Xyloglucan mobilisation in cotyledons of developing plantlets of *Hymenaea courbaril* L. (Leguminosae-Caesalpinoideae)

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Abstract

Many seeds contain storage compounds that are used by the embryo/plantlet as a source of nutrients after germination. In seeds of *Hymenaea courbaril*, a leguminous tree, the main reserve consists of a structurally unusual xyloglucan stored in thickened walls of the cotyledon cells. The present work aimed to study *H. courbaril* xyloglucan metabolism during and after germination in order to compare its degrading system with the other known xyloglucan containing seeds. Polysaccharide degradation occurred after germination between 35 and 55 days after planting. The activities of α-xylosidase, β-glucosidase, β-galactosidase and XET rose during the period of xyloglucan disassembling but a low level of endo-β-glucanase activity was detected, suggesting that this XET has high affinity for the oligosaccharides. The pH optimum of β-galactosidase was different from the α-xylosidase, β-glucosidase and XET optima suggesting that the former may be important in the control of the mobilisation process. A tentative model for xyloglucan disassembling in vivo is proposed, where β-galactosidase allows the free oligosaccharides to bypass a transglycosylation cycle and be disassembled by the other exo-enzymes. Some ecophysiological comparisons among *H. courbaril* and other xyloglucan storing seeds are discussed. © 2000 Published by Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Many legume seeds are known to accumulate carbohydrates as storage compounds. Usually, the first reserves to be broken down during germination are the raffinose family oligosaccharides, whereas cell wall polysaccharides such as galactomannan [1], galactan [2] and xyloglucan [3,4] are thought to be reserves for the growing plantlet, being degraded after germination [5]. Among legumes, some members of the subfamily Caesalpinoideae are known to accumulate xyloglucan in the cotyledons [6–9]. These polymers are present in storage cell walls of species such as tamarind (*Tamarindus indica*) [6], *Copaifera langsdorffii* and *Hymenaea courbaril* [4,8]. Seed storage xyloglucan have a cellulosic β-(1,4)-glucan backbone, branched at some points with α-(1,6)-xylpyranosyl (forming with the glucose of the backbone a disaccharide assigned X) or β-(1,2)-D-galactopyranosyl-α-(1,6)-D-xylpyranosyl (forming a trisaccharide assigned L) [10]. Except for the absence of terminal fucosyl units α-(1,2)-linked to the β-D-galactopyranosyl groups, there is a remarkable similarity between seed storage xyloglucans and structural xyloglucan from primary walls of dicotyledons [11]. The basic polymer molecule is composed of heptasaccharide repeating units of XXXG with variation in the substitution with galactose generating oligosaccharides structures like XLXG, XXLG and XLLG.
Although Leguminosae seems to be the principal family to possess storage xyloglucan in seeds, a member of Tropaeolaceae (*Tropaeolum majus*) is the xyloglucan-containing system best studied to date. The work by Reid and collaborators has shown that the rate of xyloglucan mobilisation in this species coincided with the increase and later decrease in the levels of four hydrolytic enzymes: endo-β-glucanase, β-galactosidase (β-gal), α-xylosidase (α-xyl) and β-glucosidase (β-glc) [12]. Since then, the hydrolases were purified to homogeneity and some of their properties were studied. Endo-β-glucanase was purified by Edwards et al. [13], and further studies on its mode of action on xyloglucan were performed by Fanutti et al. [14], demonstrating that this enzyme is a xyloglucan-endo-transglycosylase (XET).

As far as we know, the only legume which had its hydrolases studied is *Copaifera langsdorffii*. Buckeridge et al. [4] inferred the presence of these enzymes through the detection of glucose, galactose and xylose released by crude enzymic extracts containing endogenous water soluble xyloglucan. They also detected the exo enzymes (β-gal, α-xyl and β-glc) using artificial substrates.

We now present data on the mobilisation of the xyloglucan of *H. courbaril*, a native tropical legume known to accumulate up to 40% of the seed dry weight as xyloglucan [8]. Recently a new oligosaccharide was isolated and identified in this seed [15]. The presence of this oligosaccharide makes this polysaccharide unique among others described in the literature, possibly with implications in the metabolism of the cell wall.

### 2. Material and methods

#### 2.1. Seed origin and germination conditions

Seeds of *H. courbaril* L. were provided by the Seed Department of the Institute of Botany at São Paulo. The quiescent seeds had an average mass of 4.74 ± 1.18 g and only seeds between 3 and 6.5 g were used in the experiment to avoid variations caused by very different seed sizes. The seeds were scarified individually by abrading the seed coat with sand paper, weighed, soaked in commercial sodium hypochlorite solution for 5 min, washed with tap water for 10 min, soaked in distilled water for 12 h and planted in vermiculite. Trays with the seeds regularly spaced were kept at 25°C under a 12 h photoperiod. The regular distribution of the seeds in the trays allowed the calculation of the relative dry weight (i.e. the weight of the embryo or the pair of cotyledons divided by the weight of the quiescent seed), because the initial weight of each seed was known. Every 5 days, 20 seeds were collected until the abscission of the cotyledons which occurred 75 days after planting.

Of the 20 seeds collected, 15 were used for dry mass measurements, two were used for microscopic observations and three were subjected to carbohydrate extraction and analyses and measurement of enzyme activities. The seeds used for the studies of mass allocation were dissected and had their cotyledons and embryo dried for 24 h at 80°C and weighed for the determination of dry weight. The values were expressed in relative dry weight as a result of the difference in the size of the seeds used.

#### 2.2. Light microscopy

The seeds collected for microscopic observations were fixed with p-formaldehyde (4%/glutaraldehyde (2.5%) for 24 h at 5°C, dehydrated in a series of ethanol (70, 80, 95 and 100%, successively) at room temperature, included in Paraplast and 8 μm sections were obtained [16]. For staining with iodine, the sections were covered with a solution of I₂/KI (0.5/1%) and photographed immediately.

#### 2.3. Biochemical analyses

All polymeric or oligomeric xyloglucan used in this work was obtained as described in [15]. The seeds collected for biochemical analyses were cut into pieces of approximately 5 mm and the pool was separated into two groups: one was dried as described before, ground and stored at −20°C until usage for carbohydrate analyses; the other was homogenised in 50 mM sodium acetate buffer (pH 5) with NaCl (200 mM), centrifuged (10 000 × g, 10 min) and the supernatant was used as crude extract for determination of enzyme activities.

Before measurements of the hydrolases activities could be made, the optimal pH for each one was determined. For the determination of pH optima, crude extract obtained as described above was incubated in citrate-phosphate buffer in pHs rang-
ing from 2.6 to 7.6 (with increases of 0.2 units). Sodium acetate buffer (pH 5) was used in all assays in the rest of the work. In the viscometric assays, the fall in viscosity, characteristic of endo activity, was observed only when xyloglucan oligosaccharides were added to the assay medium (see below). Therefore, limit digest xyloglucan oligosaccharides were added to the assay and the enzyme named XET.

The choice of substrates was based on the knowledge gathered after the studies by Reid and co-workers [12–14,17]. For measuring α-xyl activity, the release of pentose (measured according to [18]) from cellulase limit digest oligosaccharides (obtained as in [15]) was used. The enzymatic extract (20 µl) was incubated with 25 µl of sodium acetate buffer (100 mM pH 5.0) and 20 µl of a 20 mg ml⁻¹ oligosaccharide solution for 24 h at 30°C and the amount of pentose released was measured. β-gal was assayed against pNP-β-D-galactopyranoside (Sigma) [10 µl of crude extract, 10 µl of sodium acetate (100 mM pH 5.0) and 10 µl of 50 mM substrate, incubated for 30 min at 40°C]. β-glc was assayed similar to β-gal, but using pNP-β-D-glucopyranoside as substrate. XET was assayed viscometrically using purified H. courbaril xyloglucan as substrate (400 µl of 1.2% xyloglu-
can, 10 µl of a 20 mg ml⁻¹ oligosaccharide solution, 50 µl of enzymatic extract and 60 µl of 1 M sodium acetate pH 5, incubated at 30°C and the flow through a 0.2 ml pipette was measured every 5 min). The activity of endo-glucanase was measured as described above but without addition of oligosaccharides. It was calculated as the slope of the line obtained from the logarithm of the flow time versus incubation time.

2.4. Xyloglucan extraction and analysis

For extraction of polysaccharides, 100 mg of dried seeds were extracted with ethanol (4 × 750 µl of 80% ethanol, 80°C, 10 min) and then with water (4 × 1 ml, 80°C, 180 min). The insoluble material was extracted with 4 M KOH with 26 mM sodium borohydride (3 × 1 ml, 180 min, room temperature). The alkali extracted polymer was neutralised with acetic acid and both water and alkali extracted polysaccharides were dialysed against distilled water and freeze dried. The polysaccharides were hydrolysed with sulphuric acid according to Ref. [19] or hydrolysed with cellulase (Megazyme-Australia) according to Ref. [15]. The monosaccharide products of acidic hydrolysis were analysed by HPAEC-PAD (Dionex) using isocratic elution (23 mM NaOH, 0.8 ml min⁻¹) for 20 min with a PA-1 column (CARBOPAK). The proportions among monosaccharides were corrected according to detector sensitivity to each monosaccharide, calculated by using equimolar standards. The xyloglucan oligosaccharides were analysed in a gradient of sodium acetate from 75 to 116.5 mM in isocratic NaOH (150 mM) for 40 min (1 ml min⁻¹) using the same column.

3. Results

3.1. Mass allocation and morphologic characteristics

Fig. 1 shows the changes in the dry mass of the cotyledon and embryo. The first drop in the cotyledon dry weight occurred between 10 and 20 days and was as a result of the seed coat natural detachment. Germination of most of the seeds occurs 35 days after planting and during the next 20 days the embryo dry mass increases while the mass of the cotyledons decrease. The primary
leaves expand to their full length by day 45. It is only after germination that xyloglucan degradation takes place. Mobilisation starts after day 40 being complete by day 70, when only residual amounts of xyloglucan are left into severely shrunken cotyledons. Xyloglucan solubility increases from days 25 to 45, prior to degradation.

3.2. Light microscopy

Fig. 2 shows transversal cuts of the storage parenchyma of cotyledons of *H. courbaril* at different stages of xyloglucan mobilisation. In quiescent seeds, the appearance of the thick storage cell walls is smooth and protein bodies were observed into the cytoplasm (Fig. 2a). At the beginning of xyloglucan disassembling (day 40) the smooth appearance of the cell wall had disappeared. The staining with iodine was altered and lamellae could be observed. At the same time, starch could be seen in the cytoplasm and the thickness of the wall increased two fold (Fig. 2b). After nearly complete mobilisation (day 60) few xyloglucan could be detected by iodine staining (Fig. 2c) and some starch remained in the cytoplasm. Walls were much thinner, accounting for only 10% of the initial thickness.

3.3. Biochemical analyses

For the optimisation of assay conditions, the pH optima of the hydrolases were determined (Fig. 3). β-gluc, α-xyl and XET have maximal activities around pH 4.5, although the latter was active in a broad range of pHs (from 4 to 7). Exception was made for β-gal, which presented a sharp peak of activity at pH 3.2 and less than 30% of its activity at pH 5.0, in which the other hydrolases are most active.

In the XET/endo-β-glucanase assay, the presence of xyloglucan oligosaccharides was required for the detection of activity (Fig. 4). Although a mixture of oligosaccharides with different structures and molecular weights was used, an apparently Michaelis-Menten kinetics was obtained, which led us to consider the oligosaccharides as substrates for the transglycosylation reactions.

Using the conditions mentioned above, a time course of the activity of each enzyme was obtained (Fig. 5). Although rigidly controlled conditions of light, temperature and water availability were
used, high variability was observed on the enzyme activities during the time course. Fig. 5 shows that β-glc and β-gal activities were already present in quiescent seeds (but rise during xyloglucan disassembling) whereas α-xyl and XET activities were not detected at the beginning and rose during and after germination, respectively. The cellulase activity raised together with transglycosylation activity, indicating that the *Hymenaea* XET has both activities, like *Nasturtium* XET [14] or contamination of the enzyme extract with xyloglucan which, during the incubation, generates oligosaccharides.

### 3.4. Carbohydrate analysis

The changes in xyloglucan structure following germination and plantlet growth were probed by monosaccharide and limit digest oligosaccharides analyses by HPAE chromatography. Table 1 shows monosaccharide analysis of the xyloglucan extracted with hot water and alkali from the cotyledons of quiescent seeds and from 30, 45 and 75 days old cotyledons. The proportion of arabinose in the water-extracted polymer increased after mobilisation to a proportion close to the one found in the alkali extracted polysaccharide. This arabinose is probably present in xyloglucan molecules, because acid hydrolysis of cellulase limit digest oligosaccharides, which hydrolysed nearly 70% of the xyloglucan from *H. courbaril*, yields arabinose in approximately the same proportions (data not shown). Galactose and xylose also showed changes in proportion. Their proportion (amount of monosaccharides for each of the four glucoses) dropped from 1.2 and 3.2, respectively, during imbibition, to 0.9 and 2.9 after 30 days and rose back to 1.1–1.2 and 3–3.3, respectively, following mobilisation. These data can be compared with the analyses of the limit digest oligosaccharides (Fig. 6), which show that the peak assigned to oligosaccharide XXLG dramatically decreases at the beginning of xyloglucan disassembling, when compared to the other peaks. Together with the change in solubility (see Fig. 1), these data suggest that xyloglucan was somehow processed before complete mobilisation to the embryo.

### 4. Discussion

#### 4.1. Seedling growth and xyloglucan mobilisation

The seeds of the tropical legume *H. courbaril* take approximately 30 days to germinate. They are large seeds and water imbibition takes about one
third of the germination period (10 days), the rest consisting of metabolic activities that culminate in radicle protrusion. Only after germination, the mobilisation of xyloglucan from the cotyledons starts. This could be concluded from the reduction of the cotyledon average dry mass with an increase in the dry mass of the embryo (Fig. 1), the reduction of the cell wall thickness after this period, the appearance of starch in the cell (indicating a large input of carbohydrate into the cell — see Fig. 2) and also by the rise in the activities of the enzymes involved in xyloglucan disassembling (Fig. 5). Despite the presence of other reserves in the cotyledons of quiescent seeds (sucrose, raffinose family oligosaccharides and protein bodies, data not shown), it is only during xyloglucan mobilisation that the dry mass of the embryo changes significantly.

The aspect of the cotyledon cell walls after xyloglucan mobilisation (Fig. 2C) indicates that some structural elements of the wall are not mobilised after germination. The presence of two populations of xyloglucan with different extractability can be suggested from the data in shown in Table 1. Most of the xyloglucan present in quiescent seeds can be extracted with hot water.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Proportion of monosaccharides obtained by acidic hydrolysis of the polysaccharide extracted from the seed powder with water and KOH (4 M)(^a)</th>
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<td></td>
<td>Days after planting</td>
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<td>0</td>
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<td>Water</td>
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<td>Gal</td>
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\(^a\)The area of the glucose peak was considered 4 and the other areas were calculated proportionally. All the areas were corrected for the different sensitivity of the detector for each monosaccharide, using an equimolar standard.
4.2. Biochemical aspects of xyloglucan disassembling

Three facts suggest that a modification in the fine structure of the xyloglucan occurs prior to mobilisation: (1) the change in polymer solubility that made the polymer more easily extractable immediately before disassembling; (2) change in the appearance of the cell wall; and (3) change in the polymer structure. The existence of enzymes with exo-activities, especially β-gal, which is the only one reported to hydrolyse the polymer [20], could explain the changes in the fine structure of the xyloglucan prior to mobilisation. Although a family of β-galactosidases occurs in seeds, only some of its members were purified and characterised [21,22] and they show differences in specificity. The diversity of β-galactosidases and their modes of action suggest a high complexity in the process of degalactosylation of the xyloglucan. For example, the β-gal from C. langsdorffii [22] was shown to hydrolyse galactose from XLG and XLLG, but not from XXLG. The characterisation of this purified enzyme led to similar results for Hymenaea concerning the pH optimum for activity, i.e. a peak at pH 3.2.

As a pH optimum around 5 is expected for wall enzymes [23], the difference observed between the pH optima for β-galactosidase (pH 3.2) and the other enzymes (α-xyl, β-glc and XET) suggest that β-gal activity may limit, under certain circumstances, xyloglucan disassembling. This can be important in the control of the process, because the disassembling cannot proceed without the retrieval of the galactose [24]. The acidic growth theory [25] and the model for the control of cell expansion based on the differences of pH optima of the enzymes involved in the process [26] are two previous examples of regulation of cell wall metabolism based on pH.

The requirement of xyloglucan oligosaccharides for detection of viscosity decrease in the assay system for endo-β-glucanase suggests that in H. courbaril, the enzyme responsible for the release of xyloglucan fragments from the cell wall is a xyloglucan endo-transglycosylase (XET). However, differently from the enzyme purified from T. majus, this XET has a very low activity against purified polymer in the absence of oligosaccharides.
4.3. An improved model for storage xyloglucan disassembling

In some aspects, our data appear to differ from what has been observed by Edwards et al. [12] in cotyledons of *T. majus*, in which the activities of the four enzymes overlap. On the basis of *T. majus* system, Buckeridge and Reid [5] proposed a model for xyloglucan degradation in which the polymer would enter a linear disassembling process which would lead from polymer to monomer directly, beginning with the action of one XET (with endoglucanase activity at low concentrations of oligosaccharides). The fragments released would be hydrolysed by the sequential action of β-gal, α-xyl and β-glc, producing free galactose, xylose and glucose.

It is possible that in species of the Leguminosae, the xyloglucan degrading system works differently from the *T. majus* one. Thus, some changes in this model can be suggested to explain the results presented here and by Buckeridge et al. [4], obtained with other seeds (from legumes) that store xyloglucan (*H. courbaril*, *C. langsdorffii*). This new tentative model (Fig. 7) proposes that seed xyloglucan degradation occurs in at least three steps: (1) transglycosylation; (2) degalactosylation; and (3) oligosaccharide disassembling. Such a model is not linear, but would have two different cycles (transglycosylation and disassembling) which would be linked by the action of β-gal. In a condition of low β-gal activity, the fragments generated by the XET would not be disassembled and the rise in their concentration would lead to an increase in endo-transglycosylation activity, halting hydrolysis and avoiding an excessive rise in the input of carbohydrate into the cell. After the increase in β-gal activity, these fragments of xyloglucan would be degalactosylated and subsequently enter the second cycle of degradation, releasing free monosaccharides.

With only a few cell wall storage systems biochemically studied so far, it is difficult to suggest a general model that describes the disassembling of the storage xyloglucan in every seed. As different species are exposed to different environmental conditions, this is likely to reflect distinct strategies of adaptation, requiring diverse physiological and biochemical characteristics. As *H. courbaril* is a tropical tree with seeds much larger than *T. majus*, the disassembling of xyloglucan at the same time in all the cells might lead to an imbalance of the source/sink relation, therefore resulting in an overload of the transport system towards the embryo. Indeed, the accumulation of starch inside cotyledon cells suggests that the input of carbohydrate into the cell is greater than the output. If the proposed model is correct, the presence of transglycosylation even at low oligosaccharide concentration (contrarily to *T. majus*) might serve to make the disassembling system of *H. courbaril* partially reversible, unless β-gal is active. This would delay degradation and possibly synchronise it with a relatively slower pace of mobilisation (30 days from start to end of degradation). This contrasts with *T. majus* which, being a faster grower, degrades xyloglucan within an interval of only 5 days [12].

![Fig. 7. Tentative model proposed for the in vivo disassembling of the storage xyloglucan in seeds. The polysaccharide is first hydrolysed by the XET characterising a period of Transglycosylation. Only when certain galactose residues at XLXG and XLLG are hydrolysed (degalactosylation), the remaining oligosaccharides of higher fragments containing XXLG and XXXG enter the final cycle of disassembling in which glucose and xylose are attacked by β-glucosidase and α-xylosidase to produce free monosaccharides.](image-url)
tions used in this work to detect enzyme activities, these positional differences in activities were probably lost and instead of a sharp peak of activity, a smooth rise and a plateau were observed.

Distinct ecological strategies could also explain some differences between these two plants. Since T. majus is herbaceous and germinates in areas with higher light intensity than H. courbaril, its plantlets would have to grow faster in order to achieve reproductive success in less than a year. H. courbaril plantlets, on the other hand, grow slower under the canopy and may stay under shadowed conditions for much longer periods. This requirement of an internal source of carbohydrate for longer periods could explain the larger seed found in tropical trees, the greater amount of xyloglucan and the longer period for its mobilisation as well as the biochemical differences among the xyloglucan disassembling systems.

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