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Investigation of parietal polysaccharides from *Retama raetam* roots

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This study characterizes the cell wall hemicellulose and pectins polymers of *Retama raetam*. This species develops a particularly important root system and is adapted to arid areas. The cellulose, hemicelluloses and pectins were extracted. The cellulose remains the major component of the wall (27% for young roots and 80% for adult roots), hemicelluloses (14.3% for young roots and 3.6% for adult roots) and pectins (17.3% for young roots and 4.1% for adult roots). The monosaccharidic composition of water soluble extracts determined by gas liquid chromatography (GLC) and completed by infrared (FTIR) spectroscopy of hemicellulosic shows the presence of xylose as a major monosaccharide in the non-cellulose polysaccharides (47.8% for young roots and 59.5% for adult roots). These results indicate the presence of the homogalacturonans and rhamnogalacturonans in pectin. This study constitutes the preliminary data obtained in the biochemical analysis of the parietal compounds of the roots of a species which grows in an arid area in comparison with those of its aerial parts.

Key words: *Retama raetam*, roots, cell wall, investigation, polysaccharides, monosaccharidic.

INTRODUCTION

The cell wall compartment is the subject of many studies because of the important roles that it plays in the vegetal cell (growth, protection, defence against the phyto pathogenes). The components of the cell wall are subjected to applied research since they constitute 80% of the vegetal biomass (Robert and Roland, 1989). The structural diversity of plant cell wall polysaccharides has brought lots of application in many diverse domains. For

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example, polysaccharides can be used in the metal biosorption (Hachem et al*.,* 2012) or in the cholesterol biosorption (Copikova et al*.,* 2013). Some hemicelluloses present immune-stimulating properties (Caffall and Mohnen, 2009) or inhibiting properties of cellular proliferation (Barbat et al., 2010).

Few studies have been conducted in relation to the walls of roots (Leucci et al., 2008). This is the reason a biochemical study of the walls of the roots of *R. raetam* was undertaken. This species belonging to the Fabaceae family has a very productive vertical and horizontal root system which can reach 20 m. This in turn increases substantially the stabilization of the soil. Moreover, the *Retama* species contributes to the bio fertilisation of poor grounds because of their aptitude to associate with fixing nitrogen bacteria *Rhizobia* (Boulila et al., 2009; Selami et al*.,* 2014). Therefore, the genus of the *Retama* is included in a re-vegetation program for degraded areas in semi-arid Mediterranean environments (Caravaca et al*.,* 2003). This preliminary study on the biochemical analysis of the parietal compartment of *R. raetam* roots could help in developing future valorization strategies of cell wall polysaccharides (bioactives molecules, cellular signalisation).

MATERIALS AND METHODS

Biological materials

Seeds, stems and leaves of *R. raetam,* were collected in Oran, Algeria, in 2012, and identified by Pr. M. Kaid Harche, University of Sciences and Technology, Mohamed Boudiaf. A voucher specimen (F 2652) was deposited at the Herbarium of the Department of Biotechnology, University of Sciences and Technology, Mohamed Boudiaf, Oran, Algeria.

Plant samples consist of *R. raetam* young and adult roots. In the case of the young roots, plantlets were obtained from 10 days old germinations. Two centimeter long fragments were excised from the above apical zone of roots. For adult roots, 2 cm long-fragments were excised from the differentiated zone of 5 year old plants growing in natural conditions (USTO university campus). The samples were collected in March 2012.

Extraction of cell wall polysaccharides

The sequential and selective extraction of parietal polysaccharides of the roots of *R. raetam* was carried out according to Bailey (1967) and Carpita (1984). The extractions were carried out under magnetic agitation and the residues were separated from the supernatants by filtration on sintered glass (porosity 3). Dialysis was carried out in a Spectrapor membrane whose cutting threshold lies between 6000 and 8000 Da. The steps are presented in Figure 1.

Colorimetric assay of total sugars

The uronic acid contents of the polysaccharidic fractions were determined following the meta-hydroxydiphenyl method (Blumenkrantz and Asboe Hansen, 1973). Glucuronic acid was used as standard. The determination of the content of neutral sugars of polysaccharidic fractions was carried out by the method with phenol/H_2SO_4 (Dubois et al., 1956) with glucose and xylose as standards. A correction by calculation was made in order to take into account the interferences due to the presence of the uronic acids as established by (Montreuil et al., 1963):

$[ON] = (DO_{Phenol} - b^* [AU]) / a$
$[AU] = (DO_{MHDP} -(a' / a) * DO_{Phenol}) / ((ab'-a'b) / a)$
[ON]: Concentration in neutral sugars
[AU] = uronic acids concentration
a = Slope (Glc) of the assay of the neutral sugars
b = slope (GalA) for the assay of the neutral sugars
$DO_{Phenol} = a [ON] + b [AU]$
a' = Slope (Glc) for the assay of the uronic acids
b' = slope (GalA) for the assay of uronic acids
$DO_{MHDP} = a' [ON] + b' [AU]$.

Qualitative analysis by gas liquid chromatography (GLC)

Prior to analysis, polysaccharidic fractions were submitted to methanolysis. The methylglucosides released obtained were then derivatized by trimethylsilylation.

Preparation of themethyl glycosides trimethylated derivatives

The analysis was carried out on 200 to 500 µg of freeze-dried polysaccharide powder, added to an internal standard, mesoinositol (MI), at a rate of 10% of the quantity of polysaccharide. This method was used for the direct analysis of crude plant powders (Marga et al*.,* 1995). It can also be used for the study of the monosaccharidic composition of the residues of extraction.

Methanolysis

Monosaccharides were released as methylglycosides by adding 1 mL of 1 M hydrochloric methanol to the anhydrous polysaccharide sample. After 24 h, at 80°C in a sealed tube, the methanolisis was stopped by evaporation of the hydrolyzate under a nitrogen flux. The residue was dissolved in 1 mL methanol and then delipidated by three successive extraction with heptane (3 x 1 mL). Finally, it was evaporated again under nitrogen flux.

Trimethylating

The methylglycosides were then trimethylated with 100 µL of N,O-bis-trim ethylsilyl- trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS) in pyridine (100 µL), in the darkness during 2 h at 27°C. These samples were maintained at -20°C during 24 h and then directly injected in GLC.

Analysis of the trimethylsilylation derivatives by gas liquid chromatography

Methylglycosidestrimethylsylilated have been identified by GLC by comparison with authentic samples using a Perichrom PR 2100 chromatograph equipped with a capillary tube (0.32 mm by 60 m) OPTIMA® 1-Accent 0.25 µM (Macherey-Nagel) and with a flame ionization detector (FID). The carrier gas is nitrogen under a pressure of 75 kPa. The temperature of the injector was fixed at 260°C. The rise in the temperature of the furnace has been programmed from 130 to 210°C at a rate of $2°C.min^{-1}$, with a 5 min mitigation at 190°C, then from 210 to 260°C at a rate of $5°C.min^{-1}$.

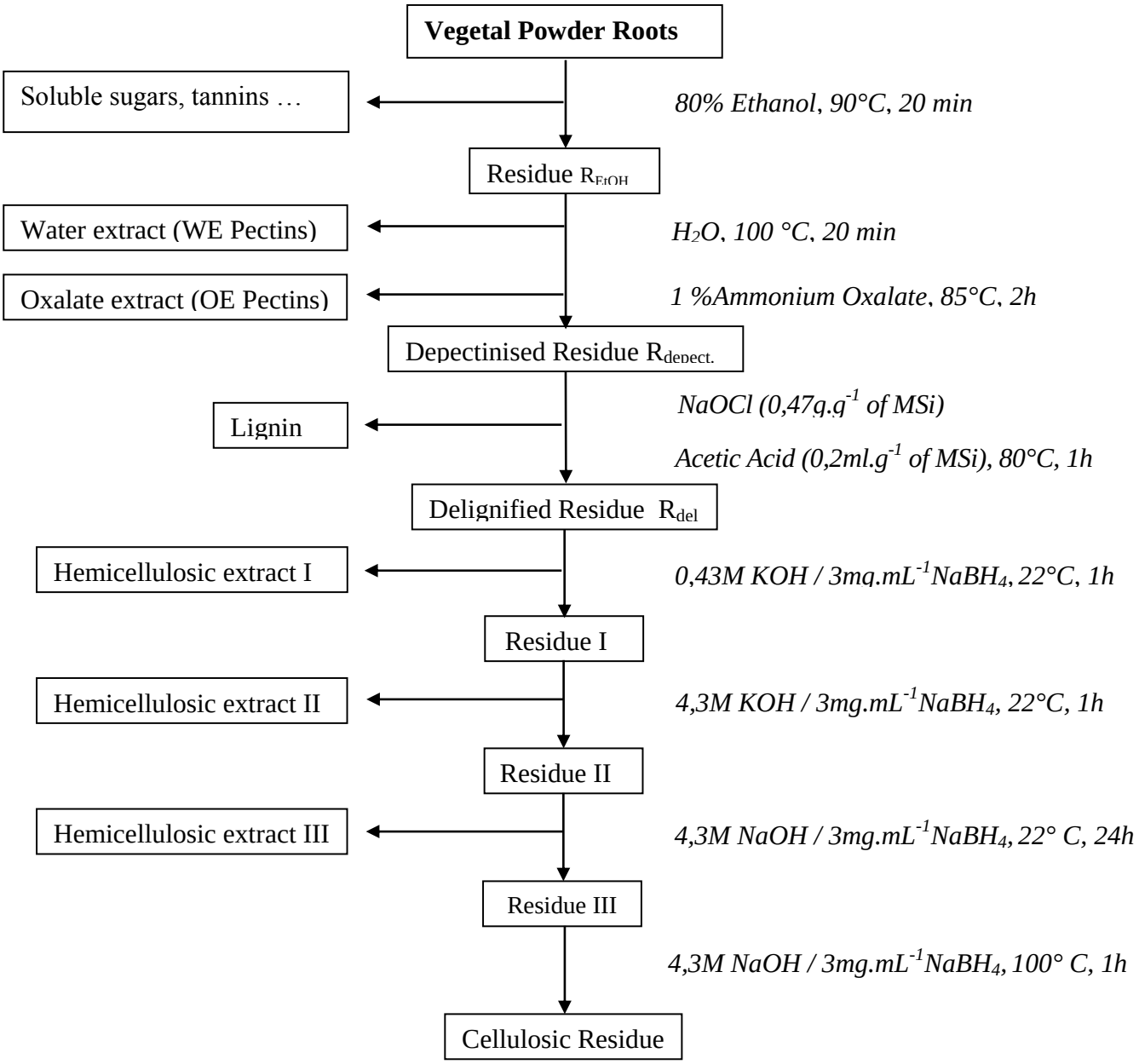

Figure 1. Selective and sequential extraction protocol of cell wall polysaccharides adapted from Bailey (1967) and Carpita (1984).

The chromatograph has been controlled by the software Winilab III (Perichrom). The results were expressed as a molar percentage after correction of the areas of the chromatographic peaks by the response factors.

Evaluation of the degree of methylesterification of pectins

A biochemical assay of methanol was carried out after saponification on extracted pectic fractions (WE and OE) in order to determine the degree of methylesterification of pectins. The methanol compound was determined by visible UV spectrometry: the coupling of Purpald with methanol produced by the enzymatic oxidation of methanol led to the formation of a purple compound dosed at 550 nm as described by (Anthon and Barrett, 2008).

The methanol concentration was estimated by reference to a methanol standard (0 and 40 µg.L $^{-1}$). 10 mg of WE and OE were mixed with 1 ml of 0.1 M NaOH in order to saponify the ester functions. The suspension was maintained to 4°C during 15 h. After neutralization of excess NaOH by adding hydrochloric acid, the suspended particles were eliminated by centrifugation (12000 *g*, 5 min, and 4°C). The supernantant constitute the sample for methanol assay. A volume of 100 µL (or diluted sample) or standard was added to 90 µL of 200 mM potassium phosphate buffer, pH 7, then 10 µL of enzymatic

Table 1. Extraction yields of extracted fractions from young roots (YR) and adult roots (AR). All fractions were dialysed before lyophilisation and weighting.

Parameter		Extracted Quantity in g	Extraction yields expressed in percent of dry mass	Uronic acid content (%)
Ethanolic extract	YR	0.3	14.6	Nd
	AR	1	6.4	Nd
WE	YR	0.2	9.7	16.2
	AR	0.4	2.6	15.6
OE	YR	0.1	7.6	32.5
	AR	0.2	1.5	20.8
Hemicellulosic Fractions	YR	0.2	14.3	11.1
(I , II and III)	AR	0.6	3.6	12.4
Cellulosic fractions	YR	0.5	27	Nd
	AR	12.8	80	Nd

WE, water extract; OE, oxalate extract; YR, young roots; AR, adult roots; Nd, not detected.

alcohol oxydase solution (Alcohol oxidase from *Pichia pastoris*, Sigma) freshly prepared in the same phosphate buffer. The samples was homogenized, then incubated for 10 min at 30°C. Then 200 µL of Purpald at 5 $mg.mL^{-1}$freshly were prepared in 0.5 M NaOH were added. The samples were homogenized then incubated for 40 min at 30°C. Finally, 600 µL of water were added. After homogenisation, the absorbance was measured with a UV-Visible spectrophotometer (Shimadzu, PharmaSpec UV-1700). The results were expressed in percentage of the molar quantity of methanol in relation to the molar quantity of galacturonic acid of the extract determined by GLC.

Characterization by infrared (IR) spectroscopy

Polysaccharidic fractions were characterized by infrared (IR) spectroscopy with a 1000 FT-IR Perkin Elmer Spectrum spectrometer in the 400 to 4000 cm^{-1} frequency range.

RESULTS AND DISCUSSION

Yield of extraction of polysaccharidic components

The quantities of *R. raetam* root parietal components are represented in Table 1. The compounds extracted by ethanol represent 14.6% of the mass of the young roots and 6.4% of the adult roots. This difference in percentage was probably due to a more efficient elimination in adult roots of pigments, tanins or suberin. The WE fractions were relatively important compared to OE, and contained from 15.6 to 32.5% of uronic acids. The OE fractions was represented as 7.6% against 9.7% for young roots and 1.5% against 2.6% for adult roots expressed in percentage dry mass. The percentage of pectic substances in adult roots seemed lower than that of young roots. This could be explained by the inaccessibility of these polymers since strong linkages (covalent) between lignins, hemicelluloses and these pectins may exist; this impair the attack by chemical treatment.

Young roots showed a high hemicellulose content compared to adult roots (14.3% against 3.6%), and were mainly composed of contained neutral oses (89% in average). Conversely, the percentage of cellulose in adult roots (80%) was definitely higher than that in young roots (27%). However, the total extraction yields were higher for adult roots (94% of the DW) than for young roots (73% of the DW). It can be noticed that cellulose represented the majority (80%) of the cell wall, the hemicellulosic fraction represented 14.3% for young roots and 3.6% for adult roots. Hemicelluloses were less accessible in the secondary walls lignified tissues (Harche et al.,1989).

The percentages of the pectin fractions WE and OE were relatively low for young and adult roots (9.7 and 2. 6%) respectively, for WE and 7. 6% and 1.5% for OE. This could be explained by the presence of covalent links between pectins and other parietal components**,** but also linkages between pectins exist (rhamnogalacturonans II). According to Thibault and Saulnier (1991), the extraction of pectins (which form an eggbox structure) could be improved by a chelating agent of metals like ammonium oxalate (Golovchenko et al., 2012).

Xu et al*.* (2007) have recorded losses in weight of about 24% of the initial mass, following the treatment of depectinisation with hot water of the leaves of some Poaceae.

Determination of the monosaccharidic composition by GLC

The filtrates and the residues resulting from the various extractions have been characterized by GLC (Table 2).The analysis of the WE and OE of young and adult roots, confirmed their pectic nature by the high rate of galacturonic acid (13.4 and 14.6% for the WE of the young and adult roots respectively, 28.6% and 19% at the OE respectively). The rhamnose rates varied from 5

Table 2. Monosaccharidic composition of the extracts and the residues of extraction, from *R.raetam* young roots (YR) and adult roots (AR)

Fractions	Monosaccharides (% molar)							
	Ara	Rha	Xyl	Man	Gal	Glu	Gal A	Glc A
Young roots (crude extract)	23.9	6.6	9.1	2.2	10.7	30.2	15.4	1.0
Adult Roots (crude extract)	21.3	6.2	15.7	0.2	2.5	44.7	6.9	1.7
WE YR	19.4	7.9	8.0	5.0	17.5	25.2	13.4	3.4
WE AR	9.9	5.8	2.6	0.8	4.7	62.2	14.6	1.2
OE YR	19.0	10.2	12.0	0.2	11.9	17.3	28.6	0.2
OE AR	14.0	9.8	7.1	0.5	7.8	40.9	19.0	1.0
Hemicelluloses YR	30.6	11.5	47.8	0.4	17.9	10.0	10.3	1.4
Hemicelluloses AR	8.0	6.4	59.5	0.7	6.1	4.9	11.7	1.0
CelluloseYR	Nd	Nd	3.2	3.5	Nd	90.1	3.2	Nd
Cellulose AR	Nd	Nd	4.9	Nd	5.8	86.6	2.7	Nd

WE, water extract; OE, oxalate extract; YR, young roots; AR, adult roots; Nd, not detected.

to 10%, this led us to suppose that these fractions also contain rhamno-galacturonans which could be substituted by side chains of arabinans, of galactans and/or arabino-galactans. Other identified monosaccharides (mannose, glucuronic acid) were detected at low rates (less than 5%). Consequently, the ratio Gal A/Rha was decreased (2.8% to 1.9% for OE of young and adult roots respectively). This is typical of more ramified structural pectic features.

The hemicellulosic fractions extracted with KOH were rich in xylose (from 47.8 to 59.5 %) which indicated the presence of xylans. The presence of arabinose (8 to 30.6%) indicated the presence of arabinoxylans. Galactose and galacturonic acid were also present (from 6 to 17.9% and 10.3 to 11.7%, respectively); this could be explained by a co-extraction of pectic polymers.

The final residue of the extraction was largely composed of glucose (86 to 90%), monosaccharide characteristic parietal glucans such as cellulose. The presence of low levels of remaining xylose (3 to 5%) suggests that hemicellulosic polymers were co-extracted almost quantitatively during the extraction. According to Chaa et al*.,* (2008), the presence in these features of xylose and arabinose, after the various treatments of depectinisation seems to indicate that hemicellulosic polymers were co-extracted during this stage of depectinisation. Xu et al*.* (2007), noted that the treatment of depectinisation by hot water solubilizes, in addition to pectins, low molecular weight polysaccharides such as galacto-arabinoxylans and also recorded weight losses of about 24% of the initial mass after hot water treatment; the hemicelluloses fractions represented 35% of initial mass and the lignins 10.4%.

The presence of glucose in the WE of young roots and OE of adult roots with contents of 25.2, 62.2, 17.3 and 40.9% respectively suggested that this detected monosaccharides comes from the hydrolysis of the callose, a polysaccharide which is present in the walls of young and mature sieved tube (Currier, 1957). In fact, an amylase treatment has shown a persistence of glucose in the WE of adult roots (85% before treatment and 50% after) and in the OE of the adult roots (88% before treatment and 33.1% afterwards). These results make it possible to argue that in *R. raetam* roots, pectins were strongly related to the callose as observed in the pollen of Nicotiana (Kroh and Knuiman, 1982). We can also suggest that the strong proportion of glucose in the two fractions probably comes from the residual hydrolysis of intra-parietal saccharose. In fact, the conveying of saccharose in fabaceae was primarily performed by means of the apoplasmic pathway (Dinant and Lemoine, 2010). The hemicellulosic fractions extracted with KOH were rich in xylose (from 47.8 to 59.5%) which indicated the presence of xylans. The presence of arabinose (8 to 30.6%) indicated the presence of arabinoxylans (Chaa et al., 2008). Galactose was also present (from 6 to 17.9%); this could be explained by a co-extraction of pectic polymers. They also contained galacturonic acid at 10.3 to 11.7%. Nevertheless, the presence of xyloglucan features in the hemicellulosic fractions was not to be excluded because the Xyl/Glc ratio was from 5 to 12 for young and adult roots respectively.

The composition of the cellulose to glucose residue (Brannavall, 2007) confirms the selectivity of the extraction of other cell wall polysaccharides and thus the good efficiency of the extraction protocol implemented.

Degree of methylesterification (DM) of the pectins extracted from young and adult roots

The DM was established by the relation between the quantity of released methanol during saponification (UV-visible spectrophotometry assay after derivation with Purpald) and the galacturonic quantity of acid previously was determined by GLC. The values obtained were in

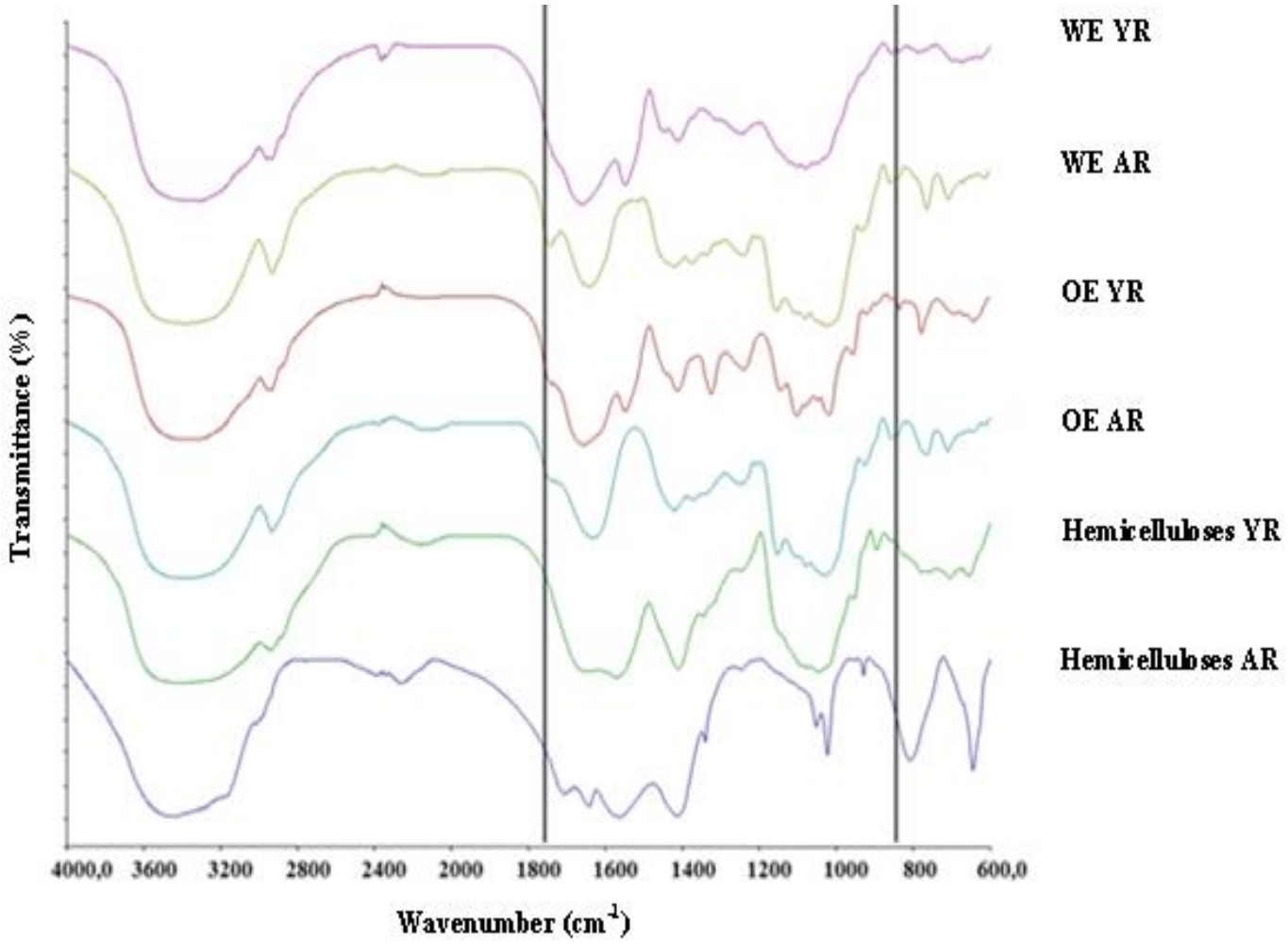

Figure 2. Infrared spectrum of polysaccharides obtained after different extraction steps.

conformity with the extraction strategy: in the pectic fractions (WE), the DMs were often high between (63 and 79%). A strong DM was often correlated with a small proportion of structure of the "egg box" type within pectins. Consequently, these were more easily extracted by hot water. In the case of the pectic fractions with low DM ranging between 50 to 62%, structural features rich in homogalacturonans might be present.

Characterization by infrared spectroscopy

The various parietal polysaccharide fractions were analysed by infrared (IR) spectroscopy (Figure 2). Some parietal polysaccharides have in their structure characteristic chemical groups which can be highlighted (Taboada et al., 2010). They are listed in Table 3.

The comparison of the different spectra (Figure 2) showed, in the case of the pectic fractions WE and OE from YR and AR, the presence of an intense band around 1742 cm^{-1} which corresponded to the elongation band for the C=0 of esters and acids. This band may be attributed to acidic oses esterified or not that are mainly found in pectins and in a lesser proportion in hemicelluloses (Habibi, 2004). Nevertheless,the disappearance of this band was observed in hemicelluloses spectra (YR and AR) after extraction with NaOH. The hemicelluloses spectra also showed typical arabinose absorption bands with low intensity in the vicinity of 1077 to 1154 cm^{-1} because of vibrations C-O-C (Peng and Wu, 2010; Chaa et al., 2008). This might be due to the presence of pectic substances with arabinans inside chains.

The spectra of hemicellulosic fractions of YP and AR showed absorption bands between 1017 and 1101 cm^{-1} with a maximum in the vicinity of 1051 cm^{-1} corresponding to elongation vibrations of linkage inside the cycle and C-OH. These signals are characteristic of xylans. This suggests that the xylose is the main constituent under the form of pyrans (Peng and Wu, 2010). The pectins and hemicelluloses spectra presented other bands around 1545-1570 cm^{-1}. This could be due to the possible presence of residual lignin (Xu et al., 2007).

Table 3. Attribution of the main bands observed on the spectra IR of the various polysaccharidic fractions obtained

Vibration	Attribution	Frequency cm^{-1}
$\sqrt{}_{(OH)}$	Polysaccharides and hydratation water	3313-3383
$\sqrt{}_{(CH)}$	Polysaccharides	2930-2935
$\sqrt{}_{(C=O)}$	Esters and acids	1742
Liaisons absorbed hydrogenes and water		1629-1656
Residual Lignine		1545-1570
$\delta_{(CH)}$ et $\delta_{(CH2)}$ et $\delta_{(O-H)}$	Polysaccharides	1240-1420
$\sqrt{}_{(COC)}$	Skeletal Pyranose	1077-1154
$\sqrt{}_{(C-C)}$	Polysaccharides	1017-1101
$\sqrt{}_{(C1-H)}$	β-D-Xylose	834-859

Conclusion

This preliminary biochemical study of the parietal compounds in the roots gives some information about the polysaccharidic composition of the cell walls of the young and adult roots of *R. raetam*. The ponderal dosage indicates that the cellulose remains a major component of the polysaccharides of the wall (27% for young roots and 80% for adult roots) compared to hemicelluloses (14.3% for young roots and 3.6% for adult roots) and to pectins (17.3% for young roots and 4.1% for adult roots).

The analysis of gas chromomatography and infrared spectroscopy reveal the presence of altering homo-galacturonan and rhamnogalacturonan I blocks which can be substituted by galactan and arabinan side chains in the pectin fraction and the presence of arabinoxylans in the hemicellulosic fraction.

Conflict of interests

The authors did not declare any conflict of interest.

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