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Compositional changes in cell wall polysaccharides from apple fruit callus cultures modulated by different plant growth regulators

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ABSTRACT

The cell wall composition of apples callus cultures showed changes in the presence of 5 mg l^{-1} of three different plant growth regulators (PGRs), namely picloram, abscisic acid and gibberellic acid. Although the structural functions of cell walls do not generally allow for pronounced variations of the total pectin and matrix glycan content, this work provides evidence that the addition of these plant growth regulators can rule, at least partly, cell wall metabolism in apple callus cultures. The chelator- and carbonate-extracts always had the analytical characteristics of pectins, with high proportions of uronic acids, arabinose and galactose as the main monosaccharides, and a significant proportion of rhamnose, but the cross-linking glycan fractions were still rich in RG-I-like material. The application of PGRs produced shifts of uronic acid and neutral sugars between fractions. Arabinose was the neutral sugar exhibiting more variations in apple callus cell wall. Picloram and abscisic acid produced an increase of the uronic acid contents of the cell walls. The AIRs obtained from calluses treated with different PGRs did not show large amounts of high molecular weight products, as determined by size-exclusion chromatography. For the carbonate-extract only the callus treated with picloram displayed two separated peaks for products of different molecular weights. The chromatographic profiles for the 4% KOH-extract displayed two peaks for all the treatments, one very sharp with high molecular weight, and another one wider of smaller molecular weight, whereas the difference between treatments can only be appraised through the areas of the peaks. This is the first report on cell wall composition from fruit calluses supplemented with different PGRs.

1. Introduction

The primary cell wall is a dynamic structure with an essential role during plant growth and development. Whereas the general structural features of the cell wall components (mainly polysaccharides and proteins) are quite conserved [1], their proportions and arrangements differ dramatically between cells, or even within domains of the walls of the same cell, suggesting that this complexity needs to be precisely regulated [2,3]. Morphological changes occurring during development were associated to modifications and rearrangements of the cell wall components [4,5], and to a balance between synthesis and deposition of new materials in pre-existing walls [6]. These processes require cell wall proteins able to cleave or loose wall components at specific sites and timelines, thus requiring precise and fine-tuned regulations. Subtle changes in the wall components structure showed dramatic morphological alterations [7]. Plant-growth regulators (PGRs) participate in the control of several metabolic processes occurring during normal plant development [8–11]. PGRs have shown to increase the activity of various cell wall degrading enzymes [12–14].

Studies about metabolism are difficult to perform in whole plants, due to complex source-sink interactions, distinct responses to sugar signals, or close integration of a complex signaling network ruled by plant hormones, nutrients, and environmental conditions [15]. Furthermore, the research in this area was hindered because the studies with intact plant organs require vacuum infiltrations, sprays or dips of the organ in different PGR solutions. Therefore, entrance and distribution of the PGRs are uncertain due to surface diffusion barriers, with treatments differing of each organ, depending on its anatomy and morphology. For this reason, calyx and fruit cultures [16,17] or callus cultures [18] have been used to characterize different aspects of fruit metabolism. *In vitro* callus cultures have been used as a model system to study enzyme expression, changes in morphogenesis, and polysaccharide composition of callus tissues from healthy and aphid-induced galls [19]. A callus explant

Abbreviations: ABA, abscisic acid; AIR, alcohol-insoluble residue; GA_3, gibberellic acid; PGR, plant growth regulator; PIC, picloram; SEC, size-exclusion chromatography.

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Table 1
Uronic acid content (mg/g AIR) in cell wall fractions of apple calluses under different PGR treatments.[a]

Cell wall fraction	Control	+ Picloram (PIC)	+ Abscisic acid (ABA)	+ Gibberellic acid (GA_3)
W-F	2.9 ± 0.3 a	9.0 ± 0.5 b	2.7 ± 0.4 a	2.4 ± 0.4 a
CDTA-F	35.4 ± 0.4 a	81.1 ± 1.1 c	40.7 ± 0.5 b	36.0 ± 0.5 a
Na_2CO_3-F	45.5 ± 0.5 b	55.8 ± 0.7 c	65.7 ± 0.6 d	37.8 ± 0.8 a
4% KOH-F	12.8 ± 0.5 c	8.2 ± 0.2 a	9.5 ± 0.2 b	10.0 ± 0.6 b
24% KOH-F	21.0 ± 0.5 d	7.1 ± 0.4 a	16.4 ± 0.3 c	10.1 ± 0.8 b

[a] Values represent the means ± SE ($n = 3$). Different letters within each cell wall fraction (row) indicate significant ($p < 0.05$) differences between stages.

Table 2
Neutral sugar content[a] (mg/g AIR) in cell wall fractions of apple calluses under different PGR treatments.[b]

Cell wall fraction	Control	+ Picloram (PIC)	+ Abscisic acid (ABA)	+ Gibberellic acid (GA_3)
W-F	15.7 ± 0.1 b	25.2 ± 0.2 c	15.5 ± 0.4 ab	13.9 ± 0.7 a
CDTA-F	3.8 ± 0.2 a	25.1 ± 1.1 c	3.4 ± 0.2 a	7.7 ± 0.2 b
Na_2CO_3-F	17.3 ± 0.1 d	15.9 ± 0.2 c	3.9 ± 0.6 a	14.0 ± 0.7 b
4% KOH-F	105.8 ± 1.4 c	84.9 ± 0.8 a	85.6 ± 0.9 a	91.9 ± 0.6 b
4% KOH-F[c]	69.1	67.6	64.9	59.3
24% KOH-F	67.0 ± 0.5 d	52.4 ± 0.5 b	56.8 ± 1.0 c	34.9 ± 0.9 a
24% KOH-F[c]	63.4	51.9	53.6	34.1

[a] Obtained after substracting the content of uronic acids, and of agar contamination to that of total carbohydrates (see Section 3.5).
[b] Values represent the means ± SE ($n = 3$). Different letters within each cell wall fraction (row) indicate significant ($p < 0.05$) differences between stages.
[c] After discounting the starch present.

represents an artificial biological system, but provides a convenient and easy-to-handle source of plant material that can be rapidly regenerated, and from which cell walls may be obtained without difficulty. Particularly, apple callus tissue cultures can be used for this purpose [12,13,18]. Since callus cultures can continue to grow in the basal medium with no plant growth regulators (PGRs), this system allows comprehensive studies involving the addition of different metabolites at different concentrations with or without any addition of PGRs at physiological levels to the culture medium.

Although the influence of the PGRs on the composition and characteristics of the cell wall has been evaluated in different elongating organs, to the best of our knowledge there are no previous reports of these studies in plant tissues calluses, or in fruits in particular. This work aims to determine the modifications promoted in the cell wall polysaccharides of apple fruit callus cultures by the action of different PGRs. The composition and size distribution of the different pectin and cross-linking glycan fractions will help to understand the metabolic paths related to cell wall synthesis, assembly and breakdown modulated by the different PGRs.

2. Results and discussion

2.1. Composition of cell wall of apple calluses

The cell walls of cultures of apple calluses were extracted sequentially with different solvents, in order to obtain three pectic and two cross-linking glycan fractions. Their uronic acid content, neutral sugar content, and neutral monosaccharide composition are shown in Tables 1–3, respectively, under the heading "Control". Fig. 1 depicts the yields of each fraction in relation to the total alcohol-insoluble residue (AIR), whereas Fig. 2 shows the monosaccharide content of the solubilized cell wall, obtained by adding the contents of the five cell wall fractions. For the control, ca. 50% of the original AIR was extracted (Fig. 1), whereas an additional 18%

Table 3
Neutral sugar composition (mol/100 mol) of pectin and cross-linking glycan fractions of apple calluses under different PGR treatments.[a]

Fraction	Treatment	Monosaccharide						
		Rha	Fuc	Ara	Xyl	Man	Gal	Glc
W-F	Control	3	2	13	10	1	59	12
	PIC	3	1	15	8	1	63	9
	ABA	2	1	20	6	2	63	6
	GA_3	2	2	12	11	1	60	12
CDTA-F	Control	9	4	23	14	2	36	12
	PIC	10	3	26	8	1	46	6
	ABA	11	3	36	7	2	39	2
	GA_3	10	3	24	10	1	43	9
Na_2CO_3-F	Control	15	3	43	6	1	28	4
	PIC	14	3	42	6	1	31	3
	ABA	10	2	49	6	2	29	2
	GA_3	14	3	44	7	1	27	4
4% KOH-F	Control	3 (4)	2 (3)	20 (30)	9 (14)	1 (2)	12 (19)	53 (28)
	PIC	4 (5)	4 (5)	37 (46)	10 (13)	2 (3)	9 (11)	34 (17)
	ABA	4 (6)	3 (4)	41 (54)	9 (12)	2 (2)	9 (12)	32 (10)
	GA_3	4 (6)	2 (3)	28 (44)	10 (16)	2 (2)	11 (17)	43 (12)
24% KOH-F	Control	8	3	28 (30)	16	Tr.	17 (19)	28 (24)
	PIC	9	5	33 (34)	19	Tr.	11	23 (22)
	ABA	6	4	27 (29)	21 (22)	1	15 (16)	26 (22)
	GA_3	8	3	30 (31)	19	Tr. (1)	16	24 (22)

[a] In parentheses, the values after discounting the starch present are reported.

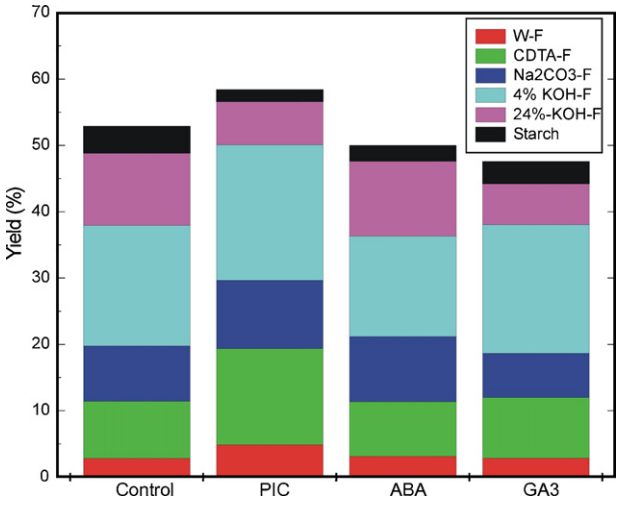

Fig. 1. Accumulated yields of the different fractions of cell wall polysaccharides from apple calluses grown in a medium enriched with picloram (PIC), abscisic acid (ABA) and gibberellic acid (GA_3). Starch is indicated separately.

was recovered as the final residue. A small amount of starch was present in the AIR (ca. 4% of the AIR, 0.2% of the fresh weight of calluses, Fig. 1), mostly concentrated on the 4% KOH-F, with less in the 24% KOH-F. The W-F product usually represents the loosely bound pectins, according to its usual analytical features (high proportions of uronic acids, Ara, Gal > Rha, Xyl as the main neutral monosaccharides [20]). The current results do not follow that trend: the uronic acid content was low (Table 1), lower than that of neutral sugars (Table 2), whereas Gal appeared in high amounts, higher than those for Ara, Xyl, and Glc (the other monosaccharides appearing in significant proportions, Table 3). On the other hand, CDTA-F and Na_2CO_3-F had the analytical characteristics of pectins, with higher proportions of uronic acids, Ara and Gal as the main monosaccharides, and a significant proportion of Rha (Tables 1 and 3). An increase of the Ara/Gal ratio (Fig. 3) as the extraction progresses was observed for the control culture. The cross-linking glycan fractions, extracted with increasing alkali concentrations, were still rich in RG-I-like material, as shown by their significant amount of uronic acids (Table 1), as well as by their high proportion of Ara and Gal. However, as expected, the presence of xyloglucans was suggested by the increasing amounts of Xyl and Glc. The Ara/Gal ratio for both

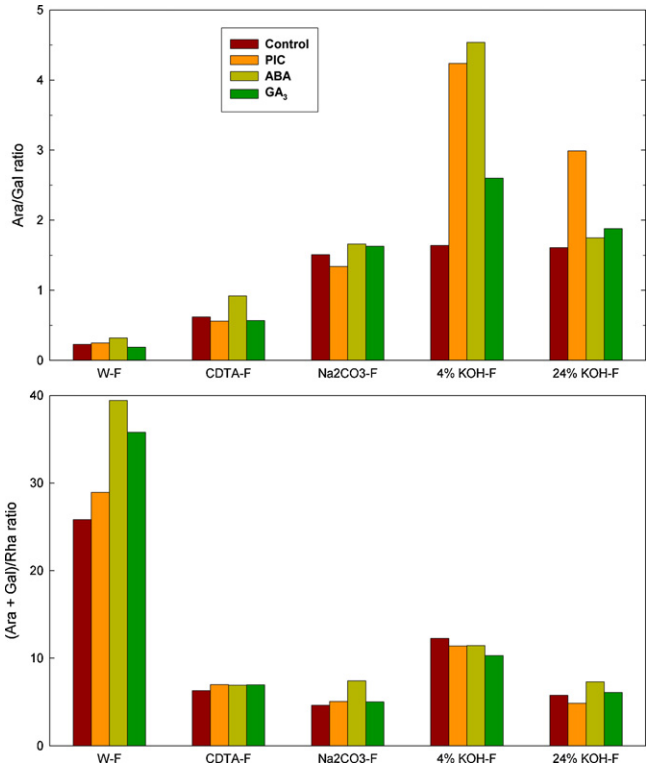

Fig. 3. Ara/Gal and (Ara + Gal)/Rha ratios for cell wall pectins and cross-linking glycans. Contiguous bars represent the fractions obtained from the cell walls of apple calluses grown in a medium enriched with picloram (PIC), abscisic acid (ABA) and gibberellic acid (GA_3).

fractions was similar to that observed for the Na_2CO_3-F (Fig. 3). Arabinose was the main neutral sugar composing the non-cellulosic cell wall of apple calluses (Fig. 2), when the starch present was discounted. Starch is usually scarce in non-organogenic calluses: Thorpe and Meier [21] reported the presence of 0.05% starch (with respect to fresh weight) in non-shoot forming tobacco callus cultures, and up to 0.4% in shoot forming tissue at the peak stage. The current apple calluses contain 0.2% of starch. The 4% KOH-F concentrated most of the starch present. The presence of small percentages of Man and Fuc in the cross-linking glycan fractions (together with

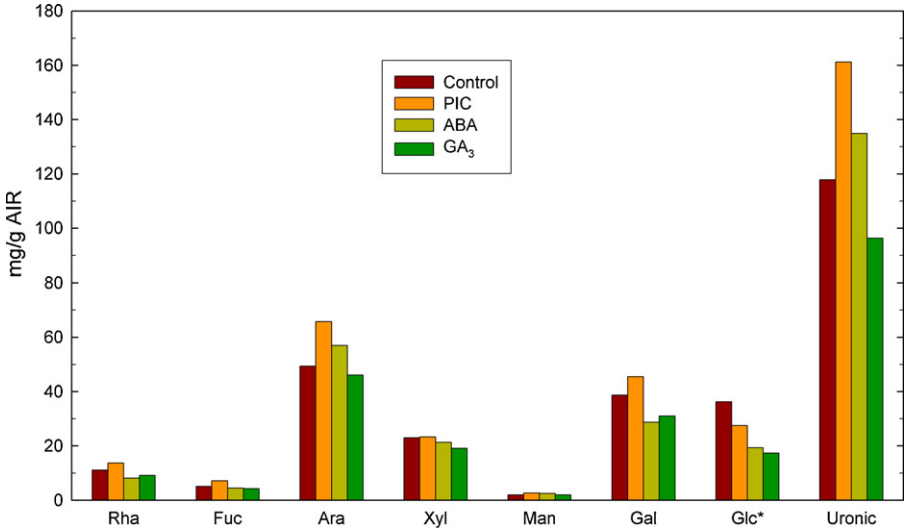

Fig. 2. Amount of monosaccharides (mg/g AIR) solubilized from the cell walls of apple calluses grown in a medium enriched with picloram (PIC), abscisic acid (ABA) and gibberellic acid (GA_3). The figures indicate the sum of the monosaccharides extracted by each of the five extraction media. Glc* indicates non-starch Glc.

Glc and Xyl) could be related to the existence of mannans, galactomannans, galactoglucomannans and to fucosylated xyloglucans, all typical components of hemicellulose fractions. The degree of branching of RG-I appeared to be similar for both cultures in the four fractions, as suggested by the (Ara + Gal)/Rha ratios (Fig. 3).

2.2. Effect of PGR treatment

With the aim of analyzing the possible effect of PGRs on the composition of the polysaccharides of the cell wall of apple calluses, their cultures were exposed to supplements of 4-amino-3,5,6-trichloro-pyridine-2-carboxylic acid (PIC), abscisic acid (ABA) and gibberellic acid (GA_3), and their cell walls extracted in the same fashion as those for the control culture (Section 2.1). Tables 1–3 and Figs. 1–3 show the corresponding results. For the fractions W-F and CDTA-F, a considerable increase of the neutral sugar and uronic acid levels are observed for the culture grown with PIC (Tables 1 and 2), whereas no major changes were observed for the cultures grown with ABA and GA_3. The tightly bound pectins appearing in Na_2CO_3-F contained more uronic acids (~45%) than the control when treated with ABA, 18% less when treated with GA_3 and 20% more when treated with PIC. The neutral sugar contents, on the other hand, were not affected by the treatments with PIC and GA_3, but showed a marked decrease in the ABA treatment (Table 2). For the cross-linking glycan fraction 4% KOH-F, a slight reduction in uronic acid (22–36%) and neutral sugar (13–20%) content was observed for any PGR treatment (Tables 1 and 2). A similar qualitative result was produced for the other matrix glycan fraction (24% KOH-F), though the actual degree of decrease was greater: GA_3 showed the larger effect in decreasing neutral sugars and PIC decreasing uronic acids (Tables 1 and 2), whereas ABA showed the smallest effect on either the uronic acids or neutral sugar contents. The decrease in neutral sugar content of 4% KOH-F proceeded from a lower starch accumulation (Table 2) when PGRs were added. The cultures grown with PIC accumulated 31% of the starch of the control, and those grown with ABA 65% (in terms of fresh weight). GA_3 had a negligible effect. For shoot-forming tobacco callus cultures, a decrease in starch accumulation after GA_3 treatment has been reported [22]. The different uronic acid content in the five fractions as a result of the PGR action differed from that observed by Yeo et al. [11], who did not find PGR-mediated differences in the uronic acid content in cell walls of cultures of *Phaseolus vulgaris*. Tanimoto and Huber [23] detected lower levels of neutral sugars in the cell walls of bean roots in active growth when treated with GA_3. In cell cultures of *P. vulgaris*, Yeo et al. [11] found differences in the total sugar content of low molecular weight pectins, but not in high molecular weight pectins or in cross-linking glycans when supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin. Previous reports indicated high arabinan and low galactan contents in meristematic regions of carrot apexes with high cell density, or cell culture suspensions supplemented with 2,4-D [24]. However, the low levels of (1,4)-β-galactan were measured in walls of dividing cells, whereas they accumulated in walls of cells induced for elongation, where the (1,5)-arabinan was almost absent. The occurrence and location of side chains of pectic polysaccharides in RG-I can be modulated by metabolic differentiation of plant cells, as also shown in a complete chemical and histochemical study about a tomato fruit mutant [25,26]. In the present work, the increase in Ara/Gal ratio occurred for all the pectin fractions as the extraction process advanced (Fig. 3). It appears to be only slightly affected by the PGR addition. On the other hand, the (Ara + Gal)/Rha ratio increased in W-F for every PGR (Fig. 3), whereas negligible variations were observed for the remaining fractions. In other systems, like bean roots, a decrease in the Ara/Gal ratio by the addition of GA_3 was observed [23]. Kikuchi et al. [27] found that the pectin side chains of carrot calluses modified during the change from an embryogenic state to a non-embryogenic state showed an Ara/Gal ratio correlated with the size of the cell groups. On the other hand, a very low Ara/Gal ratio was observed in "nolac" ('non-organogenic callus with loosely attached cells') calluses of tobacco, characterized by the little cell adhesion [28] and the absence of Ara side chains in RG-I. This did not happen in any of the treatments of the apple calluses (Table 3 and Fig. 3), and suggested the importance of the arabinan presence in cementing the calluses cells. The cell walls of tomato mutants which showed poor cell–cell adhesion had a sharply different location and distribution of the arabinan chains than those of the regular wild-type [25,26]. As pointed out earlier (Section 2.1) the loosely bound cross-linking glycans (4% KOH-F) were significantly rich in Ara, more than Gal or Xyl (Table 3). The levels of Ara and Glc showed important changes between treatments: in the KOH 4%-soluble fraction, the Ara content of calluses treated with ABA almost doubled that appearing in the control (Table 3 and Fig. 3), whereas those treated with PIC and GA_3 showed a smaller, but similar significant effect. The non-starch Glc present diminished with the treatments, especially with those of ABA and GA_3 (Table 3). The Ara increase could be related to the scarce activity of the α-L-arabinofuranosidase in PIC-treated cell walls [12]. The decrease in non-starch Glc content in 4% KOH-F by PGR addition (Table 3) originated a net decrease in the total Glc extracted from the cell walls (Fig. 2), suggesting that the PGRs may have induced larger networks of Glc-containing hemicelluloses which thus are not extracted by alkali. The effect on other glycosyl hydrolases [12] could be responsible of these changes, as well as other processes, such as polysaccharide synthesis or modification.

The application of PGRs produced only a moderate increase in the Xyl level in 24% KOH-F, but this occurred at expenses of some xylose lost in the pectic fractions, suggesting once more that Xyl-containing polysaccharides (xyloglucans) became less extractable when PGRs are added. No major variations in the Ara or Glc contents by PGRs addition were observed in this fraction, though the Ara/Gal ratio appears to be increased by the PIC treatment (Fig. 3). The (Ara + Gal)/Rha ratios were only very slightly affected by PGR addition (Fig. 3), although this ratio appears to be higher in the KOH 4%-F than in the KOH 24%-F, suggesting more branching in the former RG-I. The PGRs only showed some effect on this index for the W-F pectins. A slightly larger effect of ABA over this index was observed in most fractions.

The variation in the levels of Ara between treatments was more evident in some fractions than in others. This could be an indication of the existence of different populations of Ara-containing polysaccharides within the cell wall [20]. Thus, the Ara in the KOH 24%-F could be less susceptible to the enzymatic degradation, keeping its levels relatively stable between treatments. Redgwell et al. [29] found high molecular weight arabinogalactans very strongly associated to the cellulose in fruits of different species. Ponce et al. [20] detected a similar condition in Japanese plum, whereas Brummell et al. [30] found substantial amounts of pectins in alkaline extracts of peach fruit. The amount of arabinogalactans appears to be dependent on the system and on the PGR. For example, the application of indolacetic acid stimulated a decrease in both Ara and Gal contents of epicotyls of *Vigna angularis*, in agreement with the growth induction [31].

The size-exclusion chromatography (SEC) of the pectin fractions is shown in Fig. 4. In CDTA-F a noticeable peak corresponding to intermediate size molecules was observed for the different treatments (Fig. 4A–D), being slightly more defined in the control. The peak appearing for the same fraction in the treated calluses was wider, showing a partial extension to higher and lower molecular weight products (*i.e.*, more dispersion). ABA showed a small shift of the peak towards a higher molecular weight (Fig. 4C), whereas the GA_3 curve showed the most notable dispersion towards low molecular weight products. It has been shown that the pectins of

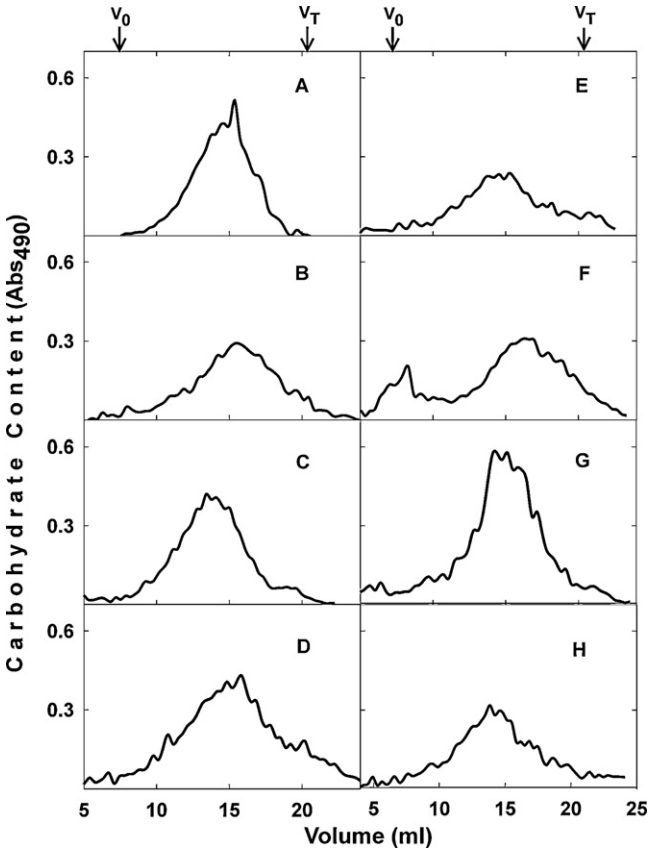

Fig. 4. Size exclusion chromatography profiles from the cell walls of apple calluses grown in a medium enriched with picloram (PIC), abscisic acid (ABA) and gibberellic acid (GA_3), using Sepharose CL-2B. (A–D) CDTA-F and (E–H) Na_2CO_3-F corresponding to control (A and E), PIC (B and F), ABA (C and G) and GA_3 (D and H). V_0, void volume; V_T, total volume.

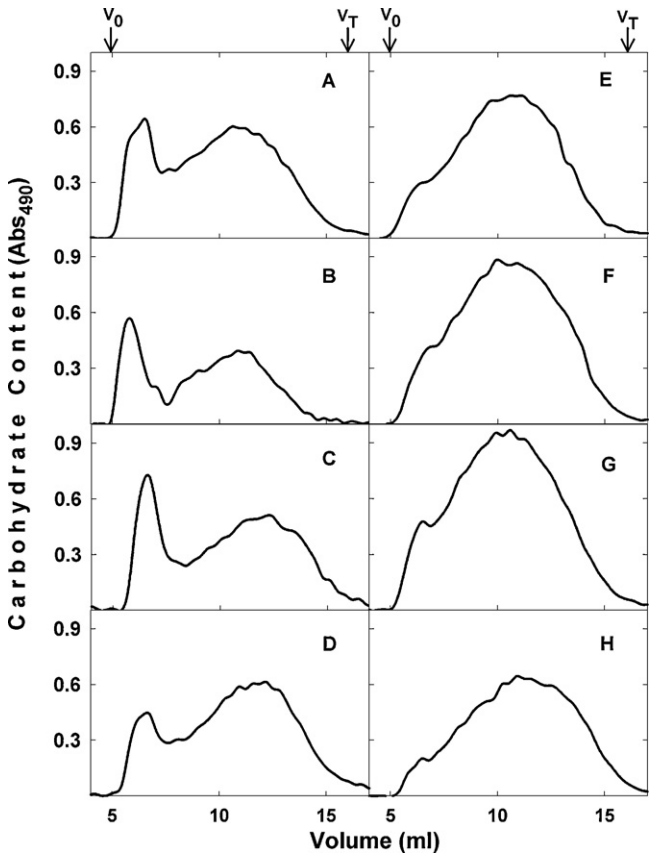

Fig. 5. Size exclusion chromatography profiles from the cell walls of apple calluses grown in a medium enriched with picloram (PIC), abscisic acid (ABA) and gibberellic acid (GA_3), using Sepharose CL-6B. (A–D) KOH 4%-F and (E–H) KOH 24%-F corresponding to control (A and E), PIC (B and F), ABA (C and G) and GA_3 (D and H). V_0, void volume; V_T, total volume.

GA_3-treated roots showed species with higher molecular weight than non-treated counterparts, both in the elongation area and in the maturation basal area [23]. Nevertheless, the AIRs obtained from calluses treated with different PGRs did not register a larger presence of high molecular weight products, as also found by Ponce et al. [20] for unripe stages of Japanese plums, but unlike the findings for other fruits [32–35], for which immature samples display high molecular weight polyuronides in this fraction. Helped by deesterification, Na_2CO_3 releases pectins held together to the wall by covalent binding. This treatment can also break other bonds, yielding predictably pectins of relatively lower molecular weight. For Na_2CO_3-F, the calluses control displayed a peak of intermediate molecular size, similar to that found in CDTA-F, though less sharp (Fig. 4E). The callus treated with GA_3 gave a similar result (Fig. 4H), though with a slight displacement towards larger molecular species. In the extract derived from a callus worked up with ABA, a larger peak was observed, with similar average molecular weight, but accompanied by smaller percentages of molecules of higher and lower sizes (Fig. 4G). On the other hand, the callus treated with PIC displayed two peaks, one with molecules of high molecular weight, and another involving smaller species, even smaller than those of previous treatments (Fig. 4F). The high molecular weight peak observed of this fraction could be associated with a very low activity of the glycosyl hydrolases in callus treated with PIC [12]. In the glycan fraction 4% KOH-F, the chromatographic profiles displayed two peaks for all the treatments (Fig. 5A–D), one very sharp with high molecular weight, concurrent with the exclusion volume (V_0), and another one wider of smaller molecular weight. This distribution resembled that observed in other systems, as the immature stages of Japanese plum [20], which did not contain starch. In apple calluses, the difference between treatments could only be appraised through the magnitude of the peaks. The high molecular weight peak showed decreasing magnitudes in the order ABA > control > PIC > GA_3, with the peak from ABA-treated cultures being 66% larger than with GA_3. On the other hand, the wider peak corresponding to smaller molecules showed decreasing magnitudes following the order: control > GA_3 > ABA > PIC, with the peak of the control samples being 50% larger than that from the PIC-treated culture. The first peak of this fraction matched that found on the same fraction of melon [35] and pea [36]. In those systems, this peak corresponded to high molecular weight polysaccharides, rich in Ara, Gal and with less Rha. Similar patterns have been found in hemicelluloses extracted with low alkali concentrations [37–39]. Some models of primary cell wall propose macromolecules covalently bound to noncellulose polysaccharides [40–42]: xyloglucans to arabinogalactans, arabinogalactans to pectins and pectins to structural proteins. It has been stressed that many polyuronides constituting the cell wall can be extracted under relatively mild conditions, without the concomitant release of xyloglucans, arabinogalactans or other hemicelluloses. However, the possibility of covalently bound pectins, arabinogalactans and xyloglucans cannot be excluded. The unusual coextraction of high molecular weight xyloglucans with Ara/Gal-rich polymers with mild alkaline conditions, which can break labile bonds, could be suggesting these covalent bonds between arabinogalactans and xyloglucans. The current conclusions should not be affected by the starch present in this fraction, as its proportion was quite small (8–16%). For the cross-linking glycans strongly bound to the cellulose (KOH 24%-F),

the chromatographic profiles did not change substantially between treatments (Fig. 5E–H), showing in all the cases a wide peak of intermediate molecular size, and a small shoulder eluting earlier. This shoulder was observed for all the treatments, though it was more marked in the treatment with ABA.

The current study attempted to determine the overall effect of the PGRs on the cell wall composition and structure. These effects could be either direct (synthesis or breakdown of cell components) or indirect (*e.g.*, an induction of cell differentiation, thus originating altered cell wall compositions). It should be stressed that the current approach reflects the average values of different cells which may eventually differ in their wall structures and/or composition [43], and that the wall composition arises from a delicate balance between synthesis and breakdown of cell wall components.

3. Experimental

3.1. General experimental procedures

All the reagents used in this work were of analytical grade, and from well-known sources (Merck Co. or Sigma-Aldrich Co.). Gas chromatography (GC) was carried out using a Hewlett Packard 5890 apparatus equipped with a FID and an HP 3395 integrator. Total sugars were determined using the colorimetric method of phenol–sulfuric acid using glucose as the standard [44]. Uronic acids were quantified using the *m*-hydroxydiphenyl method of Filisetti-Cozzi and Carpita [45], using galacturonic acid as standard. Both results were converted to anhydrous units. The proportion of neutral sugars was determined after discounting the uronic acid content to that of total sugars. For this purpose, the phenol–sulfuric acid reaction was carried out with galacturonic acid, showing a 0.28 absorbance ratio against the same mass of glucose [20]. In all cases, three repetitions of each determination were carried out and the results expressed as averages ± their standard errors. When the fractions were insoluble in water, they were previously digested using the method of Ahmed and Labavitch [46], in order to pursue further colorimetric dosages. Starch was determined qualitatively by staining with I_2, and quantitatively by an enzymatic method involving α-amylase, amyloglucosidase and *o*-dianisidine [47].

3.2. Statistical analysis

For uronic acid and neutral sugar content, statistical significance was determined by one-way ANOVA with the PC-SAS software package. The model assumptions of homogeneity of variance and normality were probed by means of Levene's and Shapiro–Wilk's tests, respectively. When these assumptions were not satisfied, data were transformed into ranks for further analysis. When a significant *F*-value was found, treatment means were compared using the Tukey's studentized range test ($p < 0.05$).

3.3. Origin of explants and culture treatments

Each explant consisted of a 5-mm apple-tissue cube obtained from the enlarged fleshy receptacle of a 75-day immature apple (*Malus domestica* Borkh. 'Anna') grown in an orchard in the city of Corrientes (Province of Corrientes, Argentina). The cultures were initiated as explained previously [12]. Glass tubes containing 3 ml of basal Murashige and Skoog [48] medium (MS medium) with 3% sucrose as the energy source were used as the non-PGR control. The pH of the medium was adjusted to 5.8 prior to the addition of the 0.65% agar. Tubes were covered with aluminum foil and autoclaved at 0.101 MPa for 20 min. Then, the apple calluses were placed individually in the tubes, which were sealed with Resinite AF50® and incubated at 27 ± 2 °C in the darkness.

The treatments consisted in additions of PGRs added to the basal medium at a concentration of 5 mg l^{-1}. The PGRs tested were 4-amino-3,5,6-trichloro-pyridine-2-carboxylic acid (picloram, PIC), abscisic acid (ABA) and gibberellic acid (GA_3). Three independent replicates per PGR treatment were prepared. After 30 days, actively growing calluses were harvested, frozen in liquid nitrogen and stored at −70 °C until used. Moderate levels of PGRs (5 ppm) were used, equivalent to those applied in a previous study [12].

3.4. Isolation of the cell wall materials

The isolation of the alcohol-insoluble residue (AIR), and from it, the different cell wall fractions was carried out as previously described [20], but using calluses that were mortar-homogenized at 4 °C (ca. 20 g). The loosely bound, ionically and tightly bound pectins (W-F, CDTA-F, and Na_2CO_3-F, respectively) were isolated, as well as loosely and tightly bound cross-linking glycans (4% KOH-F, and 24% KOH-F, respectively). All the fractions were exhaustively dialyzed and lyophilized.

3.5. Size-exclusion chromatography (SEC)

In order to analyze the size distribution of the polysaccharides, the fractions were analyzed by gel chromatography, using Sepharose® CL-2B (for pectins) and Sepharose® CL-6B (for cross-linking glycans), as depicted by Ponce et al. [20].

3.6. Analysis of the constituting monosaccharides

The polysaccharides were hydrolyzed using 2 M trifluoroacetic acid (TFA) at 120 °C for 90 min, and converted to the alditol acetates in order to be analyzed by GC. When agar interference was suspected, its proportion was estimated by quantifying 3,6-anhydrogalactose (3,6-AnGal): this sugar is destroyed by a regular hydrolysis but it can be stabilized by a reductive hydrolysis procedure. The procedure of Stevenson and Furneaux [49] was used, modified by using a second period of hydrolysis of 2 h. In a separate experiment, it was determined that the agar used for the cultures showed an experimental 3,6-AnGal/Gal ratio of 0.89. The proportion of Gal present in agar was discounted from the results of the cell wall; thus, the tables exclude this contamination. The resulting alditols were determined by GC on a capillary fused silica SP-2330 column (30 m × 0.25 mm i.d., 0.20 μm film thickness). The carrier gas was N_2 at 1 ml min^{-1}/15 psi (split ratio 1:80). The oven was held isothermally at 220 °C, and the injector and FID were held at 240 °C.

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