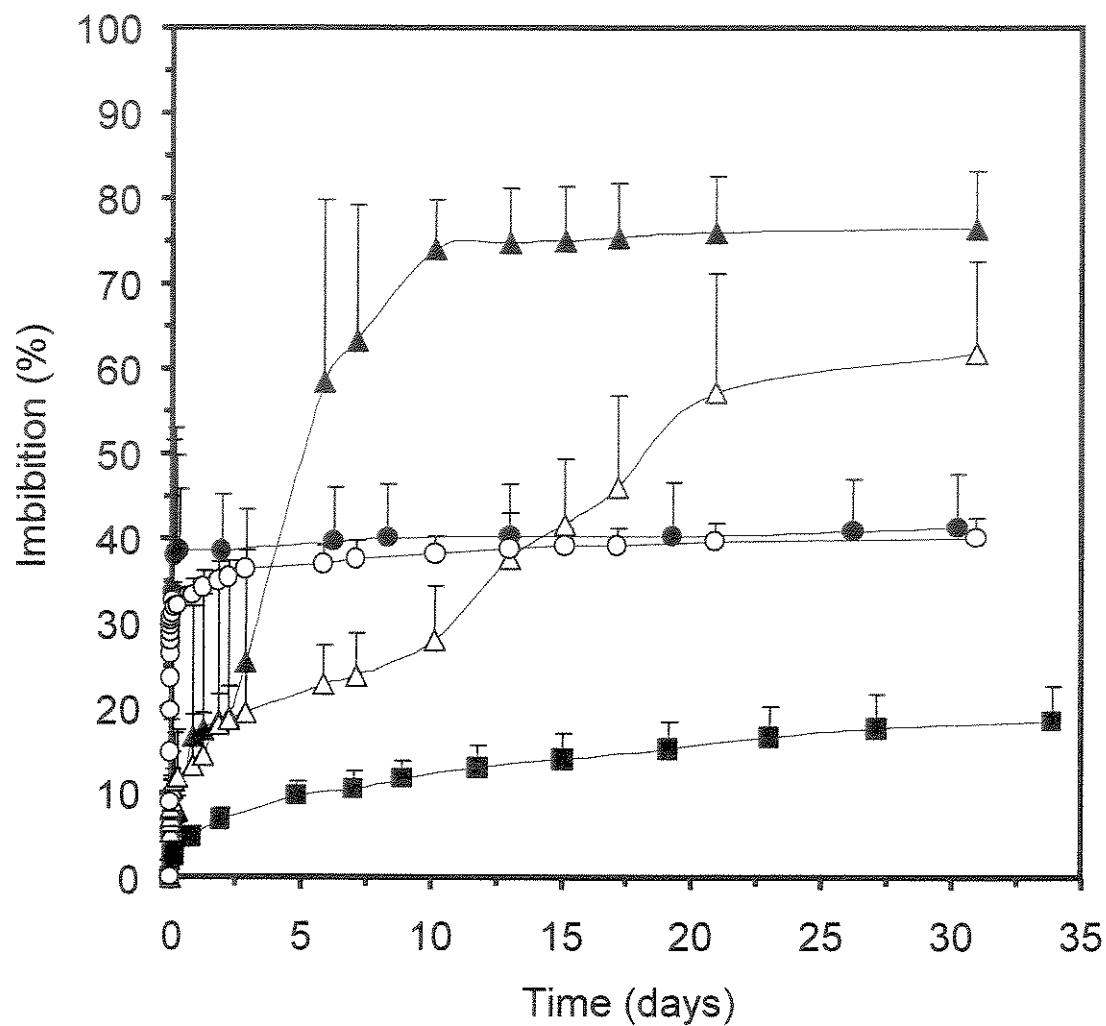


Fig. 6. Time course of *in vitro* imbibition of cellulose (○), 100% xyloglucan (■), and composites of pectin:cellulose (30:70%, ●) and xyloglucan:cellulose (20:80%, ▲; 40:60%, △). The pectin and xyloglucan were both extracted from seed coat and cotyledons, respectively, of seeds of *H. courbaril*. Bars represent the standard deviation among five measurements.

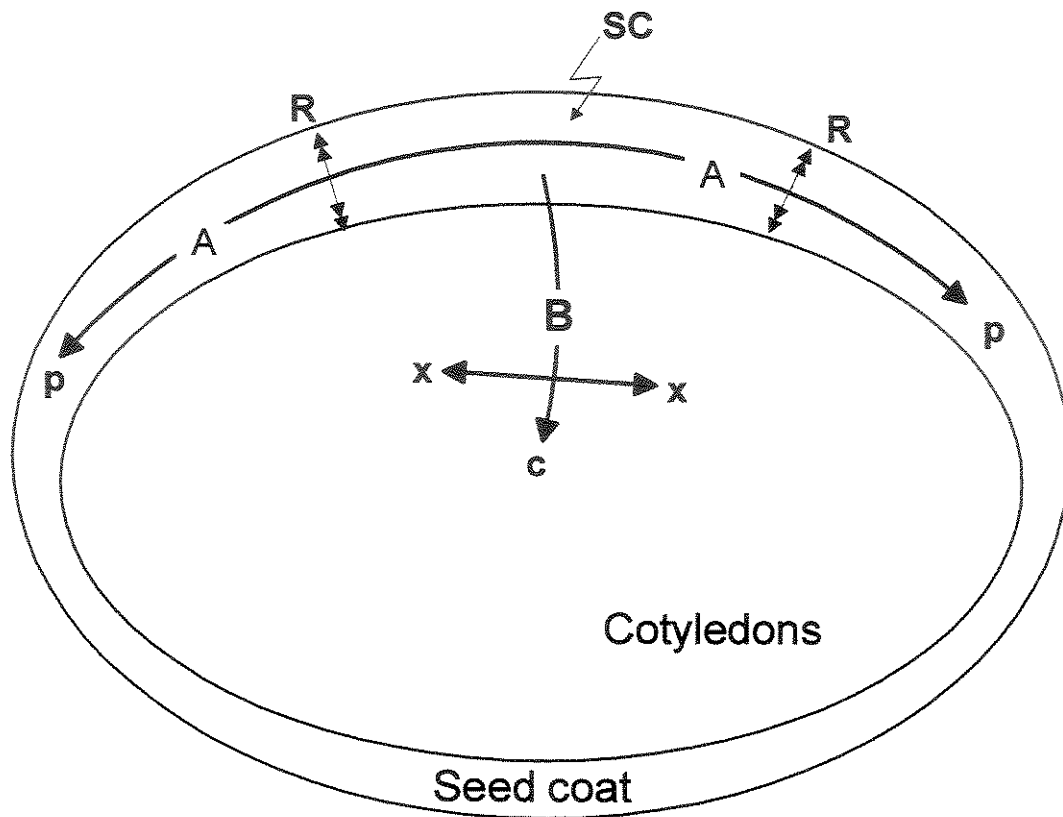


Fig. 7. A model for water uptake by seeds of *H. courbaril*. After scarification (SC), the water follows route A, which moves faster in the coat than enters the cotyledons, driven by pectin-rich walls (p) in the seed coat (parenchymatic layer). This behaviour promotes the expansion of seed coat (double-head arrows) and consequently induces ruptures (R) of the palisade layer, thus offering new openings to water intake. After the seed coat is saturated with water, water flows by route B, driven both through intercellular channels (c) and xyloglucan containing storage walls (x).

Table 1 – Dry weight of seed parts in *Hymenaea courbari* L.

	Seed parts		
	Cotyledons	Coat	Embryonic axis
Dry weight (g)	3.37 (\pm 0.27)	1.40 (\pm 0.33)	0.02 (\pm 0.01)
Proportion (%)	70.61 (\pm 5.97)	28.97 (\pm 5.99)	0.42 (\pm 0.23)

Numbers in parenthesis represent the standard deviation of weight of parts of 30 seeds.

Table 2 - Composition of water soluble polysaccharides from cotyledons and seed coat of *Hymenaea courbaril*.

<i>Extractions /</i>	Yield	Uronic	Monosaccharides (%)					
Seed part	extraction	Acids						
	(%) ^a	(%) ^b	Glc <i>p</i>	Gal <i>p</i>	Xil <i>p</i> ^g	Fuc <i>p</i>	Ara <i>f</i>	Rha <i>p</i>
<i>Water extracts</i>								
Cotyledons	54 (±8.1) ^c	2 (±1.3)	53	11	35	tr	nd	nd
Seed coat	13 (±0.4)	43 (±0.9)	27	10	35	tr	28	nd
<i>Oxalate/Alkali extracts</i>								
Seed coat								
(oxalate 0.5 M)	58 (±2.1)	67 (±0.5) ^d	11	57	tr	nd	26	nd
(NaOH 0.1 M)	6 (±0.8)	61 (±2.6)	13	7	52	nd	29	nd
(NaOH 1.0 M)	4 (±1.4)	37 (±2.1)	11	4	59	nd	26	nd
(NaOH 4.0 M)	2 (±0.5)	tr ^e	29	10	37	tr	24	nd
(residue)	31 (±1.1)	nd ^f	48	5	23	nd	24	tr

^a data given as % of dry mass; ^b in relation to yield extraction; ^c standard deviation (n=3); ^d percentage of each fraction;

^e traces (<0.5%); ^f not detected ; ^g these fractions presented trace contamination with mannose according to analysis by GC-MS.

Table 3 - Linkage analysis of Oxalate / Alkali cell wall fraction of the seed coat of *H. courbaril*.

Deduced glycosidic linkage		Percentage of total peak area			
		Oxa	0.1 M	1.0 M	4.0 M
<i>Ara f</i>	Terminal-	-	-	1.8	2.0
	3-	-	0.8	1.1	1.0
	5-	5.9	9.0	13.1	9.3
	2,4-	-	1.4	3.9	-
	3,5-	3.7	3.0	4.3	4.2
	4,6-	-	-	-	0.5
	2,5/3,5-	2.1	-	-	-
<i>Xyl p</i>	Terminal-	-	-	2.2	2.3
	4-	10.9	36.2	38.1	34.9 ^a
	2,4-	-	12.3	14.9	11.7
<i>GalA p</i>	Terminal-	-	8.5	11.4	5.2
	4-	21.9	17.5	2.2	0.6
<i>Gal p</i>	Terminal-	3.7	2.4	0.3	1.9
	6-	-	-	-	1.0
	4,6-	-	-	-	0.5
	3,6-	-	-	0.2	1.0
<i>Rha p</i>	2,4-	-	-	-	2.0
<i>Man p</i>	4-	9.8	1.8	0.7	3.0
<i>Glc p</i>	4-	29.0 ^b	7.1	-	8.9 ^c
	4,6-	4.0	-	1.0	8.5

^a 2-Xylp low contamination; ^b there might be a limited contamination with starch from the fruit endocarp; ^c 4-GlcAp low contamination.

5. CAPÍTULO 2

Artigo 2 – The role of cotyledons on the establishment and growth of seedlings of *Hymenaea courbaril* L. under different environmental conditions

The role of cotyledons on the establishment and growth of seedlings of *Hymenaea courbaril* L. under different environmental conditions.

Henrique Pessoa Dos Santos¹ & Marcos Silveira Buckeridge^{2, 3}

¹ Departamento de Biologia Vegetal – UNICAMP / Seção de Fisiologia e Bioquímica de Plantas – IBt. Actual address: Rua Livramento, 515, Caixa Postal 130, CEP 95700-000, Bento Gonçalves, RS, Brazil. (henrique@cnpuv.embrapa.br)

² Seção de Fisiologia e Bioquímica de Plantas, Instituto de Botânica, Caixa Postal 4005, CEP 01061-970, São Paulo, SP Brazil. (msbuck@usp.br)

³ To whom correspondence should be addressed.

RESUMO

Hymenaea courbaril tem sido considerada uma espécie tolerante à sombra, porém até o presente momento o conhecimento sobre as estratégias de estabelecimento de suas plântulas sob o dossel de matas tropicais é muito restrito. Esta espécie apresenta sementes grandes (aprox. 5 g) e cotilédones globóides, ricos em um polissacarídeo de reserva de parede celular (xiloglucano) que corresponde a aproximadamente 40% do peso seco das sementes. O objetivo do presente trabalho foi descrever a importância desta reserva cotiledonar (xiloglucano) para o estabelecimento e crescimento de plântulas de *Hymenaea courbaril*. Para isto, foram adotadas como estratégias a retirada dos cotilédones, durante o período de mobilização de xiloglucano, e o crescimento das plântulas sob diferentes condições de luz e temperatura. Em todas as condições de luz e temperatura a mobilização das reservas de xiloglucano foi altamente relacionada com o crescimento e desenvolvimento da parte aérea das plântulas. Estes dados foram evidenciados também pelo acompanhamento da radioatividade injetada em um cotilédone na forma de [^{14}C]-sacarose, a qual foi principalmente direcionada para as folhas em expansão. A ausência das reservas de xiloglucano promoveu também a antecipação do estabelecimento da fotossíntese com reduzida área foliar (50% menor), o que limitou o potencial fotossintético e o crescimento das plântulas no interior da floresta. Em conjunto, estes resultados são as primeiras evidências na definição das reservas cotiledonares de xiloglucano como uma vantagem ecológica para o estabelecimento de plântulas de *H. courbaril* em condições de sombra das florestas tropicais.

ABSTRACT

Although *Hymenaea courbaril* has been considered as a shade tolerant species, the present knowledge about what strategies are adopted for seedling establishment in the understorey are restrict. This species presents large seeds (ca. 5 g) with globoid cotyledons, rich in a storage cell wall polysaccharide (xyloglucan) that corresponds to about 40% of the dry mass of the seed. The goal of the present work was to describe the importance of this cotyledon reserve (xyloglucan) for establishment and growth of seedlings of *H. courbaril*. Cotyledons excision, during the mobilisation of xyloglucan, and growth under different environmental conditions were the experimental strategies. In the light and temperature conditions used, the mobilisation of xyloglucan reserves was highly correlated to growth and development of the seedlings shoot. This observation was confirmed by restriction of shoot growth in seedlings without cotyledons during the period of xyloglucan mobilisation as well as by the observation that [^{14}C]-sucrose radioactivity (injected in one cotyledon) was mainly directed to expanding leaves. The absence of xyloglucan reserves also promoted an anticipation of establishment of photosynthesis instead of leaf area expansion (50% less), which resulted in restriction of the photosynthetic potential and growth of seedlings in the understorey of the forest. Altogether these results are the first evidence suggesting that xyloglucan reserves may be an ecological advantage to establishment of *H. courbaril* seedlings in the shade conditions of the tropical forest.

Key words: cotyledon, xyloglucan, photosynthesis, establishment, *Hymenaea*

INTRODUCTION

Studies to elucidate the importance of cotyledons by excision experiments have been the subject of many researches (Carelli & Magalhães, 1981; Veirskow, 1985; Wulff, 1986; Sassaki & Felipe, 1992). Except for a few exceptions, these studies have mainly demonstrated the role of cotyledons in the growth of commercial and/or annual species.

The excision of cotyledons tend to promote a reduction of growth, plant height, leaf expansion, and changes in carbon allocation which affect the root:shoot ratio. McConnaughay & Coleman (1999), working with the annual plant species (*Abutilon theophrasti*, *Chenopodium album* and *Polygonum pensylvanicum*), produced results that support a model of plasticity in biomass allocation in response to limiting resources and suggested that the carbon allocation is a good indicator of availability of resources. This has been applied to understand plant's ability or performance to grow in shaded forest understorey or open land (Whitmore, 1996; Cao & Ohkubo, 1998). DeLucia *et al.* (1998) studied the hypothesis that the pattern of biomass allocation varied predictably with shade-tolerance rank. These authors did not find consistent variation of biomass allocation among species but the results were consistent with shade-growth strategies, which maintained high specific leaf area and leaf area ratio. In general within species, however, shade-grown individuals allocate relatively more biomass to shoots than to roots (lower root:shoot ratio) and have greater ratios of total leaf area per unit area (Kitajima, 1994). These are considered to be adaptive phenotypic responses to shading because they increase the ratio of photosynthesis to respiration at the whole plant level and contribute to the maintenance of a positive carbon budget and maximisation of growth in the shade (Givnish, 1988).

Hymenaea courbaril L. (Leguminosae-Caesalpinioideae) is a leguminous tree present in forests of Central and South America. This species has been shown as a potential dominant in the rain forest principally due to shade (Souza & Válio, 1999) and drought (Gerhardt, 1993) tolerances. According to Swaine & Whitmore (1988)

nomenclature, *H. courbaril* can be considered as a typical climax species, which correspond to an extreme of the actual trends in seedling classification under wide and continuous gradient of shade tolerance (Kitajima, 1996; Whitmore, 1996).

Although *H. courbaril* has been considered a shade tolerant species until now the strategies adopted by the seedlings to survive under closed canopy were not described. This species presents large seeds (ca. 5 g) with globoid cotyledons, rich in a storage cell wall polysaccharide (xyloglucan) that corresponds to about 40% of the dry mass of the seed (Buckeridge & Dietrich, 1990). The germination of seeds of *H. courbaril* is epigeal (Flores & Benavides, 1990) and the initial development is mainly supported by mobilisation of a storage xyloglucan until a period of approximately two months after germination (Tin   *et al.*, 2000). The mobilisation of this reserve has been shown to display a biochemical complex with the co-ordinated action of four enzymes that completely degrade xyloglucan and produces a drastic decline of dry mass of cotyledons with concomitant increase of transitory starch and embryo growth (Buckeridge *et al.*, 2000a; Tin   *et al.*, 2000). Although the studies with xyloglucan suggest that this reserve compound can be an adaptive advantage to the establishment of seedlings of *H. courbaril* to shade-conditions, no experiments have been performed to understand the relationships between xyloglucan mobilisation and seedling growth at different environment conditions.

As proposed by Kitajima (1996), to understand some of the ecophysiological aspects of the relationship between the type of storage compound, shade tolerance and growth rate, it is essential to examine the association and interaction among multiple seedling traits and duration of seed reserve dependence. It is also instructive to follow parameters such as size, morphology and seedling growth as well as seedling growth rate.

Considering that few studies have been focused on the behaviour of tree seedlings in relation to carbon metabolism and allocation during the heterotrophy-autotrophy transition period (Escobar-Guti  rrez *et al.*, 1998), the focus of the present work was to describe the development and growth of seedlings of *H. courbaril* with and without cotyledons during the mobilisation of xyloglucan (the principal period of

loss of cotyledons dry mass, Tiné *et al.*, 2000), under different environmental conditions. We found that seedlings growing under higher temperatures and light intensities (greenhouse) developed faster. This was consistent with the speed of mobilisation of xyloglucan in the cotyledons. These data, plus the fact that excision of cotyledons prior to xyloglucan mobilisation strongly affected shoot development, suggest that a large parcel of this growth is dependent on the amount of carbon transferred from cotyledons to seedling. These results were also consistent with a relation between storage mobilisation and the establishment of photosynthesis by the new leaves formed by the seedling, since without cotyledons both C assimilation as well as light harvesting efficiency (as measured by Fv/Fm) were anticipated considerably.

MATERIAL AND METHODS

Seeds of *H. courbaril* were obtained from two trees growing in a gallery forest at São João da Boa Vista county (22° 00' S; 47°18' W), São Paulo, Brazil. Seeds were stored for four years in dry and cold conditions (RH 35%, 8°C). They were separated according to the fresh mass of dormant seeds (6.48% of water content) corresponding to the statistical mode (4.6-5.3 g). Seeds were placed between two sheets of wet paper until germination was visible (0.5 cm of radicle). Germinated seeds were placed in pots (2 L) with a mixture of organic soil and vermiculite (2:1 v/v) and the pots were placed in three different environmental conditions which are described below.

The environments were chosen principally by differences in temperature, radiation, light quality and air humidity (shown in Table 1). Growth-room: pots were randomly arranged on shelves provided with 10 fluorescent lamps (60 watts) and 4 incandescent lamps (40 watts). The photoperiod throughout the experiment was 12/12 hours and the maintenance of temperature was performed with the help of an air conditioning system with continuous air change. The purpose of using these conditions was to maintain plants under more constant temperature, humidity and

intermediate light intensity (around $200 \mu\text{mol.m}^{-2}.\text{s}^{-1}$). Greenhouse: this experimental conditions had the purpose of obtaining higher levels of radiation and temperature (table 1). Forest: we chose a set of conditions in the forest that are very similar to the ones where young *Hymenaea courbaril* seedlings are found naturally. Pots were placed inside the forest without gaps which were left under natural conditions of radiation, temperature and red:far red ratio (table 1).

The seedlings in all experiments were watered once daily. The red:far red ratios (R/FR, 655-665/725-735 nm ratio) and photosynthetically active photon flux density (PPFD, 400-700 nm) were recorded on a horizontal surface with SKR 110 and SKP 210 sensors (Skye Instruments Ltd., Llandrindod Wells, U.K), respectively. The measurement of temperature and air humidity of different environments were obtained with a Hygrothermograph (Oakton 08368-60, Cole-Parmer Instrument Co., Chicago, USA). Environments, as followed with the Hygrothermograph, could be described as varying periodically in respect to temperature and humidity.

Under growth-room conditions, Tiné *et al.* (2000) described that xyloglucan mobilisation occurred after seedling emergence between 35 and 55 days, beginning when the eophyll turned visible between the cotyledons, usually 30 days after germination and followed by a drastic change in cotyledons dry mass. In all experiments to test the effect of cotyledons excision during xyloglucan mobilisation in seedling growth, its were taken out when the eophyll turned visible (~ 2 mm outside cotyledons).

During growth, five plants per treatment (with and without cotyledons) in each environment were harvested at day 24 (at cotyledons excision), 36 and 49 after the beginning of seed imbibition, and were submitted to the following analyses: a) dry mass of the cotyledons and seedling parts (shoot and root); b) measurement of the height of the seedling; c) leaf area; and d) time necessary for expansion of different leaves. The variables a, b and c, were determined at days 24,36 and 49 and leaf area was determined before drying using a portable leaf area meter (LI-3000A, LI-COR, Inc., Lincoln, USA). The leaf emergence (variable d) was recorded daily.

All mass values were determined after drying at 70°C/72 h. On the basis of the results of leaf area and total mass measurements the leaf area ratio (LAR, total leaf area divided by the total seedling mass) was calculated. To each harvest date the relative growth rate of the seedlings (RGR) and the relative mobilisation rate of reserves (RMR) were calculated as follows: $RGR = [\ln(\text{mean final mass of the axes}) - \ln(\text{mean initial mass of the axes})] \cdot [(\text{final time}) - (\text{initial time})]^{-1}$ and $RMR = [\ln(\text{mean final mass of the cotyledons}) - \ln(\text{mean initial mass of the cotyledons})] \cdot [(\text{final time}) - (\text{initial time})]^{-1}$.

Differences among treatments (presence or absence of cotyledons in three environments) in each sample date were subjected to analysis of variance with the ANOVA procedures of a statistical analysis software (SANEST). When the *F*-value was significant at $P < 0.05$, the multiple comparison Tukey test ($P < 0.05$) was performed.

In order to follow the photosynthesis establishment during the heterotrophy-autotrophy transition, another set of 40 plants (20 without cotyledons, as described above) were grown under the same conditions as the growth-room. Evaluations of biochemical and photochemical performances were confined to the eophyll, which was capable to reach full expansion during the reserve-dependent period. The evaluations were performed once a week since the start of the eophyll expansion until the fall of the cotyledons.

Photosynthesis was determined following leaf gas exchange curves (net photosynthesis) at different light intensities and CO₂ concentration using a portable infrared gas analyser LI-6400 (LI-COR, Inc., Lincoln, USA) equipped with a led-source chamber (LI-6400-02B) and a CO₂-mixer (LI-6400-01). The light curves were obtained by fixing leaf temperature (25 °C) and CO₂ concentration (340 µmol.mol⁻¹) and within the PPFD range of 500 and 0 µmol.m⁻².s⁻¹. Using the same fixed temperature, but with the saturate PPFD (200 µmol.m⁻².s⁻¹, determined from the light curves), the CO₂ curves were obtained within a range of 0 and 1000 µmol.mol⁻¹. Furthermore, the photochemical performance of eophylls was assessed by measures of variable fluorescence/maximum fluorescence ratio (Fv/Fm) of chlorophyll from

photosystem II (PSII) using a modulated fluorometer (OS5-FL, Opti-Sciences Inc., Tyngsboro, USA). Measurements were performed in dark adapted leaves (during 20 min) supplying a saturating flash (0.8s) of red light ($7000 \mu\text{mol.m}^{-2}.\text{s}^{-1}$).

The carbon distribution was assessed in nine 35 days old seedlings growing in the growth-room. Uniformly labelled $[^{14}\text{C}]$ -sucrose solution ($5 \mu\text{l}$, $0.5 \mu\text{Ci}$) was applied, with a Hamilton syringe ($25 \mu\text{l}$), into the medium part of one cotyledon in each plant. Two non treated seedlings were kept as control. Feeding of $[^{14}\text{C}]$ -sucrose occurred between 9:00 and 10:00 o'clock a.m. After 72 h, seedlings were harvested and separated into the following parts: treated cotyledon, untreated cotyledon, roots, hypocotyl, eophyll, first metaphyll and top shoot (last internode including the apical part and the second metaphyll with initial development). The seedling parts were dried at 70°C for 72 h and after determination of dry mass, they were ground to fine powder. Samples ranging from 20 mg to 60 mg of each seedling part were suspended in 5 ml scintillation fluid (Ultima Goldtm, Packard BioScience B.V., The Netherlands) and the radioactivity was counted in a liquid scintillation counter (Tri-Carb 2100 TR, Packard BioScience B.V., The Netherlands). An external standard ratio using non-radioactive material from similar seedling parts was used to correct for quenching. Results were converted to percentages of the total recovered activity. The relative sink strength of the seedling parts was calculated by dividing the percentage radioactivity by the dry mass of the respective part (Palit, 1985). In order to follow the radioactivity in the structural fraction 50 mg of root, hypocotyl and the first metaphyll were also submitted to an alcohol extraction (four times in ethanol 80%, 80°C). After centrifugation ($10\,000 g$, 10 min) the alcohol insoluble materials were dried, powdered, suspended in 5 ml scintillation fluid and counted as described above.

RESULTS

The three environmental conditions in which the experiments were performed were different principally regarding to PPFD (22 to 670 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) and R/FR ratios (0.5 to 1.4), although tendencies to increasing average humidity and decreasing average temperature were observed for greenhouse, growth-room and forest respectively (Table 1). Furthermore, temperature and humidity in the greenhouse and forest were inversely correlated to each other ($r = -0.83$ and -0.79 respectively) whereas in the growth-room the humidity varied independently ($r = -0.07$) of the temperature, due to the control of temperature by air conditioning.

In the greenhouse and growth-room, the light quality was similar to an open environment (R/FR = 1.2 and 1.4 respectively), while in the forest a high shade condition was present (R/FR = 0.5).

Seedling development

The diagrams in Figure 1 represent the events that took place during the development of seedlings of *Hymenaea courbaril* under the three environmental conditions used. It can be seen that development was delayed in seedlings growing in the forest and growth-room in relation to the greenhouse. Although the effect increased throughout the period of observation, the differences appear to have started since seedling emergence, which was possibly related to temperature, and provoking an average delay of the eophyll development of approximately 2.5 and 5 days in growth-room and forest, respectively, in relation to greenhouse (Fig. 1). In the experiments of cotyledons excision, these delays were taken into consideration so that the excision was performed at 22 days (greenhouse), 24 days (growth-room) and 27 days (forest). Although seedlings showed the same morphology, in all cases cotyledons excision strongly affected the speed of growth (Figs. 1 and 2B).

In the forest and growth-room respectively, cotyledon excision provoked 12 and 10.5 days of delay in the production of the first metaphyll in relation to 5 and 4.6 days of non excised control seedlings. These delays were higher than the ones

observed in the greenhouse, which has little difference mainly regarding the first metaphyll (ca. 2.5 days) in relation to non excised seedlings (Fig. 1).

In all environments, in spite of the delay observed for each one, fifty percent of seedlings lost their cotyledons when the second metaphyll started to expand (asterisks in Fig. 1). Therefore, the half lives of the cotyledons were 34, 38 and 43 days respectively for seedlings growing in the greenhouse, growth-room and forest. The average remaining mass of the cotyledons was 278 ± 48 mg (Fig. 2A).

Seed germination occurred under the same conditions, independently of the further treatment. Thus, up to 15 days after the beginning of imbibition, we assumed that all seedlings were at similar stages of development and the influence of the different environmental conditions started thereafter. Consequently, the analysis of the diagram in Figure 1 indicates that the period of dependence of cotyledon reserves was 19, 23 and 28 days for greenhouse, growth-room and forest respectively.

Dry mass

Figure 2 shows the comparison of the storage mobilisation (Fig. 2A) and initial seedling growth (Fig. 2B). A direct relationship between the two phenomena ($r = -0.99$, -0.99 and -0.96 for forest, growth-room and greenhouse respectively) can be clearly seen in these figures. In the forest, there was a delay in the mobilisation of the storage compounds from the cotyledons to the axis. In comparison with seedlings growing in the growth-room and greenhouse, which showed higher relative mobilisation rate (RMR, Table 2) between 24 and 36 days, the seedlings growing in the forest showed a peak of RMR only in the period between 36 and 49 days after imbibition.

The absolute dry mass of whole seedlings increased slowly until 24 days in all environmental conditions, but the relative growth rate (RGR) was lower in the following periods. The observation of these higher RGR values at early stages of development was probably due to a small initial size of embryo in dormant seeds of *H. courbaril* (0.015 g), which grew very fast in this initial period. Furthermore, after

the period of xyloglucan mobilisation, seedling growth tended to stop, as can be seen by the slower RGR observed between 36 and 49 (Table 2).

Although in all cases an effect of cotyledon excision has been observed, it was much stronger when seedlings were growing in the greenhouse. This growth reduction was clearly related to the absence of reserves during the xyloglucan mobilisation period in the cotyledons. On the other hand, the effect of the absence of cotyledons was relatively smaller when seedlings were growing in the forest (Table 2). The seedlings grew much slower in the forest in relation to the greenhouse or growth-room (Figs. 1 and 2B), which can support the low effect of the cotyledon absence during the period of observation.

At the end of the period of storage mobilisation (average 38 days), the seedlings start to depend on photosynthesis and light availability for growth so that, except for seedlings grown without cotyledons in the forest, RGRs became similar among seedlings grown with or without cotyledons (Table 2). In the growth-room ($194 \mu\text{mol.m}^{-2}.\text{s}^{-1}$) and in the greenhouse ($670 \mu\text{mol.m}^{-2}.\text{s}^{-1}$), the seedlings without cotyledons were able to maintain a slow growth and development.

Root:shoot ratio

In the forest and in the growth-room a higher root:shoot ratio was observed in comparison to greenhouse at 24 days, i.e. before storage mobilisation period (Fig. 3). However, during this period, in the forest and the growth-room, a reduction of root:shoot ratio was observed (Figs. 3A-C), denoting a tendency to increasing allocation to aerial parts of the seedling.

Cotyledons strongly affected the investment in shoot development, except for seedlings growing in the greenhouse. These seedlings maintained root:shoot ratio around 0.2, i.e. investing 80% in the shoot, in both with and without cotyledons treatments (Fig. 3C). On the other hand, seedlings with cotyledons growing in the forest and growth-room invested approximately 85% in the shoots whereas seedlings without cotyledons decreased investment in shoots to approximately 75% (Figs. 3A, 3B). These results suggest that the storage compounds are apparently

more relevant for seedling development in lower levels of radiation (forest mainly). Also, under lower radiation levels, the cotyledons reserves of seedlings of *Hymenaea courbaril* contribute principally to shoot growth and after they reach full photosynthetic establishment, a higher proportion of photoassimilates are driven to root growth. This was confirmed by injection of [^{14}C]-sucrose in one of the cotyledons at 35 days, during the period of maximal RMR in the growth-room (see Table 2). The determination of radioactivity distribution was performed after 3 days (Table 3). Only 30% of the total radioactivity injected remained in the cotyledon whereas almost 60% of the radioactivity was present in aerial parts of the seedling (hypocotyl, eophyll, first metaphyll and top shoots) and 10% was present in the roots (Table 3). It is interesting to note that no radioactivity was detected in the untreated cotyledon, characterising absence of carbon flux from the embryonic axis into cotyledons and from one to another.

An estimate of the relative sink intensity was obtained by dividing the percentage of radioactivity per dry mass in every seedling part. The results shown in Table 3 clearly indicate that a higher sink intensity is related to development of aerial parts as compared to the roots. Furthermore, 17, 23 and 36% of the radioactivity were found in the insoluble fraction of roots, hypocotyl and first metaphyll respectively (Table 3), indicating a highest carbon incorporation in structural compounds of leaves.

Seedling height

In all environments the excised seedlings were smaller than seedlings with cotyledons (Fig. 3D-F). The height of seedlings without cotyledons growing in the forest and growth-room increased up to 8 and 11 days after excision respectively, and then practically stopped (Fig. 3D and 3E). However, the excised seedlings grown in the greenhouse were able to maintain a slower but continuous increase of height throughout the experiment (Fig. 3F), which indicates the photosynthetic contribution of the present leaves. It is interesting to note that even growing under very low PPFD conditions ($22 \mu\text{moles.m}^{-2}.\text{s}^{-1}$) seedlings of *Hymenaea courbaril* did not show etiolated

growth. This can be concluded from the observation that seedling height correlated better with the number of inter-nodes than with their length (compare Figs. 3 D-F with Fig. 1). This also corroborates the faster development observed in greenhouse conditions in comparison with forest and growth-room.

Leaf area

Figure 4 shows the changes in leaf area of seedlings of *Hymenaea courbaril* cultivated with and without cotyledons in the forest, growth-room and greenhouse. A comparison of the effects in eophyll, first metaphyll and total leaf area at 50 days old seedlings can be used to evaluate the effect of cotyledons and environmental conditions in *H. courbaril* seedling development. Eophyll development was strongly affected by cotyledon excision, limiting leaf area by 37, 45 and 60% (Figs. 4A-C) for greenhouse, growth-room and forest respectively. The development of the first metaphyll was affected in a similar fashion as the eophyll, but in this case the limitation was stronger, reaching 100% in the forest.

Considering total leaf area, the strongest effect of cotyledon excision was on leaves of seedlings growing in the forest, where the limitation of total leaf area was of ca. 77%. For the other environmental conditions, where light intensity increased considerably (Table 1), the limitation was progressively smaller (60% for growth-room and 50% for greenhouse).

Under the conditions found in the forest, the presence of the cotyledons was crucial to maintain a minimal level of leaf expansion so that the seedling tolerates a rather shadowed environment. On the other hand, under conditions where light intensity and temperature were higher (greenhouse and growth-room), photosynthesis appears to have had a more important role in development, resulting in seedlings that were taller and with greater total leaf area. This has to be analysed also under the light of seedling development, i.e. whereas 50 days old seedlings (with cotyledons) grown in the greenhouse produced 4 leaves, in the forest only the eophyll and the first metaphyll were fully expanded after 50 days (Fig. 1).

Calculations of leaf area ratio (LAR) for seedlings of *H. courbaril* growing in the three environments with and without cotyledons are shown in Figure 5. LAR was significantly different regarding the presence or absence of cotyledons only in the forest. Furthermore, despite the lower rate of development in the forest, 50 days old seedlings with cotyledons presented levels of LAR that were higher than in the growth-room and similar to the greenhouse environments. In this aspect, cotyledons appeared to be more important in lower levels of irradiation.

Photosynthesis

In order to understand the contribution and relationship between storage mobilisation in the cotyledons and photosynthesis establishment, seedlings were grown under conditions of growth-room. Net photosynthesis under different light intensities demonstrated that eophylls of *H. courbaril* saturated at about $200 \mu\text{mol} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$. The maximum assimilation rates for fully expanded eophylls from seedlings with and without cotyledons were both around $5.5 \mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ (Fig. 6A).

We also examined the net photosynthesis under different CO_2 concentration by 54 days old eophylls of seedlings growing with and without cotyledons (Fig. 6B). Although the CO_2 concentration increased almost three times the internal concentration (ca. $800 \mu\text{mol CO}_2 \cdot \text{mol}^{-1}$) the eophylls were capable to reach only 30% more ($2 \mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$) in the net assimilation.

Despite the similarity of assimilation levels observed for fully expanded eophylls (ca. $5.5 \mu\text{mol CO}_2 \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), the seedlings without cotyledons reached higher maximum saturation earlier than the ones with cotyledons. This can also be seen by following the changes in light compensation points, maximum net assimilation rates (A_{max}) and F_v/F_m ratio reached during expansion of the eophylls in the two treatments (Fig. 7). The leaves of excised seedlings not show also the typical red colour during the first steps of expansion.

The F_v/F_m ratio, which evaluates the performance of photosystem II, increased linearly from 32 to 46 days (from 0.70 to 0.77) in developing eophylls of seedlings without cotyledons. However, in eophylls of intact seedlings the

photosystem II increased the performance only after 40 days (Fig. 7B). Thus, the eophylls of *H. courbaril* established their light harvesting systems as well as CO₂ assimilation concurrently with the mobilisation process, and the overlap period between reserve mobilisation and photosynthesis establishment (heterotrophy to autotrophy transition) could be characterised as a transition period from 30 to 45 days.

DISCUSSION

Paulilo & Felipe (1998) and Souza & Válio (1999) studied plantlets of *Hymenaea courbaril* and considered it as a shade tolerant species. However, no work has been performed with the aim of understanding the mechanisms that led this species to establish in the understorey of the tropical rain forest. Seedlings of *H. courbaril* can be classified as shade tolerant due to several reasons. One is the fact that it presents large seeds (ca. 5 g per seed), which, from the point of view of ecophysiology, has been considered as associated with the capacity of the species to adapt to shaded environment (Foster, 1986; Westoby *et al.*, 1992). Seed size has also been associated with the functional type of the cotyledons (Kitajima, 1996). Seedlings that develop under very low light intensities usually contain larger amounts of reserves so that the amount of carbon stored is, after mobilisation to developing leaves, sufficient to guarantee that the new individual will be capable to perform photosynthesis. This is the case of *H. courbaril*, that has non photosynthetic cotyledons and at the same time store large amounts of a carbon in a cell wall storage polysaccharide, xyloglucan (Buckeridge & Dietrich, 1990). Such behaviour can be contrasted with species capable to develop quickly under higher light intensities such as *Schyzolobium parahybum* (Malavasi & Malavasi, 2001). In Leguminosae, some species have foliaceous photosynthetic cotyledons and the proportion of carbon reserve is either very low (e.g. in *Piptadenia gonoacantha* and *Anadenanthera falcata*) or is high, but in the form of a cell wall polysaccharide (galactomannan) in the endosperm (see Buckeridge *et al.*, 2000b for a review).

In *Hymenaea courbaril*, the cotyledons clearly have a storage function and their storage compounds are mobilised to the developing seedling. Tiné *et al.* (1997) have shown that not only xyloglucan, but also raffinose series oligosaccharides (more related to germination) and proteins (as post germinative reserves) are present in relatively high proportions (microscopically observed). The latter is important as a source of nitrogen for the growing seedling and xyloglucan and raffinose as a carbon source.

Tiné *et al.* (2000) demonstrated that in cotyledons of *H. courbaril*, xyloglucan is mobilised after germination and its degradation is concurrent with the development of the seedling. The results shown in the present work confirm and extend these observations. We have shown that even when seedlings develop under contrasting environmental conditions, xyloglucan mobilisation takes place after emergence of the eophylls and the pace of mobilisation is synchronised with eophylls and the first metaphyll expansion as well as seedling development (compare Figures 1 and 2A). It is interesting to note that the environmental factors influencing these synchronised phenomena probably change depending on the developmental stage. In other words, during germination and until the point where the eophylls become photosynthetically active, the main environmental factor influencing the physiological and biochemical processes appears to be temperature. A second phase follows when both processes, storage mobilisation in the cotyledon and photosynthesis in the developing eophylls, are active at the same time and influenced by temperature and light. Our results indicated that the start and duration of this overlap period depends on the growth rate of the seedling, which is determined by the environmental conditions. After this overlap period follows a third phase, which is when the cotyledons fall and the seedling becomes dependent exclusively on photosynthesis (compare Figures 2 and 7). In this last phase, light is relatively more important as an environmental factor for seedling development. It is very likely that these three phases are directly related to the RGRs observed, since in the three different environments, seedling RGR increased during storage mobilisation (Table 2). In the greenhouse, where development was

faster, the duration of our experiment permitted a further observation of a drastic fall in RGR after mobilisation finished (Table 2).

Several authors have shown that the proportion of subterranean to aerial parts (root:shoot ratio) decreases with development of seedlings adapted to shaded environments, which correspond the increase in leaf area ratio (Huante and Rincón, 1998; DeLucia *et al.*, 1998; Foster, 1986; Lei & Lechowicz, 1998; Westoby *et al.*, 1992; Kitajima, 1996). We demonstrated that in *H. courbaril* the aerial parts were the main carbon sink tissues, since in experiments where radioactive sucrose was injected in one of the cotyledons during storage mobilisation (Table 3) radioactivity was directed mainly to the shoots. It is interesting to note that after the storage mobilisation period, this pattern of carbon partitioning seems to invert. Souza & Válio (1999) found that radioactive sucrose injected into the leaves of 140 days old seedlings of *H. courbaril* (i.e. long after mobilisation period) was directed mainly to the lower parts of the seedling. This probably explains the fact that under lower light intensity (i.e. in the forest and growth-room) the root:shoot ratio decreased during storage mobilisation period whereas no difference was observed for seedlings growing under higher light intensity in the greenhouse (Figure 3A-C). On the basis of the results described above it can be suggested that during the initial phases of seedling establishment the carbon reserves (xyloglucan) are used to built the photosynthetic shoots which, after becoming capable to produce sugars, start to direct carbon towards the root system.

Our results show that the total leaf area of seedlings that developed with cotyledons is approximately twice the leaf area of the ones that grew without them. This suggests that this species, which is thought to be well adapted to shadowed conditions, uses xyloglucan as part of a strategy to produce larger leaf area which provides means to avoid a negative carbon budget (Foster, 1986; DeLucia *et al.* 1998). The synchronism between storage mobilisation and construction of the photosynthetic apparatus is clearly indicated by the results of the experiments of evaluation of the light harvesting capacity of the leaves (eophylls) as well as the CO₂ assimilation capacity (see Figures 6 and 7). The fact that light saturation occurred at

200 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ with a rather low light compensation point (11,8 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$) are both indications that *H. courbaril* presents a high degree of tolerance to the shade. These findings agree with the discoveries of Whitmore (1996) and Kitajima (1994) who found similar performance of photosynthetic systems of several tropical shade-tolerant climax trees. Altogether these results are the first evidences targeting the cotyledon xyloglucan reserves as an ecological advantage to establishment of *H. courbaril* seedlings in shade conditions of tropical forest.

ACKNOWLEDGEMENTS

This study was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo-FAPESP. The authors thank Mrs A. M. Baroni and L. I. V. Amaral for technical assistance with photosynthesis and radioactivity analyses.

REFERENCES

- Buckeridge, M.S. & Dietrich, S.M.C. 1990. Galactomannan from Brazilian legume seeds. *Revista Brasileira de Botânica* 13: 109-112.
- Buckeridge, M.S.; Santos, H.P. & Tiné, M.A.S. 2000a. Mobilisation of storage cell wall polysaccharides in seeds. *Plant Physiology and Biochemistry* 38: 141-156.
- Buckeridge, M.S.; Dietrich, S.M.C. & Lima, D.U. 2000b. Galactomannans as the reserve carbohydrate in legume seeds. In: Gupta, A. K. and Kaur, N. Carbohydrate reserves in plants – Synthesis and Regulation, 283-316. Elsevier, Amsterdam, The Netherlands.
- Cao, K.F. & Ohkubo, T. 1998. Allometry, root/shoot ratio and root architecture in understory saplings of deciduous dicotyledoneous trees in central Japan. *Ecological Research* 13: 217-227.

- Carelli, M.L.C. & Magalhães, A.C. 1981. Efeito da remoção dos cotilédones no desenvolvimento das plântulas e na atividade da redutase de nitrato em soja (*Glycine max* L.Merr.). *Revista Brasileira de Botânica* 4: 43-47.
- DeLucia, E.H.; Sipe, T.W.; Herrick, J. & Maherali, H. 1998. Sapling biomass allocation and growth in the understory of deciduous hardwood forest. *American Journal of Botany* 85: 955-963.
- Escobar-Gutiérrez, A.J.; Daudet, F.A.; Gaudillère, J.P.; Maillard, P. & Frossard, J.S. 1998. Modelling of allocation and balance of carbon in walnut (*Juglans regia* L.) seedlings during heterotrophy-autotrophy transition. *Journal of Theoretical Biology* 194: 29-47.
- Flores, E.M. & Benavides, C.E. 1990. Germinación y morfología de la plántula de *Hymenaea courbaril* L. (Caesalpinaceae). *Revista de Biología Tropical* 38: 91-98.
- Foster, S.A. 1986. On the adaptive value of large seeds for tropical moist forest trees: a review and synthesis. *The Botanical Reviews* 52: 260-299.
- Gerhardt, K. 1993. Tree seedling development in tropical dry abandoned pasture and secondary forest in Costa Rica. *Journal of Vegetation Science* 4: 95-102.
- Givnish, T.J. 1988. Adaptation to sun and shade, a whole-plant perspective. *Australian Journal of Plant Physiology* 15: 63-92.
- Huante, P. & Rincón, E. 1998. Responses to light changes in tropical deciduous woody seedlings with contrasting growth rates. *Oecologia* 113: 53-66.
- Kitajima, K. 1994. Relative importance of photosynthetic traits and allocation patterns as correlates of seedling shade tolerance of 13 tropical trees. *Oecologia* 98: 419-428.
- Kitajima, K. 1996. Cotyledon functional morphology, patterns of seed reserve utilization and regeneration niches of tropical tree seedlings. In Swaine, M.D.(ed.), *Ecology of Tropical Forest Tree Seedlings - Man and the Biosphere series*, 17: 193-210. UNESCO/Parthenon, Paris, France.
- Lei, T.T. & Lechowicz, M.J. 1998. Diverse responses of maple saplings to forest light regimes. *Annals of Botany* 82: 9-19.

- Malavasi, U.C. & Malavasi, M.M. 2001. Leaf characteristics and chlorophyll concentration of *Schyzolobium parahybum* and *Hymenaea stilbocarpa* seedlings grown in different light regimes. *Tree Physiology* 21: 701-703.
- McConnaughay, K.D.M. & Coleman, J.S. 1999. Biomass allocation in plants: ontogeny or optimality? A test along three resource gradients. *Ecology* 80: 2581-2593.
- Palit, P. 1985. Translocation and distribution of ^{14}C -labelled assimilate associated with growth of jute (*Corchorus olitorius* L.). *Australian Journal of Plant Physiology* 12: 527-534.
- Paulilo, M.T.S. & Felipe, G.M. 1998. Growth of the shrub-tree flora of the Brazilian cerrados: a review. *Tropical Ecology* 39: 165-174.
- Sassaki, R.M. & Felipe, G.M. 1992. Remoção dos cotilédones e desenvolvimento inicial de *Dalbergia miscolobium*. *Revista Brasileira de Botânica* 15: 5-16.
- Souza, R.P. & Válio, I.F.M. 1999. Carbon translocation as affected by shade in saplings of shade tolerant and intolerant species. *Biologia Plantarum* 42: 631-636.
- Swaine, M. & Whitmore, T.C. 1988. On the definition of ecological species groups in tropical rain forests. *Vegetatio* 75: 81-86.
- Tiné, M.A.S.; Cortelazzo, A.L. & Buckeridge, M.S. 2000. Xyloglucan mobilisation in cotyledons of developing plantlets of *Hymenaea courbaril* L. (Leguminosae-Caesalpinoideae). *Plant Science* 154: 117-126
- Veirskow, B. 1985. Pea seedling growth and development regulated by cotyledons and modified by irradiance. *Physiologia Plantarum* 65: 79-84.
- Westoby, M.; Jurado, E. & Leishman, M. 1992. Comparative evolutionary ecology of seed size. *Tree* 7: 368-372.
- Whitmore, T.C. 1996. A review of some aspects of tropical rain forest seedling ecology with suggestions for further enquiry. In Swaine, M.D. (ed.), *Ecology of Tropical Forest Tree Seedlings* - Man and the Biosphere series, 17: 3-39. UNESCO/Parthenon, Paris, France.
- Wulf, R. 1986. Seed size variation in *Desmodium paniculatum*. II effects on seedling growth and physiological performance. *Journal of Ecology* 74: 99-114.

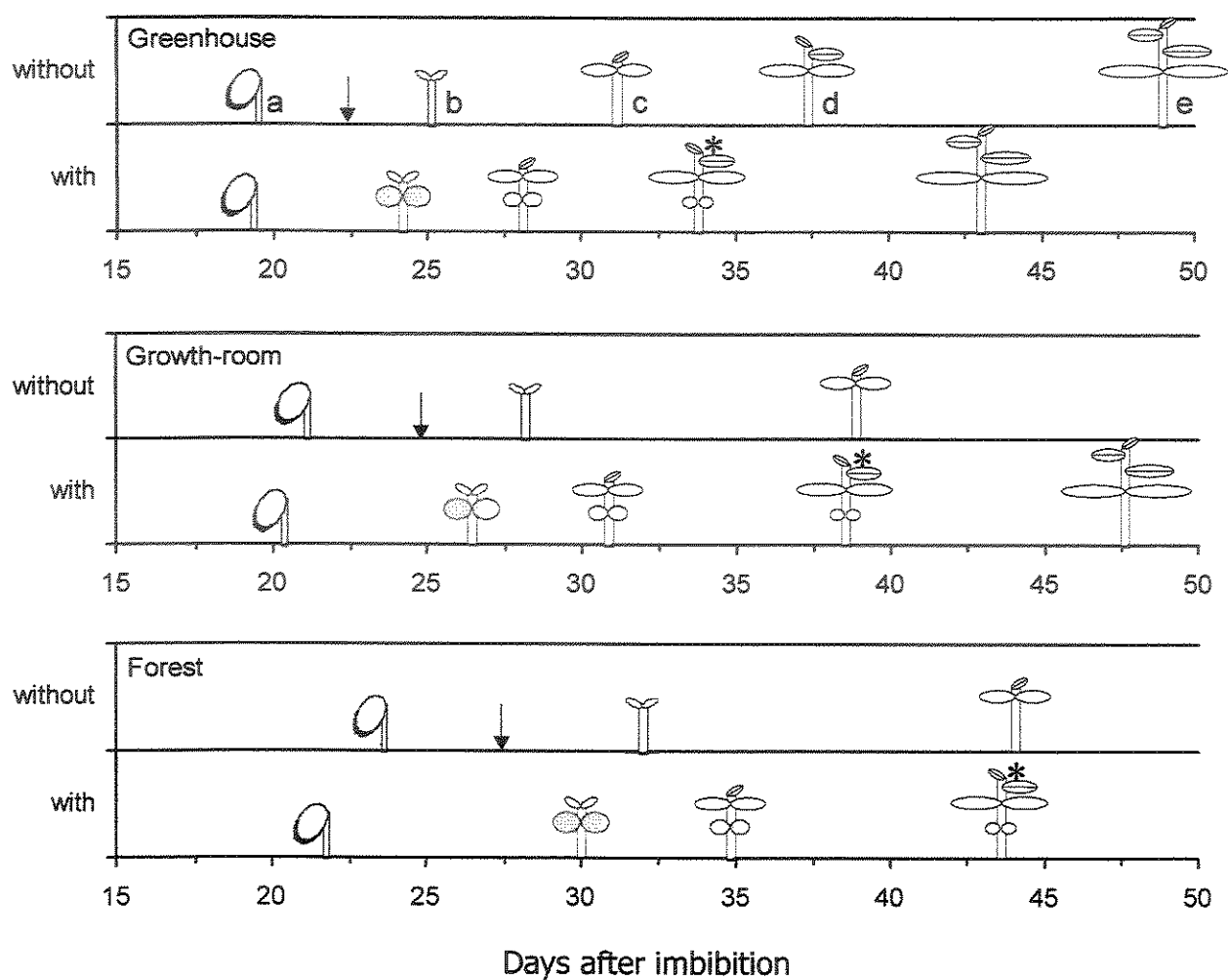


Fig. 1. Schematic representation of development of seedlings of *Hymenaea courbaril* growing in greenhouse, growth-room and forest environments. Data represent the emergence dates of seedlings (a), eophylls (b) and first (c), second (d) and third (e) metaphylls of seedlings with and without cotyledons. The cotyledons excision was performed at 22, 24 and 27 days after the beginning of imbibition (black arrows). In intact seedlings fifty percent of cotyledons felt at 34 ± 2.0 , 38 ± 2.5 and 43 ± 3.4 days, respectively in greenhouse, growth-room and forest (asterisks).

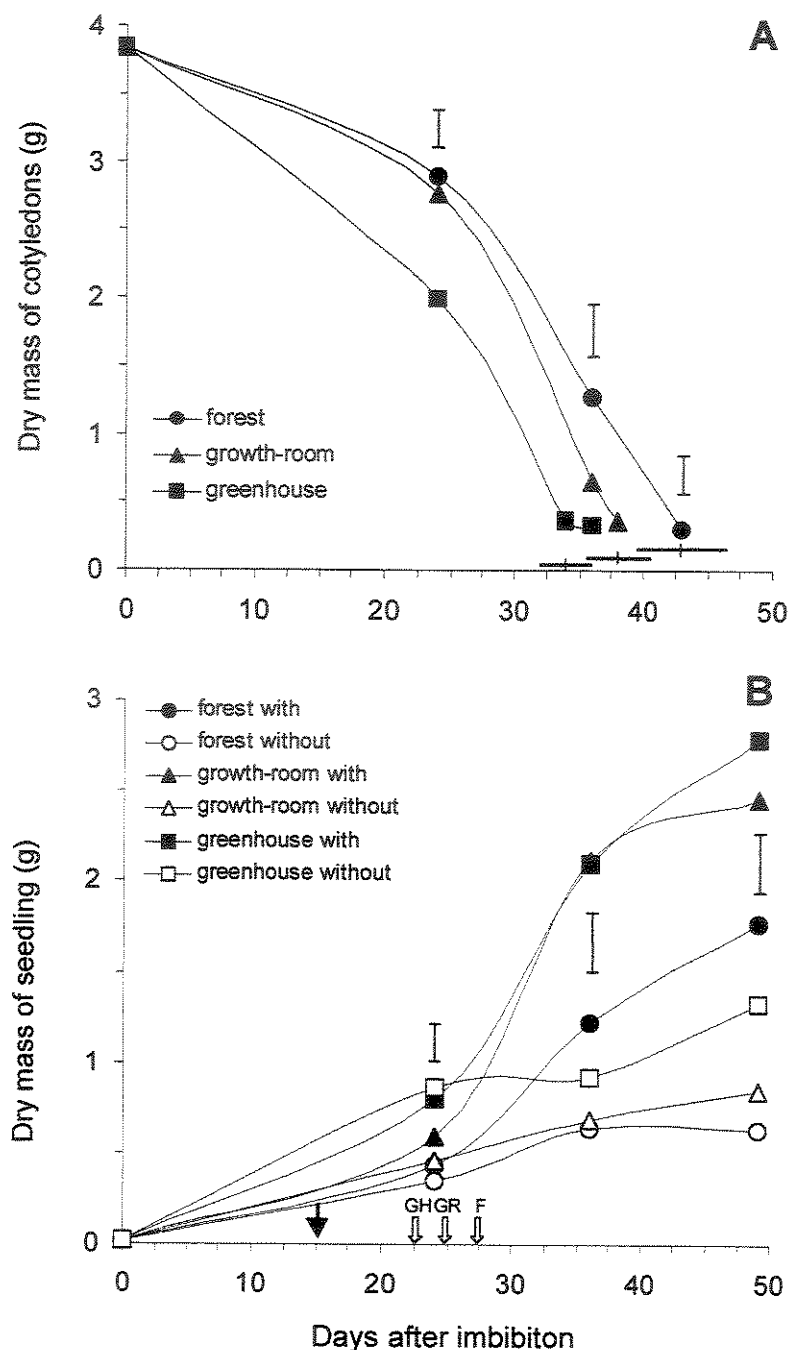


Fig. 2. Changes in dry mass of cotyledons (A) and total seedlings (B) of *Hymenaea courbaril*. The seedlings were growing in greenhouse, growth-room and forest with or without cotyledons, in which the excision was performed 22, 24 and 27 days after the beginning of imbibition, respectively (GH, GR and F arrows in B). The germination was 15 days after imbibition in all environments (black arrow). The cotyledons felt with an average residue mass of 278 ± 48 mg at 34 ± 2.0 , 38 ± 2.5 and 43 ± 3.4 days, respectively in greenhouse, growth-room and forest (horizontal bars in A). Vertical bars represent $\text{LSD}_{5\%}$ Tukey test ($n=5$) among treatments.

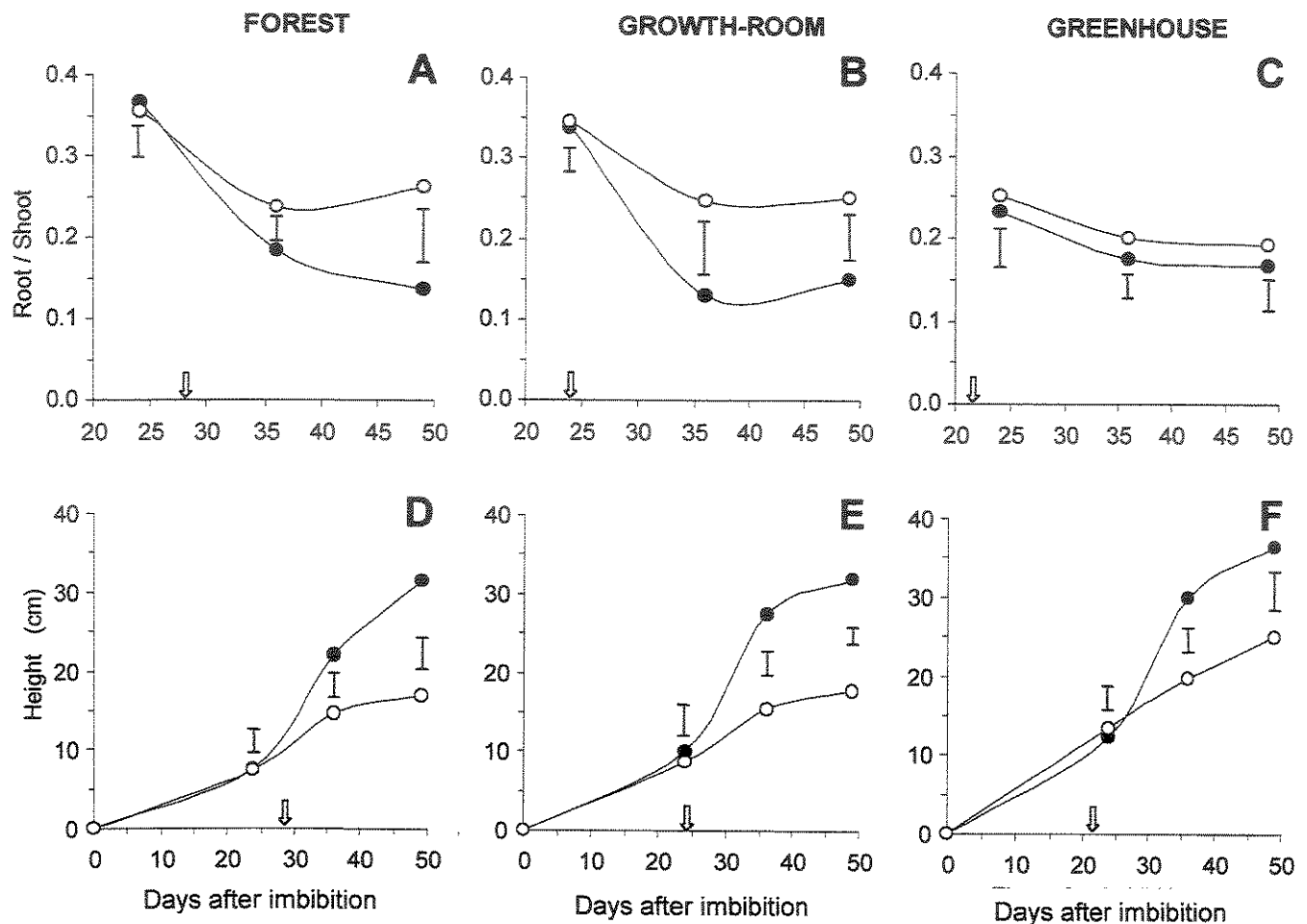


Fig. 3. Root:shoot ratio (A-C) and height (D-F) of seedlings of *Hymenaea courbaril* growing with (full symbols) or without (empty symbols) cotyledons in forest (A,D), growth-room (B,E) and greenhouse (C,F), respectively. The excision of cotyledons was performed 22, 24 and 27 days after the beginning of imbibition, in greenhouse, growth-room and forest, respectively (arrows). Vertical bars represent LSD_{5%} Tukey test ($n=5$) among treatments.

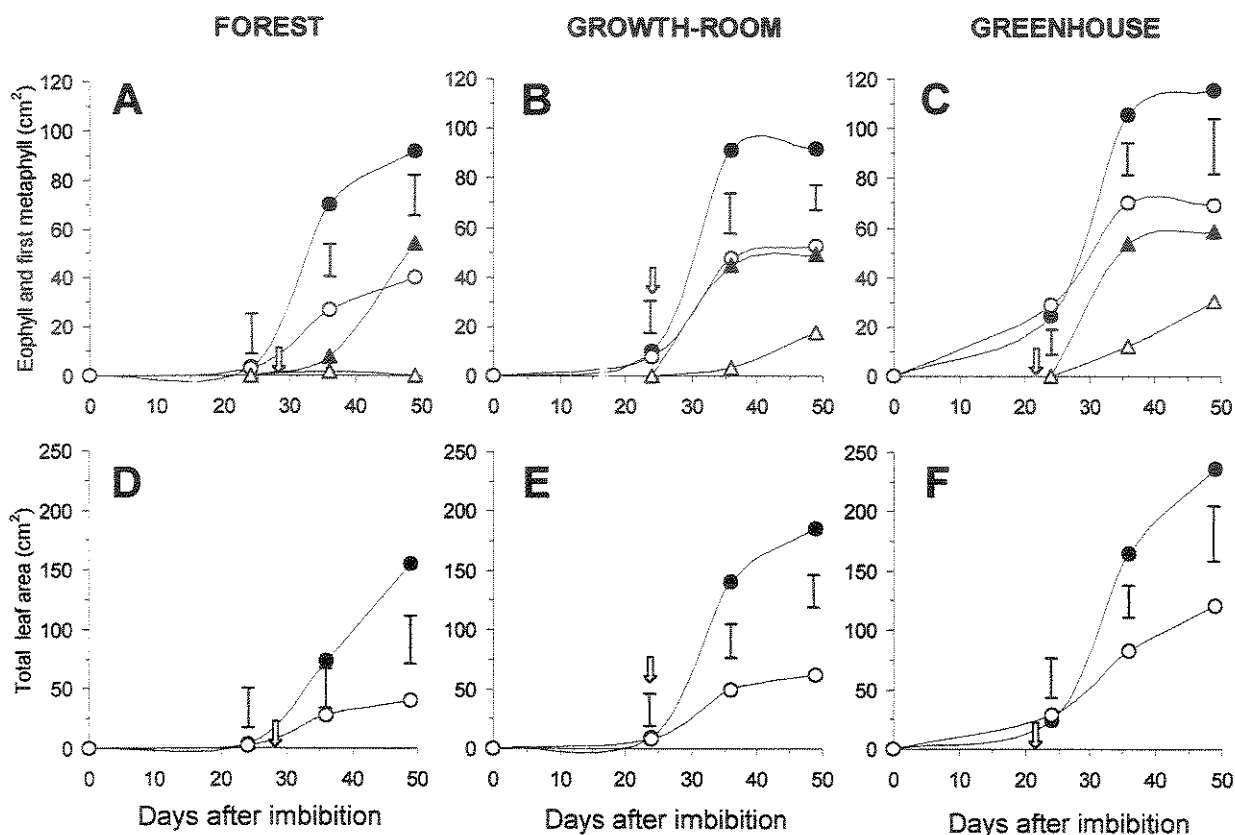


Fig. 4. Changes in leaf area of seedlings of *Hymenaea courbaril* growing with (full symbols) or without (empty symbols) cotyledons in forest (A,D), growth-room (B,E) and greenhouse (C,F), respectively. A,B and C represent the area of eophylls (circles) and of first metaphyll (triangles), in the different treatments. D, E and F represent the summation in area of all leaves present per seedlings in each treatment. The excision of cotyledons was performed 22, 24 and 27 days after the beginning of imbibition, in greenhouse, growth-room and forest, respectively (arrows). Vertical bars represent LSD_{5%} Tukey test ($n=5$) among treatments.

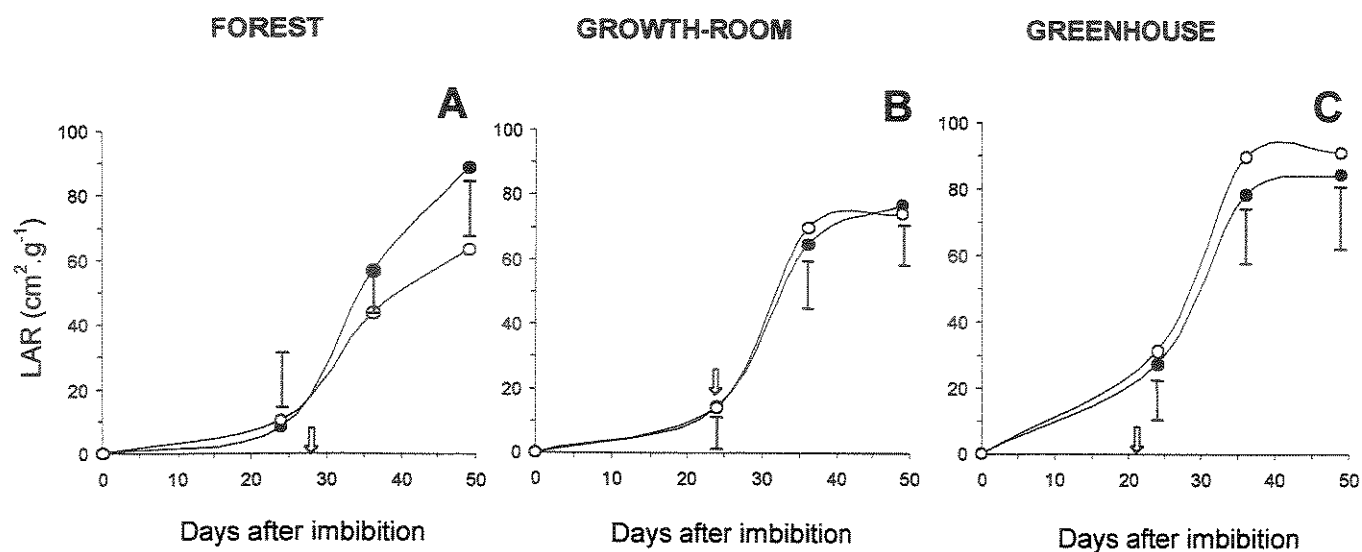


Fig. 5. Leaf area ratio (LAR) of seedlings of *Hymenaea courbaril* growing with (full symbols) or without (empty symbols) cotyledons in forest (A), growth-room (B) and greenhouse (C), respectively. The excision of cotyledons was performed 22, 24 and 27 days after the beginning of imbibition, in greenhouse, growth-room and forest, respectively (arrows). Vertical bars represent LSD_{5%} Tukey test ($n=5$) among treatments.

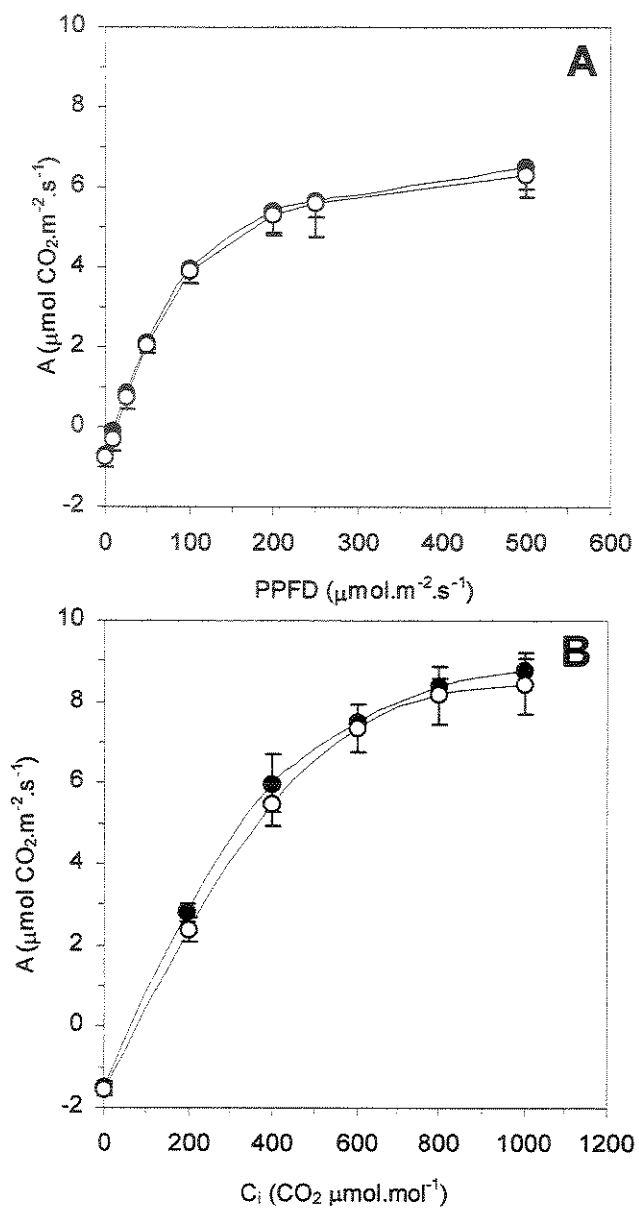


Fig. 6. Response of net photosynthesis to (A) photosynthetic photon flux density (PPFD) and to (B) internal concentration of CO_2 (C_i) by 54 days old eophylls of seedlings of *Hymenaea courbaril* growing with (full symbols) or without (empty symbols) cotyledons in growth-room. Vertical bars represent the standard deviation ($n=20$).

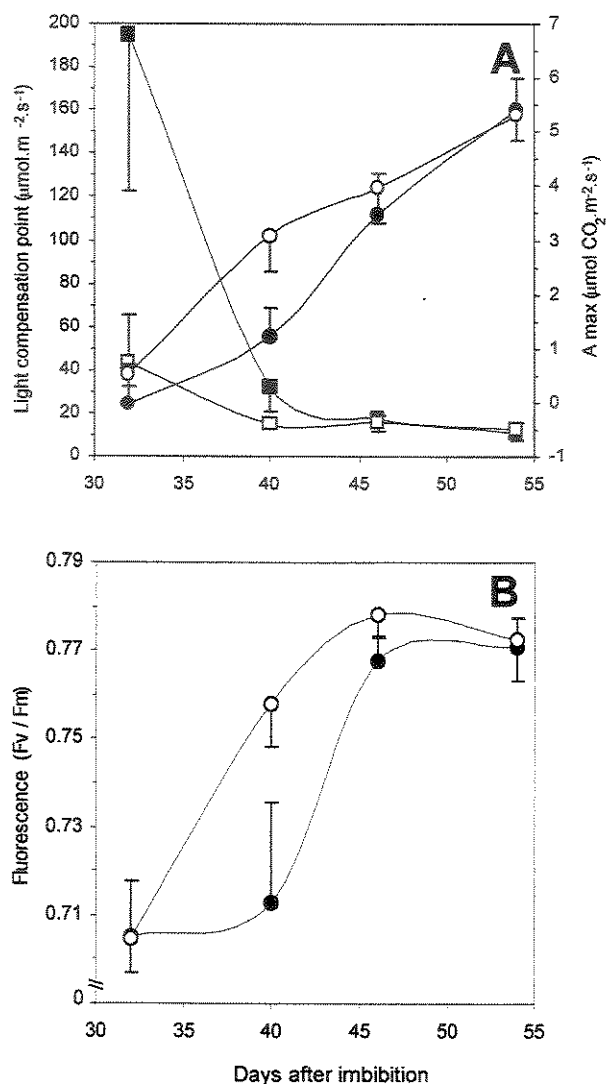


Fig. 7. Changes in light compensation point (squares) and maximum net photosynthesis (Amax, circles) (A) and fluorescence (B) of 32, 40, 46 and 54 days old eophylls of seedlings of *Hymenaea courbaril* growing with (full symbols) or without (empty symbols) cotyledons in growth-room. The maximum net photosynthesis was measured using $200 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of PPFD and $350 \mu\text{mol.mol}^{-1}$ of CO_2 . Vertical bars represent the standard deviation ($n=20$).

Table 1 - Characteristics of the three different environmental conditions where the seedlings of *Hymenaea courbaril* were grown.

Environments	Parameters			
	R / FR*	Air humidity (%)	PPFD** ($\mu\text{mol.m}^{-2}.\text{s}^{-1}$)	Air temperature (°C)
Greenhouse	1.2	59 ± 19	670	28 ± 6
Growth room	1.4	61 ± 18	194	23 ± 2
Forest	0.5	76 ± 10	22***	22 ± 2

(*) Red / Far red ratio (655-665/725-735 nm) measured at the same time as PPFD (Photosynthetically active photon flux density). (**) PPFD measured at about 12 o'clock. (***) The forest environment showed also sunflecks within the range of 200 and 700 $\mu\text{mol.m}^{-2}.\text{s}^{-1}$ PPFD.

Table 2 - Relative mobilisation rate (RMR) of the cotyledons reserves and relative growth rate (RGR) of seedlings of *Hymenaea courbaril* growing in the forest, growth-room and greenhouse conditions with and without attached cotyledons. Data are given as $\text{mg.g}^{-1}.\text{day}^{-1}$.

Variable	Treatments		Periods after imbibition of seeds (days)		
	Environments	Cotyledons	0 – 24	24 – 36	36 – 49
RMR	Greenhouse	With	-28	-142 *	-6 **
	Growth-room	With	-14	-122	-45 **
	Forest	With	-12	-70	-114 **
RGR	Greenhouse	With	161	80	22
	Greenhouse	Without	169	4	27
	Growth-room	With	151	106	9
	Growth-room	Without	142	33	15
	Forest	With	140	85	27
	Forest	Without	129	53	1

(*) Calculated using the difference in dry mass of cotyledons at the 24 - 34 period. (**) Calculated using the difference in dry mass of cotyledons at the 34 - 36, 36 - 38 and 36 - 43 periods for greenhouse, growth-room and forest respectively.

Table 3 - Relative distribution of [^{14}C]-sucrose among different tissues from 35 days old seedlings of *Hymenaea courbaril* grown in growth-room. The radioactive sucrose was injected ($5\mu\text{l}$, $0.5\mu\text{Ci}$) in one cotyledon (treated) and the ^{14}C -scintillation was read after 72h. Dry tissues of root, hypocotyl and first metaphyll were submitted to an alcohol extraction (ethanol 80%, 80°C) and the ^{14}C -scintillation of insoluble material was read. The numbers represent the average of nine replicates (seedlings) \pm standard deviation.

Tissues	Relative radioactivity		
	% of total counts per tissue	Relative sink strength *	alcohol insoluble (%)
Top shoot **	15 ± 2.9	41 ± 7.3	-
First metaphyll	24 ± 6.8	22 ± 8.7	36 ± 5.3
Eophyll	6 ± 2.9	2 ± 1.3	-
Hypocotyl	14 ± 4.5	7 ± 3.7	23 ± 6.9
Untreated cotyledon	0 ± 0.0	0 ± 0.0	-
Treated cotyledon	30 ± 8.3	20 ± 5.1	-
Roots	10 ± 6.2	8 ± 4.2	17 ± 3.8

(*) Percentage of total counts per g of dry mass. (**) Represent the last internode including the apical part and the second metaphyll with initial development.

6. CAPÍTULO 3

Artigo 3 - **The control of storage xyloglucan degradation in cotyledons of *Hymenaea courbaril* L. resembles the auxin-induced growth of primary cell wall**

The control of storage xyloglucan degradation in cotyledons of *Hymenaea courbaril* L. resembles the auxin-induced growth of primary cell wall

**Henrique Pessoa dos Santos¹, Eduardo Purgatto², Helenice Mercier³
& Marcos Silveira Buckeridge^{4,5}**

- ¹ Departamento de Biologia Vegetal – UNICAMP / Seção de Fisiologia e Bioquímica de Plantas – IBt. Actual addresses: Rua Livramento, 515, Caixa Postal 130, CEP 95700-000, Bento Gonçalves, RS, Brazil. (henrique@cnpuv.embrapa.br)
- ² Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Universidade de São Paulo.
- ³ Departamento de Botânica. Instituto de Biociências. Universidade de São Paulo, Caixa Postal 11461, CEP 05422-970, São Paulo, SP, Brazil. (hmercier@usp.br)
- ⁴ Seção de Fisiologia e Bioquímica de Plantas, Instituto de Botânica, Caixa Postal 4005, CEP 01061-970, São Paulo, SP, Brazil. (msbuck@usp.br)
- ⁵ To whom correspondence should be addressed.

RESUMO

Hymenaea courbaril é uma espécie leguminosa arbórea presente em florestas tropicais, cujas plântulas apresentam cotilédones ricos em um polissacarídeo de reserva de parede celular (xiloglucano). A importância deste tipo de reserva para o crescimento e estabelecimento, bem como o controle de sua mobilização têm sido pouco investigados. Neste trabalho foi demonstrado que a mobilização de xiloglucano está fortemente relacionada com o desenvolvimento da parte aérea das plântulas, sendo que a sua degradação ocorreu somente após o início da expansão dos eófilos. Durante a mobilização desta reserva, observou-se o incremento da atividade das hidrolases de xiloglucano e um aumento na concentração de amido e de açúcares livres (sacarose, glucose e frutose) nos cotilédones. A mobilização de xiloglucano foi inibida pelo corte da parte aérea das plântulas, pelo crescimento de plântulas no escuro e pelo tratamento com ácido naftilftalâmico, um inibidor do transporte polar de AIA. As análises de AIA endógeno, nos cotilédones, revelaram que sua concentração variou de acordo com os tratamentos e com a atividade das hidrolases de xiloglucano, indicando que a auxina apresenta uma relação direta com processo de mobilização de xiloglucano de reserva. Isto também foi evidenciado na análise de cotilédones isolados durante o período de degradação de xiloglucano e em presença de ácido 2,4-Diclorofenoxiacético (10^{-6} M), quando a taxa de degradação de xiloglucano foi semelhante à observada em cotilédones presos à plântula. Considerando estes resultados, mais o fato de que o pH ótimo das hidrolases é ácido, é razoável sugerir que o metabolismo de xiloglucano de reserva em cotilédones de *H. courbaril* tenha mantido, durante a evolução, características semelhantes ao mecanismo de crescimento de paredes primárias, induzido por auxina. Este controle hormonal apresenta uma grande importância ecofisiológica para esta espécie, pois sincroniza o crescimento e a degradação das reservas de acordo com a disponibilidade de luz do ambiente, aumentando a eficiência no uso das reservas pelas plântulas crescidas no interior das florestas tropicais.

ABSTRACT

Hymenaea courbaril is a leguminous tree species present in tropical forests, which have cotyledons enriched with a storage cell wall polysaccharide (xyloglucan). The importance of this kind of reserve for seedling growth and establishment as well as the control of its mobilisation, has been poorly investigated. In this work we found that xyloglucan mobilisation is strictly controlled by the development of aerial parts of the seedling, with the start of its degradation occurring only after the beginning of eophyll expansion. During the mobilisation of this reserve an increase in activity of related hydrolases and in the concentration of starch and free sugars (sucrose, glucose and fructose) were observed in the cotyledons. Xyloglucan mobilisation was inhibited by excision of the shoot, by darkness and by treatment with *N*-1-naphthylphthalamic acid at 200 μ M, an inhibitor of polar transport of IAA. Analyses of endogenous IAA in the cotyledons revealed that its change in concentration is followed by xyloglucan hydrolase activities, indicating that auxin is directly related to xyloglucan mobilisation process. The same can be said about cotyledons isolated during xyloglucan mobilisation and treated with 10^{-6} M 2,4-Dichlorophenoxyacetic acid, which showed a similar mobilisation rate as observed in attached cotyledons. Considering these results plus the fact that the pH optima of the hydrolytic enzymes are slightly acidic, it is reasonable to suggest that storage xyloglucan metabolism maintained, during evolution, features that resemble auxin induced growth of primary cell walls. This hormonal control presents an ecophysiological importance for this species by promoting synchronism between growth and reserve degradation. This is likely to increase the efficiency of carbon reserves utilisation by the growing seedling in the understorey of the rain forest.

INTRODUCTION

The presence and mobilisation of xyloglucans following seed germination were first reported in the 19th century for seeds of *Impatiens balsamina*, *Tropaeolum majus* and *Cyclamen europaeum* (Heinricher, 1888; Reiss, 1889). Later on in the 1960s, the botanical distribution of xyloglucans has been reviewed by Kooiman (1960) who used the ability of xyloglucan to stain with iodine (showing a distinctive blue colour) as a form of detection of these polymers in seeds.

Seed xyloglucans have a main β -D-(1 \rightarrow 4)-glucan backbone branched with α (1 \rightarrow 6) linked D-xylopyranosyl or β -D-galactopyranosyl(1 \rightarrow 2)D-xylopyranosyl residues (White & Rao, 1953). Except for the absence of terminal fucosyl units α -L-(1 \rightarrow 2)-linked to the β -D-galactosyl groups, there is a remarkable similarity between seed reserve xyloglucan and structural xyloglucan from primary cell walls of dicotyledonous tissues (Hayashi, 1989).

The reserve function of xyloglucan in cotyledons have been demonstrated for seeds of *Tropaeolum majus* (Edwards *et al.*, 1985), *Tamarindus indica* (Reis *et al.*, 1987), *Copaifera langsdorffii* (Buckeridge *et al.*, 1992), *Hymenaea courbaril* (Tin   *et al.*, 2000), showing that xyloglucan mobilisation *in vivo* is accompanied by the rise and fall of the activities of four hydrolases: β -galactosidase, endo- β -(1 \rightarrow 4)-glucanase (or XET), α -xylosidase and β -glucosidase.

Reid & co-workers isolated the four main enzymes responsible for xyloglucan degradation in *Tropaeolum majus*. They are *i)* xyloglucan-specific endo- β -(1 \rightarrow 4)-D-glucanase or xyloglucan endo-transglycosylase (XET) (Edwards *et al.*, 1986; Fanutti *et al.*, 1993); *ii)* a β -galactosidase with high specificity towards xyloglucan (Edwards *et al.*, 1988); *iii)* a xyloglucan-specific oligosaccharide-specific α -xylosidase or oligoxyloglucan exo-xylohydrolase (Fanutti *et al.*, 1991) and *iv)* a transglycosilating β -glucosidase (Crombie *et al.*, 1998).

On the basis of these results plus other studies on the mode of action of the XET (Edwards *et al.*, 1986; Fanutti *et al.*, 1993), Crombie *et al.* (1998) proposed a model for xyloglucan mobilisation in nasturtium. In this model, the four enzymes are

thought to work in a concerted fashion producing galactose, glucose and xylose. In nasturtium, XET and β -galactosidase are the only enzymes capable of attacking the polymer. Under low concentrations of xyloglucan oligosaccharides (acceptors), the hydrolytic activity predominates over XET (Fanutti *et al.*, 1993). Thus, when in contact with high molecular weight xyloglucan, hydrolysis predominates, producing oligosaccharides which are promptly attacked by the exo-glycosidases (α -xylosidase and β -glucosidase), therefore reducing the polymer to its monosaccharide constituents.

Reis *et al.* (1987) described cytochemically the digestion of the xyloglucan-containing cell walls of *Tamarindus indica* cotyledons. Using the techniques of iodine staining and a gold-probe prepared by complexing *Escherichia coli* β -galactosidase with gold particles, they were able to study xyloglucan mobilisation in cotyledonary cell walls at the ultrastructural level with great specificity. They observed the presence of an inner and an outer wall, which are not degraded and become more apparent following xyloglucan mobilisation. Also, a fibrous material was left after mobilisation, showing that not all the wall is mobilised. They also found that as xyloglucan is degraded, the proportions among monosaccharides (glucose:xylose:galactose) do not change significantly.

In *Copaifera langsdorffii*, the mobilisation of the cotyledonary cell wall storage has been observed cytochemically, physiologically and biochemically by Buckeridge *et al.* (1992). They have studied two different populations, from two different biomes (forest and savana) and did not find apparent differences in xyloglucan mobilisation between seeds of the two environments. Recently, a β -galactosidase was purified from cotyledons of *Copaifera* (Alcântara *et al.* 1999). Differently from nasturtium β -galactosidase, this enzyme showed very high specificity towards certain xyloglucan oligosaccharides and was not active on the polymer at all. Furthermore, its pH optimum showed a very sharp peak at 3.2, out of the optima of the other hydrolases that are around 4.5.

In cotyledons of *Hymenaea courbaril*, Tiné *et al.* (2000) showed that xyloglucan is mobilised after germination. Concomitantly to xyloglucan disassembling, fructose,

glucose and sucrose are produced (Tin  , 1997). Furthermore, Tin   *et al.* (2000) detected the same four enzymes activities found in nasturtium. They also found evidence for the presence of transglycosilation activity (XET) in this species. As in *Copaifera*, all β -galactosidase activity that can be detected using *p*-nitrophenyl-galactopyranoside had pH optimum at 3.2, whereas the other hydrolases detected were active at 4.5. The results obtained for β -galactosidases of *Copaifera* and *Hymenaea* suggest that this enzyme might be one of the important steps in the control of seed storage xyloglucan metabolism in legumes.

Although some work has been performed on the mechanism of xyloglucan mobilisation, very little is known about the control mechanisms involved in the process. The only report was provided by Hensel *et al.* (1991), who demonstrated that 2,4-D (an synthetic auxin) was capable to induce xyloglucan mobilisation in excised cotyledons of *Tropaeolum majus*. On the other hand, the effects of auxin on primary cell wall fucosylated xyloglucan have been studied more extensively, the principal effects of the hormone being i) activation of H^+ transport to the extracellular medium (possibly related to activities of ATPases), in which the lowering of local pH favours the action of glycosidases and expansins (Taguchi *et al.* 1999, Cosgrove, 2000) and ii) activation or alteration of gene expression so that enzymes (mainly glycosidases) increase their activities or are synthesised *de novo*.

In the present work, we investigated some aspects of the effect of auxin on the mobilisation of xyloglucan in cotyledons of growing seedlings of *Hymenaea courbaril*. Our results indicate that auxin is involved in xyloglucan metabolism, being produced in the aerial parts of the growing seedling so that the pace of growth possibly controls storage mobilisation in the cotyledons. The results resemble evidences commonly used to support the effect of auxin on plant growth and development. This suggests that this mechanism might have been preserved during evolution through the transference of auxin control of wall xyloglucan metabolism in the growing tissues to cell wall storage mobilisation in cotyledons.

MATERIAL AND METHODS

Material and experimental conditions

In all experiments we employed size-selected seeds (5.5 – 6.0 g) of *Hymenaea courbaril*, collected in São João da Boa Vista county (22°00' S; 47°18' W), São Paulo, Brazil. These seeds were stored for four years under dry and cold conditions (RH 35%, 8°C) in the Botanical Institute of São Paulo, Brazil. Seeds were scarified with sand paper on the lateral position (in relation to the embryo), surface-sterilised for 15 min at 10 fold-diluted commercial hypochlorite bleach, rinsed, and placed in trays between two sheets of wet paper (at 30°C) until germination was visible (0.5 cm of radicle). The time in all experiments was registered in relation to the beginning of imbibition, in days (days after the start of imbibition).

Germinated seeds were placed in pots (1.5 L) with a mixture of washed sand and vermiculite (2:1, v/v) and the pots were placed in a growth chamber, under shelves provided with 10 fluorescent lamps (60 watts) and 4 incandescent lamps (40 watts) reaching around $200 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ of photosynthetic active light intensity. The photoperiod throughout the experiments was 12/12 hours with constant temperature (25 °C) and relative humidity (60%). Every fifteen days, 50 ml per pot of a complete Hoagland solution was added to avoid mineral deficiency.

Experiments and treatment descriptions

This work was performed with two complementary experiments. The first one compared the xyloglucan mobilisation process in detached and attached cotyledons. The attached cotyledons were followed in intact seedlings (control), in the darkness and with shoot excision above the cotyledon insertion (excised). With these procedures we intended to characterise xyloglucan mobilisation process on intact plants or in plants growing without light stimulus or shoot sink, as well as to verify whether and when the isolated cotyledons are able to start/maintain the mobilisation process. With these experiments it was also possible to test whether cotyledons are able to respond to endogenous auxin.

The control plants were grown in pots (as described above) and without growth restrictions. In the darkness treatment the pots were placed (when the emergence of seedlings started, ca. 19 days) in a black paper box (to avoid light) located in the same growth chamber conditions. The shoot excision was performed when the development of eophylls started (about 34 days) by cutting the entire shoot above the cotyledons insertion (epicotyl and eophylls).

Excision of cotyledons was performed at 7, 19, 26, 34 and 41 days after the beginning of imbibition, from plants grown as in the control. The excision dates were chosen on the basis of the results obtained by Tiné *et al.* (2000). This experiment had the aim to isolate the cotyledons before, during and after the start of xyloglucan mobilisation. The excised cotyledons were surface-sterilised for 15 sec in 70% ethanol containing 1% (v/v) polyoxyethylenesorbitan monooleate (Tween 80), and placed in groups of 15 in sterile Petri dishes (20 x 140 mm). The solution of incubation (15 ml per dish) was composed by freshly distilled water, 1 mM CaCl_2 , 300 mg.L^{-1} Ampicillin plus 2,4-Dichlorophenoxyacetic acid (2,4-D) at 10^{-4} , 10^{-5} , 10^{-6} M or water (as a control). The excised cotyledons were kept in the same growth chamber of seedlings to maintain the same temperature regimes, but in darkness to avoid light oxidation of auxin. The change of incubation solution and the surface-sterilisation were performed daily to avoid differences in hormonal concentration and fungal or bacterial attack.

The second experiment was performed only with attached cotyledons in seedlings submitted to different treatments. They were performed aiming to separate the sink strength, endogenous IAA and light effects on the xyloglucan mobilisation. The treatments were: intact seedlings (intact), shoot excised seedlings (excised), shoot excised seedlings with light protected cotyledons (excised LPC), shoot apex excised seedlings (top shoot excised) and intact plants treated with *N*-1-naphthylphthalamic acid at 200 μM (NPA 200 μM), an inhibitor of polar transport of IAA. The intact plants were grown under the same conditions of light, temperature and humidity as used for the control plants in the first experiment. Shoot excision was performed as in the first experiment. However, in the top shoot excised, the

plants were grown with the expansion of eophylls but without apex of the last internode (second). In the light protected cotyledons (LPC) treatment, the cotyledons were wrapped with aluminium foil before the start of xyloglucan mobilisation (ca. 26 days). At the same time the NPA treatment was performed by applying a ring of lanolin paste containing 200 μ M NPA (concentration chosen according to Reed *et al.*, 1998; and Sieburth, 1999). The paste was applied, after warming with hot water, on epicotyl surface 1 cm above the cotyledon insertion, using the tip of a Pasteur pipette. The amount of paste applied was around 3mg. Applications of [3 H]IAA or cold IAA (in lanolin) at 10^{-6} , 10^{-5} and 10^{-4} M concentrations to seedlings which had the top or whole shoot excised were unsuccessful. Thus, these results were not considered in our analyses.

In all experiments and treatments samples of seedlings (first experiment) and/or cotyledons (first and second experiments) were collected once a week. After measurements of leaf area all material were stored at -80°C .

Dry mass and leaf area of seedlings

The seedlings of the first experiment were divided into leaves, stem (nodes and internodes) and roots. Leaf area was analysed using a digital system (Skye Instruments Ltd., Llandrindod Wells, U.K) and together with the other seedling parts, they were dried at 70°C (72 h) and weighed to determine dry mass. The analyses were performed separately on five seedlings per sample.

Dry mass of cotyledons and xyloglucan determination

Ten cotyledons per sample (first experiment), were divided in two groups, in which five were used for dry mass and carbohydrate determinations and the other five were used for enzyme analyses. Samples of the first group were dried at 70°C (72 h), weighed (dry mass), powdered and divided into three sub-samples (100 mg each). These were subjected to xyloglucan extraction in 30 ml of water at 80°C for 8 h. After filtration through a nylon cloth, centrifugation was performed (10 000 g , 30 min, 5°C) followed by precipitation with 3 volumes of ethanol. The precipitate was

stored overnight at 5 °C, collected by centrifugation, freeze-dried and weighed. The water soluble polysaccharides originated from cotyledons, produced a freeze dried fluffy material that was mostly (more than 95%) xyloglucan (Buckeridge *et al.*, 1992). This freeze dried material (xyloglucan) was weighed and the content was calculated in relation of dry mass of cotyledons.

Starch, sucrose and monosaccharide analyses

The same powdered cotyledons used for xyloglucan extraction were submitted to starch and soluble sugars measurements. The starch analyses were performed according to an enzymatic technique described by Arêas & Lajolo (1980). One hundred mg of each powdered cotyledon were weighed and submitted to four extractions with 0.5 ml of 80% ethanol (80 °C, 20 min). After each extraction, the insoluble material was pelleted by centrifugation (10 000 *g*, 15 min) and the supernatants were pooled and saved for analysis of soluble sugars. The pellets were dried at room temperature and 1ml of amyloglucosidase (28 unit.ml⁻¹) was added, followed by incubation at 37 °C for 3 h. The glucose released was measured by mixing 0.1 ml of sample with 1.5 ml of the complex GOD/POD/ABTS [glucose oxidase/oxidase at 1.5 unit.ml⁻¹ and 2,2'-azino-bis (3-ethylbenz-thiazoline-6-sulfonic acid) at 0.5 mg.ml⁻¹, all from Sigma Chem. Co.] and incubating at 37 °C for 15 min. This reaction was read at 540 nm, and using a standard curve with glucose, the proportion of starch present in the cotyledons was calculated. Glucose released was adjusted (-10%) to the mass of linked glucose that is present in starch.

To measure the soluble sugars (sucrose, glucose e fructose) the alcohol supernatants were dried, suspended in water (1ml), filtered (Millipore 0.25) and analysed by High Performance Anion Exchange Chromatography (HPAEC) on a CarboPak PA-1 column (Dionex Corporation, Sunnyvale, Ca, USA) using a gradient elution with 200 mM NaOH and water (20 min). Sugars were detected by a Pulsed Amperometric Detector (PAD; Dionex). Detector responses were compared with the standards of glucose, fructose and sucrose at 25, 50, 75, 100, 150 and 200 µM. The

standard curve for each sugar was used to calculate carbohydrate contents in the cotyledons.

Determination of enzyme activities

Samples of cotyledons were weighed, cut into small pieces and pooled to compose three sub-samples (0.5 g each). These sub-samples were homogenised for 15 sec (Ultra-turrax, T25, IKA-Labortechnik, Germany) with 500 mM NaAc pH 5. The homogenised sub-samples were kept at 5 °C for 20 min and after centrifugation (10 000 *g*, 10 min) the supernatants were separated. Protein concentration was estimated according to Bradford (1976) and the activities of β -galactosidase, β -glucosidase, α -xylosidase (only in the second experiment) and xyloglucan endo- β -transglycosylase (XET) were performed. All enzyme activity measurements were adapted from Tiné *et al.* (2000) and adjusted to microplates of Elisa spectrophotometer.

The determination of β -galactosidase and β -glucosidase activities were performed by addition of 10 μ l of extract, 40 μ l of distilled water and 50 μ l of *p*-nitrophenil- β -galactopiranoside (*p*NP- β -Gal) or *p*-nitrophenil- β -glucopiranoside (*p*NP- β -Glc), respectively per microplate well. Incubation was at 40 °C for 10 min and the reaction was stopped by addition of 200 μ l of 0.1 N Na₂CO₃ and the absorbance was read at 405 nm.

The α -xylosidase activity was measured by addition of 50 μ l of extract, 50 μ l of xyloglucan oligosaccharides (XGO, 1%) and 50 μ l of water per microplate well. The plates were incubated at 30 °C (24 h). After incubation, 200 μ l of fresh *p*-bromoaniline were added, followed by incubation at 70 °C for 10 min and 1 h at room temperature in the darkness. The plates were read at 520 nm. To avoid the interference of free endogenous pentoses each analysis had a second plate that was not incubated in both 30 °C (24 h) and 70 °C (10 min) and the absorbance differences were subtracted. Xylose (5-30 μ g) was used as a standard.

For determination of xyloglucan endo- β -transglycosylase (XET) activities the mixture of incubation was composed of 5 μ l of XGO (1%), 20 μ l of extract and 20 μ l

of xyloglucan (XG at 0.1%) per well and incubation for 2 h at 30 °C. Enzyme reaction was stopped by addition of 20 µl of 1N HCl. This was followed by addition of 150 µl of distilled water, 50 µl of 20% Na₂SO₄ and 40 µl of KI/I₂ diluted in water (1:10) and after incubation for 10 min at room temperature, the mixture was read at 630 nm. As a control, we used the same extracts without incubation (to avoid endogenous xyloglucan interference) and the reagents were added, at the same volumes, but in the following order: HCl (to stop XET), extract, XGO, XG, water, Na₂SO₄ and KI/I₂. The control plate was read together with the incubated plates and the relative differences in absorbances were considered as the XET activity. All enzymatic measurements were made in two replicates.

Measurements of endogenous IAA

The levels of free endogenous IAA were determined by two distinct techniques. In the first experiment an Enzyme Linked Immuno Sorbent Assay (ELISA) technique was employed in which the IAA is detected by an specific antibody (Peres *et al.*, 1997). In the second experiment a gas liquid chromatography-mass spectrometer with a scan ion modulation (GC-SIM-MS) technique was employed to confirm ELISA data and to compare to other treatments.

For IAA determination by ELISA, the same samples used for enzyme activity were used. These samples were from: 1) attached cotyledons in control (34, 41 and 48 days); 2) in darkness (34, 41 and 48 days); 3) and in shoot excised seedlings (41, 54, 61 and 68 days), and 4) detached cotyledons (34 and 41 days). Each previously ground sample was divided into three composed sub-samples of 1g that were individually powdered using liquid nitrogen and submitted to an alcohol extraction. This was performed by addition of 3 ml of methanol per sub-sample and stirring for 60h in the darkness at 4 °C. During this extraction step 100 µl of [³H]IAA (0.5 µCi.ml⁻¹) was added to each sample as an internal standard which was used to determine the extraction and purification yield of IAA. The extracts were filtered in nitrocellulose [0.45 mm (Millex-HV) and 0.22 mm (Millex-GS)] followed by Sep-pak C-18 column, previously activated with 80% methanol. The filtered sample was dried, suspended in

0.2 ml.L⁻¹ formic acid pH 3.0 and submitted to IAA purification by high performance liquid chromatography (HPLC) on a C-18 column using a gradient elution with 0.2 ml.L⁻¹ formic acid pH 3.0 and methanol (45 min) . The radioactive fractions were pooled, dried, methylated with diazomethane, resuspended in water and distributed onto a microplate of ELISA spectrophotometer pre-conditioned with the specific antibody to Me-IAA, according to Peres *et al.* (1997). The IAA concentration was calculated by the use of a standard curve of Me-IAA and the relative optical density.

The GC-SIM-MS analyses of free endogenous IAA were performed according to Chen *et al.* (1988). Five fresh cotyledons (per date and treatment) were chopped into small pieces, weighed and divided into three sub-samples (0.5 g). Each sub-sample was ground (Ultra-turrax T25, IKA-Labortechnik, Germany) adding 4 ml of 65% isopropanol with 0.2 M imidazole buffer pH 7. As an internal standard [¹³C₆]IAA was added (0.5 µg.sub-sample⁻¹). After homogenisation for 1 h at 5 °C, the extracts were centrifuged at 5 000 *g* for 5 min and the supernatants were diluted 6 times in water to reduce the isopropanol concentration. The diluted extracts were applied to a pre-conditioned aminopropyl column (Sep-Pak NH₂) and sequentially washed with hexane, ethyl acetate, acetonitrile, and methanol (2 ml of each). The IAA was eluted from the aminopropyl column by addition of 3 ml of 2% acetic acid in methanol. The eluted samples were neutralised with 20% NH₄OH (20 µl.ml⁻¹), freeze-dried, resuspended in 100 µl of methanol and purified in a high performance liquid chromatography (HPLC) using a C-18 column which was eluted with a gradient elution of acetonitrile and 1% acetic acid. The fractions corresponding to IAA were neutralised by 20% NH₄OH, dried and resuspended in 100 µl of methanol. They were methylated by addition of 1ml of ethereal diazomethane (prepared according to Peres *et al.*, 1997). The methylated samples were nitrogen dried, resuspended in ethyl acetate (30 µl) and analysed by a gas liquid chromatography (6890 series) – mass spectrometer (5973 series, Agilent Technologies, Inc. EUA) using a column of medium polarity (HP1701, Hewlett Packard, USA) in the scan ion modulation (SIM) mode. The detection area of endogenous IAA (130) and internal standard (136) ions

and the initial concentration of the internal standard were used to calculate the IAA concentration in the cotyledons as described by Cohen *et al.* (1986).

RESULTS

Storage mobilisation in the cotyledons and early seedling growth

The decay curve of dry mass of the cotyledons of *H. courbaril* decreased in two phases. First step was from zero to 30 days, when protein bodies and raffinose series oligosaccharides are mobilised and the second was from 30 to 50 days, when xyloglucan is degraded (Figs. 1A and 2A). In excised cotyledons from growing seedlings at different stages of germination/establishment the decrease of dry mass (Fig. 1A) or xyloglucan contents (Fig. 2A) were observed only when it happened after 34 days. On the other hand, when cotyledons were excised at 41 days and incubated in water, they were capable to maintain xyloglucan degradation with a slow, but similar tendency as observed in the attached cotyledons (Fig. 2A).

The addition of 10^{-6} M 2,4-D after 41 days significantly increased xyloglucan degradation, similar to attached cotyledons (Fig. 2A). Exogenous 2,4-D was active on xyloglucan mobilisation only at concentration of 10^{-6} M and in the same period as the attached cotyledons. Incubation of detached cotyledons in 2,4-D at 10^{-4} and 10^{-5} M failed to evoke any detectable xyloglucan breakdown.

The drastic dry mass loss and xyloglucan mobilisation in the cotyledons were directly related to the increase in dry mass of the seedling, mainly with the shoot and expansion of eophylls (Figs. 1 and 2A). This relationship was clearer from the observation of seedlings of *H. courbaril* growing after excision of aerial parts or in the darkness. The latter prevented the increase in total leaf area (Fig. 1D) and dry masses of whole seedlings (Fig. 1B) and their aerial parts (Fig. 1C). Furthermore, darkness promoted a reduction of xyloglucan mobilisation (Figs. 1A and 2C respectively).

Another group of seedlings, subjected to excision of aerial parts at 34 days (when xyloglucan degradation starts), showed a strong delay in xyloglucan

mobilisation (Fig. 2C). This inhibitory effect was followed by a significant reduction of seedling growth (Fig. 1B). Approximately 15 days after excision, the aerial parts started to grow again, producing new leaves (Figs. 1C and 1D), which was followed by an increase in xyloglucan mobilisation (Fig. 2C).

Starch, soluble sugars and xyloglucan metabolism

As can be observed by dry mass and xyloglucan changes during mobilisation, the excision of cotyledons promoted an increase in the amounts of starch. Moreover, addition of 10^{-6} M 2,4-D to excised cotyledons at a period of advanced mobilisation (41 days), promoted an even higher increase in starch synthesis (Fig. 2B), suggesting that this phenomenon might be dependent on xyloglucan mobilisation.

Seedlings growing in the darkness and with the excision of the aerial parts showed an induction of starch synthesis, accumulating relatively higher amounts of starch in the cotyledons than the control plants (Fig. 2D). In the excised seedlings, the accumulated starch was mobilised when growth of aerial parts re-started.

Analysis of the free sugars showed that in attached cotyledons, fructose and glucose increased at the same time as xyloglucan was being degraded, peaking at 48 days and decreasing rapidly afterwards (Table 1, control seedling). Sucrose was also present during germination period, decreasing quickly up to 26 days and increasing again during xyloglucan catabolism. When seedlings were grown in the darkness or the aerial parts were excised, a strong reduction in the concentration of free sugars was observed (Table 1). The re-start of xyloglucan mobilisation after excision of aerial parts was also confirmed by a parallel increase in glucose, fructose and sucrose in the cotyledons (Table 1).

The analysis of detached cotyledons showed that only those ones isolated after 34 days were capable to maintain an increase in free sugars, reaching higher levels when excision occurred at 41 days (Table 1). Likewise, the 2,4-D treated cotyledons (at 41 days) showed high free-sugar contents during xyloglucan mobilisation, which support the data of xyloglucan degradation and starch synthesis observed in the same

cotyledons. It is important to note that isolated cotyledons tended to show a higher sucrose:monosaccharide ratio than attached ones.

Xyloglucan hydrolases

In attached cotyledons, all hydrolase activities related to xyloglucan mobilisation increased at the same period of the most intense changes in dry mass and carbohydrates contents. In all treatments, the onset of the activities of XET, α -xyl (assayed only in the second experiment), β -gal and β -glc occurred after 30 days (Figs. 3 and 4).

In cotyledons detached at 19 days (experiment 1), the activities of the three enzymes did not increase at the same rate as in the attached cotyledons. However, the cotyledons isolated after 34 days and kept in water thereafter, showed significantly lower activities of both endo- and exo-enzymes (Figs. 3B, 3D and 3F). This occurred concomitantly with the reduction of xyloglucan and with the increase of free sugars and starch in the cotyledons (Table 1, Fig. 2). The enzyme activities in cotyledons detached at 41 days were also stimulated by 10^{-6} M 2,4-D, mainly regarding XET (Fig. 3B).

The dark-grown seedlings showed reduction of all hydrolase activities in relation the light-growth seedlings (Figs. 3A, 3C and 3E). It was also observed after excision of the aerial parts of the seedling in both experiments (Figs. 3 and 4).

To evaluate the isolated effect of auxin on the activity of xyloglucan hydrolases in the presence of sink-strength, top shoot excision or NPA treatments were performed. Although after top shoot excision no differences in hydrolase activities as well as in IAA contents have been observed (Figs. 4 and 5B), when NPA (an inhibitor of polar auxin transport) was used, all hydrolase activities were strongly reduced (Fig. 4).

Endogenous auxin

Aiming to test the hypothesis that there is a relationship between auxin increase and xyloglucan mobilisation in the cotyledons, we sought to measure the endogenous levels of auxin in detached cotyledons or in attached cotyledons of

seedlings submitted to shoot and top shoot excision and polar auxin transport inhibition (NPA). Our results showed that auxin levels increased at the same period of the xyloglucan mobilisation in the cotyledons of *H. courbaril* (Fig. 5). It increased exponentially in cotyledons of intact plants (ca. 8 fold), whereas in cotyledons detached at 34 days as well as in attached cotyledons from shoot excised (kept in the light or in the dark) and NPA-treated seedlings, no increase was observed (Fig. 5). Although these results suggest that the cotyledons of *H. courbaril* are not able to produce endogenous auxin, this possibility can not be excluded, since in the light-kept cotyledons (NPA or shoot excised) a small increase in IAA was observed throughout the experiment (Fig. 5B). In the darkness, only a slight increase in endogenous auxin was measured, whereas in seedlings where aerial parts were excised, the endogenous auxin increased, but with a delay compatible with the observed re-growth of aerial parts (compare Figs. 5A and 1D).

DISCUSSION

According to Bewley & Black (1994) there are two possible mechanisms by which the embryonic axis can control storage mobilisation in seeds. One is through the action of axis as a sink organ, metabolising the products of storage compounds (e.g. sucrose), avoiding feedback mechanisms by their end products, and another is through the delivery of a signal (e.g. hormone) to modulate storage mobilisation.

Sink strength and xyloglucan mobilisation

Considering the first mechanism, our results demonstrated that the embryonic axis plays an important role in xyloglucan mobilisation, by establishment of a sink (mainly the expanding leaves) of its degradation products. In this process, light seems to play an important role by stimulation of growth of aerial parts and the establishment of sink strength, as previously proposed by Chory (1993). This was clearly demonstrated by following xyloglucan mobilisation after isolation of the

cotyledons at different stages of seedlings development and in attached cotyledons of seedlings growing in the darkness as well as after excision of the shoot. These treatments clearly affected sink strength, reducing xyloglucan mobilisation and increasing starch and soluble sugar concentrations while degradation was active. These results indicated that the steady decrease in cotyledon dry mass after excision of aerial parts might be related to a limited storage mobilisation activity. This probably sustained further development of the aerial parts and after full growth of the new aerial parts was re-established, the complete shrinking of the cotyledons was observed (not shown).

The reduction of the rate of xyloglucan mobilisation by excision of aerial parts or by isolation of cotyledons from the seedlings was related to reduction in activity of all xyloglucan hydrolases (Figs. 3 and 4). This may be explained by the reduction of sink-strength, which can be seen by the high levels of starch produced (Fig. 2D), and a consequent feed-back inhibition. The hydrolase/transglycosylase activities of XET of *Hymenaea courbaril* are dependent on the oligosaccharide concentration present in the apoplast (Tin   *et al.*, 2000). Furthermore, the β -galactosidase of cotyledons of *Hymenaea courbaril* is strongly inhibited by galactose ($K_i=3.7$ mM, Alc  ntara, 2000). A strong inhibitory effect was also observed on hydrolase activities when cotyledons of shoot excised seedlings were covered with aluminium foil (Fig. 4). Although, the kind of light involved, in terms of wave length or energy, on this stimulus remains to be investigated, these results permit to speculate that darkness has an inhibitory effect on the xyloglucan mobilisation, so that the cotyledons have a mechanism to sense light availability.

Our results suggest that accumulation of starch might be dependent on xyloglucan mobilisation. Indeed, transitory starch has been observed previously in seeds that mobilise galactomannan (Reid, 1971, McCleary, 1983, Buckeridge & Dietrich, 1996) and have been regarded as an important step in the regulation of carbon flow in leaves (Ludewig *et al.*, 1998). It is thought that transitory starch is synthesised as a response to the production of high amounts of carbon during storage cell wall polysaccharide mobilisation (Buckeridge *et al.*, 2000). Although the

presence of starch during the xyloglucan mobilisation in cotyledons of *H. courbaril* had been microscopically observed by Tiné *et al.* (2000), our results show for the first time that a pattern of relationship with starch similar to galactomannan containing seeds occurs in the xyloglucan containing cotyledons of *H. courbaril*. In the case of cotyledons of *H. courbaril* the accumulation of starch may be therefore considered as a temporary regulator of the relationship between source (xyloglucan degradation) and sink (synthesis/transport of sucrose to aerial parts) intensities and therefore it can avoid potentially adverse effect of accumulation of high concentration of reducing sugars (Geiger *et al.*, 2000).

Auxin and xyloglucan mobilisation

Considering the second mechanism proposed by Bewley & Black (1994), in which storage mobilisation might be controlled by hormones, our experiments have shown that auxin appears to be involved in the control of xyloglucan mobilisation in the cotyledons. One evidence favouring this hypothesis is that the contents of IAA in the cotyledons varied in step with the changes in hydrolase activities (Figs. 3, 4 and 5). Furthermore, this increase in IAA in the cotyledons could be prevented by, 1) excision of aerial parts; 2) growth in the darkness and 3) use of NPA, a potent inhibitor of polar auxin transport. As the addition of 10^{-6} M 2,4-D to excised cotyledons was enough to induce an increase in xyloglucan degradation, starch biosynthesis and at the same time to promote xyloglucan hydrolase activities, it can also be suggested that these events are possibly under control of auxin as has been observed for primary (Hoson, 1993; Valero & Labrador, 1995; Kotake *et al.*, 2000; Catalá *et al.*, 1997, 2000) and storage cell walls (Hensel *et al.*, 1991). Furthermore, similarly to what was observed for *T. majus* by Hensel *et al.* (1991), we also observed that cotyledons became sensitive to auxin only during the *in vivo* period of mobilisation of xyloglucan.

Altogether, the results confirm that the IAA present in the cotyledons during xyloglucan mobilisation is produced in the developing shoot, which is considered the main site of auxin biosynthesis (Bartel, 1997). It has to be noted that during

xyloglucan mobilisation, IAA seems to be produced mainly in the eophylls, since the excision of the shoot apex had a limited effect on IAA production (Fig. 5B).

The levels of IAA in the cotyledons are mainly related to polar auxin transport, as we confirmed by NPA treatment, and not to hydrolysis of IAA conjugates as has been described for seedlings of *Pinus sylvestris* (Ljung *et al.*, 2001). The behaviour of NPA-treated seedlings demonstrated clearly that the increase in xyloglucan hydrolase activities was strongly dependent on polar auxin transport from the developing shoot. This treatment promoted a negative regulation of all hydrolase activities (Fig. 4).

More recently, studies have demonstrated the importance of the polar auxin transport in many aspects, as to expansion of hypocotyl of cucumber (Shinkle *et al.*, 1998), *Arabidopsis* (Jensen *et al.*, 1998) and tomato (Kraepiel *et al.*, 2001) as well as to development of lateral roots (Reed *et al.*, 1998) and of leaf and cotyledon vein in *Arabidopsis* (Sieburth, 1999). This polar auxin transport has also been considered as a co-ordinator of the rhythmicity in the extension rate oscillations of the first internode in *Arabidopsis* (Jouve *et al.*, 1999). However, the majority of the results about the mode of action of auxin have been described as influencing the metabolism of the primary cell wall mainly in herbaceous seedlings.

The only work in which a relationship between auxin and storage cell wall metabolism had been studied was in cotyledons of *Tropaeolum majus* (Hensel *et al.*, 1991), which is also a herbaceous species. To our knowledge, the present work is the first evidence of a relationship between *in vivo* levels of IAA and storage xyloglucan mobilisation in the cotyledons of *Hymenaea courbaril*, a climax tree from the Neotropical Forest. Among the possibilities for further studies are the influence on veins establishment in the cotyledons, the influence on the expression of cell wall related genes and on apoplastic pH since an acid optimum-pH (3.2 - 4.5) of activity of these enzymes in cotyledons of *H. courbaril* (Tin   *et al.*, 2000; Alc  ntara, 2000) and in *C. langsdorffii* (Alc  ntara *et al.*, 1999) have been demonstrated.

Synchronism and Ecological function

Considering the results on the effects of sink and auxin on carbohydrate metabolism in the cotyledons, it can be suggested that both light and aerial parts have a strong relationship with the catabolism of the storage cell wall xyloglucan, transient accumulation of starch and sucrose, as summarised in Figure 6. As *Hymenaea courbaril* is a shade-tolerant species (Souza & Válio, 1999) this kind of correlation highlights the existence of a synchronism among the rates of storage mobilisation/products utilisation, depending on light intensity.

Jensen *et al.* (1998) e Shinkle *et al.* (1998) demonstrated that red light is capable to induce polar transport of auxin in *Arabidopsis* and cucumber respectively. However, Kraepiel *et al.* (2001) showed that in tomato, the polar transport was independent of light. Our results showed that darkness strongly inhibited polar auxin transport in seedlings of *Hymenaea*. Also, in experiments where seedlings were grown under different conditions of Red/Far red ratios (Capítulo 2, Tab. 1), mobilisation was faster in increasing proportions of red light (Capítulo 2, Fig. 2). This suggests that seedlings of *Hymenaea courbaril* responses in a similar way as demonstrated for *Arabidopsis* and cucumber. Considering the light influence on auxin polar transport, the hormonal control shows an ecophysiological importance for seedlings of *Hymenaea courbaril* by promoting synchronism between growth and reserve degradation under variable light conditions (Fig. 6). This is likely to increase the efficiency of carbon reserves utilisation by the growing seedling in the understorey of the rain forest.

Altogether, our results highlight the possibility that the presence of cell wall polysaccharides as storage compounds in seeds appears to be a result, during the evolution, of a mechanism of transference of function as has been suggested by Buckeridge *et al.* (2000). In this process, not only the polymer, but also the related metabolism could have been subjected to selective pressure during evolution. Therefore, it is reasonable to suggest that some features of the mechanism of primary cell extension have been maintained in the storage cell wall metabolism

during evolution, so that the actual catabolism of xyloglucan and its control mechanisms resemble the auxin induced growth of primary cell walls.

ACKNOWLEDGEMENTS

This work was supported by Fundação de Amparo a Pesquisa do Estado de São Paulo-FAPESP (BIOTA-FAPESP 98/05124-8). The authors thank the FAPESP for granting a doctoral fellowship to HPS (98/02775-8) and CNPq for research fellowship to MSB. The authors also thank Mrs A. M. Baroni, L. I. V. Amaral and V. Tamaki for technical assistance in the auxin procedures.

REFERENCES

- Alcântara, P.H.N. 2000. Isolamento e caracterização das enzimas xiloglucano endotransglicosilase e β -galactosidase do catabolismo do xiloglucano de reserva dos cotilédones de *Hymenaea courbaril* L. (Leguminosae-Caesalpinioideae). Ph.D. thesis. UNIFESP, São Paulo 98p.
- Alcântara, P.H.N.; Dietrich, S.M.C. & Buckeridge, M.S. 1999. Xyloglucan mobilisation and purification of a (XLLG/XLXG) specific β -galactosidase from cotyledons of *Copaifera langsdorffii*. *Plant Physiology and Biochemistry* 37:653-663.
- Arêas, J.A.G. & Lajolo, F.M. 1980. Determinação enzimática específica de amido, glicose, frutose e sacarose em bananas pré-climatéricas e climatéricas. *Anais de Farmácia e Química de São Paulo* 20: 307-318.
- Bartel, B. 1997. Auxin biosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* 48:51-66.
- Bewley, J.D. & Black, M. 1994. *Seeds - Physiology of Development and Germination*. Second edition. Plenum press, New York.
- Bradford, M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of Protein-Dye Binding. *Analytical Biochemistry* 72: 248-254.

- Buckeridge, M.S. & Dietrich, S.M.C. 1996. Mobilisation of the raffinose family oligosaccharides and galactomannan in germinating seeds of *Sesbania marginata* Benth. (Leguminosae-Faboideae). *Plant Science* 117: 33–43.
- Buckeridge, M.S.; Rocha, D.C.; Reid, J.S.G. & Dietrich, S.M.C. 1992. Xyloglucan structure and post-germinative metabolism in seeds of *Copaifera langsdorffii* from savana and forest populations. *Physiologia Plantarum* 86: 145-151.
- Buckeridge, M.S.; Santos, H.P. & Tiné, M.A.S. 2000. Mobilisation of storage cell wall polysaccharides in seeds. *Plant Physiology and Biochemistry* 38: 141-156.
- Catalá, C.; Rose, J.K.C. & Bennett, A.B. 1997. Auxin regulation and spatial localization of an endo-1,4- β -D-glucanase and xyloglucan endotransglycosylase in expanding tomato hypocotyls. *The Plant Journal* 12: 417-426.
- Catalá, C.; Rose, J.K.C. & Bennett, A.L. 2000. Auxin-regulated genes encoding cell wall-modifying proteins are expressed during early tomato fruit growth. *Plant Physiology* 122: 527-534.
- Chen, K.; Miller, A.N.; Patterson, G.W. & Cohen, J.D. 1988. A rapid and simple procedure for purification of Indole-3-Acetic Acid prior to GC-SIM-MS analysis. *Plant Physiology* 86: 822-825.
- Chory, J. 1993. Out of darkness: mutants reveal pathways controlling light-regulate development in plants. *Trends in Genetics* 9:167-172.
- Cohen, J.D.; Baldi, B.G. & Slovin, J.P. 1986. $^{13}\text{C}_6$ -[benzene ring]-indole-3-acetic acid: a new internal standard for quantitative mass spectral analysis of indole-3acetic-acid in plants. *Plant Physiology* 80: 14-19.
- Cosgrove, D.J. 2000. Expansive growth of plant cell walls. *Plant Physiology and Biochemistry* 38: 109-124.
- Crombie, H.J.; Chengappa, S., Hellyer, A. & Reid, J.S.G. 1998. A xyloglucan oligosaccharide-active, transglycosylating β -D-glucosidase from the cotyledons of nasturtium (*Tropaeolum majus* L.) seedlings-purification, properties and characterization of a cDNA clone. *Plant Journal* 15: 27-38.

- Edwards, M.; Dea, I.C.M.; Bulpin, P.V. & Reid J.S.G. 1985. Xyloglucan (amyloid) mobilisation in the cotyledons of *Tropaeolum majus* L. seeds following germination. *Planta* 163: 133–140.
- Edwards, M.; Dea, I.C.M.; Bulpin, P.V. & Reid J.S.G. 1986. Purification and properties of a novel xyloglucan-specific endo- β -(1,4)-D-glucanase from germinating nasturtium seeds (*Tropaeolum majus* L.). *Journal of Biological Chemistry* 261: 9489-9494.
- Edwards, M.E.; Bowman, J.L.; Dea, I.C.M. & Reid, J.S.G. 1988. A β -D galactosidase from nasturtium (*Tropaeolum majus* L.) cotyledons. *Journal of Biological Chemistry*, 263: 4333-4337.
- Fanutti, C.; Gidley, M.J. & Reid, J.S.G. 1991. A xyloglucan oligosaccharide specific α -D-xylosidase or exo-oligoxyloglucan- α -xylohydrolase from germinated nasturtium (*Tropaeolum majus* L.) seeds. Purification, properties and its interaction with a xyloglucan-specific endo- β -(1,4)-glucanase and other hydrolases during storage xyloglucan mobilisation. *Planta* 184: 137–147.
- Fanutti, C.; Gidley, M.J. & Reid, J.S.G. 1993. Action of a pure xyloglucan endo-transglycosylase (formerly called xyloglucan-specific endo-(1,4)- β -D-glucanase) from the cotyledons of germinated nasturtium seeds. *Plant Journal* 3: 691–700.
- Geiger, D.R.; Servaites, J.C. & Fuchs, M.A. 2000. Role of starch in carbon translocation and partitioning at the plant level. *Australian Journal of Plant Physiology* 27: 571-582.
- Hayashi, T. 1989. Xyloglucan in the primary cell wall. *Annual Review of Plant Physiology and Plant Molecular Biology* 40, 139-168.
- Heinricher E. 1888. Zur Biologie der gattung *Impatiens*. *Flora Jena* 71 .
- Hensel, A.; Brummell, D.A.; Hanna, R.; MacLachlan, G. 1991. Auxin-dependent breakdown of xyloglucan in cotyledons of germinating nasturtium seeds. *Planta* 183: 321-326.
- Hoson, T. 1993. Regulation of polysaccharide breakdown during auxin-induced cell wall loosening. *Journal of Plant Research* 106: 369-381.

- Jensen, P.J.; Hangarter, R.P. & Estelle, M. 1998. Auxin transport is required for hypocotyl elongation in light-growth but not dark-grown *Arabidopsis*. *Plant Physiology* 116: 455-462.
- Jouve, L.; Gaspar, T.; Kevers, C.; Greppin, H. & Agosti, R.D. 1999. Involvement of indole-3-acetic acid in the circadian growth of the first internode of *Arabidopsis*. *Planta* 209:136-142.
- Kooiman, P. 1960. On the occurrence of amyloids in plant seeds. *Acta Botanica Neerlandica* 9:208-219.
- Kotake, T.; Nakagawa, N.; Takeda, K. & Sakurai, N. 2000. Auxin-induced elongation growth and expressions of cell wall-bound exo- and endo- β -glucanases in barley coleoptiles. *Plant Cell Physiology* 41: 1272-1278.
- Kraepiel, Y.; Agnès, C.; Thiery, L.; Maldiney, R.; Miginiac, E. & Delarue, M. 2001. The growth of tomato (*Lycopersicon esculentum* Mill.) hypocotyls in the light and in darkness differentially involves auxin. *Plant Science* 161 : 1067 - 1074
- Ljung, K.; Östin, A.; Lioussanne, L. & Sandberg, G. 2001. Developmental regulation of indole-3-acetic acid turnover in scots pine seedlings. *Plant Physiology* 125: 464-475.
- Ludewig, F.; Sonnewald, U.; Kauder, F.; Heineke, D.; Geiger, M.; Stitt, M.; Müller-Rober, B.T.; Gillissen, B.; Kuhn, C. & Frommer, W.B. 1998. The role of transient starch in acclimation to elevated atmospheric CO₂. *FEBS Letters* 429:147-151.
- McCleary, B.V. 1983. Enzymic interactions in the hydrolysis of galactomannan: the role of exo-mannanase. *Phytochemistry* 22: 649-658.
- Peres, L.E.P.; Mercier, H.; Kerbauy, G.B. & Zaffari, G.R. 1997. Endogenous levels of IAA, cytokinins and ABA in shootless orchid and a rootless bromeliad determined by means of HPLC and ELISA. *Revista Brasileira de Fisiologia Vegetal* 9: 169-176.
- Reed, R.C.; Brady, S.R. & Muday, G.K. 1998. Inhibition of auxin movement from the shoot into the root inhibits lateral root development in *Arabidopsis*. *Plant Physiology* 118: 1369-1378.
- Reid, J.S.G. 1971. Reserve carbohydrate metabolism in germinating seeds of *Trigonella foenum-graecum* L. (Legum.). *Planta* 100: 131-142.

- Reis, D.; Vian, B.; Darzens, D. & Roland, J.C. 1987. Sequential patterns of intramural digestion of galactoxyloglucan in tamarind seedlings. *Planta* 170: 60-73.
- Reiss, R. 1889. Über die Natur der Reservecellulose und über ihre Auflösungsweise bei der Keimung der Samen. *Landwirtsch Jahrb* 18: 711–765.
- Shinkle, J.R.; Kadakia, R. & Jones, A.M. 1998. Dim-red-light induced increase in polar auxin transport in cucumber seedlings. *Plant Physiology* 116: 1505-1513.
- Sieburth, L.E. 1999. Auxin is required for leaf vein pattern in Arabidopsis. *Plant Physiology* 121: 1179-1190.
- Souza, R.P. & Válio, I.F.M. 1999. Carbon translocation as affected by shade in saplings of shade tolerant and intolerant species. *Biologia Plantarum* 42: 631-636.
- Taguchi, T.; Uraguchi, A. & Katsumi, M. 1999. Auxin- and acid-induced changes in the mechanical properties of the cell wall. *Plan Cell Physiology* 40: 743-749.
- Tiné, M.A.S. 1997. Mobilização de xiloglucano de reserva de sementes de *Hymenaea courbaril* L. UNICAMP. M.Sc Thesis, 85 p.
- Tiné, M.A.S.; Cortelazzo, A.L. & Buckeridge, M.S. 2000. Xyloglucan mobilisation in cotyledons of developing plantlets of *Hymenaea courbaril* L. (Leguminosae-Caesalpinoideae). *Plant Science* 154: 117-126
- Valero, P. & Labrador, E. 1995. Effect of auxin on cell wall glycanhydrolytic enzymes in epicotyls of *Cicer arietinum*. *Physiologia plantarum* 93: 764-770.
- White, E.V. & Rao, P.S. 1953. *J. Am. Chem. Soc.* 75 2617, Apud Kooiman, (1960).

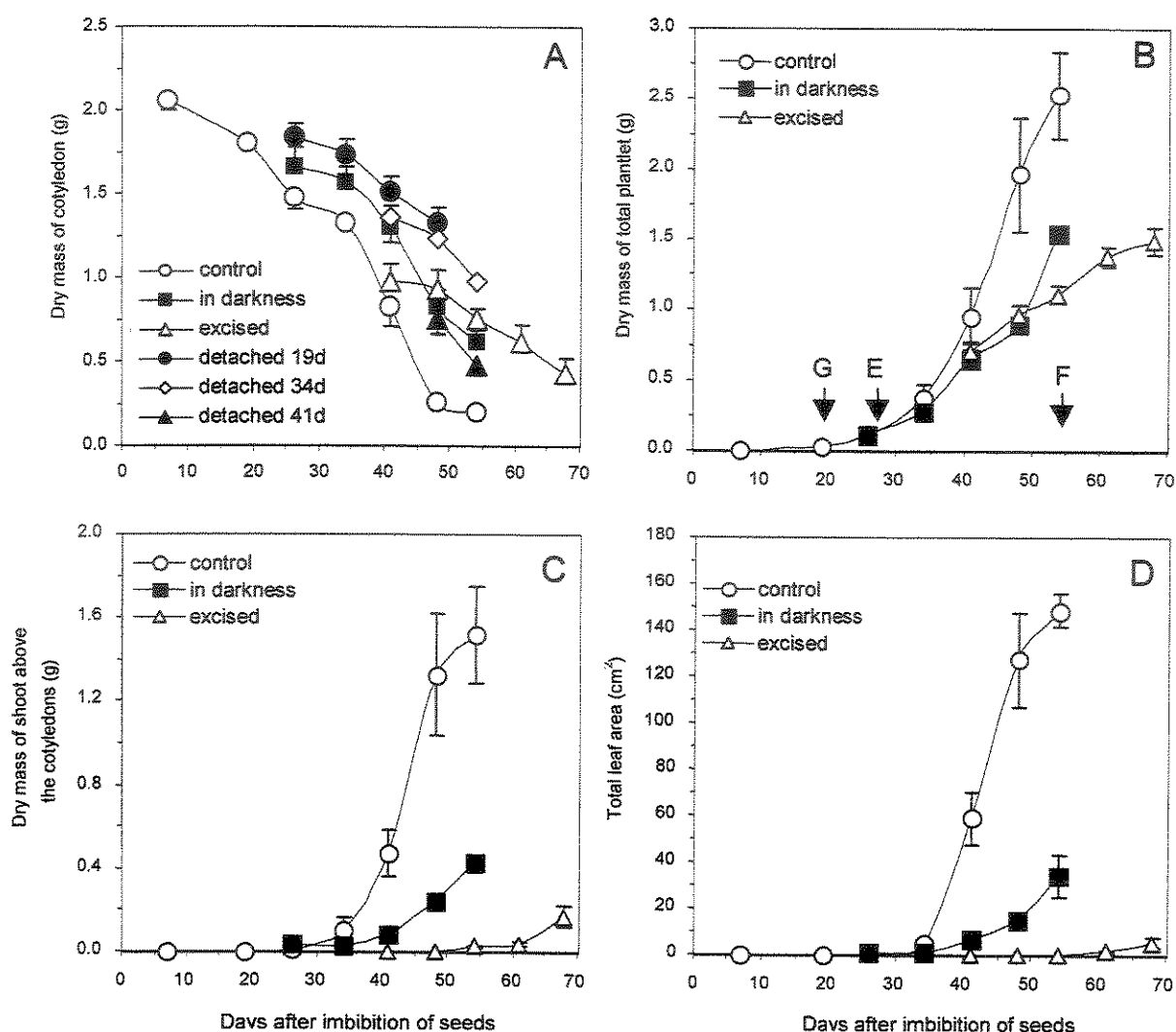


Fig. 1. Dry mass of detached and attached cotyledons (A), of whole seedlings (B) or only the shoot above the cotyledons insertions (C) and the total leaf area of seedlings of *Hymenaea courbaril*. Seedlings with cotyledons grown under control conditions (control), in the darkness or with shoot excised above the cotyledons insertions (excised). Cotyledons were detached at 19, 34 and 41 days and maintained in water. The letters at plot B refer to germination (G) and emergence (E) of seedlings and fall of cotyledons (F). Bars represent standard deviation of the mean of five replicates.

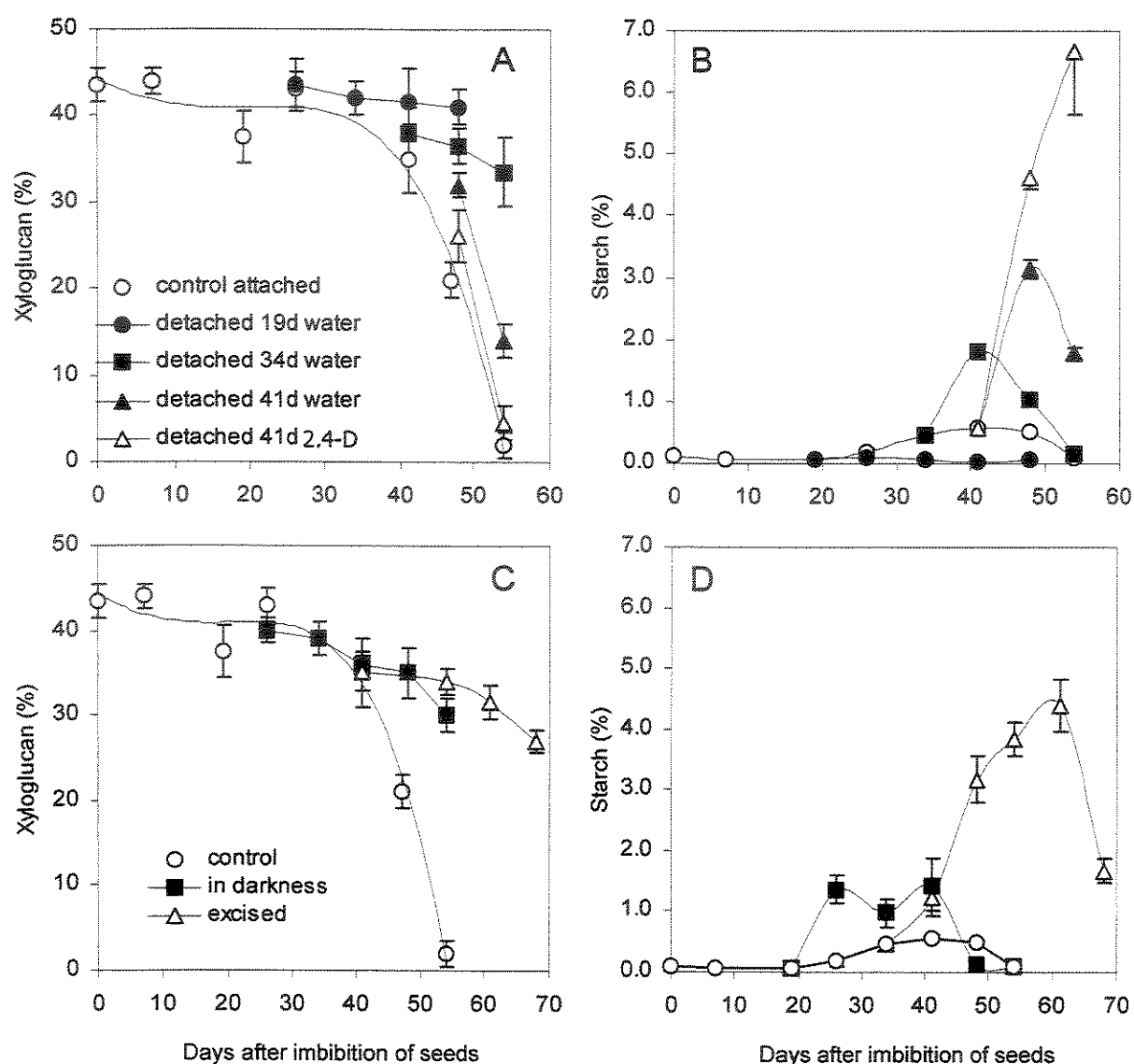


Fig. 2. Contents of xyloglucan and starch in detached cotyledons (A, B) and in cotyledons attached to the seedlings (C, D) of *Hymenaea courbaril*. The attached (legends at C) were from seedlings grown under control conditions (control), in the darkness or with shoot excised above the cotyledons insertions (excised). Cotyledons were detached at 19, 34 and 41 days and maintained in water or 10^{-6} M 2,4-D (41 days only). Bars represent standard deviation of the mean of three composed replicates.

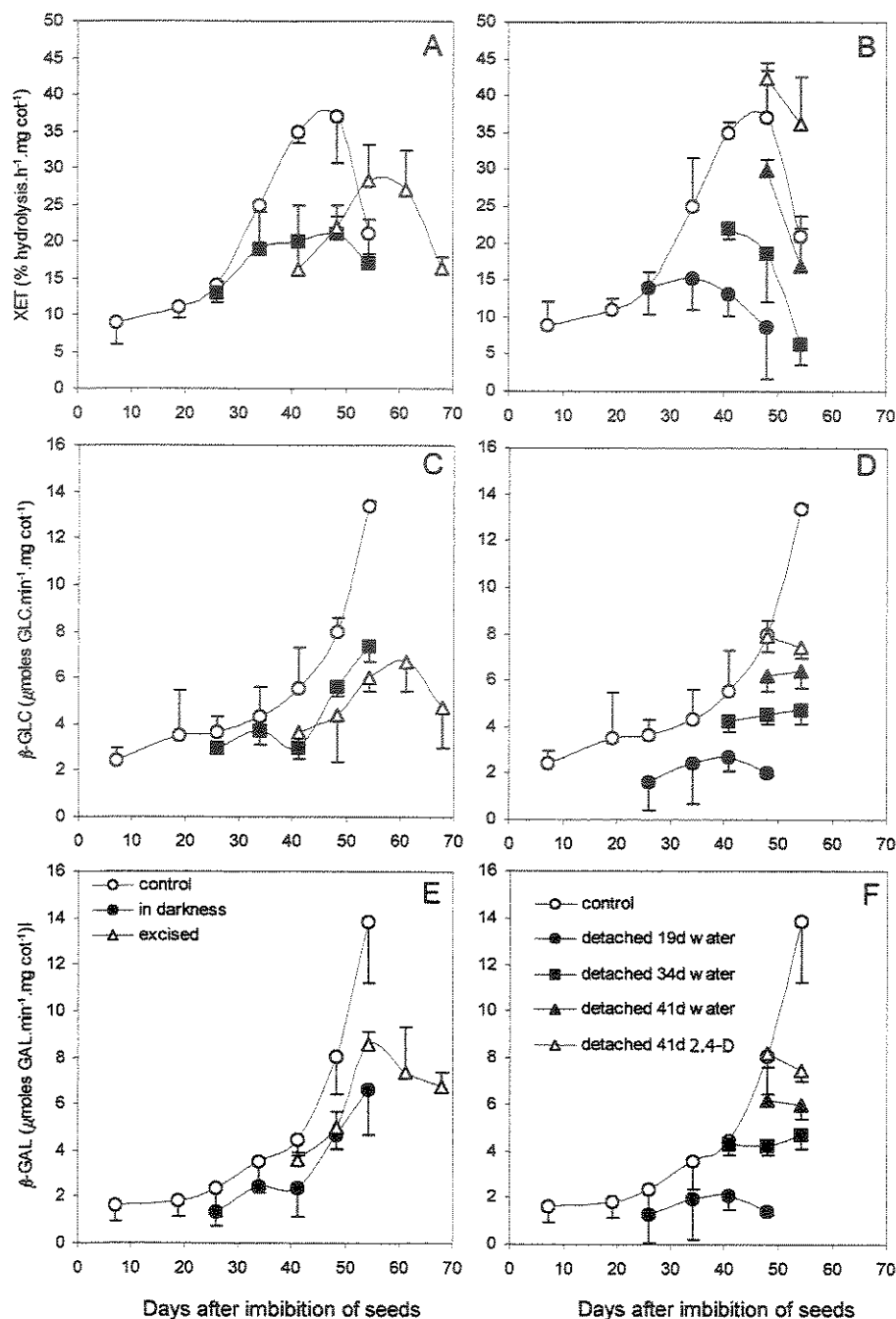


Fig. 3. Specific activities of xyloglucan hydrolases in attached (A, C and E) and detached cotyledons (B, D and F) of *Hymenaea courbaril* during seedling development. The attached (legends at E) were from seedlings grown under control conditions (control), in the darkness or with shoot excised above the cotyledons insertions (excised). The detached (legends at F) were from developing seedlings at 19, 34 and 41 days and maintained in water or 10^{-6} M 2,4-D (41 days only). A, B = xyloglucan endo- β -transglycosylase; C, D = β -glucosidase; E, F = β -galactosidase. In all plots, the activities observed in attached cotyledons (control) were added as a reference. Bars represent standard deviation of the mean of three composed replicates.

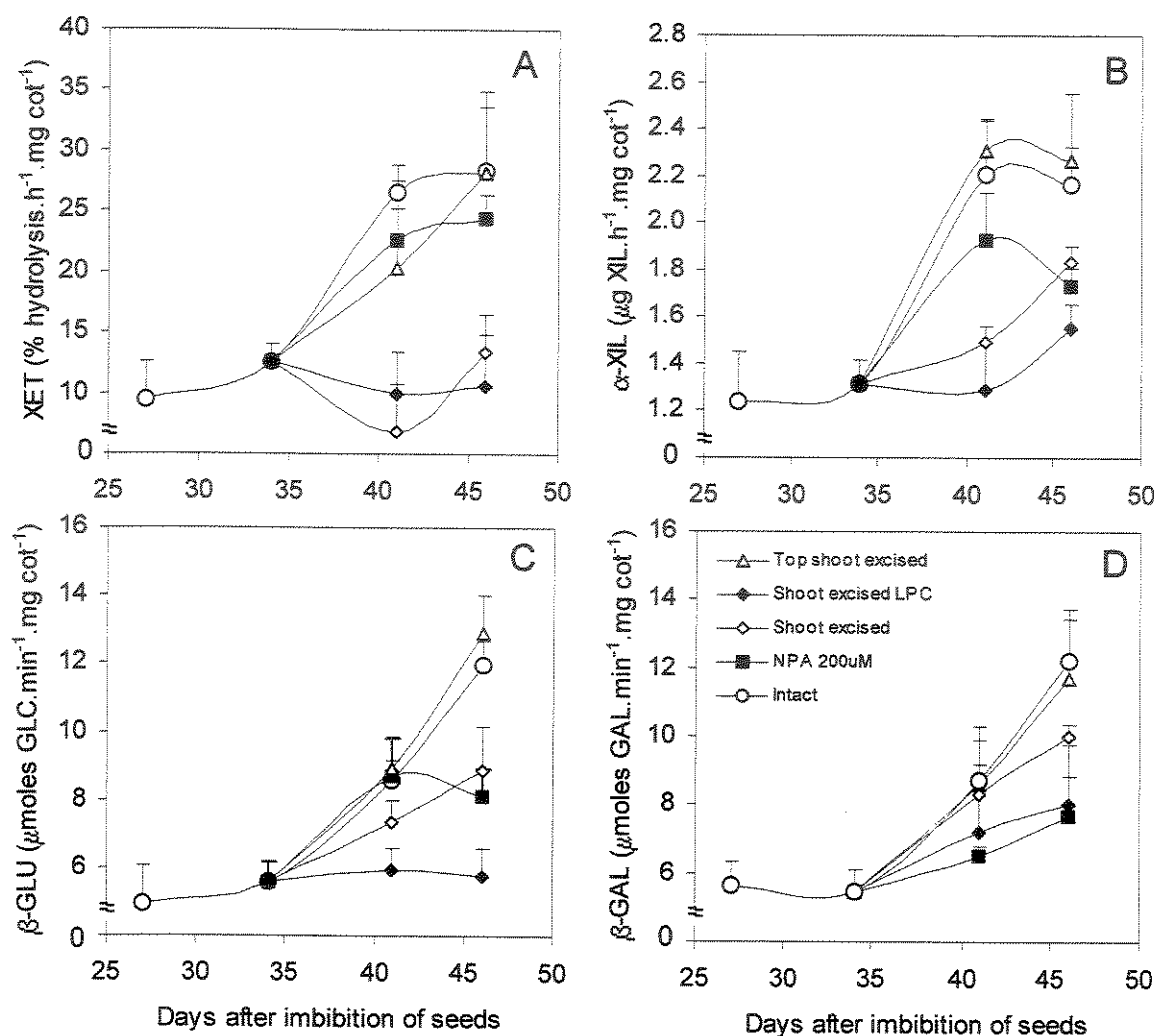


Fig. 4. Specific activities of xyloglucan hydrolases in attached cotyledons of *Hymenaea courbaril* seedlings grown at control conditions without excision treatment (intact) and intact seedlings treated with *N*-1-naphthylphthalamic acid at 200 μ M (NPA 200 μ M); and in shoot excised plants (excised), shoot excised plants with light protected cotyledons (Shoot excised LPC) and shoot apex excised plants (Top shoot excised). A = xyloglucan endo- β -transglycosylase; B = α -xylosidase; C = β -glucosidase; D = β -galactosidase. In all plots, the activities observed in intact seedlings were added as a reference. Bars represent standard deviation of the mean of three composed replicates.

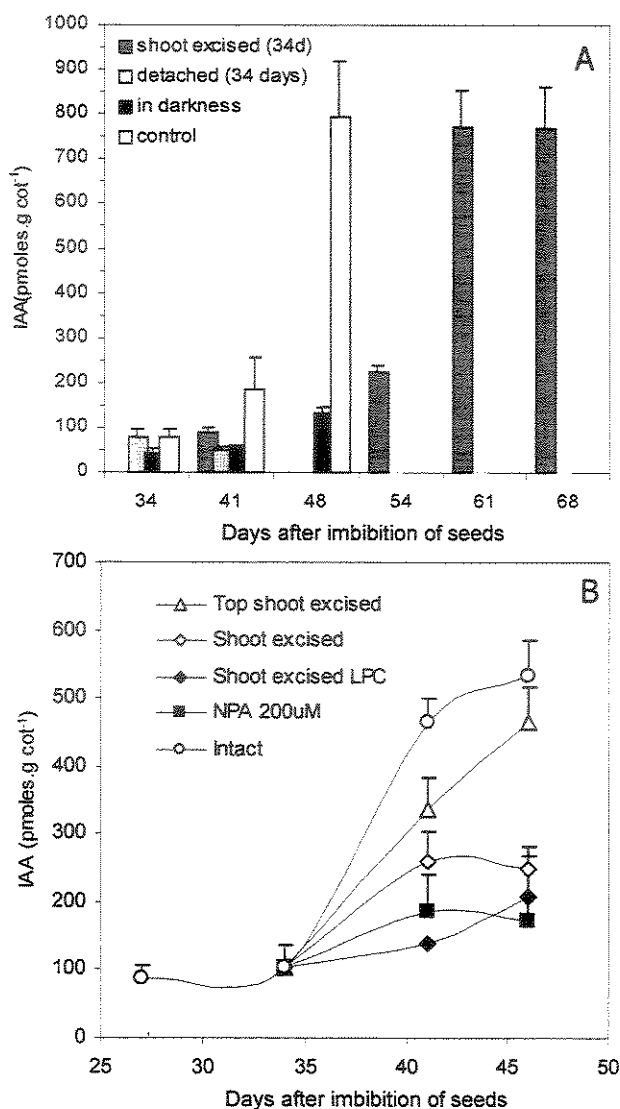


Fig. 5. Concentration of endogenous indol-3-acetic acid (IAA) observed by (A) ELISA technique (Peres *et al.*, 1997) and (B) by GC-SIM-MS technique (Chen *et al.*, 1988). In A, the IAA was measured in the attached cotyledons from intact seedlings of *Hymenaea courbaril* grown in light, darkness or with excised shoot above the cotyledons insertions (excised) at 34 days. The only isolated cotyledons employed in this analysis as a reference were the ones detached at 34 days. In B, the IAA was also measured in attached cotyledons of intact seedlings of *Hymenaea courbaril* grown in light and in intact seedlings treated with *N*-1-naphthylphthalamic acid at 200 μ M (NPA 200 μ M). In the last technique, the IAA was also evaluated in shoot excised seedlings at 34 days (excised), in shoot excised seedlings with light protected cotyledons (Shoot excised LPC) and in seedlings with the shoot apex excised (Top shoot excised). Bars represent standard deviation of the mean of three composed replicates.

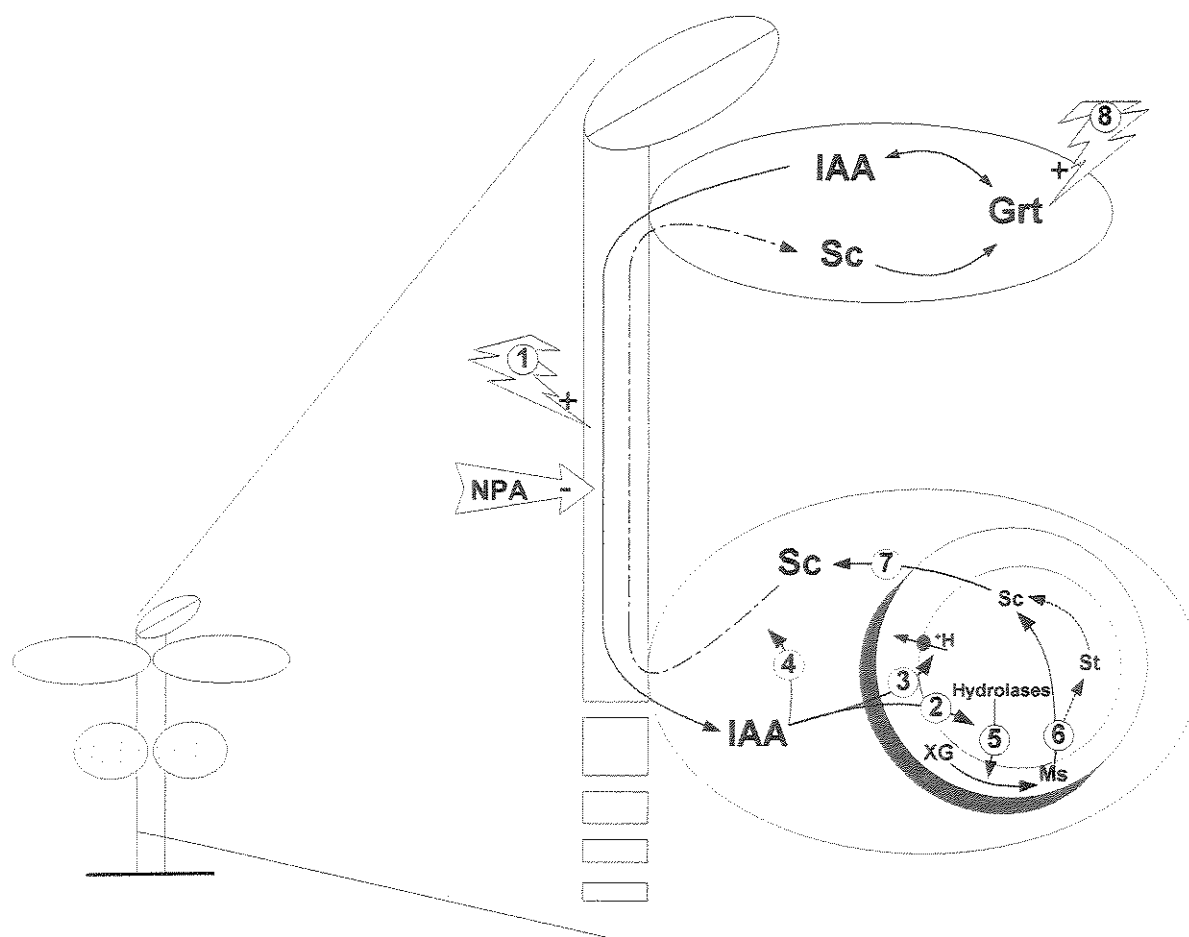


Fig. 6. Schematic representation of synchronism between xyloglucan mobilisation and shoot development in seedlings of *Hymenaea courbaril*. Auxin (IAA) is produced in expanding leaves and transported to cotyledons by polar transport, which can be stimulated by red light (1) and inhibited by *N*-1-naphthylphthalamic acid (NPA). In the cotyledons the IAA may act on the modulation of expression of the gene related to cell wall hydrolases (2); on H^+ -pump activity reducing the apoplastic pH (3) or on the establishment of vascular system (4). Following the xyloglucan (Xg) degradation (5) in storage cell walls of cotyledons by the concerted action of the hydrolases, monosaccharides (Ms) are transported to the cytoplasm, where they are metabolised (6) to sucrose (Sc) and starch (St). The sucrose produced is driven mainly to growing shoot (7). In the shoot, light stimulates (8) growth (Grt), using sucrose as a carbon backbone, which results in IAA availability to the cotyledons and consequently stimulate the xyloglucan mobilisation.

Table 1 - Contents of glucose, fructose and sucrose ($\mu\text{g} \cdot \text{mg}^{-1}$ cot) detected by HPAEC in alcohol extracts of detached and attached cotyledons of seedlings of *Hymenaea courbaril*. The attached cotyledons were from seedlings grown under control conditions (control), in the darkness or with shoot excised above the cotyledons insertions (excised) at 34 days. The detached cotyledons were isolated at 19, 34 and 41 days after beginning of seed imbibition and maintained in water or 2,4-D (10^{-6}M). All data represent the analyses of the same three samples of cotyledons used to starch determination.

Days	Treatments																				
	Control seedling			Seedling in darkness			Shoot excised 34d			Detached 19d water			Detached 34d water			Detached 41d water			Detached 41d 2,4-D		
	GLC	FRU	SUC	GLC	FRU	SUC	GLC	FRU	SUC	GLC	FRU	SUC	GLC	FRU	SUC	GLC	FRU	SUC	GLC	FRU	SUC
7	0.9	1.3	5.3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	0.5	1.7	5.7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
26	0.4	1.8	0.6	0.3	1.4	0.9	-	-	-	0.4	1.7	8.2	-	-	-	-	-	-	-	-	-
34	1.9	5.8	0.6	0.4	3.3	3.1	-	-	-	0.3	1.4	7.5	-	-	-	-	-	-	-	-	-
41	5.2	10.4	3.7	2.0	6.6	3.0	3.1	5.5	4.4	0.2	0.8	4.2	1.1	5.8	24.7	-	-	-	-	-	-
48	22.1	23.1	6.0	1.9	4.9	4.6	4.4	8.6	1.4	0.1	0.3	0.6	1.5	7.9	22.2	4.5	20.9	41.0	18.4	32.9	33.8
54	16.3	13.8	5.3	2.5	7.2	7.7	2.0	7.2	2.3	-	-	-	0.2	0.6	0.1	8.5	31.8	3.7	0.89	5.73	0.94
61	-	-	-	-	-	-	4.6	9.0	4.4	-	-	-	-	-	-	-	-	-	-	-	-
68	-	-	-	-	-	-	6.0	12.2	4.6	-	-	-	-	-	-	-	-	-	-	-	-

Traces represent not analysed points, due to treatments procedures or sample availability.

7. DISCUSSÃO GERAL

A emergência e o estabelecimento são estágios críticos no ciclo de vida de uma planta (Silvertown *et al.*, 1993). Atributos de plântulas, tais como, tamanho, função e posição dos cotilédones e a quantidade de reserva, podem ser cruciais na capacidade de estabelecimento em diferentes ambientes. Este estudo de atributos constitui a descrição morfológica de plântula ou tipo de plântulas, principalmente no âmbito ecofisiológico. Recentemente, um grande interesse tem sido direcionado para o entendimento evolutivo, funcional e ecológico destes atributos para a história de vida de uma planta (Kitajima, 1996; Garwood, 1996). Kitajima (1996), revisando a proposta de Garwood (1996), quanto ao tipo de plântulas, estabeleceu 5 categorias, baseadas na forma (papiráceo, coreáceo e globóide), na posição (epígea ou hipógea) e na permanência da casca (criptocotiledonar ou fanerocotiledonar), as quais estão relacionadas com a função dos cotilédones (fotossíntese ou reserva) e com a adaptação à disponibilidade de luz (Fig. 5). Na função reserva, os cotilédones são espessos (devido a grande quantidade de reservas), não expandem como folhas e caem em um tempo relativamente curto, em geral dentro de dois meses (Kitajima, 1992). Contudo, quando os cotilédones apresentam a função fotossintética eles apresentam pouca espessura, expandindo-se e diferenciando-se em uma folha durante o estabelecimento e permanecendo presos à plântula por um longo período (Kitajima, 1992, 1996).

De acordo com esta classificação, as plântulas de *Hymenaea courbaril* podem ser consideradas, em relação aos cotilédones, como fanerocotiledonar, epígea e globóide (Compare Fig. 1, revisão bibliográfica, com a Fig. 5, a seguir). Recentes estudos têm demonstrado que este tipo de plântula se encontra, na maioria dos casos, entre espécies tolerantes à sombra ou, segundo a classificação de Swaine & Whitmore (1988), entre as espécies clímax (Kitajima, 1992, 1994, 1996; Garwood, 1996, Ibarra-Manríquez, 2001). Isto, reforça a classificação da espécie *Hymenaea*

courbaril como sendo uma espécie clímax, como tem sido citada na literatura (Paulilo e Felipe, 1998; Souza & Válio, 1999).

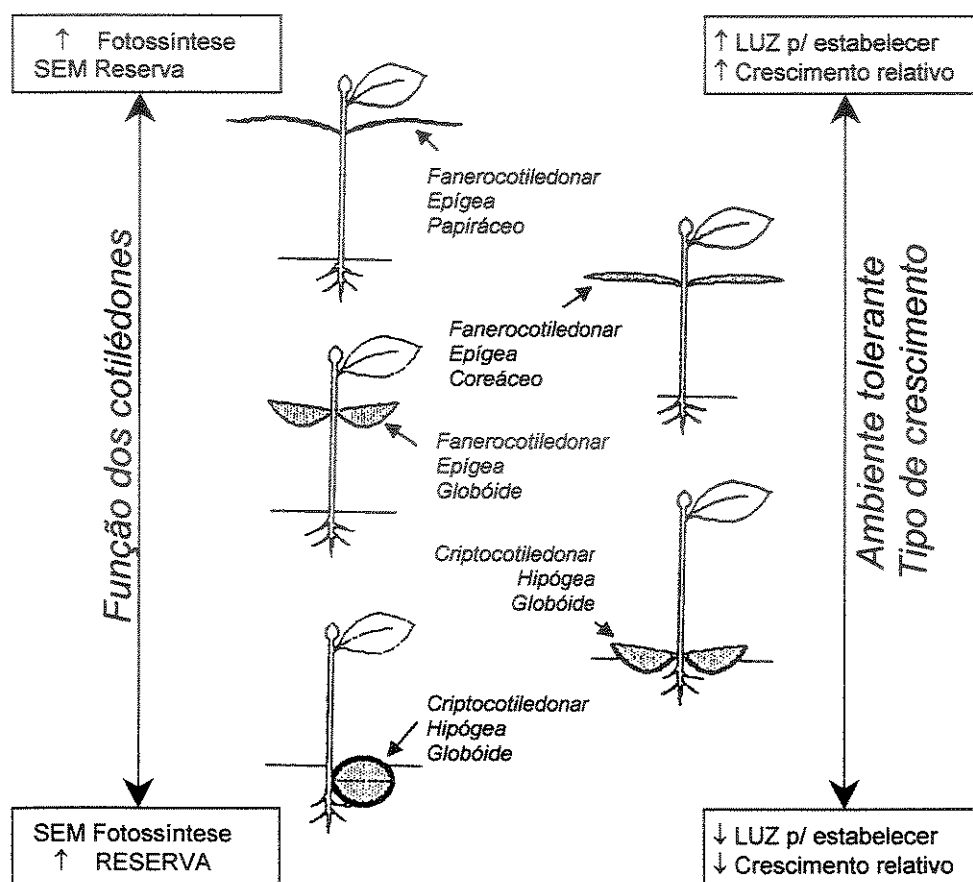


Figura 5 – Categorias de plântulas baseadas no tipo de cotilédono (papiráceo, coreáceo e globóide), persistência da casca (fanerocotiledonar ou criptocotiledonar) e a posição (epígea ou hipógea). Considerando a provável combinação destes parâmetros foram estabelecidos cinco tipos de plântulas: criptocotiledonar, hipógea e reserva (CHR); criptocotiledonar, epígea e reserva (CER); fanerocotiledonar, epígea e fotossintetizante (PEF); fanerocotiledonar, epígea e reserva (PER); fanerocotiledonar, hipógea e reserva (PHR). Adaptado de Kitajima (1996), Garwood (1996) e Ibarra-Manríquez *et al.* (2001).

Apesar da classificação funcional de plântulas apresentar fortes tendências na distribuição sucessional de espécies, nenhum estudo leva em consideração o tipo de reserva e o modo como esta reserva é mobilizada durante o estabelecimento. No caso de *Hymenaea courbaril*, o tipo de reserva cotiledonar pode, de algum modo, ter

contribuído para que esta espécie atingisse uma ampla distribuição geográfica, nas regiões tropicais do Continente Americano (Lee & Langenheim, 1975), sobrevivendo com sucesso aos diferentes tipos de estresses durante a germinação e estabelecimento das plântulas. De acordo com Lee & Langenheim (1975), as seis variedades desta espécie (*courbaril*, *altissima*, *longifolia*, *villosa*, *stilbocarpa* e *subsessilis*) podem ser encontradas nos mais variados ecossistemas (secos ou úmidos, sombreados ou abertos), porém predominam em regiões de florestas tropicais. *Hymenaea courbaril* também tem sido considerada e demonstrada como sendo uma espécie tolerante à seca (Lee & Langenheim, 1975; Gerhardt, 1993).

Desde a descoberta de que sementes de *Hymenaea courbaril* apresentam um “amilóide” nas paredes celulares (Schleiden, 1938 e Vogel & Schleiden, 1939 apud Kooiman, 1960), foi suposto que este poderia ser uma reserva de carbono para o estabelecimento inicial desta espécie. Estas afirmações foram feitas com base nos trabalhos de Heinricher (1888) e de Reiss (1889) para *Impatiens balsamina*, *Tropaeolum majus* e *Cyclamen europaeum*. Estudos recentes têm demonstrado claramente que este “amilóide”, hoje identificado como xiloglucano, corresponde à principal reserva de carbono localizada nas paredes celulares dos cotilédones de *Hymenaea courbaril* (Buckeridge & Dietrich, 1990; Tiné *et al.*, 2000a; Buckeridge *et al.*, 2000). Com base em nossos resultados (capítulos 1, 2 e 3), podemos inferir que este polissacarídeo de parede pode estar contribuindo para outras funções, além de reserva, as quais podem ter proporcionado condições para que esta espécie se adaptasse às condições ambientais predominantes nas florestas tropicais.

Considerando o tamanho das sementes (aprox. 5g) a embebição deve ser controlada para que os processos de mobilização sejam sincronizados com o crescimento do eixo embrionário. É neste ponto que as reservas de xiloglucano começam a contribuir, pois, como demonstrado em nosso primeiro trabalho (Capítulo 1), as propriedades hidrodinâmicas deste polissacarídeo controlam o avanço da água. Este “tamponamento” de água não permite que curtos períodos de disponibilidade de água ativem a germinação, assim como também dificulta a perda da água que já está embebida na matriz da semente (proteção ao dessecamento). Sendo assim, o

xiloglucano desempenha uma função na embebição de água, antes mesmo da função reserva, a qual pode proporcionar garantias ao estabelecimento da planta de *H. courbaril* desde os primeiros contatos da semente com o solo.

Durante as etapas de germinação e emergência, pode-se observar que o xiloglucano não foi importante como fonte de reserva (Capítulo 2 e 3), sendo o crescimento suportado principalmente por outras reservas como oligossacarídeos da série rafínosica e proteína (Tin  , 1997; Buckeridge & Dietrich, 1996). Os nossos resultados demonstraram que a reserva de xiloglucano torna-se importante somente a partir do in  cio do desenvolvimento dos e  filos, direcionando os produtos de sua degrada  o para a parte a  rea at   o t  rmino da expans  o do primeiro met  filo (Cap  tulo 2). Isto ficou evidente quando destacamos os cotil  dones no in  cio da mobiliza  o desta reserva, limitando a expans  o dos e  filos e a emiss  o do primeiro met  filo, principalmente sob condi  o  es limitantes de luz. No entanto, pl  ntulas sem cotil  dones sob ilumina  o continuam o seu crescimento e desenvolvimento, reduzindo a essencialidade da reserva de xiloglucano para o crescimento inicial. Contudo, quando se observa as condi  o  es de luz em que as pl  ntulas de *H. courbaril* se estabelecem em seu ambiente natural (1-2% da radia  o solar), fica evidente a import  ncia da reserva de xiloglucano. Nestas condi  o  es, os nossos resultados demonstraram que o xiloglucano    essencial para o estabelecimento de uma   rea foliar que seja capaz de suportar o crescimento sob o dossel da mata (Cap  tulo 2). Deste modo, a reserva de xiloglucano se apresenta como um "capacitor"¹ para o estabelecimento de pl  ntulas de *Hymenaea courbaril* sob o dossel de florestas tropicais.

Uma vez que esta reserva foi relacionada com o desenvolvimento da parte a  rea e principalmente com a expans  o das primeiras folhas, nossos resultados

¹ Este termo    utilizado em Eletr  nica, na denomina  o de um componente que apresenta a fun  o de armazenar energia sob a forma de um campo eletrost  tico, em um ponto do circuito el  trico. A analogia feita no texto considera o xiloglucano como um armazenador de energia que, quando disponibilizada, possibilita   s pl  ntulas de *Hymenaea courbaril* se estabelecerem em ambientes sombreados.

sugerem um mecanismo de inter-controle entre o desenvolvimento destes tecidos e o processo de mobilização de xiloglucano (capítulo 3). Assim, pode-se sugerir que a auxina exerce controle sobre a mobilização deste polissacarídeo de reserva, apresentando uma grande analogia aos processos que ocorrem durante a expansão de paredes primárias (Hayashi, 1989). Com base neste mecanismo, quando está se desenvolvendo a parte aérea aumenta a disponibilidade da auxina, uma vez que os tecidos em crescimento da parte aérea são a principal fonte desse hormônio (Bartel, 1997). Como consequência disto, ocorre a degradação de xiloglucano e a disponibilidade de carbono dos cotilédones para o crescimento da parte aérea.

De acordo com alguns estudos realizados com o transporte polar de auxina (Shinkle *et al.*, 1998; Jensen *et al.*, 1998), pode-se sugerir que os níveis deste hormônio nos cotilédones de *H. courbaril* podem estar sendo mediados pela intensidade e/ou qualidade de luz disponível no ambiente. Ou seja, quando as plântulas estão se desenvolvendo sob condições de baixa radiação (<vermelho) o transporte de auxina para os cotilédones e a mobilização das reservas de xiloglucano serão reduzidos, o que permitirá uma manutenção das plântulas sob o dossel por mais tempo. Em contrapartida, quanto maior a disponibilidade de radiação, como ocorre com a abertura natural de clareiras, maior é o estímulo de expansão da parte aérea (Chory, 1993) e, conseqüentemente, maior será a degradação de xiloglucano devido a maior produção e transporte de auxina (Shinkle *et al.*, 1998; Jensen *et al.*, 1998) para os cotilédones. Todo este inter-controle pode ser evidenciado nos resultados do capítulo 2, onde as plântulas crescidas na mata (menor radiação e baixa relação vermelho/vermelho extremo, V/Ve) apresentaram menor taxa de mobilização e crescimento, em relação as demais plântulas crescidas na sala de crescimento e na casa de vegetação (sob maior radiação e alta relação V/Ve).

Embora este estudo sugere um favorecimento na taxa de crescimento e na taxa de mobilização de xiloglucano pela disponibilidade de radiação, as plantas de *H. courbaril* não apresentam o hábito de se estabelecerem em ambientes abertos. Possivelmente, isto não ocorra devido as plântulas desta espécie apresentarem uma baixa taxa de crescimento relativo, em relação às espécies pioneiras, e também por

apresentarem uma baixa saturação luminosa da fotossíntese ($200 \mu\text{mol.m}^{-2}.\text{s}^{-1}$), o que resulta em fotoinibição sob alta intensidade de luz. Todos estes parâmetros caracterizam a espécie *H. courbaril* como “clímax típica de dossel”, de acordo com a classificação citada por Gandolfi *et al.* (1995), sendo que as plântulas crescem e se desenvolvem no sub-bosque, utilizando as reservas de xiloglucano, e quando atingem a idade adulta irão compor a condição emergente no dossel florestal.

Todos os resultados deste estudo apontam para a idéia de que a reserva de xiloglucano foi selecionada evolutivamente, em detrimento de outras (p.ex. amido), por apresentar-se como uma matriz multifuncional (controle de água e reserva) e metabolicamente interrelacionada às condições de luz do ambiente.

De acordo com Lambers *et al.* (1998), a capacidade de um indivíduo ocupar um certo nicho ecológico depende do potencial que cada um apresenta em ultrapassar os “filtros” históricos, fisiológicos e bióticos (Fig. 6). O filtro histórico se refere às barreiras de dispersão das espécies para um determinado local e, uma vez no local, os filtros fisiológicos e bióticos irão determinar quais espécies irão compor a flora. Considerando que a África é o provável centro de origem do gênero *Hymenaea*, e que a maioria das 14 espécies apresenta uma ampla distribuição Neotropical, estendendo-se desde o centro do México, Antilhas, até o norte da Argentina (Lee & Langenheim, 1975), pode-se inferir que as diferenças de ocupação nestes locais estão mais relacionadas aos filtros fisiológicos e bióticos. Com relação ao filtro fisiológico, o xiloglucano apresenta-se como “capacitor” no estabelecimento de plântulas da espécie *H. courbaril*, em ambientes de floresta, como descrito anteriormente. Além disto, em função do metabolismo análogo ao de paredes primárias, envolvendo o transporte polar de auxinas (Capítulo 3) e a inter-relação com a disponibilidade de luz (Capítulo 2), a degradação desta reserva proporciona um sincronismo muito maior entre a degradação e a utilização das reservas de carbono. Este sincronismo possibilita uma maior eficiência no uso das reservas e, consequentemente, aumenta a probabilidade de sobrevivência das plântulas sob o dossel.

Outro ajuste fisiológico importante que as plântulas de *H. courbaril* apresentam para se desenvolverem em florestas tropicais é a capacidade fotossintética das primeiras folhas. Os resultados demonstram que as primeiras folhas desta espécie apresentam um ponto de compensação de $11,8 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ (Capítulo 2), o que é equivalente aos níveis de radiação encontrados sob o dossel de matas

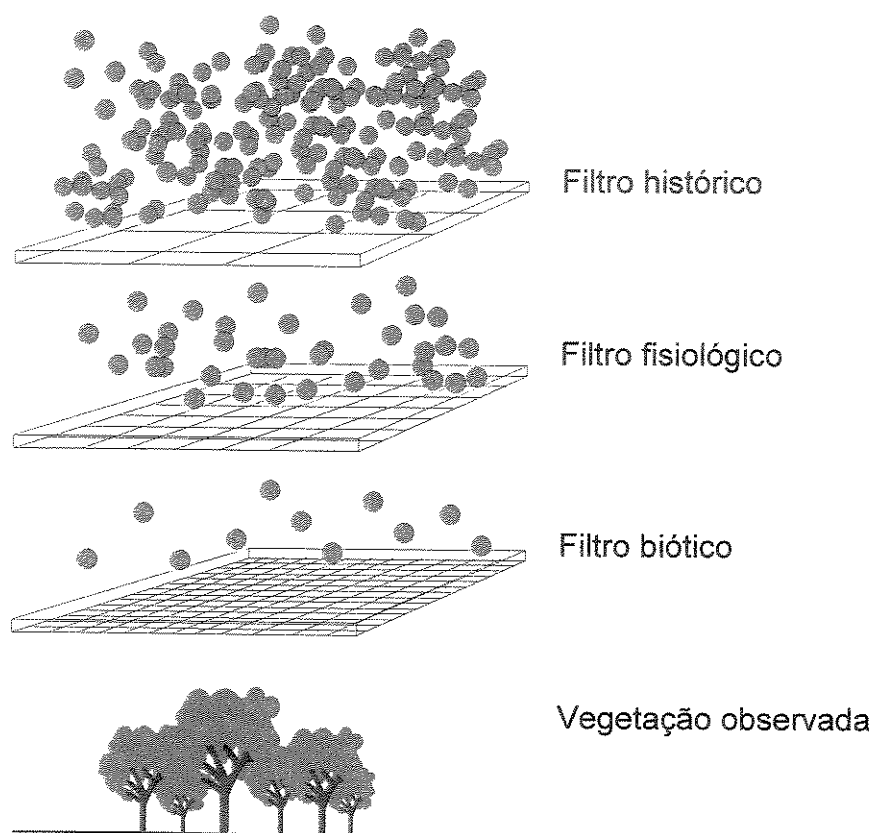


Figura 6 – Filtros histórico, fisiológico e biótico que determinam a composição de espécies vegetais em um determinado local. O filtro histórico se refere à distribuição, considerando as barreiras que uma determinada espécie enfrenta para atingir um determinado local. Os filtros fisiológico e biótico estão relacionados com a capacidade de aclimação e adaptação que uma espécie apresenta para enfrentar os fatores abióticos e bióticos, respectivamente, de um determinado local. Adaptado de Lambers *et al.* (1998).

tropicais (Canhan, 1989). Sob este mesmo enfoque, os resultados de plântulas crescidas com e sem cotilédones demonstram que as reservas de xiloglucano atuam diretamente sobre o potencial fotossintético através do incremento da proporção de área foliar durante os primeiros estágios de estabelecimento (Capítulo 2).

A reserva de xiloglucano, apesar de ser um parâmetro fisiológico, atua também, de modo indireto, na capacidade de superação ao filtro biótico. Isto ficou claro em plantas crescendo sem a disponibilidade de xiloglucano (sem cotilédones, Capítulo 2). Nestas plântulas, devido à antecipação do estabelecimento fotossintético, as folhas não apresentaram a coloração púrpura, que é característica dos primeiros estádios de expansão foliar desta espécie (Fig. 7). De acordo com Lambers *et al.* (1998), esta coloração está relacionada com a maior proporção de antocianina em relação a clorofila, com as espécies tolerantes à sombra e com o controle de herbivoria durante a expansão foliar. Neste caso, a reserva de xiloglucano também está atuando como um “capacitor”, pois na sua ausência a plântula direciona os seus recursos disponíveis para o estabelecimento fotossintético em detrimento à expansão e à proteção.

Estas respostas foliares em relação à presença ou ausência de xiloglucano aparentam ser um mecanismo mediado pela disponibilidade de carboidratos, uma vez que a presença dos cotilédones deve elevar a concentração de açúcar nas folhas em expansão. De acordo com Pego *et al.* (2000), esta concentração de açúcares pode reprimir a expressão de genes de componentes fotossintéticos. A sua redução conduz a uma ativação da expressão de genes e ao incremento da capacidade fotossintética, como pode estar ocorrendo nas plantas sem as reservas de xiloglucano. Todo este processo foi recentemente descoberto em plantas herbáceas (p.ex. *Arabidopsis* e tomate), sendo relacionado tanto com a importação de hexoses como de sacarose, e tem sido denominado de “sugar-sensing” (Smeekens, 2000).

Uma outra característica que auxilia a superação do filtro biótico é que as reservas de xiloglucano são relativamente menos predadas, em relação as reservas de amido. De acordo com a literatura, mamíferos (p.ex. pacas e macacos) atacam os

frutos para se alimentarem do amido presente na polpa (Lewinsohn, 1980; Galetti & Pedroni, 1994). Ao mesmo tempo em que se alimentam, estes animais escarificam as sementes, o que possibilita a embebição e a germinação. No entanto, após o ataque

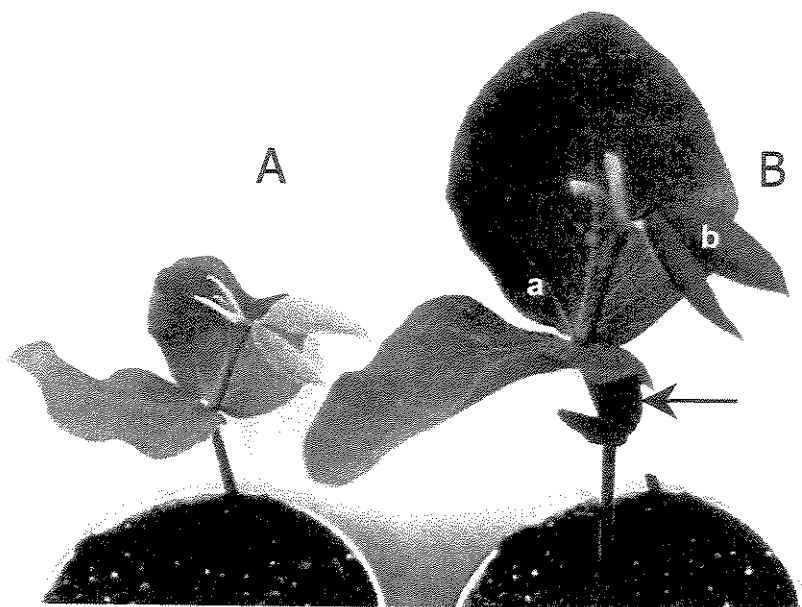


Figura 7 – Comparação fenológica de plântulas crescidas, em casa de vegetação (descrição Capítulo 2), sem (A) e com (B) os cotilédones durante o período de mobilização das reservas de xiloglucano. Plântulas com 32 dias de idade, em referência ao início da embebição das sementes. A retirada dos cotilédones (seta) foi realizada quando os eófilos (a) tornaram-se visíveis (22 dias após a embebição). (b) primeiro metáfilo.

destes animais as sementes ficam com muitos resíduos de amido da polpa, o que favorece o ataque de fungos e a perda da viabilidade das sementes. Dentro deste enfoque, Oliveira *et al.* (1995) descobriram que formigas *Mycocepurus goeldii* apresentam uma alta interação com *H. courbaril*, pois elas retiram os resíduos de amido das sementes evitando o ataque de fungos e elevando o percentual de germinação. Esta interação, aparentemente, só foi viável em função das reservas de carbono das sementes não serem amido mas sim o xiloglucano. Além disso, Lewinsohn (1980) cita que as sementes de *H. courbaril* apresentam um elenco restrito de predadores, quando comparado a outras espécies Leguminosas, sendo estes altamente específicos e estratégicos para conseguirem romper as barreiras de

proteção impostas por esta espécie. Dentre as principais barreiras, citadas por este autor, destacam-se a dureza da casca dos frutos e das sementes após a maturação. Entretanto, apesar de não ser abordado neste estudo, a reserva de xiloglucano também pode estar contribuindo nas restrições ao ataque de pragas, principalmente por ser uma molécula estruturalmente complexa para ser digerida, necessitando o ataque de no mínimo uma enzima endo-hidrolase e três exo-hidrolases. Isto pode, em parte, explicar o ataque preferencial à polpa dos frutos (rica em amido) em relação às sementes (ricas em xiloglucano), pelos principais predadores de sementes de *Hymenaea* (larvas de coleópteros cucurliónídeos e escolitídeos, e lepdópteros ficitídeos), como citado por Lewinsohn (1980). Este autor também destaca que sementes de *Copaifera lagsdorffii*, que também são ricas em xiloglucano, apresentam o mesmo e restrito elenco de predadores, o que reforça a hipótese de que as reservas de xiloglucano podem estar contribuindo contra a predação. De um modo geral, podemos sugerir que o xiloglucano de reserva possa ser considerado um "elemento chave", dentro dos processos evolutivos, para que plântulas de *H. courbaril* tenham superado os filtros fisiológicos e bióticos impostos pelas florestas tropicais.

Uma vez que esta reserva está presente na parede celular dos cotilédones e que todo o processo de degradação se assemelha ao metabolismo que está ocorrendo com xiloglucano de paredes primárias, fica evidente que ela possa ter evoluído de paredes primárias através do mecanismo de transferência de funções, como proposto por Buckeridge & Reid (1996) e Buckeridge *et al.* (2000). Neste mecanismo, o xiloglucano teria uma função primária como reserva (Capítulo 2) e, pelo menos, uma secundária, tal como controle de embebição (Capítulo 1) e controle de predação (discutido anteriormente). De acordo com Buckeridge *et al.* (2000), o mecanismo pelo qual as reservas de xiloglucano se formaram, ao longo da evolução, demandou algumas transformações chave no metabolismo e nas propriedades deste polissacarídeo. Inicialmente, teve que haver uma mudança genética para promover uma separação temporal da expressão das sintases (biossíntese, na maturação dos frutos) em relação às hidrolases (degradação, após a germinação das sementes),

pois ambas ocorrem simultaneamente na expansão de paredes primárias. Esta alteração genética ocorreu apenas na matriz de hemicelulose (xiloglucano) e com algumas alterações na estrutura fina. Entre estas alterações estão a eliminação da fucose, pois os xiloglucanos de reserva quase não apresentam este monossacarídeo (Tin  , 2002). A fucose tem sido considerada importante na intera  o entre pol  meros de xiloglucano ou entre xiloglucano e celulose, nas paredes prim  rias (Levy *et al.*, 1991; Lima & Buckeridge, 2001), o que n  o favoreceria a mobiliza  o de xiloglucano em paredes de reserva. No caso de *Hymenaea courbaril*, estudos recentes sobre a estrutura fina do xiloglucano de reserva t  m demonstrado tamb  m certas peculiaridades. Em 1997, Buckeridge *et al.*, descobriram que em xiloglucanos de reserva de *H. courbaril* era poss  vel obter uma s  rie nova de oligossacar  deos (XXXXG) em rela  o ao que j   se conhecia de sementes de *Tamarindus indica* (XXXG), quando hidrolizado com celulase. Deste estudo ainda ficaram alguns oligos desconhecidos (XXXXG + 1 galactose sem posi  o definida), que foram recentemente elucidados por Tin   (2002). Estes oligossacar  deos novos de *H. courbaril* provavelmente conferem maior intera  o com celulose, quando comparado com xiloglucano de reserva de outras esp  cies (Lima & Buckeridge, 2001). Neste caso, em fun  o da reduzida propor  o de celulose nestas paredes de reserva (possivelmente apenas na parede prim  ria, Buckeridge *et al.*, 2000; Tin   *et al.*, 2001), esta capacidade de intera  o deve resultar em uma elevada capacidade de "empacotamento", que como conseq   ncia aumenta a capacidade de estoque de reservas por unidade de volume. Al  m disto, a a  o da enzima XET de cotil  dones de *Hymenaea courbaril* tem sido demonstrada como sendo altamente espec  fica    estrutura fina do xiloglucano de reserva desta esp  cie, n  o atacando xiloglucanos de reserva extra  dos de outras esp  cies (p.ex. *Tamarindus indica*) (Minhoto, 2002).

De modo geral, o xiloglucano de reserva presente nos cotil  dones de *Hymenaea courbaril* apresentou v  rias etapas evolutivas, em rela  o a outras esp  cies que armazenam este polissacar  deo, as quais culminaram em uma reserva altamente especializada e multifuncional, do ponto de vista bioqu  mico, fisiol  gico e ecol  gico, como demonstrado ao longo deste trabalho.

8. BIBLIOGRAFIA

- Alcântara, P.H.N. 2000. Isolamento e caracterização das enzimas xiloglucano endotransglicosilase e β -galactosidase do catabolismo do xiloglucano de reserva dos cotilédones de *Hymenaea courbaril* L. (Leguminosae-Caesalpinioideae). Tese de doutorado. UNIFESP, São Paulo, 98p.
- Alcântara, P.H.N.; Dietrich, S.M.C. & Buckeridge, M.S. 1999. Xyloglucan mobilisation and purification of a (XLLG/XLXG) specific β -galactosidase from cotyledons of *Copaifera langsdorffii*. *Plant Physiology and Biochemistry* 37:653-663.
- Bartel, B. 1997. Auxin biosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* 48:51-66.
- Behe, M. 1997. *Caixa Preta de Darwin : O desafio da Bioquímica à teoria da evolução*. Traduzido do original "Darwin's Black Box: The biochemical challenger to evolution" por Ruy Jungmann. Ed. Jorge Zahar. 300 p.
- Bewley, J.D. & Black, M. 1994. *Seeds - Physiology of Development and Germination*. Second edition. Plenum press, New York.
- Buckeridge, M.S. 1988. Galactomananos de sementes de leguminosas: ocorrência e metabolismo. Dissertação de mestrado, Escola Paulista de Medicina, São Paulo, 144 p.
- Buckeridge, M.S. & Dietrich, S.M.C. 1990. Galactomannan from Brazilian legume seeds. *Revista Brasileira de Botânica* 13: 109-112.
- Buckeridge, M.S. & Dietrich, S.M.C. 1996. Mobilisation of the raffinose family oligosaccharides and galactomannan in germinating seeds of *Sesbania marginata* Benth. (Leguminosae-Faboideae). *Plant Science* 117:33-43.
- Buckeridge, M.S. & Reid, J.S.G. 1994. Purification and properties of a novel β -galactosidase or exo- β -(1,4)-galactanase from the cotyledons of germinated *Lupinus angustifolius* L. seeds. *Planta* 192:502-511.

- Buckeridge, M.S. & Reid, J.S.G. 1996. Major cell wall polysaccharides in legume seeds: Structure, catabolism and biological functions. *Ciência e Cultura Journal of Brazilian Association for the Advancement of Science* 48:153-162.
- Buckeridge, M.S.; Crombie, H.J.; Mendes, C.J.M.; Reid, J.S.G.; Gidley, M.J. & Vieira, C.C.J. 1997. A new family of oligosaccharides from the xyloglucan of *Hymenaea courbaril* L. (Leguminosae) cotyledons. *Carbohydrate Research* 303:233-237.
- Buckeridge, M.S.; Panegassi, V.R. & Dietrich, S.M.C. 1995. Storage carbohydrate mobilisation in seeds of *Dimorphandra mollis* Benth. (Leguminosae) following germination. *Revista Brasileira de Botânica* 18:171-175.
- Buckeridge, M.S.; Rocha, D.C.; Reid, J.S.G. & Dietrich, S.M.C. 1992. Xyloglucan structure and post-germinative metabolism in seeds of *Copaifera langsdorffii* from savannah and forest populations. *Physiologia Plantarum* 86:145-151.
- Buckeridge, M.S.; Santos, H.P. & Tiné, M.A.S. 2000a. Mobilisation of storage cell wall polysaccharides in seeds. *Plant Physiology and Biochemistry* 38:141-156.
- Buckeridge, M.S.; Tiné, M.A.S.; Santos, H.P. & Lima, D.U. 2000b. Polissacarídeos de reserva de parede celular em sementes. Estrutura, metabolismo, funções e aspectos ecológicos. *Revista Brasileira de Fisiologia Vegetal* 12(ed. especial) : 137-162.
- Canham, C.D. 1989. Different responses to gaps among shade-tolerant tree species. *Ecology* 70:548-550.
- Carpita, N.C. & Gibeaut, D.M. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of cell wall during growth. *Plant Journal* 3:1-30.
- Chory, J. 1993. Out of darkness: mutants reveal pathways controlling light-regulate development in plants. *Trends in Genetics* 9:167-172.
- Cosgrove, D.J. 1999. Enzymes and other agents that enhance cell wall extensibility. *Annual Review of Plant Physiology and Plant Molecular Biology* 50:391-417.
- Cosgrove, D.J. 2000. Expansive growth of plant cell walls. *Plant Physiology and Biochemistry* 38:109-124.

- Crawshaw, L.A. & Reid, J.S.G. 1984. Changes in cell-wall polysaccharides in relation to seedling development and the mobilisation of reserves in the cotyledons of *Lupinus angustifolius* Cv. Unicrop. *Planta* 160:449-454.
- Crombie, H.J.; Chengappa, S.; Hellyer, A. & Reid, J.S.G. 1998. A xyloglucan oligosaccharide-active, transglycosylating β -D-glucosidase from the cotyledons of nasturtium (*Tropaeolum majus* L.) seedlings—purification, properties and characterization of a cDNA clone. *Plant Journal* 15:27-38.
- Eda, S. & Kato, K. 1978. An arabinoxylglucan isolad from the midrib of the leaves of *Nicotiana tabacum* L. *Agricultural and Biology Chemistry* 42: 351-357.
- Edwards, M.E.; Bowman, J.L.; Dea, I.C.M. & Reid, J.S.G. 1988. A β -D galactosidase from nasturtium (*Tropaeolum majus* L.) cotyledons. *Journal of Biological Chemistry* 263:4333-4337.
- Edwards, M.E.; Dea, I.C.M.; Bulpin, P.V. & Reid, J.S.G. 1985. Xyloglucan (amyloid) mobilisation in the cotyledons of *Tropaeolum majus* L. seeds following germination. *Planta* 163:133-140.
- Edwards, M.E.; Dea, I.C.M.; Bulpin, P.V. & Reid, J.S.G. 1986. Purification and properties of a novel xyloglucan-specific endo- β -(1,4)-D-glucanase from germinating nasturtium seeds (*Tropaeolum majus* L.). *Journal of Biological Chemistry* 261:9489-9494.
- Fanutti, C.; Gidley, M.J. & Reid, J.S.G. 1991. A xyloglucan oligosaccharide specific α -D-xylosidase or exo-oligoxyloglucan- α -xylohydrolase from germinated nasturtium (*Tropaeolum majus* L.) seeds. Purification, properties and its interaction with a xyloglucan-specific endo- β -(1,4)-glucanase and other hydrolases during storage xyloglucan mobilisation. *Planta* 184:137-147.
- Fanutti, C., Gidley M.J. & Reid J.S.G. 1993. Action of a pure xyloglucan endo-transglycosylase (formerly called xyloglucan-specific endo-(1,4)- β -D-glucanase) from the cotyledons of germinated nasturtium seeds. *Plant Journal* 3:691–700.
- Flores E.M. & Benavides, C.E. 1990. Germinación y morfología de la plántula de *Hymenaea courbaril* L. (Caesalpinaceae). *Revista de Biología Tropical* 38:91-8.

- Fry, S.C.; York, W.S.; Albersheim, P.; Darvill, A.; Hayashi, T.; Joseleau, J.P.; Kato, Y.; Lorences, E.P.; MacLachlan, G.A.; Mcneil, M.; Mort, A.J.; Reid, J.S.G.; Seitz, H.U.; Selvendran, R.R.; Voragen, A.G.J. & White, A.R. 1993. An unambiguous nomenclature for xyloglucan-derived oligosaccharides. *Physiologia Plantarum* 89:1-3.
- Galetti, M. & Pedroni, F. 1994. Seasonal diet of capuchin monkeys (*Cebus apella*) in a semideciduous forest in southeast Brazil. *Journal of Tropical Ecology* 10:27-39.
- Garwood, N.C. 1996. Functional morphology of tropical tree seedlings. In Swaine, M.D.(ed.), *Ecology of Tropical Forest Tree Seedlings*, pp.59-129. Man and the Biosphere series, vol. 17. UNESCO/Parthenon, Paris, France.
- Gerhardt, K. 1993. Tree seedling development in tropical dry abandoned pasture and secondary forest in Costa Rica. *Journal of Vegetation Science* 4:95-102.
- Gibbs, P.E.; Oliveira, P.E. & Bianchi, M.B. 1999. Postzygotic control of selfing in *Hymenaea stigonocarpa* (Leguminosae-Caesalpinioideae), a bat-pollinated tree of Brazilian cerrados. *Int. J. Plant Sci.* 160: 72-78.
- Gidley, M.J.; Lillford P.J.; Rowlands, D.W.; Lang, P.; Dentini, M.; Crescenzi, V.; Edwards, M.; Fanutti C. & Reid, J.S.G. 1991. Structure and solution properties of tamarind-seed polysaccharide. *Carbohydrate Research* 214: 299-314.
- Hayashi, T. 1989. Xyloglucans in the primary cell wall. *Annual Review of Plant Physiology and Plant Molecular Biology* 40:139-168.
- Hayashi, T.; Marsden, M.P.F. & Delmer, D.P. 1987. Pea xyloglucan and cellulose. V. Xyloglucan-cellulose interactions *in vitro* and *in vivo*. *Plant Physiology* 83: 384-389.
- Hayashi, T.; Wong, Y.S. & MacLachlan, G.A. 1984. Pea xyloglucan and cellulose. II. Hydrolysis by pea endo-1,4- β -glucanases. *Plant Physiology* 75:605-610.
- Heinricher E. 1888. Zur Biologie der gattung *Impatiens*. *Flora Jena* 71.
- Hensel, A.; Brummell, D.A.; Hanna, R.; MacLachlan, G. 1991. Auxin-dependent breakdown of xyloglucan in cotyledons of germinating nasturtion seeds. *Planta* 183: 321-326.

- Ibarra-Manríquez, G., Ramos, M. M. & Oyama, K. 2001. Seedling functional types in a lowland rain forest in Mexico. *American Journal of Botany* 88:1801-1812.
- Jensen, P.J., Hangarter, R.P. & Estelle, M. 1998. Auxin transport is required for hypocotyl elongation in light-growth but not dark-grown *Arabidopsis*. *Plant Physiology* 116: 455-462.
- Kitajima, K. 1992. Relationship between photosynthesis and thickness of cotyledons for tropical tree species. *Functional Ecology* 6: 582-589.
- Kitajima, K. 1994. Relative importance of photosynthetic traits and allocation patterns as correlates of seedling shade tolerance of 13 tropical trees. *Oecologia* 98: 419-428.
- Kitajima, K. 1996. Cotyledon functional morphology, patterns of seed reserve utilization and regeneration niches of tropical tree seedlings. In Swaine, M.D.(ed.), *Ecology of Tropical Forest Tree Seedlings*, pp. 193-210. Man and the Biosphere series, vol. 17. UNESCO/Parthenon, Paris, France.
- Kooiman, P. 1960. On the occurrence of amyloids in plant seeds. *Acta Botanica Neerlandica* 9:208-219.
- Kooiman, P. 1961. The constitution of *Tamarindus*-amyloid. *Recueil des Travaux Chimiques des Pays-Bas* 80:849-865.
- Lambers, H., Chappin III, F. S. & Pons T. L. 1998. Plant Physiological Ecology. Cap. 1. Assumptions and Approaches, pgs 1-9; Cap. 8. Life cycles: environmental influences and adaptations, pgs 352-375. Springer-Verlag, New York.
- Lee, Y. & Langenheim, J. H. 1975. Systematics of the genus *Hymenaea* L. (Leguminosae, Caesalpinoideae, Detarieae). University of California Press, Berkeley, Los Angeles.
- Levy, S.; York, W. S.; Struik-Prill, R.; Meyer, B. & Staehelin, L.A. 1991. Simulations of the static and dynamic molecular conformations of xyloglucan. The role of the fucosylated sidechain in surface-specific sidechain folding. *The Plant Journal* 1:195-215.

- Lewinsohn, T.M. 1980. Predação de sementes em *Hymenaea* (Leguminosae: Caesalpinoideae): Aspectos ecológicos e evolutivos. Universidade Estadual de Campinas, Dissertação de mestrado, Campinas, 193 p.
- Lima, D. U. & Buckeridge, M. S. 2001. Interaction between cellulose and storage xyloglucans: the influence of the degree of galactosylation. *Carbohydrate Polymers* 46:157-163.
- Lima, N.N.; Reicher, F.; Correa J.B.C.; Ganter, J.L.M.S. & Sierakowski, M.R. 1993. Partial structure of a xyloglucan from the seeds of *Hymenaea courbaril* var. *stilbocarpa* (jatobá). *Ciência e Cultura Journal of Brazilian Association for the Advancement of Science* 45:22-26.
- Maillard, P.; Deléens E.; Daudet, F.A.; Lacoite, A. & Frossard, J.S. 1994. Carbon economy in walnut seedlings during the acquisition of autotrophy studied by long-term labelling with $^{13}\text{CO}_2$. *Physiologia Plantarum* 91:359-268.
- Mayer, A.M. & Poljakoff-Mayber, A. 1975. *The germination of seeds*. Exeter, Pergamon Press.
- McCann, M.C. & Roberts, K. 1991. Architecture of the primary cell wall. In: Lloyd, C.W. (Ed.) *The cytoskeletal basis of plant growth and form*. London, Academic Press, 109-129.
- Meier, H. & Reid, J.S.G. 1982. Reserve polysaccharide other than starch in higher plants. In: Loewus FA and Tanner W, eds. *Plant carbohydrates*. New York: Springer, 418-71.
- Minhoto, M.J. 2002. O efeito da temperatura na ação da xiloglucano endo-beta-transglicosilase sobre xiloglucanos de reserva. Universidade Estadual de Campinas, Dissertação de mestrado, Campinas, 63 p.
- Oliveira, P. S.; Galetti, M.; Pedroni, F. & Morellato, L.P.C. 1995. Seed cleaning by *Mycocepurus goeldii* ants (Attini) facilitates germination in *Hymenaea courbaril* (Caesalpiniaceae). *Biotropica* 27:518-522.
- Paulilo, M.T.S. & Felipe, G.M. 1998. Growth of the shrub-tree flora of the Brazilian cerrados: a review. *Tropical Ecology* 39:165-174.

- Pego, J.V., Kortstee, A.J. Huijser, C & Smeekens, S.C.M. 2000. Photosynthesis, sugars and the regulation of gene expression. *Journal of Experimental Botany* 51(special issue): 407-416.
- Reid, J.S.G. 1985. Structure and function in legume-seed polysaccharides. In: Brett, C. (Ed.) *Biochemistry of the plant cell walls*. Cambridge, Cambridge Univ. Press., 259-268.
- Reid, J.S.G. & Bewley, J.D. 1979. A dual rôle for the endosperm and its galactomannan reserves in the germinative physiology of fenugreek (*Trigonella foenum-graecum* L.), an endospermic leguminous seed. *Planta* 147:145-150.
- Reis, D.; Vian, B.; Darzens, D. & Roland, J.C. 1987. Sequential patterns of intramural digestion of galactoxyloglucan in tamarind seedlings. *Planta* 170:60-73.
- Reiss R. 1889. Über die Natur der Reservecellulose und über ihre Auflösungsweise bei der Keimung der Samen. *Landwirtsch Jahrb* 18:711-765.
- Rose, J.K.C. & Bennett, A.B. 1999. Co-operative disassembling of the cellulose-xyloglucan network of plant cell walls: parallels between cell expansion and fruit ripening. *Trends in Plant Science* 4:176-183.
- Shinkle, J.R., Kadakia, R. & Jones, A.M. 1998. Dim-red-light induced increase in polar auxin transport in cucumber seedlings. *Plant Physiology* 116:1505-1513.
- Silvertow, J., Franco, M., Pisanty, I. & Mendonza, A. 1993. Comparative plant demography. Relative importance of life-cycle components to the finite rate of increase in woody and the herbaceous perennials. *Journal of Ecology* 81:465-476.
- Smeekens, S. 2000. Sugar-induced signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* 51:49-81.
- Souza, R.P. & Válio, I.F.M. 1999. Carbon translocation as affected by shade in saplings of shade tolerant and intolerant species. *Biologia Plantarum* 42:631-636.
- Swaine, 1996. (Ed.) *Ecology of Tropical Forest Tree Seedlings*. Foreword, pp. xxi-xxviii. Man and the Biosphere series, vol. 17. UNESCO/Parthenon, Paris, France.
- Swaine, M. D. & Whitmore, T. C. 1988. On the definition of ecological species groups in tropical rain forest. *Vegetatio* 75: 81-86.

- Taguchi, T., Uraguchi, A. & Katsumi, M. 1999. Auxin- and acid-induced changes in the mechanical properties of the cell wall. *Plan Cell Physiology* 40: 743-749.
- Taiz, L. & Zeiger, E. (1998) *Plant Physiology*. 2nd ed. Sinauer Associates, Inc. Massachusetts, USA.
- Terry, M.E.; Jones, R.L. & Bonner, B.A. 1981. Soluble cell wall polysaccharides released from pea stems by centrifugation. 1. Effect of auxin. *Plant Physiology* 68: 531-537.
- Tiné, M.A.S. 1997. Mobilização de xiloglucano de reserva de sementes de *Hymenaea courbaril* L. Universidade Estadual de Campinas, Dissertação de mestrado, Campinas, 85 p.
- Tiné, M.A.S. 2002. O conteúdo informacional da molécula de xiloglucano de cotilédones de *Hymenaea courbaril* reflete suas funções em nível celular. Universidade Estadual de Campinas, Tese de Doutorado, Campinas, 106 p.
- Tiné, M.A.S.; Cortelazzo, A.L. & Buckeridge, M.S. 2000. Xyloglucan mobilisation in cotyledons of developing plantlets of *Hymenaea courbaril* L. (Leguminosae-Caesalpinoideae). *Plant Science* 154:117-126
- White, E.V. & Rao, P.S. 1953. *J. Am. Chem. Soc.* 75 2617, Apud Kooiman, (1960).
- Whitmore, 1996. A review of some aspects of tropical rain forest seedling ecology with suggestions for further enquiry. In Swaine, M.D.(ed.), *Ecology of Tropical Forest Tree Seedlings*, pp. 3-39. Man and the Biosphere series, vol. 17. UNESCO/Parthenon, Paris, France.
- Whitney, S.E.C.; Brigham, J.E.; Darke, A.H.; Reid, J.S.G. & Gidley, M.J. 1998. *In vitro* assembly of cellulose/xyloglucan networks: ultrastructural and molecular aspects. *Carbohydrate Research* 307:299-309.