Phytochemistry 170 (2020) 112219

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

The role of cell wall phenolics during the early remodelling of cellulose-deficient maize cells

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ARTICLE INFO

Keywords:
Zea mays L.
Poaceae
Maize
HPLC
Hydroxycinnamates
Cell wall
p-coumaric acid
Ferulic acid
Dichlobenil

ABSTRACT

The habituation of cultured cells to cellulose biosynthesis inhibitors such as dichlobenil (dichlorobenzonitrile, DCB) has proven a valuable tool to elucidate the mechanisms involved in plant cell wall structural plasticity. Our group has demonstrated that maize cells cope with DCB through a modified cell wall in which cellulose is replaced by a more extensive network of highly cross-linked feruloylated arabinoxylans. In order to gain further insight into the contribution of phenolics to the early remodelling of cellulose-deficient cell walls, a comparative HPLC-PAD analysis was carried out of hydroxycinnamates esterified into nascent and cell wall polysaccharides obtained from non-habituated (NH) and habituated to low DCB concentrations (1.5 μM; H) maize suspension-cultured cells.

Incipient DCB-habituated cell walls showed significantly higher levels of esterified ferulic acid and *p*-coumaric acid throughout the culture cycle. In terms of cell wall fortification, ferulic acid is associated to arabinoxylan crosslinking whereas the increase of *p*-coumaric suggests an early lignification response. As expected, the level of hydroxycinnamates esterified into nascent polysaccharides was also higher in DCB-habituated cells indicating an overexpression of phenylpropanoid pathway. Due to their key role in cell wall strengthening, special attention was paid into the dimerization pattern of ferulic acid. A quantitative comparison of diferulate dehydrodimers (DFAs) between cell lines and cell compartments revealed that an extra dimerization took place in H cells when both nascent and mature cell wall polysaccharides were analysed. In addition, qualitative differences in the ferulic acid coupling pattern were detected in H cells, allowing us to suggest that 8-O-4′-DFA and 8-5′-DFA featured the ferulic acid dimerization when it occurred in the protoplasmic and cell wall fractions respectively. Both qualitative and quantitative differences in the phenolic profile between NH and H cells point to a regioselectivity in the ferulate dehydrodimerization.

1. Introduction

Primary cell walls of commelinoid monocots (also known as type II cell walls) such as maize are composed of a framework of cellulose microfibrils embedded in a hemicellulosic matrix of (glucurono)arabinoxylans and a smaller amount of pectins and glycoproteins (Carpita and Gibeaut, 1993). These cell walls are also characterised by the presence of cell wall phenolics, mainly the hydroxycinnamates, ferulic (FA) and *p*-coumaric (CA) acids, which are ester-linked to α–L-arabinosyl residues of (glucurono)arabinoxylans (Smith and Hartley, 1983; Wende and Fry, 1997).

Although phenolics are minor components of the primary cell wall (~1% of cell wall dry weight), their contribution to cell wall assembly seems to be crucial. *In vitro* experiments have demonstrated that hydroxycinnamates are susceptible to oxidative coupling in the presence of peroxidases and hydrogen peroxide, cross-linking adjacent arabinoxylan (AX) molecules and thus contributing to cell wall assembly (Geissman and Neukom, 1971; Fry et al., 2000; Fry, 2004; Encina and Fry, 2005; Parker et al., 2005; Burr and Fry, 2009). Due to its polysaccharide cross-linking role, phenolic coupling is involved in a number of cell wall properties, causing cell wall stiffening and growth cessation, promoting tissue cohesion, strengthening cell wall structure in response to biotic and abiotic stresses and hindering cell wall degradability (de O Buanafina, 2009).

Abbreviations: AX, arabinoxylan; CA, *p*-coumaric acid; DCB, 2,6-dichlorobenzonitrile or dichlobenil; DFA, dehydrodiferulate; FA, ferulic acid; H, 1.5 μM dichlobenil-habituated cultures; NH, non-habituated cultures; PDA, photodiode array detection; TFA, trifluoroacetic acid

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https://doi.org/10.1016/j.phytochem.2019.112219
Received 10 April 2019; Received in revised form 24 September 2019; Accepted 22 November 2019
0031-9422/

FA and CA are synthesised by the phenylpropanoid pathway (Vogt, 2010), the first step of which is the deamination of L-phenylalanine by phenylalanine ammonia lyase to cinnamic acid. Subsequent enzymatic steps catalysing hydroxylation and methylation produce feruloyl-CoA, which is widely accepted as being ester-linked to AXs in the endomembrane system (Fry et al., 2000; Lindsay and Fry, 2008). In this respect, evidence for *in muro* feruloylation has also been reported (Mastrangelo et al., 2009). Recently, it has been proposed that members of the Pfam family, the BAHD acyl transferases, may act as AX feruloyl transferases in rice (Piston et al., 2010), *Brachypodium distachyon* (L.) P.Beauv. (de O Buanafina et al., 2016; de Souza et al., 2018) and *Setaria viridis* (L.) P.Beauv. (de Souza et al., 2018), being involved in AX feruloylation (Withers et al., 2012; de Souza et al., 2018).

Phenolic coupling to form dehydrodiferulates (DFAs) can occur intra-protoplasmically, before or during Golgi vesicular transit to cell membrane, and/or in the cell wall (*in muro*), following polysaccharide secretion or after wall integration (Fry et al., 2000; Obel et al., 2003; Mastrangelo et al., 2009). The first dimer to be discovered was 5-5′-dehydrodiferulate (5-5′-DFA), followed by several other dimers such as 8-5′-DFA and 8-O-4′-DFA Figure 1. In addition, trimers, tetramers and larger oligoferulates have also been characterised after obtaining them through alkaline hydrolysis of cell wall polysaccharides (Ralph et al., 1994; Waldron et al., 1996; Bunzel et al., 2003, 2006; Rouau et al., 2003; Funk et al., 2005). Whether or not differences exist in the type of DFA depending on the site of dimerization (endomembrane system or cell wall) remains a subject of debate.

Due to the key role of cellulose in the cell wall structure, cellulose biosynthesis inhibitors have become valuable tools for the analysis of cell wall structure and biogenesis (Sabba and Vaughn, 1999; Vaughn, 2002; Acebes et al., 2010; Tateno et al., 2016). Although cellulose biosynthesis inhibitors are highly specific and potent herbicides, cell cultures of several species have been habituated to grow in their presence by incremental exposure over many culturing cycles. One of these compounds, dichlobenil (2,6-dichlorobenzonitrile, DCB), specifically inhibits polymerisation of glucose into β-(1,4)-linked glucans (Montezinos and Delmer, 1980), causing a reduction in the cell wall cellulose content.

The habituation of different cell cultures to DCB has shed light on the mechanisms to maintain cell wall integrity. The characterisation of cell walls from DCB-habituated cells has demonstrated that plant cells develop the capacity to cope with the inhibitor through the acquisition of a modified cell wall. Changes in cell wall composition/structure greatly depended on the type of cell wall (Type I or II) and on the degree of cellulose lacking (Shedletzky et al., 1990; Encina et al., 2001, 2002; García-Angulo et al., 2006, 2009; Mélida et al., 2009, 2015).

Maize cells habituated to high DCB concentrations (long-term habituation, 12 μM) showed a 75% reduction in cellulose. As a compensatory strategy, the major load-bearing structure was replaced by an extensive network of highly cross-linked AXs with increased weight-average relative molecular mass (Mw). Additionally, long-term habituated cells were featured by the deposition of lignin-like polymers that further stiffen the cell wall (Mélida et al., 2009, 2010a; 2010b, 2011, 2015).

In mildly cellulose deficient cells (maize cells habituated to 1.5 μM DCB) the 33% reduction in the cellulose content was compensated by means of a re-build network of low Mw AXs (de Castro et al., 2014, 2015). A detailed characterisation of hemicellulose synthesis in early habituated cells indicates that the lower Mw of AXs was not related to a lower degree of diferulate cross-linkage but to a defect in the AX chain elongation (de Castro et al., 2017). Other featuring metabolic characteristics of short-term DCB-habituation were: an increased antioxidant capacity (Largo-Gosens et al., 2016), a high level of class III-peroxidase activity and a specific apoplastic isoperoxidase profile (Martínez-Rubio et al., 2018).

So far, the DCB-habituation process teaches us about the remarkable plasticity of the primary cell wall as it involves a wide array of metabolic modifications that depends on the concentration of DCB and the length of time that cells were exposed to the inhibitor (Alonso-Simón et al., 2004; de Castro et al., 2015; Mélida et al., 2015; Largo-Gosens et al., 2016).

The aim of this work was to study the contribution of hydroxycinnamates esterified into nascent (protoplasmic fraction) and cell wall polysaccharides, to the early remodelling of cellulose-deficient cell walls habituated to low DCB concentrations. Expected changes in the phenolic prolife of protoplasmic and cell wall polysaccharides were quantitatively and qualitatively analysed by HPLC-PDA throughout the cell culture cycle. In an attempt to identify patterns in the specificity of dimerization between cell lines (non-habituated *vs* habituated, NH *vs* H) and/or cell compartments (endomembrane system *vs* cell wall) special attention was paid to changes in the amount and type of FA dehydrodimers.

Cell wall remodelling induced by the habituation to low concentrations of DCB is expected to teach us on cell wall responses under moderate abiotic stresses. By using this experimental system, a better knowledge of quick responses of plant cells under more physiological conditions than severe stress can be obtained.

2. Results and discussion

To obtain further insight into the relationship between cell wall phenolics and early cell wall remodelling in maize cells, the phenolic profile of cell wall and protoplasmic fractions was obtained from control cells (NH) and cells habituated to low DCB concentrations (1.5 μM; H) at different phases of the cell culture cycle (Tables 1 and 2).

Table 1
Hydroxycinnamate composition of the cell wall fraction from non-habituated (NH) and habituated (H) maize suspension-cultured cells at their early-log (early-logarithmic) and late-log (late-logarithmic) growth phases. CA, *p*-coumaric acid; FA, ferulic acid; 5-5′-DFA, 5-5′-diferulic acid; 8-O-4′-DFA, 8-O-4′-diferulic acid and 8-5′-DFA, 8-5′-diferulic acid. Results shown are representative of three different experiments ($\bar{X}$ ± s.d.). Different letters indicate significant differences after Student's *t*-test ($p < 0.05$) when NH *vs* H data for each growth phase were compared.

ng mg^{-1} FW	Cell wall fraction					
	Early-log growth phase		Ratio (H:NH)	Late-log growth phase		Ratio (H:NH)
	NH	H		NH	H	
CA	9.53 ± 0.11^a	109.38 ± 22.07^b	**(x 11.4)**	7.17 ± 2.24^a	106.10 ± 12.27^b	**(x 14.8)**
FA	884.97 ± 18.34^a	2422.99 ± 436.60^b	**(x 2.7)**	1186.96 ± 208.61^a	2378.40 ± 77.95^b	**(x 1.2)**
Total DFAs	298.72 ± 5.55^a	1097.34 ± 274.63^b	**(x 3.6)**	395.47 ± 30.81^a	868.88 ± 76.87^b	**(x 2.2)**
5-5′-DFA	139.18 ± 2.32^a	351.90 ± 52.54^b	**(x 2.5)**	184.81 ± 7.03^a	367.46 ± 5.74^b	**(x 2.0)**
8-O-4′-DFA	105.06 ± 5.52^a	380.28 ± 98.52^b	**(x 3.6)**	130.14 ± 12.46^a	302.88 ± 6.71^b	**(x 2.3)**
8-5′-DFA	54.48 ± 2.35^a	365.17 ± 130.58^b	**(x 6.7)**	80.52 ± 13.94^a	242.37 ± 4.73^b	**(x 3.0)**

Table 2
Hydroxycinnamate composition of the protoplasmic fraction from non-habituated (NH) and habituated (H) maize suspension-cultured cells at their early-log (early-logarithmic) and late-log (late-logarithmic) growth phases. Other details as in Table 1.

ng mg^{-1} FW	Protoplasmic fraction					
	Early-log growth phase		Ratio (H:NH)	Late-log growth phase		Ratio (H:NH)
	NH	H		NH	H	
CA	53.58 ± 21.83[a]	137.84 ± 23.47[b]	**(x 2.6)**	62.50 ± 0.43[a]	119.82 ± 56.21[a]	**(x 1.9)**
FA	3.99 ± 0.63[a]	10.85 ± 0.54[b]	**(x 2.7)**	2.99 ± 0.67[a]	11.51 ± 1.17[b]	**(x 3.8)**
Total DFAs	1.25 ± 0.45[a]	4.61 ± 0.32[b]	**(x 3.6)**	1.77 ± 0.02[a]	5.01 ± 2.20[a]	**(x 2.8)**
5-5′-DFA	0.63 ± 0.29[a]	2.73 ± 0.14[b]	**(x 4.5)**	0.52 ± 0.23[a]	2.38 ± 1.31[a]	**(x 4.6)**
8-O-4′-DFA	0.07 ± 0.01[a]	1.56 ± 0.33[b]	**(x 22.3)**	0.08 ± 0.01[a]	2.12 ± 0.80[a]	**(x 26.5)**
8-5′-DFA	0.54 ± 0.40[a]	0.33 ± 0.16[a]	**(x 0.6)**	1.18 ± 0.25[a]	0.51 ± 0.09[a]	**(x 0.4)**

2.1. Differences between alkali-extracted phenolics from non-habituated and DCB-habituated cell walls

Hydroxycinnamates ester-linked into polysaccharides already deposited in the cell wall were obtained after alkali extraction of the AIR fraction (Fry, 1983; Mastrangelo et al., 2009; Mélida et al., 2010b). HPLC analysis of alkali-extracted compounds from maize suspension-cultured cells (Figure 2 and 3, Tables 1 and 2) indicated that this fraction was composed of monomeric FA and CA and three dehydrodiferulates, namely: 5-5′-DFA, 8-O-4′-DFA and 8-5′-DFA (Table 1, Figure 2) as previously shown (Mélida et al., 2010b).

The presence of a *p*-coumaroylated cell wall has previously been associated with DCB habituation in barley and maize cultured cells (Shedletzky et al., 1992; Mélida et al., 2010b, 2015). Here, it is also reported that CA levels were significantly higher in H than in NH cells in both growth phases (Table 1) suggesting a cell wall lignification in early DCB-habituated cells. Several lines of evidences support this assumption. First, CA incorporation into the cell wall has been positively correlated with the formation of syringyl-rich lignins (Hatfield and Marita, 2010; Hatfield et al., 2017). Second, CA is mainly found acylating lignin in monocot cell walls (Ralph, 2010; Karlen et al., 2018). Third, ectopic lignification and high levels of CA have previously been reported in maize cultured cells (Mélida et al., 2015).

A comparison of cell wall phenolics obtained from NH cells in the early and late-log growth phases indicated that FA and DFA content of cell walls increased as the culture cycle progressed. However no changes in the DFA:FA ratio (0.33 in early log vs 0.33 in late-log phase; Table 1) were recorded during the cell culture cycle, rendering the positive correlation between growth cessation and phenolic cross-linking previously reported in some monocot species unlikely (Kamisaka et al., 1990; MacAdam and Grabber, 2002; Azuma et al., 2005).

Maize cells habituated short-term to DCB (H) were characterised by a significant increase in FA (and DFAs) compared to NH cell lines. In the case of H cells this mechanism seems to be related to an early cell wall stiffening strategy, since a high level of ester-linked FA precludes the availability of more phenolic side-chains to cross-link adjacent AXs. This assumption was confirmed in so far as the DFA content of H cells was on average 3.6 to 2.2-fold higher than that of NH cell walls depending on the culture phase. In agreement with this, extensive phenolic cross-linking of AXs has previously been reported in maize cells habituated to 1.5 μM DCB (de Castro et al., 2017).

In spite of having solely primary cell walls, DCB-habituated cells become lignified in response to a lack of cellulose (Mélida et al., 2015). Regarding an ectopic lignification response, the increased FA contents of H cell walls may play a pivotal role because structural evidence suggests that FA can act as nucleation sites for lignin polymerisation (Ralph, 2010; Grabber et al., 1998). FA may covalently cross-link AX to growing lignin polymers during the lignification process (Ralph, 2010; Terret and Dupree, 2019). As a result of this, FA may be incorporated into lignin, becoming resistant to alkali extraction and contributing to explain the slightly reduction in the cell wall feruloylation as H cells shift from early-log to late-log growth phases (Table 1).

Total amount of cell wall DFAs peaked in the early-log growth phase of H cells (Table 1). In consequence, the DFA:FA ratio, which may be used as an indicator of coupling intensity, was higher in the early-log phase in H cells as expected for an early cell wall stiffening strategy. In line with this, previous results using the same cell lines showed that class III-peroxidase activity also peaked in the early-log growth phase, predisposing H cells to the formation of DFAs and ectopic lignin in early

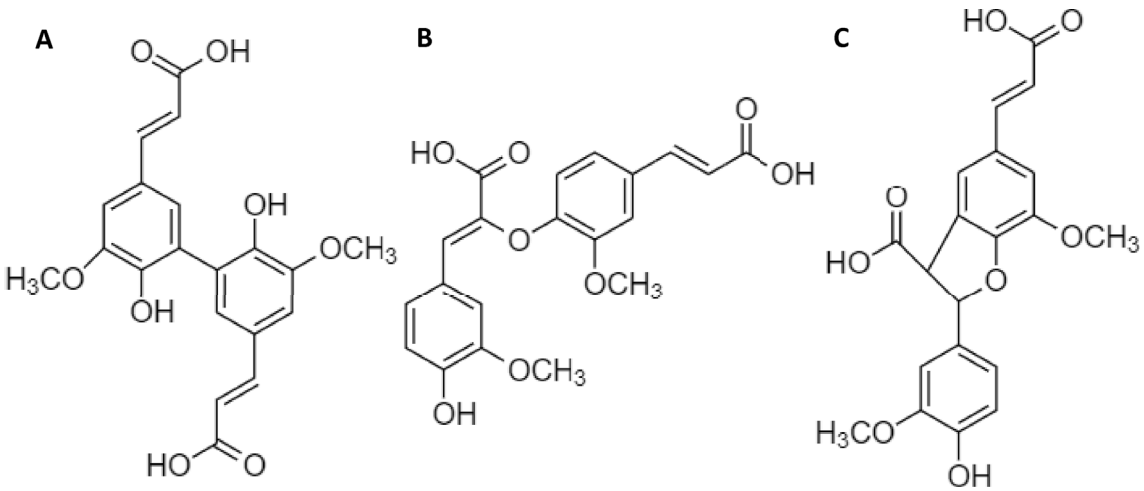

Fig. 1. Structures of the three diferulate dehydrodimers found in this work. 5-5′-diferulic acid (5-5′-DFA, **A**); 8-O-4′-diferulic acid; (8-O-4′-DFA, **B**) and 8-5′-diferulic acid (benzofuran form) (8-5′-DFA, **C**).

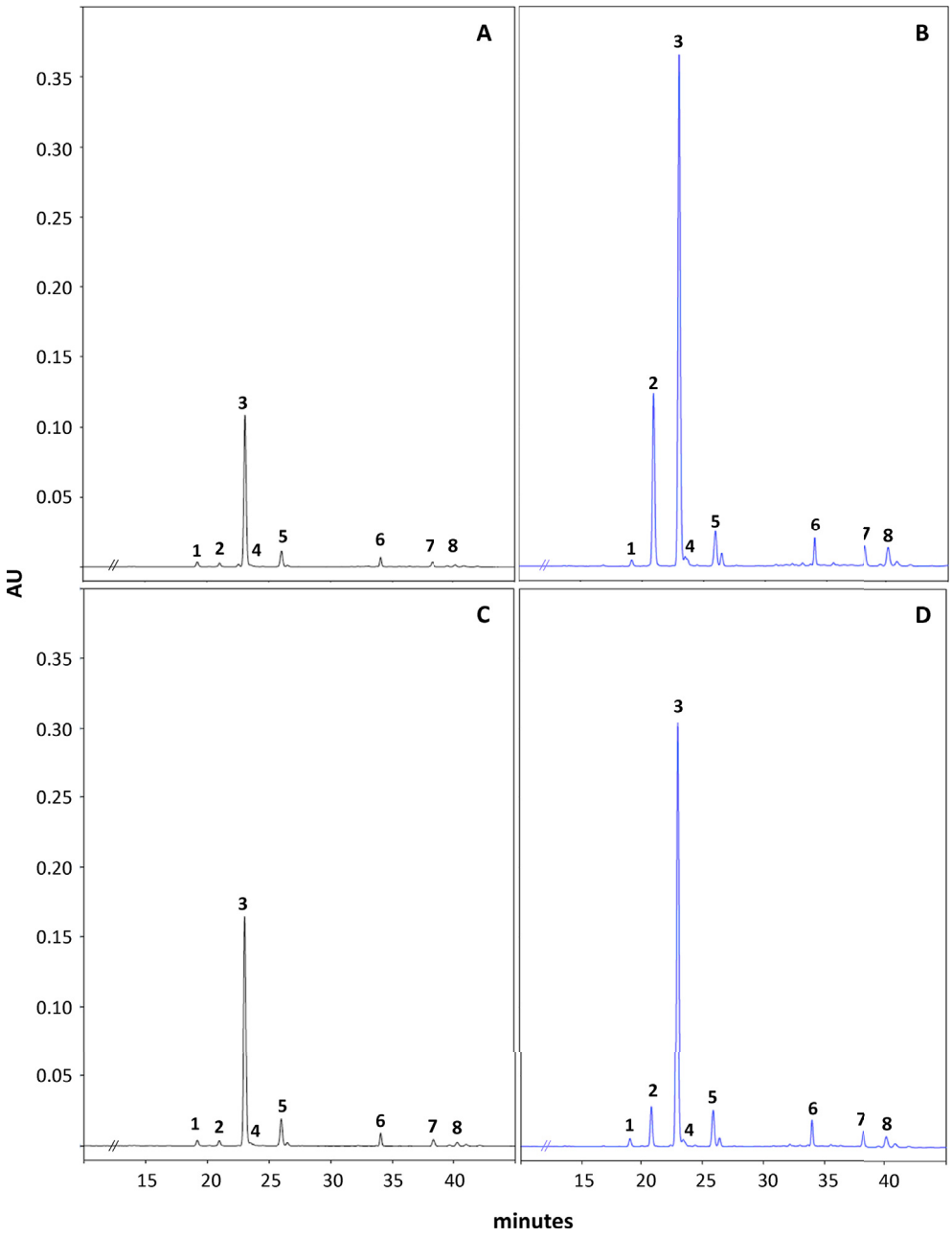

Fig. 2. Representative HPLC-PAD elution profile of phenolic compounds after alkali hydrolysis of cell walls from non-habituated (NH, **A, C**) and DCB-habituated (H, **B, D**) suspension-cultured cells corresponding at their early logarithmic (**A, B**) and late logarithmic (**C, D**) growth phase. Peaks were detected at 300 nm. 50 μl of sample was injected in a 1:10 dilution. Key to peak identity: **1**, vanillin; **2**, *trans-p*-coumaric acid; **3**, *trans*-ferulic acid; **4**, *cis-p*-coumaric acid; **5**, *cis*-ferulic acid; **6**, 5-5′-DFA; **7**, 8-O-4′-DFA; **8**, 8-5′-DFA.

stages of the cell culture cycle (Martínez-Rubio et al., 2018). The DFA:FA ratio did not vary greatly throughout the cell culture cycle of NH cells as previously shown by Mélida et al. (2010b).

2.2. Differences in alkali-extracted phenolics from the protoplasmic fraction between non-habituated and DCB-habituated suspension-cultured cells

The phenolic profile of ester-linked hydroxycinnamates obtained from newly synthesised polysaccharides in the protoplasmic fraction agreed with that obtained from mature ones (cell wall polysaccharides). As expected, FA, DFA and CA content in H protoplasm was significantly higher when compared with control cells (Figure 3, Table 2). Phenolic enrichment in H cell walls is related to a general induction of the phenylpropanoid pathway (Mélida et al., 2010a).

In cells habituated to low DCB concentrations, the fold change in FA and DFA between cell lines was similar when cell walls and protoplasm were compared (Tables 1 and 2). This was not the case for CA, as the relative increase in cell wall *p*-coumaroylation in H cells (11.4–14.8 fold change for the H:NH ratio depending on the cell culture phase) far exceeded that found in protoplasm (~2.5–2 fold change for the same ratio). In the case of maize cell walls, CA is more extensively esterified to lignin than to AXs (Iiyama et al., 1994; Ralph et al., 1994). Therefore, the increase in cell wall *p*-coumaroylation found in H cells is probably linked to incipient cell wall lignification as previously suggested in short-term and long-term habituated cells (Mélida et al., 2010b, 2015).

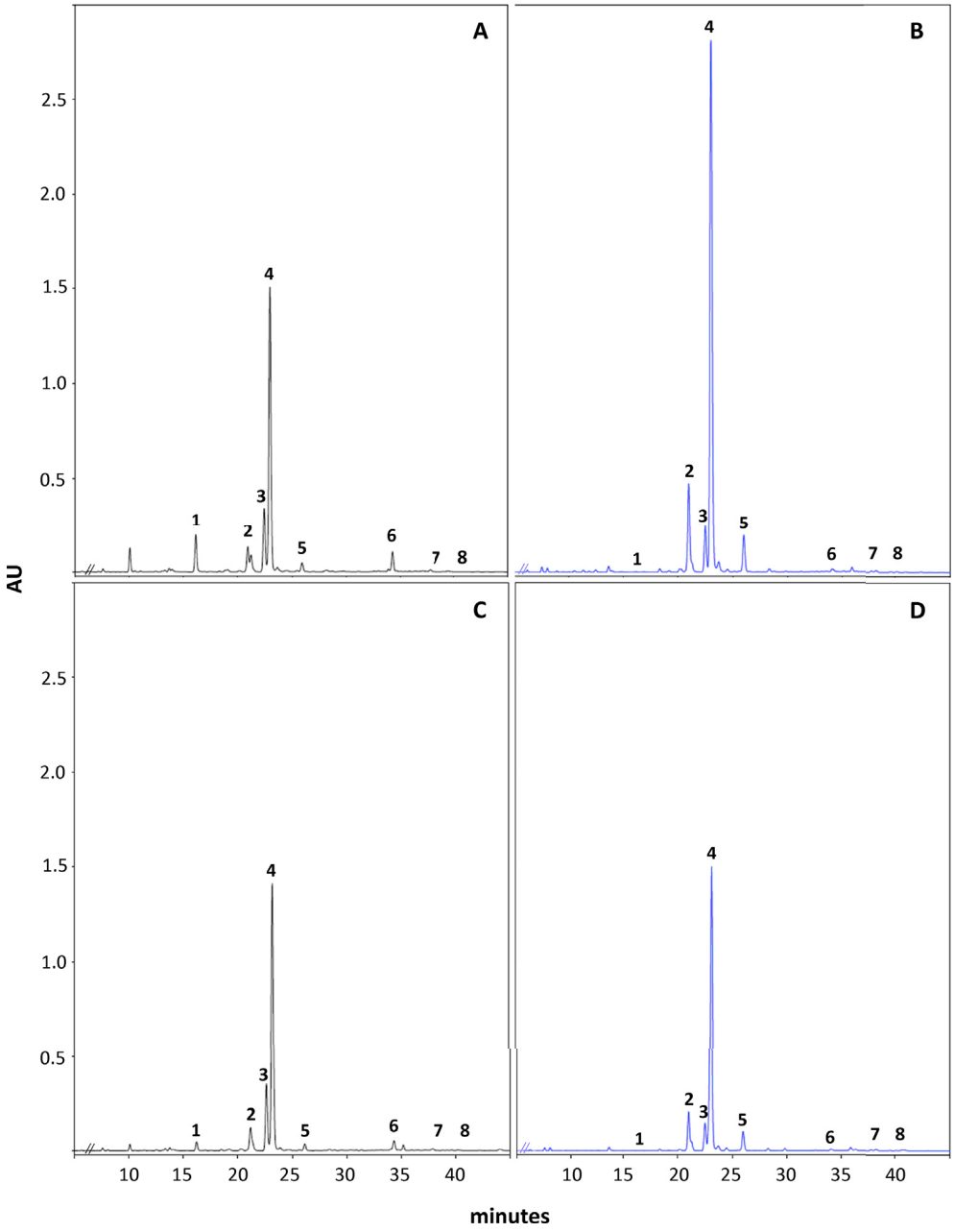

Fig. 3. Representative HPLC-PAD elution profile of phenolic compounds after alkali hydrolysis of protoplasmic fraction from non-habituated (NH, **A, C**) and DCB-habituated (H, **B, D**) suspension-cultured cells corresponding at their early logarithmic (**A, B**) and late logarithmic (**C, D**) growth phase. Peaks were detected at 300 nm. 50 µl of sample was injected without dilution. Key to peak identity as Fig. 2.

2.3. Differential pattern of FA dimerization among cell lines and cell compartments

As previously indicated, early DCB-habituated cells are characterised by an increase in FA dimerization. In this regard, previous results obtained by our group indicated not only quantitative but also qualitative differences in DFAs in H with respect to NH cells (Mélida et al., 2010b).

A DFA analysis showed that 5-5′- and 8-O-4′-DFA were the predominant coupling products in the cell wall of both cell lines. 8-5′-DFA, that was the scarcest DFA in NH cells, increased by 3 to ca. 7-fold in H cells. Therefore, 8-5′-DFA seems to present cell wall ferulate dimerization when an extra cross-linking occurs as in the case of DCB-habituated cells (Table 1). Similar results have previously been reported in maize cells habituated to high DCB concentrations (Mélida et al., 2010b).

The present study has demonstrated that besides 8-5′-DFA, 5-5′-DFA and 8-O-4′-DFA were also present in the protoplasm of NH maize cells (Table 2). Using a radiolabelling approach, Obel et al. (2003) found that intra-protoplasmic FA dimerization in wheat cultured cells was restricted to the 8-5′-DFA coupling product. Later, Lindsay and Fry (2008) demonstrated that other coupling products such as 8-8'; 8-O-4′ and 5-5′-DFA can be formed intra-protoplasmically in maize suspension-cultured cells as demonstrated here.

The relative change in DFA shows that 8-O-4′-DFA increased by 22 to 26-fold in H cells when compared to control ones. This latter result indicates that the 8-O-4′-DFA is significantly favoured when intra-protoplasmic FA dimerization occurs in H cells. In contrast, a restriction in the coupling for 8-5′-DFA was found in this same cell line (Table 2).

In summary, maize cells habituated to low DCB concentrations

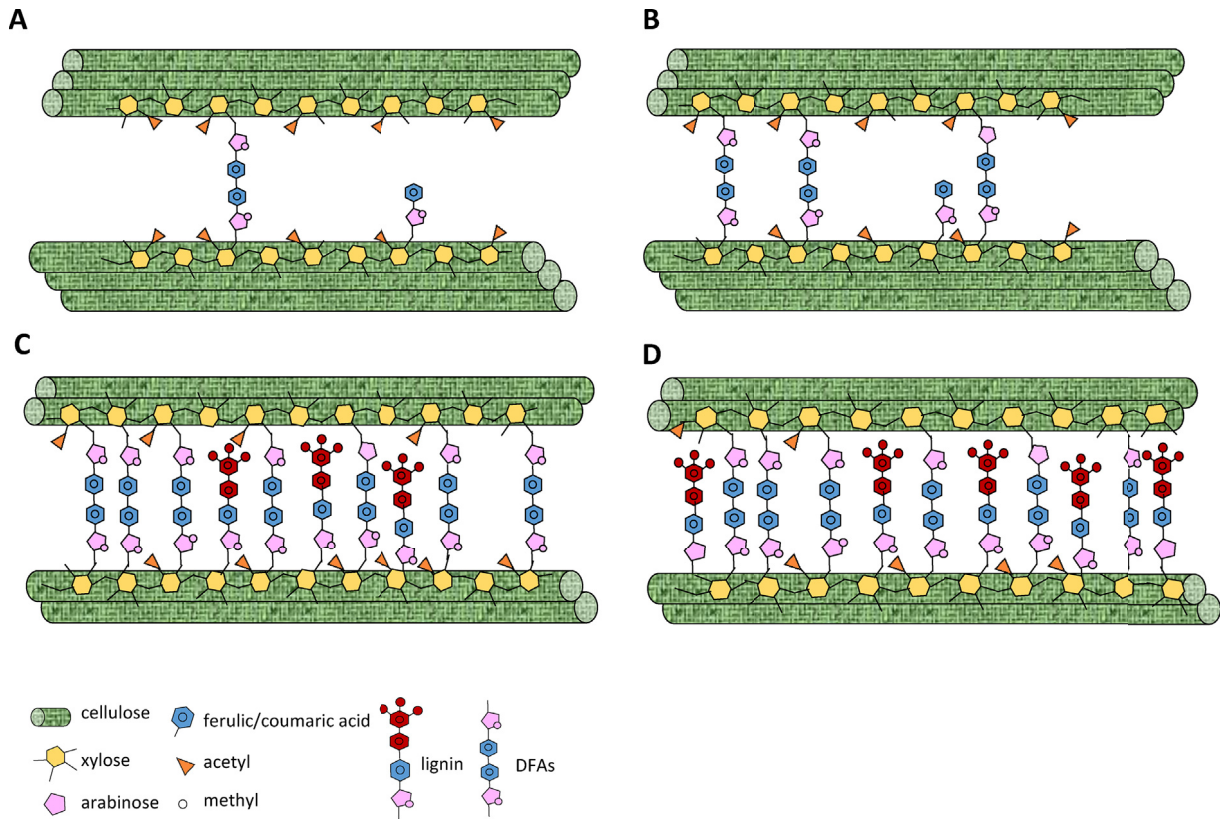

Fig. 4. Structural models of the primary cell wall of non-habituated (**A, B**) and DCB-habituated (**C, D**) maize suspension-cultured cells at their early-logarithmic (**A, C**) and late-logarithmic (**B, D**) growth phases. The model shows the molecular interactions between cellulose, arabinoxylans and hydroxycinnamic acids (phenolic compounds). Based on Gómez and McQueen-Mason (2018). Dehydrodiferulates can also be ether-linked to lignin.

compensated cellulose impoverishment by modifying cell wall architecture. DCB-driven modifications in cell wall architecture mainly consisted of the formation of a more extensive network of diferulate cross-linked AXs and the deposition of a lignin-like material (Figure 4). This modified cell wall architecture reflects a remarkable biochemical plasticity of primary cell walls in response to stresses. As demonstrated here, a significant change in the phenolic metabolism occurs during early DCB habituation. It has been proved that FA and CA accumulate in early DCB-habituated cell walls: FA seems to be more implicated in AX cross-linking whereas CA may play an important role as an ectopic lignification marker (Santiago and Malvar, 2010; Hatfield et al., 2017). As expected, the level of FA and CA esterified into nascent polysaccharides was also higher in H cells. A quantitative comparison of DFAs between cell lines revealed that an extra dimerization took place in H cells when both nascent (protoplasmic) and mature (cell wall) polysaccharides were analysed. In addition, qualitative differences in the FA coupling pattern were detected in H cells, suggesting that 8-O-4′-DFA and 8-5′-DFA presented the FA dimerization when it occurred in the protoplasmic and cell wall fractions respectively. Both qualitative and quantitative differences in the phenolic profile between NH and H cells point to regioselectivity in ferulate dehydrodimerization. This “non-random” pattern of ferulate coupling may be governed, at least partially, by the matrix (i.e. cell wall composition), and/or the kinetic properties of peroxidases (Ralph et al., 2004).

3. Materials and methods

3.1. Cell cultures

Maize (*Zea mays* L. Black Mexican sweetcorn) suspension-cultured cells were obtained as described by Lorences and Fry (1991) and subcultured every 15 days in liquid Murashige and Skoog medium (Murashige and Skoog, 1962) supplemented with 9 μM 2,4-D and 20 g l^{-1} sucrose. The cultures were cultivated at 25 °C under photoperiodic conditions (16 h light: 8 h dark; 41 μmol m^{-2} s^{-1}, GRO-lux Sylvania) in a rotary shaker (120 rpm) (Mélida et al., 2011). Maize suspension-cultured cells were stepwise subcultured in a medium supplied with increasing concentrations of DCB (Fluka). DCB was dissolved in dimethyl sulphoxide (DMSO), and the maximum concentration of DMSO added to the culture medium (0.015%; v/v) did not affect cell growth (de Castro et al., 2014). NH suspension-cultured cells were initially transferred to a medium containing 0.3 μM DCB, increasing the DCB concentration gradually up to 1.5 μM DCB (H cells) after seven subcultures. Cell growth kinetics for NH and H cells were followed as described by de Castro et al. (2014), and cells from each cell line were collected in their early (early-log) and late (late-log) logarithmic growth phases (4 and 8 day old cells for NH and 8 and 12 day old cells for H cell lines, respectively).

3.2. Phenolic acid analysis

Suspension-cultured cells (20–25 g fr. wt) were frozen, homogenised with liquid nitrogen and extracted with 70% ethanol (v/v) for 5 days at room temperature. Ethanol-extracted cells were centrifuged (4000 rpm) and the collected and vacuum-dried supernatant was considered the protoplasmic fraction. The remaining cell material was washed with 70% ethanol (x6) and acetone (x6) and then air-dried to obtain the alcohol insoluble residue (AIR).

The entire protoplasmic fraction and the AIR (10 mg) were treated with 1 ml of 1 M NaOH under N_2 for 16 h at room temperature in the dark in order to saponify the phenolic esters. After saponification, the resulting suspension was clarified by centrifugation (4000 rpm) and the supernatant was collected and acidified to pH 5.0 with concentrated trifluoroacetic acid (TFA).

The solution was partitioned against ethyl acetate (x2). The ethyl acetate phases were collected, vacuum-dried and re-dissolved in 1.0 ml of 50% methanol (v/v) for HPLC-PDA analysis. These samples were considered the phenolic acids of the cell wall and the protoplasmic fractions. In all the cases, 50 µl of each sample was injected in a 1:10 and without dilution for cell wall and protoplasmic fraction, respectively.

HPLC-PAD analyses of FA and CA monomers and ester-bound DFAs were performed using a Waters 2695 chromatograph equipped with a Waters 996 PDA following the method described by Mélida et al. (2010b). Separation was achieved using a Kromasil C18 (Teknokroma) column (250 × 4.6 mm i.d.; 5 µm particle size). The mobile phase was prepared in acidified water (1 mM TFA), and consisted of 10% acetonitrile (solvent A) and a mix of 40% acetonitrile and 40% methanol (solvent B) and followed a binary gradient elution programme: initial conditions were 90:10 (A:B), changing to 25:75 after 25 min (linear gradient), then to 0:100 after 5 min (linear gradient) and returning to initial conditions after 10 min (exponential gradient). The mobile phase flow was 1 ml min^{-1}. In order to obtain the chromatograms, elution profiles were monitored by UV absorbance at 300 nm. Peaks spectra were recorded between 220 nm and 340 nm to compare with spectra obtained from external standard compounds.

Retention times and spectra were compared with freshly prepared standard solutions of *p*-OH-benzoic acid, vanillic acid, CA, FA, 5-5′-DFA, 8-5′-DFA and 8-O-4′-DFA. Calibrations curves were used to quantify these compounds. Total FA and CA were calculated as the sum of *cis* and *trans* isomers. The total ester-linked DFA concentration was calculated as the sum of the three DFA isomers identified and quantified by this analytical procedure. Three replicates were analysed and quantified.

3.3. Statistical analysis

Results are expressed as the means and standard deviation of three different experiments. Where appropriate, data were compared using Student's *t*-test and differences with P-values < 0.05 are considered to be statistically significant.

Acknowledgements

This work was supported by grants from the Spanish Ministerio de Economía y Competitividad (AGL2011-30545-C02-02). Romina Martínez-Rubio's doctoral research was granted by the Ministerio de Educación, Cultura y Deporte by the FPU program (FPU13/03505). We are grateful to Rogelio Santiago and Rosana Malvar from Estación Biológica de Galicia-CSIC (Pontevedra, Spain), for their kind gifts of DFAs standards. We also thank to Sofía Fernández-Llamazares for providing HPLC technical support and Denis Phelps for the English revision of the manuscript.

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