Effect of water deficit on the cell wall of the date palm (Phoenix dactylifera 'Deglet nour', Arecales) fruit during development

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Original Article

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Running title: Water deficit and date palm fruit cell wall

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ABSTRACT

Date palm (Phoenix dactylifera) is an important crop providing a valuable nutrition source for people in many countries including the Middle East and North Africa. In recent years, the amount of rain in North Africa and especially in the Tunisian palm grove areas has dropped significantly. We investigated the growth and cell wall remodeling of fruits harvested at three key development stages from trees grown with or without water supply. During development, cell wall solubilisation and remodeling was characterized by a decrease of the degree of methylesterification of pectin, an important loss of galactose content and a reduction of the branching of xylan by arabinose in irrigated condition. Water deficit had a profound effect on fruit size, pulp content, cell wall composition and remodeling. The loss of galactose content was not as important, arabinose content was significantly higher in the pectin-enriched extracts from non-irrigated condition and the levels of methylesterification of pectin and *O*-acetylation of xyloglucan were lower than in irrigated condition. The lower levels of hydrophobic groups (methylester and *O*-acetyl) and the less intensive degradation of the hydrophilic galactan, arabinan and arabinogalactan in the cell wall may be implicated in maintaining the hydration status of the cells under water deficit.

Key-words: Phoenix dactylifera, cell wall, date palm, fruit development, irrigation, pectin, water deficit, xylan, xyloglucan.

INTRODUCTION

Date palm (Phoenix dactylifera L.) is a dioecious monocot crop of the commelinid clade like grasses (Poales) (APGIII 2009). It grows in arid and semi-arid regions and can develop at an elevation up to 1500 m in well-drained soils. It is cultivated in the Middle East, North Africa, parts of Central and South America, Southern Europe, India and Pakistan (Al-Farsi & Lee 2008). More than 2000 different cultivars of date palm are known over the world and more than 250 are present in Tunisia (El-Arem *et al*. 2011). Date fruit is an important food source rich in nutrients (Al-Farsi & Lee 2008) and carbohydrates (Al-Shahib & Marshall 2003; Shafiei, Karimi & Taherzadeh 2010). Moreover, date fruit has been used as a source of active components in the development of drugs (Ishurd & Kennedy 2005; Baliga *et al*. 2011). Because of its economic importance, the need to predict the gender of immature plant and to improve fruit traits, the chloroplast (Yang *et al.* 2010) and the nuclear genomes of several date palm cultivars (Khalas, Deglet nour, Medjool) have been sequenced (Al-Dous *et al.* 2011). More recently, the gene expression profiles at seven fruit development stages of the date palm cv. Khalas were also investigated but mostly focused on starch and soluble sugar metabolism (Yin *et al.* 2012).

With the reduction of rain fall and the increase of drought period, the water deficit limits the growth and distribution of natural vegetation and reduced the performance of cultivated plants (Shao *et al*. 2008). Water stress influences plant growth at various levels, from cell to community (Colom & Vazzana 2001) and affects plant growth depending on cell division, cell enlargement and cell differentiation (Correia, Coelho & David 2001; Cabuslay, Ito & Alejal 2002). Expansive cell growth is more inhibited by water stress than cell division and various physiological and biochemical processes are reduced such as photosynthesis, respiration, translocation, ion uptake, carbohydrate and nutrient metabolisms (Hsiao 1973).

Plants can undergo changes in their development and physiology under such stress as growth depends on the changes in the structure and remodeling of the cell wall (Konno *et al*. 2008).

The cell wall of land plant consists mainly of polysaccharides including pectins, hemicellulose and cellulose with structural glycoproteins and enzymes (Popper *et al.*, 2011). Pectins are complex wall polymers consisting of homogalacturonan (HG) that can be methyl- and acetyl-esterified, rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II), and xylogalacturonan (Fry 2011). HG is a polymer of repeated units of (1→4)-α-D-galacturonic acid (GalA) that can be cross-linked with calcium upon block wise action of pectin methylesterases on methylesterified HG (Micheli 2001). RG-I consists of the repeating disaccharide, (1→4)-α-D-galacturonic acid-(1→2)-α-L-rhamnose (Rha), unit with a wide variety of side chains attached to the rhamnosyl residues, ranging from monomers to large oligosaccharides such as (1→4)-β-D-galactan, (1→5)-α-L-arabinan, and/or type I arabinogalactan (Caffall & Mohnen 2009).

In most Angiosperms (flowering plants including eudicots and monocot non-commelinids), the main hemicellulose is the xyloglucan (XyG)-type. Classic XyG consists of a (1→4)-β-D-glucan backbone substituted with xylose (Xyl), galactose (Gal)-Xyl or fucose (Fuc)-Gal-Xyl motifs which correspond, according to the one letter code proposed by Fry *et al.* (1993), to X, L and F, respectively, G being the unsubtituted glucosyl (Glc) residue of the glucan backbone. However, structural differences in XyG have been shown to occur that are related to taxonomic groups including the Asteridae (Hoffman *et al.* 2005). In addition, *O*-acetylation of XyG can occur, most generally on the galactosyl residues, but its biological function is unknown (Cavalier *et al.* 2008). In monocot commelinid including Poales, Commelinales, Zingiberales and Arecales (APG III 2009), XyG can be found in low amount (Hsieh & Harris 2009) but the main polymer is the xylan-type. The backbone consists of (1→4)-β-D-xylopyranosyl residues that can be *O*-acetylated and branched with one or two L-

arabinosyl residues (Scheller & Ulvskov 2010). In addition, the xylan/arabinoxylan (AX) structure can also be substituted with D-glucuronic acid (GlcA) and/or its 4-*O*-methyl derivative (GAX) and linked to ferulic acid (Fry 2011).

It has been well documented that during fruit development, the modifications of the cell wall modulate the texture of the flesh including the softening by the action of enzymes such as pectin methylesterases, polygalacturonases, expansins and others (Rose & Bennett 1999; Giovanni 2001; Vicente *et al.* 2007a). Modification of the cell wall composition during ripening has been observed in many species (Stewart, Iannetta & Davies 2001, Brummel *et al.* 2004, Li *et al.* 2006, Cardoso *et al.* 2007, Ordaz-Ortiz, Marcus & Knox 2009). However, most of the studies focused on the influence of abiotic stresses on the cell wall modification of fruits have been carried out during post-harvest storage (under cold, light or gas treatments including ethylene, ozone or CO_2) (Barka *et al.* 2000; Rodoni *et al.* 2010; Toivonen & Hodges 2011).

To date, little information is available on the effect of water deficit on fruit development and cell wall remodeling and to our knowledge, none on the economically important date palm fruit. The aim of this study was to characterize the cell wall composition of the pulp during fruit maturation and to examine the effect of water deficit (in 2009, a total of 3.6 mm of rain was recorded between March and September in the Palm grove region of sampling) on the fruit development and its impact on the cell wall remodelling during ripening.

MATERIAL AND METHODS

Plant material

Fruits of Phoenix dactylifera var. Deglet nour (Arecaceae) were collected in 2009 in two nearby palm groves in the Kebili region (south-west of Tunisia) where the largest palm groves

of the country are located. The palm trees were submitted to two different water irrigation regimes: one with watering every 4 days and one without any supply of water. Fruits were harvested at three different developmental stages (Fig. 1) described by Baliga *et al.* (2011): 77 days after pollination (DAP) (17 jun. 2009, stage 1: khimri characterized by high cell multiplication), 140 DAP (19 aug. 2009, stage 2: khalal characterized by cell multiplication and expansion, starch accumulation) and 232 DAP (18 nov. 2009, mature edible fruit: stage 3 tamar characterized by an accumulation of soluble sugars such as glucose, fructose and sucrose and an absence of starch) (Yin *et al.* 2012). From February to November 2009, 60 mm of rain fall was recorded in the Kebili region with a total of 3.6 mm from March to September (*i.e.* period of fruit development) (El Ferchichi Ouarda, Walker & Larbi Khouja 2012; http://www.semide.tn/collect/annuaire/index/assoc/HASH0130.dir/doc.pdf). The harvest was randomised and to minimize the effects of individuals and sun exposition, fruits were collected in all directions and mixed with the harvest from five other palm trees.

Estimation of water content

Fruits were pitted and the fleshes were weighted to get the fresh weight (FW). After incubation for 48h in an oven at 60°C, the dry weight (DW) of the flesh was measured. The values are the means of 20 measurements per condition and expressed as % of FW.

Cell wall extraction

One hundred g of flesh was ground in liquid nitrogen and treated three times with EtOH 70 % at 70 °C for 15 min followed by incubation with a mixture of chloroform:methanol (1:1, vol./vol.) and acetone. The remaining insoluble material was then dried to yield the cell wall residue. One g (DW) of cell wall material was successively treated with (1) boiling water for 1h, (2) boiling ammonium oxalate 0.5% (w/vol.) for 1 h and (3) 50 mM Na_2CO_3

supplemented with 20 mM $NaBH_4$ for 20 h at 4 °C, (4) 1 M KOH and (5) 4 M KOH supplemented with 20 mM $NaBH_4$ at room temperature for 2 h as described by Ray *et al.* (2004). After each treatment, the insoluble material was collected by centrifugation and processed to the next step. The supernatants of the alkali treated samples (steps 3 to 5) were acidified to pH 5.5 with acetic acid. After dialysis against distilled water, the extracts were freeze dried. The final insoluble residue was extensively washed with distilled water and freeze-dried. The dry extracts were weighted to estimate the extraction yields. The cell wall fractionation procedure was carried out in triplicate for each condition.

Cell wall characterization

Monosaccharide analysis by gas chromatography. Monosaccharide composition of the fractions was determined by gas liquid chromatography as described by Dardelle *et al.* (2010). The values are the means of three independent biological samples.

Estimation of the degree of methylesterification (DM) of pectins by Fourier Transform Infra-Red (FT-IR) spectroscopy. Pectin-enriched fractions (boiling water and ammonium oxalate extracts) were desiccated in an oven for one week at 30°C and transferred in a vacuum jar containing silica gel before FT-IR analysis. Dry samples were analysed with an OMNI-Sampler FT-IR spectroscopy (version 5.2a) at 4 cm^{-1} resolution. The DM was calculated using the absorbance intensities at 1630 and 1740 cm^{-1} assigned to the carbonyl groups (COO^-) of GalA and its methylester, respectively with the equation described by Gnanasambandam & Proctor (2000) and Manrique & Lajolo (2002).

Eq. $DM = A_{1740} / (A_{1740} + A_{1630})$

Commercial pectins with determined DM were used as standards. Using this method, the DM of pectin from citrus fruit with 85% DM (Sigma) gave 79.9% ± 0.9 DM, pectin from

citrus fruit with 20-30% DM (Sigma) gave 24.6% ± 0.3 DM and finally pectate Na pectin from citrus with a DM = 8.6% (Sigma) gave 10.8% ± 0.9.

Dot-blot immuno-assays. Extracts (8, 4, 2 and 1 µg) were blotted onto nitrocellulose membrane and processed according to Mollet *et al.* (2000). The monoclonal antibodies (MAb) used were LM19 and LM20 that recognize weakly and highly methylesterified HG regions of pectins, respectively (Verhertbruggen *et al.* 2009a). (1→4)-β-D-galactans were probed with LM5 (Jones, Seymour & Knox 1997) and (1→5)-α-L-arabinans with LM6 (Willats, Marcus & Knox 1998) and LM13 (Verhertbruggen *et al.* 2009b). Weakly substituted xylans and more extensively branched arabinoxylans were detected with LM10 and LM11, respectively (McCartney, Marcus & Knox 2005). Arabinan from sugar beet (Megazyme), xylan from larchwood (Sigma), arabinoxylan from wheat (Megazyme), galactan of gum arabic from acacia, demethylesterified pectin and pectin with 8.6 and 85% DM from citrus fruits (Sigma) were used as controls. MAbs were purchased from Plant Probes (Leeds, UK) (www.plantprobes.net).

Analysis of XyG and xylan/arabinoxylan by matrix assisted light desorption ionisation-time of flight mass spectrometry (MALDI-TOF MS). Preparation of the oligosaccharides: One mg of pulp cell wall was incubated under agitation for 16 h at 37 °C with 500 µL of an *endo*-(1→4)-β-D-glucanase (5 units; EC 3.2.1.4; Megazyme) prepared in 10 mM ammonium acetate buffer, pH 5. *Endo*-(1→4)-β-D-xylanase (5 Units, EC 3.2.1.8, Megazyme) treatment was carried out on the insoluble residue of 500 mg pulp cell wall treated twice for 1h with boiling water to remove pectic polymers and once with 1% NaOH dissolved in 70% ethanol at 80°C for 2 h to eliminate lignins (Gabrielii *et al.* 2000). The glucanase and xylanase-resistant materials were removed by centrifugation after the addition of ethanol to reach a final concentration of 80%. The ethanol-soluble oligosaccharides were concentrated by evaporation

under a stream of air. MALDI-TOF mass spectrometry analyses of XyG and xylan/arabinoxylan derived oligosaccharides: MALDI-TOF mass spectra were acquired on a Voyager DE-Pro MALDI-TOF instrument (Applied Biosystems) equipped with a 337 nm nitrogen laser as described in Dardelle et al. (2010).

Statistical analyses

Data were compared during fruit development with a similar irrigation condition and between irrigated and non-irrigated conditions at the same harvesting stage with Prism (GraphPad Software Inc, USA) for statistical significance by ANOVA ($P < 0.05$).

RESULTS

Fruit growth, pulp, water and cell wall contents

During development, fruits increased in size (Fig. 1) and gained weight rapidly between stages 1 and 2 (from 1.3 g to 13.1 g and from 0.3 g to 6.2 g in fruits from irrigated and non-irrigated trees, respectively) (Table 1). Between stages 2 and 3, the fresh weight of the fruits did not change significantly (from 13.1 g to 12.1 g and from 6.2 g to 5.9 g) but the water content of the pulp decreased dramatically (from 71.2% and 79.9% to 23.5% and 20.4%). This was counter balanced by an increase of the dry matter of the pulp (from 3.5 g to 8.6 g and 1.0 g to 4.1 g in irrigated and non-irrigated conditions, respectively) (Table 1). Fruits collected from irrigated palm trees were larger (Fig. 1) and significantly heavier (about 2 fold) than the fruits harvested in non-irrigated palm groves (Table 1). The weights of the fresh and dry pulps were throughout development always significantly higher in the fruits from irrigated than non-irrigated trees (Table 1). During development, the weight of the cell walls decreased in both conditions, from about 70% at stage 1 to 10% at stage 3. At stage 2, the cell wall

content of the pulp was significantly the highest in non-irrigated condition (50.3 *vs* 30.7 %) (Table 1).

Yields of the pulp cell wall extracts

Loosely, ionically and covalently bound pectins were sequentially extracted with hot water, ammonium oxalate and Na_2CO_3, respectively. The extraction of the pulp cell wall by hot water treatment extracted an important amount of polymers that increased during fruit development (Fig. 2a). In contrast, the Na_2CO_3 extract showed an important yield reduction from stage 1 to 3 (from 11.5% to 0.3% and from 10.9% to 0.3% in fruits from irrigated and non-irrigated trees, respectively) (Fig. 2c). Moreover, the extraction yields obtained by hot water treatment were significantly higher in fruit from irrigated trees whereas no significant difference was noticeable in the Na_2CO_3 extracts (Fig. 2a,c). Treatment with ammonium oxalate and KOH 4 M extracted only a low percentage of polymers without any significant differences throughout fruit development and irrigation conditions (Fig. 2b,e). In contrast, treatment with KOH 1 M in non-irrigated condition solubilized higher amount of polymers compared to the irrigated condition (Fig. 2d). In both irrigation conditions, higher yields were obtained at stage 2. Finally, the yields of the remaining insoluble residues increased significantly during fruit maturation and were significantly higher in the non-irrigated condition at stages 1 and 2 (Fig. 2f).

Composition of the pectin-enriched pulp cell wall extracts

The hot water pectin-enriched fraction was mostly composed of GalA, Ara and Gal. During fruit development, the Ara and Gal contents were identical at stage 1 in the two irrigation systems. Under irrigated condition, both Ara and Gal levels decreased significantly whereas in non-irrigated condition the decrease was less noticeable (Fig. 3a). The mol% of GalA was

the highest at stage 2 in both irrigation conditions and was significantly higher in irrigated condition at stages 1 and 2 (Fig. 3a). Finally, at the full maturity stage, an important increase of Glc was detected, being significantly higher in the irrigated condition. GalA residues were originating from HGs and RG-I from irrigated and non-irrigated trees as shown by the immunoreaction with LM19 MAb that binds to unesterified HG oligogalacturonides and partially Me-HGs and LM20 that recognizes weakly Me-HGs (Fig. 4). The presence of Ara and Gal in the hot water pectin-enriched fraction revealed by GC were at least partly associated with RG-I side chains as revealed by immunodot-blot with LM13 and LM6 (may also bind to AGP) that recognizes (1-5)-α-L-arabinan and LM5, the MAb specific for (1-4)-β-D-galactan epitopes. Immunodot blot assays did not reveal any obvious difference in the labeling pattern of HG, arabinan and galactan between irrigated and non-irrigated conditions (Fig. 4, Supporting Information Fig. S1). The molar comparison of GalA and Rha indicated that most of the GalA content was composed of HG domains rather than the RG-I backbone (Fig. 5a). The later was relatively weakly branched by Ara and Gal residues in the hot water extracts (Fig. 5b).

The ammonium oxalate fractions were mostly composed of Ara, Gal, GalA and Xyl residues (Fig. 3b). The level of Ara increased from stage 1 to stage 2 and remained constant in stage 3. However, Ara was significantly higher in non-irrigated condition during fruit maturation. In contrast in irrigated condition, at stage 1, the level of Gal residues was significantly higher and decreased dramatically during fruit maturation. This was not observed in the fraction collected from fruits in the non-irrigated condition.

Estimation of the DM of HG did not show any significant difference in the ammonium oxalate fractions during development and between the two irrigation conditions (Fig. 6, Supporting Information Fig. S2). In contrast, in the hot water fraction, significant differences were detected at stages 1 and 3. At stage 1, the DM was 41.2% in irrigated condition and

27.5% in non-irrigated condition. At stage 3, the DM was 26.3% in irrigated condition and 8.8% in non-irrigated condition. At stage 2, no significant difference was measured (Fig. 6). The lack of noticeable differences between LM19 and LM20 labeling of the hot water extract (Fig. 4 and Supporting Information Fig. S1) originate probably from the overlapping binding capabilities of the antibodies to a range of DM.

In the Na_2CO_3 extract, Ara and Gal were the most abundant monosaccharides at stage 1 (Fig. 3c). The level of Gal was again significantly higher at stage 1 in irrigated condition and then decreased during fruit development in both irrigation conditions as observed in the ammonium oxalate fractions. Opposite trends were observed with Xyl with a sharp increase during fruit development but no significant differences were noticeable between the two irrigation system. The level of RG-I backbone increased importantly during fruit development (Fig. 5a). At the mature stage, nearly equimolar percentages of GalA and Rha were observed suggesting that this fraction was composed mostly of RG-I with low level of HG domains (Fig. 5a).

Composition of the hemicellulose-enriched pulp cell wall extracts

The extraction with KOH 1 M solubilized polymers enriched in Xyl, Ara and Gal (Fig. 7a). Xyl level increased during fruit maturation in both irrigation conditions. The values were significantly higher in the irrigated *vs* non-irrigated conditions. In contrast, the level of Ara decreased from stage 1 to 2 and then increased from stages 2 to 3. As in all the other extraction, the level of Gal was the lowest in the mature fruits. Similar results were obtained with the extraction with KOH 4 M except that an increase of Fuc and Glc residues were detected (Fig. 7b). In addition, higher level of Xyl and lower level of Ara were observed compared to the KOH 1 M extraction (Fig. 8). In both fractions, the ratios Ara/Xyl were higher at stage 1 and decreased in the following stages (Fig. 8). In mature fruits, the Ara/Xyl

ratio was slightly higher in non-irrigated compared to irrigated conditions. Overall, it appeared that xylans were more heavily branched in the KOH 1 M than the KOH 4 M.

Fuc and part of the Glc, Xyl and Gal residues may originate from the presence of XyG as shown by MALDI-TOF MS (Table 2, Supporting Information Fig. S3). At stage 1, the MALDI-TOF MS spectra did not display any detectable XyG fragments. At stages 2 and 3, in both irrigation conditions, the major ion was the fragment XXXG (Table 2, Supporting Information Fig. S3). Differences were noticeable in the levels of fucosylated and *O*-acetylated fragments, being significantly higher in the irrigated condition as compared to non-irrigated condition (Table 2).

The presence of branched (1→4)-β-D-xylan in the pulp was also verified by MALDI-TOF MS (Fig. 9, Supporting Information Fig. S4) and immunodot-blot assay using LM10 and LM11 Mabs (Fig. 10, Supporting Information Fig. S5). *Endo*-xylanase treatment on the crude or the depectinized pulp cell wall did not release detectable fragments. Only depectinized and delignified pulp cell wall treated by xylanase allowed the detection of ions suggesting that lignin was affecting the accessibility of the enzyme. The main fragments were composed of 6 and 7 pentoses branched with one 4-*O*-methyl-GlcA (Fig. 9). This acidic monosaccharide is not commercially available and could not be assessed in our GC analyses. Comparison of the relative abundance of the ions revealed that smaller oligosaccharides were dominantly found in irrigated condition. In non-irrigated condition, larger oligosaccharides were detected up to 10 pentoses. In the condition tested, the *O*-acetyl groups could not be assessed because of the alkali treatment used during the delignification process. LM10 is specific to unsubstituted or low substituted xylans whereas LM11 binds to arabinoxylan as well as unsubstituted xylan (McCartney *et al.* 2005). No significant difference of labeling was observed between both irrigation conditions in the 1 M and 4 M KOH extracts (Fig. 10 and Supporting Information Fig. S5).

DISCUSSION

Fruit ripening is accompanied by cell wall solubilization and remodeling

In date palm, fruit ripening induces a decrease of water content and an increase of soluble sugar content (mostly glucose and fructose) as observed in other varieties (Ahmed, Ahmed & Robinson 1995; El-Arem *et al*. 2011). Similarly, the cell wall content of the pulp decreased remarkably from 70% (w/w) at stage 1 (khimri) to only 10% (w/w) at stage 3 (tamar). In other fleshy fruits, a clear decrease of the cell wall material was also noted during development presumably due to intensive cell wall degradation and remodeling (Dawson, Melton & Watkins 1992; Vicente *et al.* 2007b, c, d). This was confirmed with an increase of the yields of the water soluble polymers and a decrease of the ammonium oxalate, Na_2CO_3 and KOH soluble extracts at stage 3 as shown during the ripening of kiwi (Actinida deliciosa) (Redgwell, Melton & Brasch 1991), guava (Psidium guajava) (Das & Majumder 2010), peach (Prunus persica) (Hegde & Maness 1996), pear (Pyrus communis) (Martin-Cabrejas, Waldron & Selvendran 1994) and chilli pepper (Capsicum annum) (Sethu, Prabha & Tharanathan 1996) fruits.

In date fruit cell wall, the major non-cellulosic polymer is pectin and not hemicellulose (compared to commelinid grasses which are composed mostly of GAX). Similar results were obtained in the leaflets and rachides of two other palm trees Phoenix canariensis and Rhopalostylis sapida. In these species, pectin was also the main component of the cell wall (Carnachan & Harris 2000; Hsieh & Harris 2009). Moreover, the cell wall composition revealed large changes in both pectins and hemicelluloses during ripening. In all the pectic fractions, a clear loss of Gal but not in the Ara content is observed. In many species, a net loss of non-cellulosic neutral sugar residues occurred during ripening, including galactose and/or

arabinose (Gross & Sams 1984; Seymour *et al.* 1987; Brummell & Harpster 2001; Pena & Carpita 2004).

The water soluble fraction is enriched in HG whereas the Na_2CO_3 extract is mainly composed of RG-I branched with arabinan, galactan and/or arabinogalactan as shown in raspberry (Rubus idaeus) (Vicente *et al.* 2007b) and blueberry (Vaccinium corymbosum) (Vicente *et al.* 2007c). At the final ripen stage, RG-I seem to be less branched especially in the Na_2CO_3 extract due to the loss of galactan side chains. Between stages 1 and 3, the level of DM of the HG in the water soluble extract decreases as observed in other fleshy fruits (Brummell & Harpster 2001; Stewart *et al.* 2001) that can lead to the degradation of the HG by *endo*-polygalacturonase (Orfila *et al*. 2001; Jiménez *et al*. 2001). In date palm, the loss of fruit firmness during maturation was shown to be correlated with an increase of polygalacturonase and galactosidase activities (Serrano *et al.* 2001). In addition, transcriptomic analyses during fruit development and ripening have shown changes in the expression profile of several remodeling cell wall proteins (Alba *et al.* 2005) including pectin methylesterases, polygalacturonases, expansin and XyG *endo*-transglucosylase hydrolase (Giovanni 2001; Fonseca *et al.* 2004; Carrari & Fernie 2006). Moreover, biochemical evidence has also shown variation in enzyme activities including pectin methylesterases, polygalacturonase, β-galactosidase, α-arabinofuranosidase, galactanase, glucanase, xylanase and others during fruit development of many species including boysenberry (Rubus idaeus x Rubus ursinus) (Vicente *et al.* 2007d), papaya (Manenoi & Paull 2007), apple (Malus domestica) (Ortiz, Graell & Lara 2011), tomato (Solanum lycopersicum) (Barbagallo *et al.* 2008) and date palm (Serrano *et al.* 2001).

The presence in the water soluble extract of higher level of glucose in the mature fruits is not correlated to starch that accumulates at the first stages of development but is then converted to soluble sugars and at the ripened stage, no starch is found (Baliga *et al.* 2011).

But instead, it may originate from cellulose and/or XyG degradation, or from the water soluble (1→3,1→4)-mixed linkage glucan found in the pulp of mature fruits of P. dactylifera var Nakhla (Ishurd *et al.* 2002). To date mixed-linkage glucan has been confirmed to occur in members of the Poales (Fry 2011) and in Equisetum (Fry *et al.* 2008) and was shown to appear transiently depending on the developmental stages. It has not been found in other land plants. The precise function of this polymer is not clear but might be implicated in reserve material and/or cell expansion (Buckeridge *et al.*, 2004).

In contrast with the pectin-enriched fractions, the yields of hemicellulose-enriched fractions were low except at stage 2 in the 1M KOH extract. Our results showed a similar trend to those found in blueberry (Vicente *et al.* 2007c), boysenberry (Vicente *et al.* 2007d) and apple with an increase of the alkali-soluble extract from 2 to 5 months after blooming then a decrease of the extraction yield to fruit maturity (Percy, Melton & Jameson 1997). At stage 2, cell elongation is intense and is likely to require important hemicellulose synthesis and remodeling.

The polysaccharides solubilized from the cell wall with alkali were composed mainly of GAX and galactan. The Xyl content increased during fruit ripening and at the mature stage, xylan polymers are less branched by Ara residues compared to immature stages suggesting the action of arabinofuranosidase. Moreover, the Ara/Xyl ratios were higher in mild-alkali compared to strong alkali treatments. These data confirm that highly branched GAX interacts probably less with cellulose microfibrils compared to weakly arabinosylated GAX, thus requiring lower alkali concentration to disrupt the GAX-cellulose hydrogen interactions (Ceusters *et al.* 2008). Another hemicellulose, the XyG was also detected. In vegetative organs of palm, hemicellulose consists of a mixture of XyG and GAX, the later being most abundant (Carnachan & Harris 2000) as observed in olive (Olea europaea) pulp (Coimbra *et al.* 1995). Interestingly, XyG was not detected at the first stage of development where active

cell division occurred. XyG profile is very comparable to the one found in the stem apex of *P. canariensis* with the three main fragments XXXG, XXFG and XLFG (Hsieh & Harris 2009).

Water deficit induces changes in the cell wall of date palm fruit

Water stress had a profound effect on the fruit size and mass. Date palm fruits are smaller, have lower water content, higher soluble solids and different soluble sugar content (Al-Yahyai & Al-Kharusi 2012) as observed in many fruits such as kiwi (Miller *et al.* 1998), mango (Mangifera indica) (Léchaudel & Joas 2007) or citrus (Citrus sinensis) (Huang, Huang & Gao 2000). In mango, early water deficit during fruit development reduces the number of cells, while water stress after cell division reduces the cell sizes (Léchaudel & Joas 2007). In contrast, in grape (Vitis vinifera), early water deficit does not affect cell division but berry size and weight reductions were caused only by a decrease of cell volume (Ojeda, Deloire & Carbonneau 2001). Cell expansion can only occur when turgor pressure is greater than the cell wall yield threshold. Water stress greatly suppresses cell expansion and cell growth due to the low turgor pressure (Jaleel *et al*. 2007). Water stress may have reduced cell division, cell elongation and remodeling of the cell wall. In irrigated condition, cell wall remodeling is much faster as witnesses with lower cell wall content at stage 2. Similarly, water soluble pectins are more abundant in irrigated condition whereas the insoluble residues are more abundant in non-irrigated condition.

Pectic side chains such as arabinans, galactans and highly branched arabinogalactans of various configuration and size, among other functions, are involved in determining the hydration status of cell wall matrix for their high water binding capability and ability to form gels (Willats *et al*. 2001; Moore *et al.* 2008a). Our data are consistent with such properties as arabinan levels are higher in non-irrigated condition as found in tobacco suspension cells subjected to saline and water stress (Iraki *et al.* 1989) and in the root of drought tolerant wheat

(Triticum durum) cultivar (Leucci *et al.* 2008). This difference may be related to a higher synthesis of arabinan side chains of RG-I or lower degrading enzyme activities of arabinofuranosidase and/or arabinanase during the fruit maturation. Studies on resurrection plants have shown that the cell walls were folded in dry state and in Myrothamnus flabellifolia and others, cell wall composition analyses revealed a higher concentration of arabinose in the dehydrated leaves, likely in the form of arabinan and/or arabinogalactan polymers associated with the pectin matrix (Moore *et al.*, 2006; Moore *et al.*, 2012). The authors suggested that the high level of RG-I associated arabinan side chains may contribute to maintaining the cell wall flexibility under water stress (Moore, Farrant & Driouich 2008b). During maturation of marama seeds (Tylosema esculentum), a decrease in content of non-cellulosic cell wall components was observed except arabinans that increased in abundance (Mosele *et al.*, 2011) Similarly, the authors hypothesised that the plasticity of arabinans may play a role in the protection of the tissues during seed desiccation. In non-irrigated condition, we also observed a lower DM of the HG in mature fruit. Removal of the hydrophobic methylester groups of HG may also increase the cell wall capability to retain water by formation of a more hydrophilic calcium linked gel structure (MacDougall & Ring 2004). In cells able to sustain dehydration such as pollen grain, HG was found to be present mainly under its low methylesterified form in the intine wall indicating that the demethylesterification process occurred before or during the dehydration process (Li *et al.*, 1995; Aouali *et al.*, 2001). Similarly, in a survey of cell wall compositional changes between hydrated and dehydrated resurrection plants, two species (Craterostigma wilmsii and Craterostigma plantagineum) among the six investigated showed a marked reduction of the DM of HG in dry state (Moore *et al.*, 2012). In the same way, the low level of *O*-acetyl groups of the XyG found in the fruit collected in non-irrigated condition may promote a more hydrophilic cell wall. Other significant changes in the cell wall were observed in the

composition of XyG with a clear reduction of fucosylated fragments in fruits submitted to water stress. The significance of this is not known but may be related to incomplete XyG biosynthesis or more degradation. In Arabidopsis, it has been observed in fucose-deficient mutants synthesizing XyG with little or no fucose that the level of *O*-acetylation was also importantly reduced suggesting that *O*-acetyltransferase may act preferably on fucosylated fragments (Perrin *et al.* 2003). Thus, the lower level of fucosylated fragments in non-irrigated condition may have impaired the transfer of *O*-acetyl groups. Finally, it appears that GAX displays a wider range of oligosaccharides after xylanase treatment from 5 to 10 pentoses in non-irrigated condition compared to 5 to 8 pentoses in irrigated condition. It may suggest that in mature fruit, the level of substitution of the xylan backbone by Ara residues is higher in non-irrigated condition. This suggestion is supported by a higher Ara/Xyl ratio in the most abundant hemicellulose-enriched fraction (i.e. KOH 1M). Interestingly, Ceusters *et al.* (2008) have shown a relationship between the fine structure of GAX (i.e. Ara/Xyl ratios) and the susceptibility to damage by high turgor pressure in several cultivars of Aechmea (Bromeliaceae) displaying high and low sensitivity to chlorenchyma cell bursting. The results indicated that low sensitive cultivars had low Ara/Xyl ratios while highly susceptible ones had higher Ara/Xyl ratios. These data support the hypothesis that GAX with low degree of substitution can cross-link more strongly cellulose microfibrils and permits the cell to withstand higher internal turgor pressure, thus preventing cell bursting. On the other hand, a high Ara/Xyl may allow more flexibility of the cell wall. In resurrection plant grasses, Moore *et al.*, (2012) also found that xylans were more arabinosylated in dehydrated plants compared to the hydrated ones. Together, these data suggested that structural changes may modulate the mechanical properties (i.e. flexibility) of the cell wall to avoid cell tearing during dehydration (Vicré, Farrant & Driouich 2004; Moore *et al.*, 2012).

In conclusion, our results reveal that during development of the date palm fruit, intensive pectin and hemicellulose solubilizations and loss of galactan side chains occurred (Table 3). Water deficit has profound effects on the fruit size, quality and cell wall composition. The cell wall of date palm fruit is more hydrophilic with lower levels of hydrophobic methyl and acetyl groups and a less extensive degradation of the hydrophilic galactan, arabinan and/or arabinogalactan polymers are observed (Table 3). Interestingly, the compositional cell wall modifications of the date palm fruit under water deficit are to some extent comparable to those observed in plants adapted to desiccation. These data highlight that changes in polysaccharide remodeling are an important process that can control the mechanical properties of the cell wall and the water status of the fruit cells under water deficit.

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4
5

Table 1. Estimation of fruit growth, water and cell wall contents of pulp from irrigated and non-irrigated date palm trees during fruit development [a,b].

	Irrigated			Non-irrigated		
	Stage 1	Stage 2	Stage 3	Stage 1	Stage 2	Stage 3
Mass of fruit (g fresh weight)	1.3 ± 0.3 a	13.1 ± 2.2 b	12.1 ± 1.0 b	0.3 ± 0.02 c	6.2 ± 0.6 d	5.9 ± 0.8 d
Mass of pulp (g fw) per fruit	1.2 ± 0.3 a	12.1 ± 2.2 b	11.2 ± 0.9 b	0.3 ± 0.02 c	5.1 ± 0.6 d	5.1 ± 0.7 d
Mass of pulp (g dry w) per fruit	0.2 ± 0.02 a	3.5 ± 0.9 b	8.6 ± 0.5 c	0.1 ± 0.01 d	1.0 ± 0.1 e	4.1 ± 0.7 f
Water content of pulp (%)	79.0 ± 6.0 a	71.2 ± 5.7 a	23.5 ± 2.7 b	72.2 ± 3 a	79.9 ± 7 a	20.4 ± 3.9 b
Cell wall content of dry pulp (%)	72.3 ± 6.9 a	30.7 ± 6.7 b	10.1 ± 0.6 c	70.9 ± 5.0 a	50.3 ± 5.4 d	10.9 ± 2.0 c

[a]Data are presented as means ± SE (n = 20 mass of fruit and pulp and n = 5 for the extraction yield).

[b]Different letters indicate significant differences ($P < 0.05$). Statistical analyses compared the values during fruit development within the same water irrigation condition and between the two water irrigation conditions at the same developmental stage.

Table 2. Relative quantification of XyG oligosaccharides obtained after *endo*-glucanase digestion of palm fruit cell wall during development from irrigated and non-irrigated conditions [ab]

Mass[c]	Composition[d]	Structure[e]	Relative abundance[f] (%)					
			Irrigated			Non-irrigated		
			1	2	3	1	2	3
791	Hex_3Pent_2	XXG	-[g]	9.4 ± 0.1 a	12.5 ± 0.2 b	-	11.2 ± 0.6 c	13.5 ± 0.5 d
953	Hex_4Pent_2	XXGG/GXXG	-	-	4.6 ± 0.02 a	-	5.2 ± 0.2 a	5.9 ± 0.4 b
1085	Hex_4Pent_3	XXXG	-	38.3 ± 2.4 a	49.9 ± 0.4 b	-	49 ± 1 b	57.7 ± 0.3 c
1247	Hex_5Pent_3	XLXG/XXLG	-	6.8 ± 0.2 a	6.7 ± 0.2 a	-	7.4 ± 0.1 b	8.4 ± 0.2 c
1393	$Hex_5Pent_3Dox_1$	XXFG	-	7.1 ± 0.3 a	6.8 ± 0.1 a	-	5.1 ± 0.2 b	-
1409	Hex_6Pent_3	XLLG	-	3.6 ± 0.4 a	1.8 ± 0.1 b	-	2.8 ± 0.3 c	-
1435	$Hex_5Pent_3Dox_1OAc_1$	XX**F**G	-	17.1 ± 0.4 a	10 ± 0.2 b	-	12 ± 0.7 c	9.8 ± 0.3 b
1555	$Hex_6Pent_3Dox_1$	XLFG	-	3.6 ± 0.4 a	2.3 ± 0.1 b	-	2.1 ± 0.1 b	4.4 ± 0.2 c
1597	$Hex_6Pent_3Dox_1OAc_1$	XL**F**G	-	13.9 ± 0.2 a	5 ± 0.2 b	-	5.1 ± 0.2 b	-
	Total fucosylated fragments		-	40.7 ± 2.5 a	24.1 ± 0.1 b	-	24.3 ± 1.1 b	14.2 ± 0.2 c
	Total *O*-acetylated fragments		-	30.1 ± 1.7 a	15 ± 0.1 b	-	17.1 ± 0.9 c	9.8 ± 0.3 d

[a]Data are presented as means ± SE (n = 3 different cell wall extracts).

[b]Different letters indicate significant difference ($P< 0.05$). Statistical analyses compared the values during fruit development within the same water irrigation condition and between the two water irrigation conditions at the same developmental stage.

[c]Mass of the fragments $[M+Na^+]^+$.

[d]Hex. hexose; Pent. pentose; Dox. deoxyhexose; OAc. *O*-acetyl substituent.

[e]XyG oligosaccharide structures described by Lerouxel *et al.,* (2002). Bold and underlined letter indicates the position of the *O*-acetyl group on the side chain.

[f]1, 2 and 3 correspond to the three development stages.

[g]not detected above the signal to noise ratio.

Table 3. Summary of the major cell wall changes observed during the ripening of date palm fruit collected from trees submitted or not to water irrigation [a]

	Irrigated			Non-irrigated		
Developmental stages	1	2	3	1	2	3
Loosely bound pectins						
DM of pectin						
Arabinose content						
Galactose loss						
Hemicellulose solubilisation						
Fucosylated and *O*-acetylated XyG						
Ara/Xyl ratio						

[a]DM. degree of methylesterification of HG, XyG. Xyloglucan, Ara. arabinose, Xyl. xylose.

List of Figures.

File includes 10 Figures

Figure 1. Comparison of the development of representative date palm fruits collected 77 (stage Khimri), 140 (stage Khalal) and 232 (stage Tamar) days after pollination (DAP) from (a) irrigated and (b) non-irrigated trees. Stage 3 corresponds to the mature fruit. Scale bars = 1 cm.

Figure 2. Yields of the sequentially extracted pulp cell wall fractions from irrigated (I) and non-irrigated (N.I) date palm trees during fruit development. (a) hot water, (b) ammonium oxalate, (c) Na_2CO_3, (d) KOH 1M, (e) KOH 4M and (f) insoluble residue. Percentage represents the weight of fractionated cell wall extract on the weight of starting cell wall material. Different letters indicate significant differences ($P < 0.05$). Statistical analyses compared the values during fruit development within the same water irrigation condition and between the two water irrigation conditions at the same developmental stage.

Figure 3. Comparison of monosaccharide composition of the pectin-enriched fractions extracted from the cell wall of pulp fruit with (a) boiling water, (b) ammonium oxalate and (c) Na_2CO_3 during development in irrigated (I) and non-irrigated (N.I) palm groves. 1, 2 and 3 represent the three developmental stages. Ara. arabinose; Rha, rhamnose; Fuc, fucose; Xyl, xylose; GalA, galacturonic acid; Man, mannose; Gal, galactose; Glc, glucose. Different letters indicate significant difference at $P < 0.05$.

Figure 4. Dot-blot immuno-assays of hot water pectin-enriched fraction extracted from the flesh of mature date palm fruit (stage 3) collected in irrigated palm groves.

8, 4, 2, and 1 µg of extract were probed with LM19 for weakly methylesterified HG, LM20 for methylesterified HG, LM6 for (1→5)-α-L-arabinan, LM5 for (1→4)-β-D-galactan, and LM13 for stretch of unbranched (1→5)-α-L-arabinan. Controls (8 µg) were (1) pectin with 85% methylesterification from citrus, (2) unesterified pectin from citrus, (3) gum arabic from acacia, (4) pectin with 8.6% methylesterification from citrus and (5) arabinan from sugar beet.

Figure 5. Comparison of (a) the ratio of the main pectic motifs (HG in grey bars and RG-I backbone in white bars) and (b) the branching patterns of RG-I in the pectin-enriched fractions extracted with H_2O (boiling water), Oxalate (ammonium oxalate) and Na_2CO_3 at stages 1, 2 and 3 in irrigated (I) and non-irrigated (N.I) palm groves.
HG (%) = (GalA – Rha) / (GalA + Rha) x 100. RG-I backbone (%) = 2 Rha / (GalA + Rha) x 100. (Ara + Gal) / Rha gives information concerning the level of substitution (galactan, arabinan and/or arabinogalactan) on the RG-I backbone. Different letters indicate significant difference at $P < 0.05$.

Figure 6. Estimation of the degree of methylesterification (DM%) of the pectin-enriched fractions extracted from irrigated and non-irrigated date palm trees during fruit development. Data are presented as means ± SE (n = 6 different spectra from 3 independent samples). Different letters indicate significant differences ($P < 0.05$). Statistical analyses compared the values during fruit development within the same water irrigation condition and between the two water irrigation conditions at the same developmental stage.

Figure 7. Comparison of monosaccharide composition of the hemicellulose-enriched fractions extracted from the cell wall of pulp fruit with (a) KOH 1 M and (b) KOH 4 M during development in irrigated (I) and non-irrigated (N.I) palm groves. 1, 2 and 3 represent

the three developmental stages. Ara. arabinose; Rha, rhamnose; Fuc, fucose; Xyl, xylose; GalA, galacturonic acid; Man, mannose; Gal, galactose; Glc, glucose. Different letters indicate significant difference at $P < 0.05$.

Figure 8. Comparison of the level of branching of the xylan backbone by arabinosyl residues in the arabinoxylan-enriched fractions (KOH 1 M and 4 M) during fruit development (stages 1, 2 and 3) in irrigated (I) and non-irrigated (N.I) palm groves. Different letters indicate significant difference at $P < 0.05$.

Figure 9. Relative quantification of GAX oligosaccharides obtained after *endo*-xylanase digestion of depectinized and delignified cell wall from mature fruit (stage 3) harvested in irrigated (I) and non-irrigated (N.I) conditions. Pen. Pentose, MeGlcA. 4-*O*-methylglucuronic acid. Data are presented as means ± SE (n = 3 different cell wall extracts). Different letters indicate significant difference ($P< 0.05$). * not detected above the signal to noise ratio.

Figure 10. Dot-blot immuno-assays of (a) 1M and (b) 4M KOH hemicellulose-enriched fractions extracted from the pulp of date palm fruit collected in irrigated palm groves. 8, 4, 2, and 1 µg were probed with LM10 for low substituted xylan and LM11 for partially substituted arabinoxylan. Controls (8 µg) were (1) xylan from larchwood and (2) arabinoxylan from wheat.

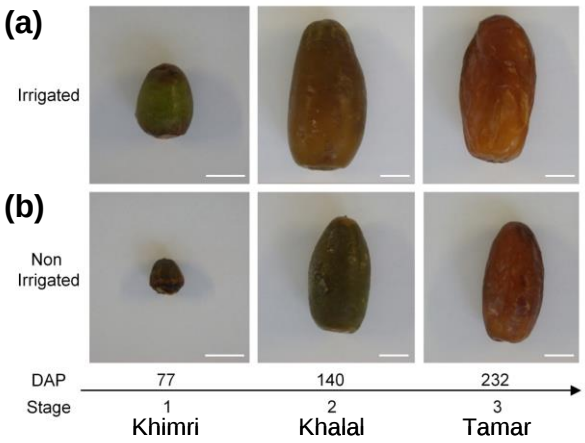

(a)
Irrigated
(b)
Non
Irrigated
DAP
77
140
232
Stage
1
2
3
Khimri
Khalal
Tamar

Figure 1

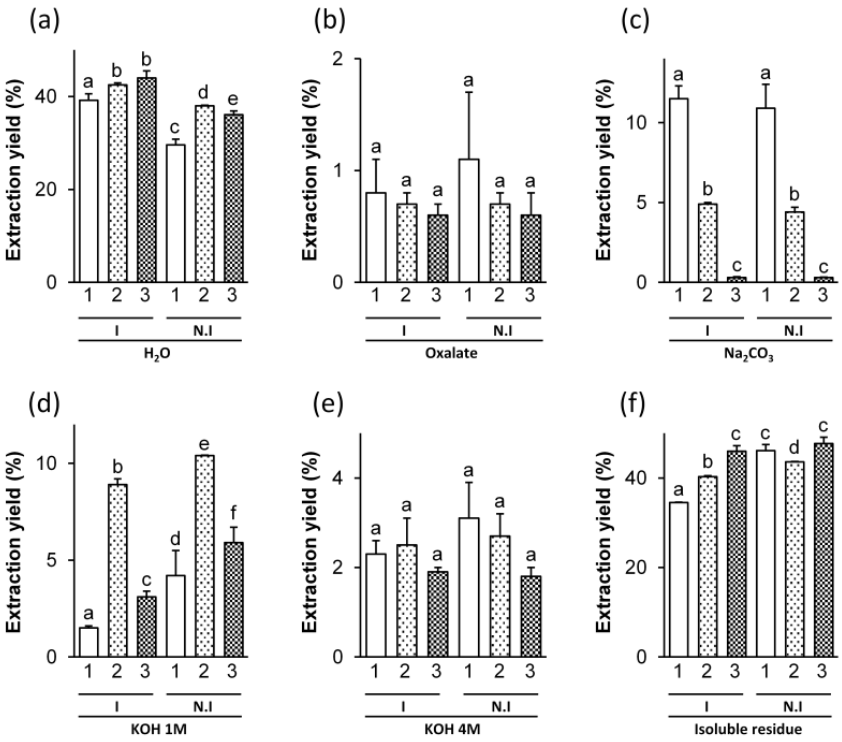
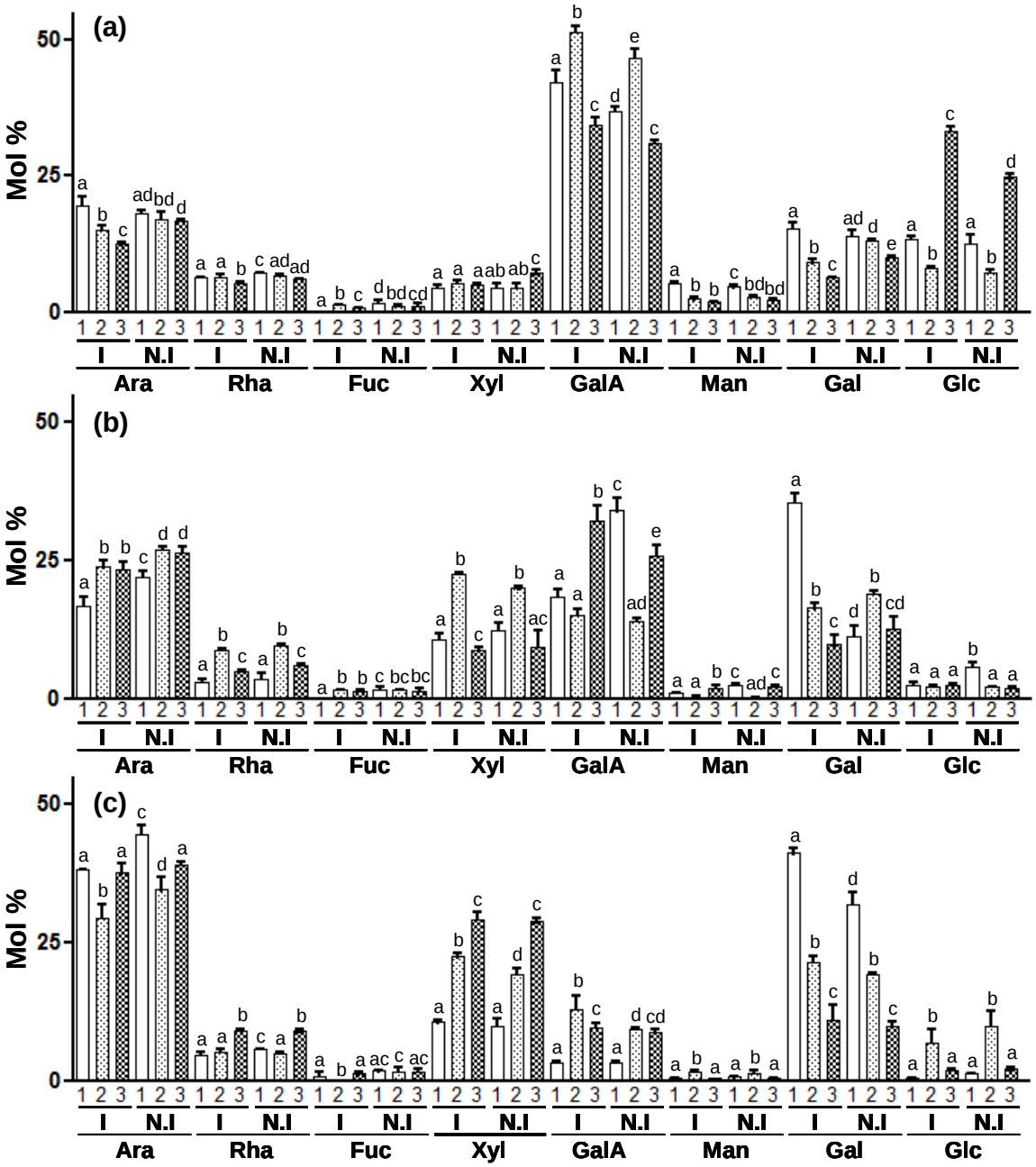

(a)
(b)
(c)
(d)
(e)
(f)
Extraction yield (%)
I
N.I
H2O
Oxalate
Na2CO3
KOH 1M
KOH 4M
Isoluble residue

Figure 2



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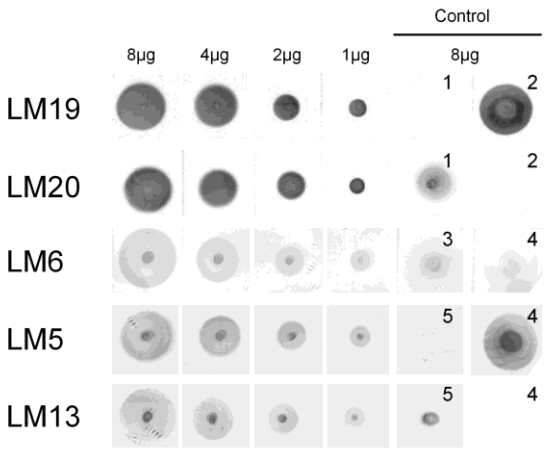

Control
8µg
4µg
2µg
1µg
8µg
LM19
LM20
LM6
LM5
LM13
1
2
1
2
3
4
5
4
5
4

Figure 4

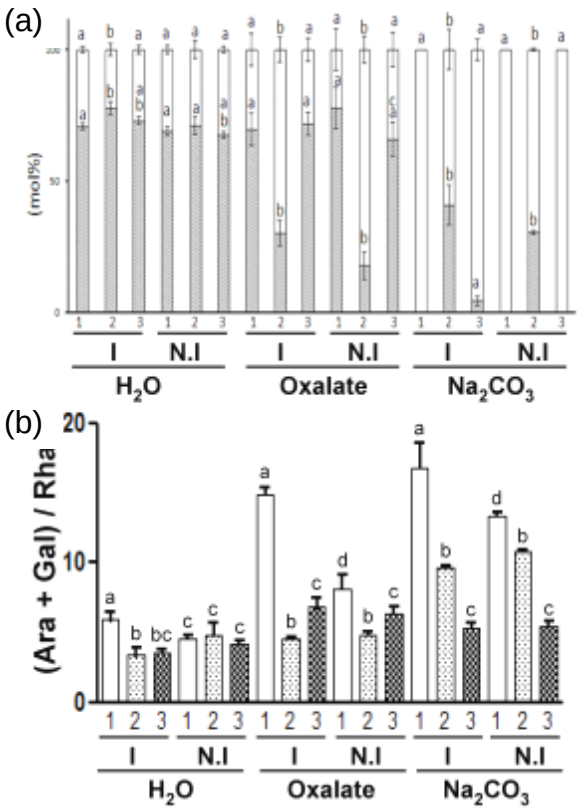


Figure 5

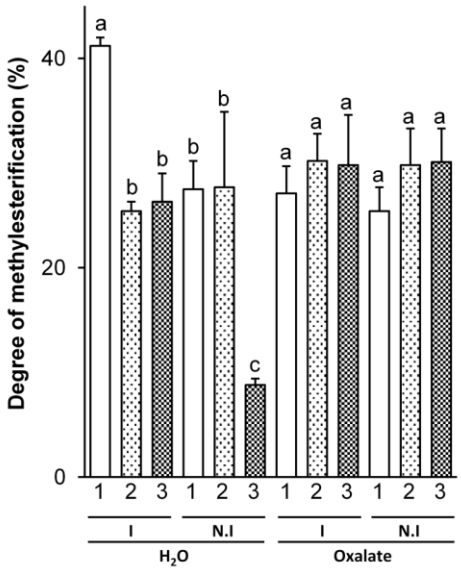

Degree of methylesterification (%)
40
20
0
a
b
b
b
b
c
a
a
a
a
a
a
1 2 3 1 2 3 1 2 3 1 2 3
I
N.I
I
N.I
H2O
Oxalate

Figure 6

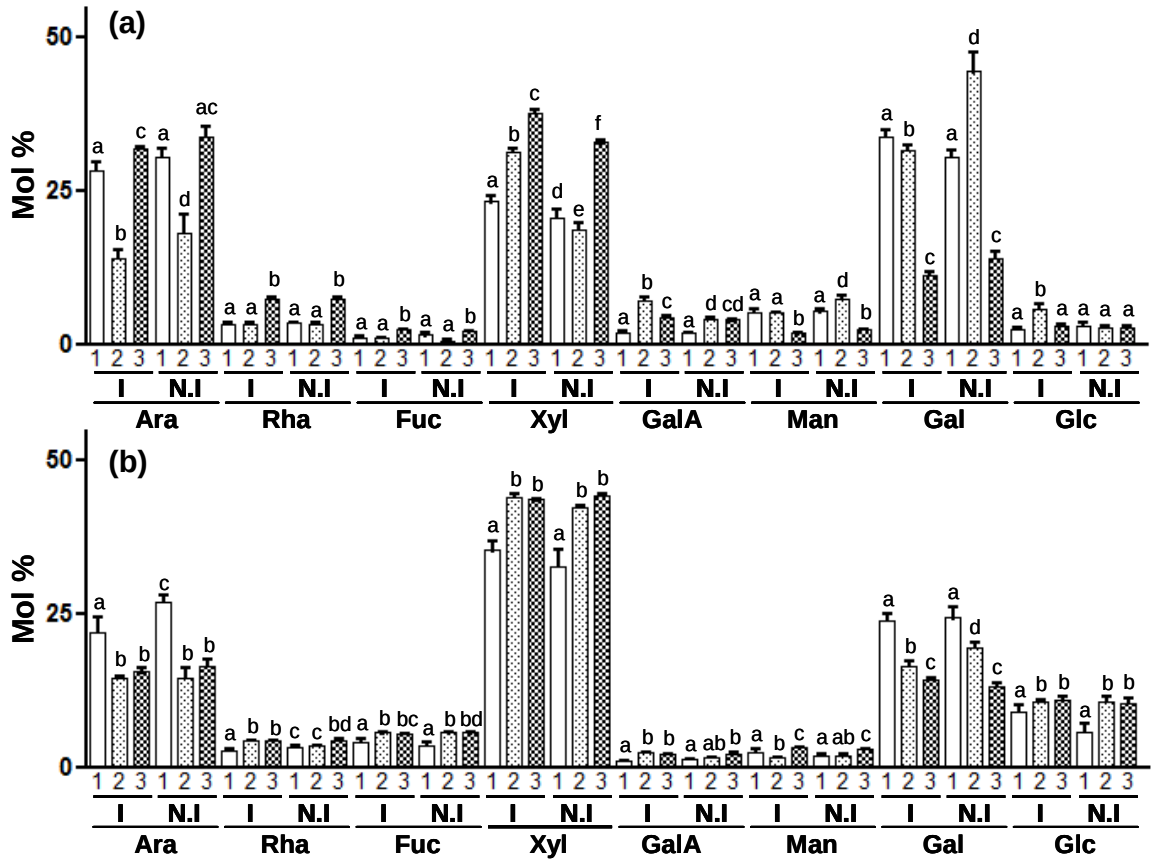
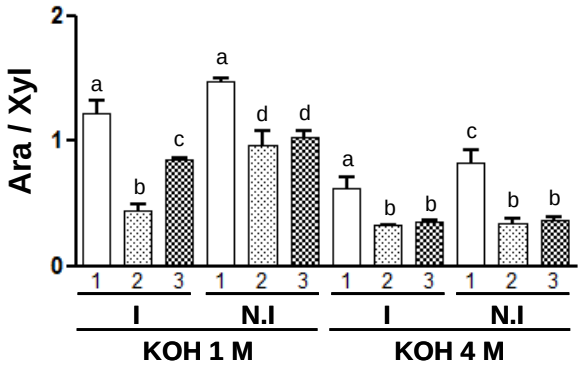


Figure 7



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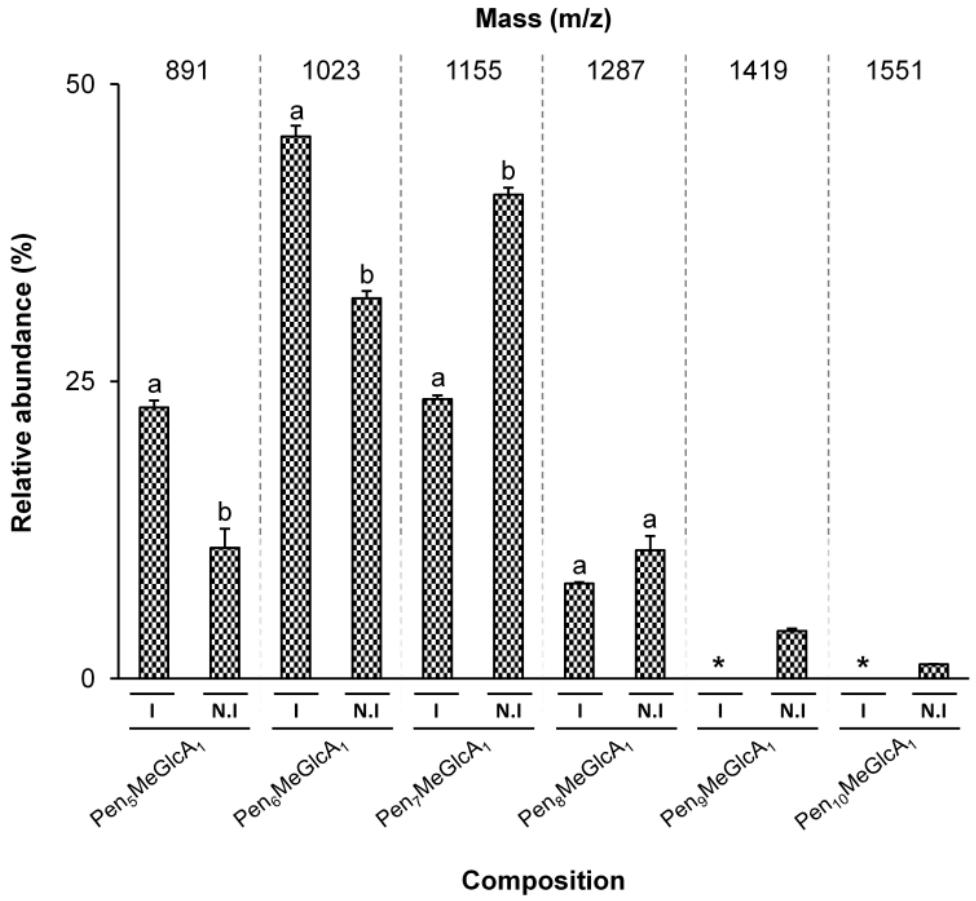
Mass (m/z)
891
1023
1155
1287
1419
1551
Relative abundance (%)
50
25
0
a
b
a
b
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b
a
a
*
*
I
N.I
Pen5MeGlcA1
Pen6MeGlcA1
Pen7MeGlcA1
Pen8MeGlcA1
Pen9MeGlcA1
Pen10MeGlcA1
Composition

Figure 9

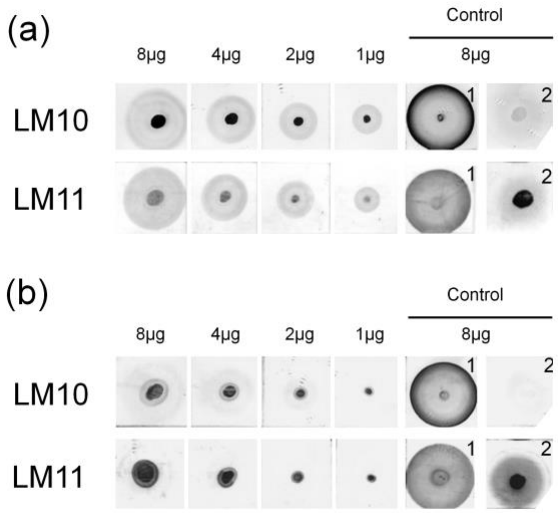
(a)
Control
8µg
4µg
2µg
1µg
8µg
LM10
1
2
LM11
1
2
(b)
Control
8µg
4µg
2µg
1µg
8µg
LM10
1
2
LM11
1
2

Figure 10

SUPPORTING INFORMATION includes 5 supporting Figures

Figure S1. Dot-blot immuno-assays of hot water pectin-enriched fraction extracted from the flesh of mature date palm fruit (stage 3) collected in non-irrigated palm groves. 8, 4, 2, and 1 µg of extract were probed with LM19 for weakly methylesterified HG, LM20 for methylesterified HG, LM6 for (1→5)-α-L-arabinan, LM5 for (1→4)-β-D-galactan, and LM13 for stretch of unbranched (1→5)-α-L-arabinan. Controls (8 µg) were (1) pectin with 85% methylesterification from citrus, (2) unesterified pectin from citrus, (3) gum arabic from acacia, (4) pectin with 8.6% methylesterification from citrus and (5) arabinan from sugar beet.

Figure S2. FT-IR spectra of (a) commercial pectins and the hot water extracts from the pulp cell wall in (b) irrigated and (c) non-irrigated conditions. In (a), commercial pectins from citrus with a: 8.6% DM, b: 20-30% DM and c: 85% DM. In (b) and (c), 1, 2 and 3 correspond to the development stages of the date palm fruits.

Figure S3. MALDI-TOF mass spectra of *endo*-glucanase-generated XyG fragments from the pulp cell wall of date palm at stage 2 and 3 in irrigated and non-irrigated conditions. Spectra from cell wall of date palm at (a) stage 2 and (b) stage 3 in irrigated condition. Spectra from cell wall of date palm at (c) stage 2 and (d) stage 3 in non-irrigated condition. The structures of the XyG fragments are shown according to the nomenclature proposed by Fry *et al.* (1993). Underlined and boldfaced letters represent *O*-acetylated side chains.

Figure S4. MALDI-TOF mass spectra of *endo*-xylanase-generated GAX fragments of the depectinized and delignified pulp cell wall of mature date palm fruit from (a) non-irrigated and (b) irrigated conditions.

Figure S5. Dot-blot immuno-assays of (a) 1M and (b) 4M KOH hemicellulose-enriched fractions extracted from the pulp of date palm fruit collected in non-irrigated palm groves. 8, 4, 2, and 1 µg were probed with LM10 for low substituted xylan and LM11 for partially substituted arabinoxylan. Controls (8 µg) were (1) xylan from larchwood and (2) arabinoxylan from wheat.

1