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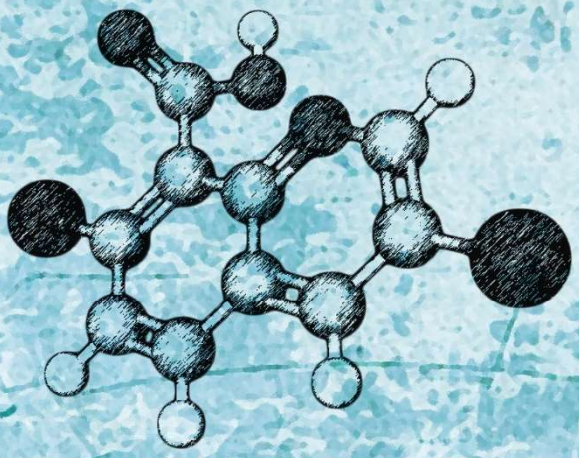
Área de Fisiología Vegetal



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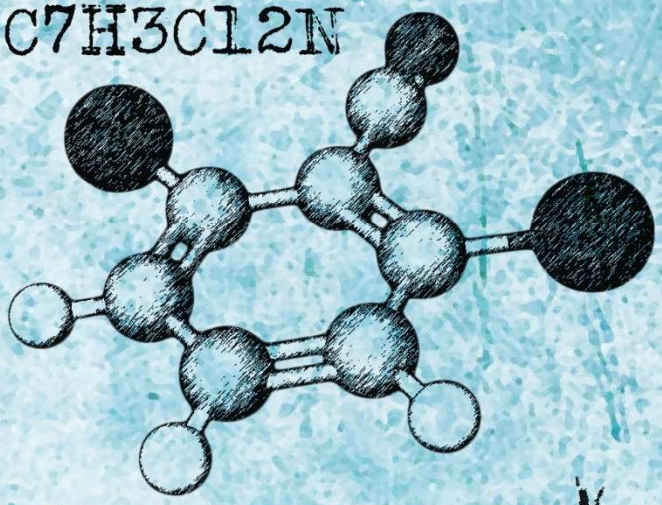

Tolerancia adquirida en la habituación de cultivos de células de plantas a diclobenil y quinclorac: modificaciones en la pared celular y estrategia antioxidante.

Asier Largo Gosens – León, 2016

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Memoria presentada por el Licenciado Asier Largo Gosens
para optar al grado de Doctor en Biología
por la Universidad de León

Fdo. Asier Largo Gosens.

VºBº
Directores:

Fdo. Dr Jesús M. Álvarez Fernández.

Fdo. Dr. Antonio E. Encina García.

A mi familia, compañeros

y amigos.

Los resultados recogidos en la presente Tesis Doctoral han sido presentados en los siguientes congresos y reuniones científicas:

A comparative study of antioxidant activities between dichlobenil-habituated callus-cultured cells of dicotyledonous (*Phaseolus vulgaris*) and monocotyledonous (*Zea mays*). A. Largo, H. Mélida, M. de Castro, P. García-Angulo, A. Alonso-Simón, J.L. Acebes, A. Encina, J. Álvarez. XVIII Congress of Federation of European Societies of Plant Biology (FESPB). Valencia. Junio 2010.

The role of antioxidant activities in the habituation of dichlobenil and quinclorac. A. Largo, H. Mélida, M.B. de Castro, P. García-Angulo, A. Alonso-Simón, A.E. Encina, J.L. Acebes, J.M. Álvarez. XII Cell Wall Meeting. Oporto (Portugal). Julio 2010.

Detección y caracterización de liginina en cultivos celulares de maíz deficientes en celulosa. A. Largo, H. Mélida, P. García-Angulo, A. Alonso-Simón, M. de Castro, J.L. Acebes, J.M. Álvarez, A. Encina. XIX Congreso de la Sociedad Española de Fisiología Vegetal (SEFV). Castellón de la Plana. Junio 2011.

Maize primary cell wall accumulates a lignin-like polymer in response to a deficiency on cellulose. A. Largo-Gosens, H. Mélida, F. Pomar, M.B. de Castro, A. Alonso-Simón, P. García-Angulo, J.L. Acebes, J.M. Álvarez, A.E. Encina. XIII Cell Wall Meeting. Nantes (Francia). Julio 2013.

Cell wall modifications during short-term habituation of maize cell suspensions to dichlobenil. M de Castro y A. Largo, A.E. Encina, J.M. Álvarez, J.L. Acebes, P. García-Angulo. XX Congreso de la Sociedad Española de Fisiología Vegetal. Lisboa (Portugal). Julio 2013.

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Mélida H, Largo-Gosens A, Novo-Uzal E, Santiago R, Pomar F, García P, García-Angulo P, Acebes JL, Álvarez J, Encina A. (2015). Ectopic lignification in primary cellulose-

deficient cell walls of maize cell suspension cultures. **Journal of Integrative Plant Biology**.57: 357-372. Doi: 10.1111/jipb.12346.

Largo-Gosens A, Encina A, de Castro M, Mélida H, Acebes JL, García-Angulo P, Álvarez JM. (2016). Early habituation of maize (*Zea mays*) suspension-cultured cells to 2,6-dichlorobenzonitrile is associated with the enhancement of antioxidant status. **Physiologia Plantarum**. 157(2): 193-204. Doi: 10.1111/ppl.12411.

Largo-Gosens A, de Castro M, Alonso-Simón A, García-Angulo P, Acebes JL, Encina A, Álvarez JM. (2016). Quinclorac-habituation of bean (*Phaseolus vulgaris*) cultured cells is related to an increase in their antioxidant capacity. **Plant Physiology and Biochemistry**. Aceptado, "*In press*". Doi: 10.1016/j.plaphy.2016.06.011.

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ABREVIATURAS

2,4-D: Ácido 2,4-difenoxiacético.
4CL: Hidroxicinamato coA ligasa.
AA: Ácido ascórbico.
ACC: Ácido 1-aminociclopropano-1-carboxílico.
AIR: Residuo insoluble en alcohol.
Api: Apiosa.
APOX: Ascorbato peroxidasa.
BSTFA: N-O-bis(trimetilsilil) trifluoroacetamida.
C3'H: *p*-cumarato 3-hidroxilasa.
C4H: Cinamato 4-hidroxilasa.
CIII-POX: Peroxidasas de la clase III.
CAD: Cinamil alcohol deshidrogenasa.
CAT: Catalasa.
CBI: Inhibidor de la biosíntesis de celulosa.
CCoAOMT: Cafeoil CoA-O-metil transferasa.
CCR: Cinamoil CoA reductasa.
CDTA: Ácido trans-1,2-diamnociclohexano-N,N,N'-N'-tetraacético.
CesA: Celulosa sintasa.
CESTRIN: *CESA trafficking inhibitor*.
COMT: Ácido cafeico/ 5-hidroxiconiferilaldehído-O-metil transferasa.
CSE: Cafeoil siquimato esterasa.
CSI: *CESA-interactive protein*.
CTL: *Chitinase-like protein*.
DCB: 2,4-diclorobenzonitrilo.
DCNB: Dicloro-2,4-nitrobenceno.
DFA: Dehidrodímeros de ácido ferúlico.
DH: Células de alubia deshabituadas.
DHA: Dehidroascorbato.
DHAR: Dehidroascorbato reductasa.
DMSO: Dimetilsulfóxido.
DTNB: Ácido 5,5-ditio-bis-2-nitrobenzoico.
DTT: Ditiotreitol.
DW: Peso seco.
EDTA: Ácido etilendiaminotetraacético.
F5H: Ferulato 5-hidroxilasa.
FDA: Diacetato de fluoresceína.
FPGS: Folipoliglutamato sintasa.
Fru: Fructosa.
FTIR: *Fourier transform infrared*.
Fuc: Fucosa.
FW: Peso fresco.
G: Unidad guayacilo.
Gal: Galactosa.
GalU: Ácido galacturónico.
GC: Cromatografía de gases.
GC-MS: Cromatografía de gases acoplada a espectrometría de masas.
Glc: Glucosa.
GlcU: Ácido glucurónico.
GPI: glucofosfatidil inositol.
GR: Glutatión reductasa.
GSH: Glutatión (forma reducida).
GSSG: Glutatión disulfuro (forma oxidada).
GST: Glutatión S-transferasa.
GT: Glicosiltransferasas.
H: Unidad *p*-hidroxifenilo.
HCN: Cianuro de hidrógeno.
HCT: *p*-hidroxicinamoil coA: siquimato/quinato *p*-hidroxicinamoil transferasa.
HPLC: Cromatografía líquida de alta resolución.
Iso-POX: Isoenzimas de CIII-POX.
JA: Ácido jasmónico.
MDA: Malondialdehído.
MDHA: Monodehidroascorbato.
MDHAR: Monodehidroascorbato reductasa.
MPI: *Maize protease inhibitor.*
NAD(P)H: Nicotinamida adenina dinucleótido (fosfato).
NH: Células de alubia no habituadas.
NH+10: Células de alubia tratadas con 10 µM de quinclorac.
NIR: *Near infrared*.
NPR1: *Non-expressor of PR1.*
OPR: 12-oxofitodienato reductasa.
PAL: Fenilalanina amonio liasa.
PCR: Reacción en cadena de la polimerasa.
POX: Peroxidasa.
PR1: *Pathogenesis-related protein1*
Q10+30: Células de alubia habituadas a 10 µM de quinclorac tratadas con 30 µM de este inhibidor.
Q15+30: Células de alubia habituadas a 15 µM de quinclorac tratadas con 30 µM de este inhibidor.
Qn: Células de alubia habituadas a crecer en presencia de "n" µM de quinclorac.
qPCR: PCR cuantitativa.
Quinclorac: Ácido 3,7-dicloroquinolino carboxílico.
RG: Ramnogalacturonano.
RGR: Tasa de crecimiento relativa.
Rha: Ramnosa.
ROS: Especies reactivas de oxígeno.

Rpm: Revoluciones por minuto.
RSA: Sistema de defensa adquirido.
RT-PCR: Retrotranscripción-PCR.
S: Unidad siringilo.
SA: Ácido salicílico.
Sac: Sacarosa.
SDS: Dodecilsulfato sódico.
SNH: Células de maíz no habituadas.
SNH+DCB(x): Células de maíz tratadas con "x" µM de DCB.
SOD: Superóxido dismutasa.
SHx: Células de maíz habituadas a "x" µM de DCB.
SuSy: Sucrosa sintasa.
TA: Ascorbato total.
TBARS: *Thiobarbituric acid reactive substances*.
Td: Tiempo de duplicación.
TFA: Ácido trifluoroacético.
TG: Glutatión total.
TNB: Ácido 5-tio-bis-2-nitrobenzoico.
UBCP: *Ubiquitin carrier protein*.
UBI: Ubiquitina.
UDP: Uridina difosfato.
UV: Ultravioleta.
Xil: Xilosa.

ÍNDICE

INTRODUCCIÓN

1. La pared celular de las plantas

La pared celular de las plantas es una estructura semirrígida de 0,1 a 10 µm de grosor que rodea los protoplastos de las células de las plantas. La pared celular proporciona forma y soporte estructural a las plantas. En acción coordinada con el citoesqueleto, limita y orienta el crecimiento de las células de las plantas ejerciendo una gran influencia en la especialización funcional de los diferentes tipos celulares y en la morfología y desarrollo de la planta (Ivakov y col., 2013; Cosgrove, 2015; Ali y Traas, 2016; Bidhendi y Geitmann, 2016). Su estructura compleja, dinámica y metabólicamente activa juega un papel esencial en las respuestas a factores ambientales, factores de estrés y en múltiples procesos fisiológicos que van desde el transporte de agua hasta la señalización celular (Popper y col., 2011; Wolf y col., 2012; Malinovsky y col.,2014; Tenhaken y col., 2015; Hamann, 2015a, 2015b; Wang y col., 2016).

La pared celular comienza a sintetizarse con la aparición de la placa celular durante la telofase (Smith, 2001). El desarrollo de la placa celular origina la lámina media constituida por polisacáridos pécticos (Aspinall, 1980). Inmediatamente, entre la membrana plasmática y la lámina media se deposita la pared celular primaria, que presenta estructura fibrilar debido a la acumulación de celulosa cristalina (Figura 1; Knox, 2008). La pared celular primaria y la lámina media están presentes en las células que conservan la capacidad de dividirse y/o elongarse. En muchos tipos celulares y sujeto a programas de desarrollo, las células pierden la capacidad de división/elongación y se especializan acumulando entre la membrana plasmática y la pared celular primaria una estructura multicapa constituida básicamente por celulosa y lignina (suberina en algunos tipos celulares), denominada pared celular secundaria (Figura 1; Schneider y Persson, 2015; Kumar y col., 2016).

1.1. Dinámica de la pared celular primaria

La pared celular primaria es una estructura compleja en cuanto arquitectura y composición. Está formada por celulosa cristalina (25-30% del peso seco) embebida en una matriz de polisacáridos complejos y glicoproteínas (70-75% peso seco), cuya composición varía dependiendo de la especie que consideremos, del tipo de tejido e incluso del grado de diferenciación del tipo celular en cuestión (Knox, 2008).

1.1.a. Composición y biosíntesis

A continuación, se van a describir los principales componentes de la pared celular primaria.

1.1.a.1. Polisacáridos

Celulosa

La celulosa es un glucano polimérico organizado en microfibrillas con estructura cristalina (Guerreiro y col., 2010). Este polisacárido se deposita en la pared celular primaria en forma de microfibrillas de entre 2 y 4 nm de diámetro, cada una de ellas formada por 36 cadenas de β-1,4-glucano, con un grado de polimerización entre 800 y 10000 (Delmer, 1999; Saxena y Brown, 2000; Lerouxel y col., 2006). Los residuos de glucosa están unidos por un enlace β-1,4-, que hace que cada residuo esté girado 180º con respecto al residuo adyacente, haciendo que el disacárido celobiosa sea realmente la unidad que se repite dentro molécula (Saxena y col., 1995). Esta disposición de la molécula, junto con la ausencia de ramificaciones laterales, es la responsable de que las moléculas de β-1,4-glucano adquieran una estructura espacial plana, permitiendo la interacción estrecha de unas cadenas con otras mediante la formación de puentes de hidrógeno. El resultado es una estructura microfibrilar mayoritariamente cristalina con algunas regiones amorfas (Somerville, 2006; Guerriero y col., 2010). Este modelo de microfibrillas de celulosa que hasta recientemente era ampliamente aceptado, hoy en día es objeto de debate. Algunos autores proponen que las microfibrillas están compuestas por 18 cadenas de β-1,4-glucano y que estas cadenas se pueden fusionar por parejas, dando lugar a una estructura más estable y resistente (Fernandes y col., 2011; Jarvis y col., 2013; Newman y col., 2013; Thomas y col., 2013). Otros autores indican que como mínimo la microfibrilla de celulosa debe estar compuesta por 24 cadenas de β-1,4-glucano (Wang y Hong, 2016).

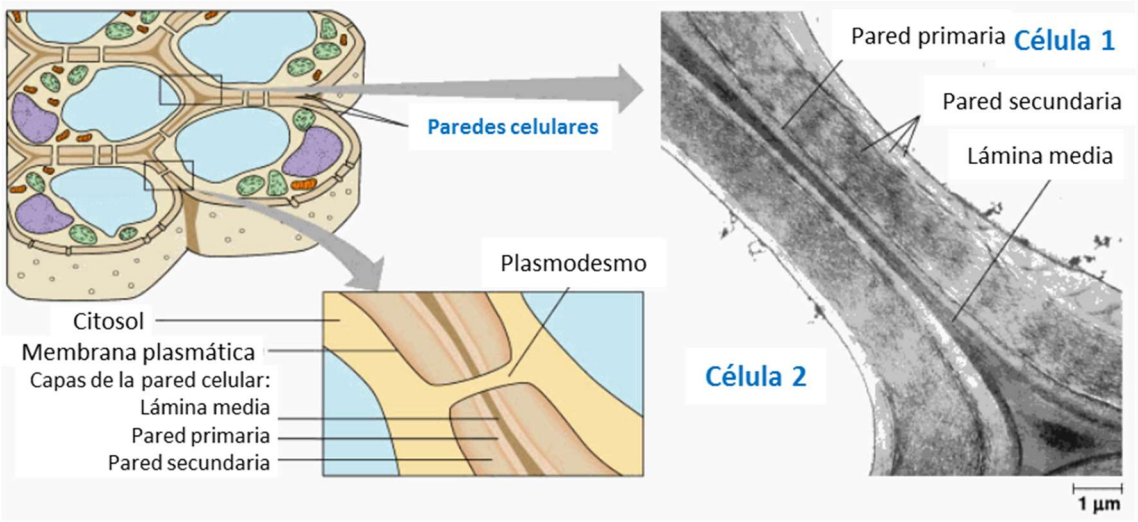


Figura 1. Representación esquemática de la pared celular vegetal. Modificado de Campbell y Reece, 2005.

En las plantas, la celulosa se sintetiza en complejos proteicos de transmembrana denominados complejos celulosa sintasa, complejos terminales o rosetas (Figura 2; Somerville, 2006; Mutwil y col., 2008; Taylor, 2008). El modelo más

extendido contempla que cada celulosa sintasa es un homohexámero que contiene en total 36 centros catalíticos (6 por cada monómero) localizados en otras tantas proteínas celulosa sintasa o proteínas CESA (Guerreiro y col., 2010). Con la aparición del nuevo modelo de microfibrillas de celulosa compuestas por 18 cadenas, se ha propuesto que, de los 6 centros catalíticos, sólo 3 cooperarían en la síntesis de celulosa (Taylor y col., 2003; Guerreiro y col., 2010; Jarvis, 2013).

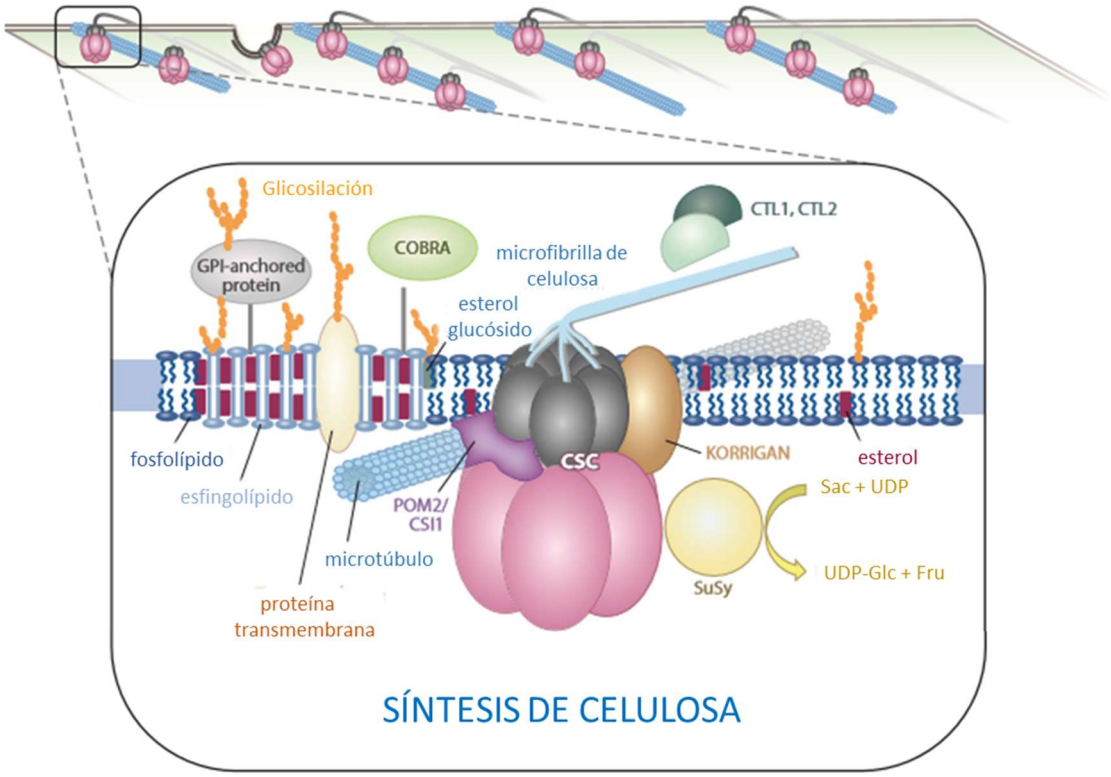


Figura 2. Representación de los complejos celulosa sintasa y de los factores que se propone regulan su biosíntesis. Los microtúbulos corticales y el aporte de UDP-Glucosa (UDP-Glc) por la enzima SuSy ("Sucrose Synthase") pueden afectar el movimiento de CSC. La composición de la membrana plasmática (presencia/ausencia de esfingolípidos y esteroles) también puede afectar a la movilidad de los CSC. Proteínas que interaccionan con los CSC como KORRIGAN (endo β-1,4-glucanasa) y CSI1/POM2 ("CESA-interactive protein") regulan esta síntesis de celulosa y, por último, proteínas extracelulares como "GPI anchored protein", incluida la proteína COBRA, y CTL1 y CTL2 ("Chitinase-like") pueden influir en la velocidad de los CSC, en la deposición de microfibrillas de celulosa y en la síntesis de otros polisacáridos. Otras abreviaturas: Sac, sacarosa; Fru, fructosa; GPI, glucofosfatidil inositol. (Modificado de McFarlane y col., 2014).

Las proteínas CESA poseen 8 dominios transmembrana que formarían un poro a través de la membrana plasmática y tienen sus centros activos, así como los extremos N y C terminales, localizados hacia el citosol (Delmer, 1999; Lerouxel y col., 2006). La síntesis de celulosa constaría de dos procesos, polimerización y cristalización, altamente coordinados, pero muy probablemente independientes (Guerreiro y col., 2010). Cada centro activo sería responsable de la polimerización de una cadena de glucano que protruiría a través del poro transmembrana hacia el

exterior de la célula para organizarse espontáneamente por unión a cadenas de glucano vecinas. El movimiento de los complejos terminales no es al azar, sino que es determinado por la polimerización de las microfibrillas de celulosa, de manera que estas se depositen en la pared celular de manera altamente ordenada (McFarlane y col., 2014). En último término, se ha propuesto que la deposición ordenada de las microfibrillas de celulosa depende de microtúbulos corticales presentes en la cara citosólica de la membrana plasmática, que servirían de guía para el movimiento de los complejos terminales (Paredez y col., 2006). Mediante la caracterización de mutantes para la biosíntesis de celulosa se ha concluido que, al menos en arabidopsis, los complejos terminales interactúan de forma transitoria o permanentemente con otras proteínas. Es el caso, entre otras, de la sacarosa sintasa (SuSy), la endo β-1,4 glucanasa (KORRIGAN) y la "CESA-interactive-protein 1" (CSI-1). Las dos últimas proteínas, asociadas a la membrana plasmática, han sido postuladas como la unión directa entre microtúbulos y complejos terminales (Figura 2; Amor y col., 1995; Nicol y col., 1998; Gu y col., 2010; Li y col., 2015). Su papel en la biosíntesis de celulosa, el tipo de unión (transitoria o no) y cómo se unen los complejos terminales está por dilucidar (Guerreiro y col., 2010; Li y col., 2015).

Las subunidades catalíticas de los complejos terminales están codificadas por genes CesA que se organizan de 6 familias multigénicas (Kumar y Turner, 2015). En el caso concreto de arabidopsis y maíz, se han identificado, respectivamente, 10 y 12 genes CesA con función característica en la formación de pared primaria o secundaria y con diferente expresión tisular (Kumar y Turner, 2015). Mediante el análisis de mutantes para la biosíntesis de celulosa se ha demostrado que durante la formación de la pared primaria en arabidopsis se requieren tres genes CesA: AtCesA1, AtCesA3 y AtCesA6/2/5/9 (Arioli y col., 1998, Fagard y col., 2000b, Scheible y col., 2001; Kaur y col., 2016). Posteriormente, durante la formación de pared celular secundaria los genes anteriormente citados son reemplazados por AtCes4, AtCesA7 y AtCesA8 (Taylor y col., 1999, 2000, Turner y Somerville, 1997). Basado en relaciones filogenéticas se ha propuesto que en maíz la función de los genes AtCesA1, AtCesA3 y AtCesA6 sería desempeñada por ZmCesA1/3; ZmCesA4/5/9 y ZmCesA6/8 respectivamente (Appenzeller y col., 2004; Kaur y col., 2016). Asimismo, durante la formación de la pared celular secundaria en maíz se requiere la expresión de los genes ZmCesA10, ZmCesA11 y ZmCesA12 (Appenzeller y col., 2004; Kaur y col., 2016). Estas proteínas CESA se coexpresan, ensamblan en el aparato de Golgi y exportan por exocitosis a la membrana plasmática donde forman parte del mismo complejo (Somerville, 2006, Desprez y col., 2007; McFarlane y col., 2014). Parece ser, además, que 3 subunidades CESA son el requerimiento estructural mínimo para que los complejos terminales tengan actividad catalítica (Guerreiro y col., 2010).

Polisacáridos matriciales: hemicelulosas y pectinas

Las hemicelulosas son polisacáridos neutros con una estructura no cristalina, compuestos principalmente por cadenas lineales de los monosacáridos xilosa,

glucosa y manosa y con cadenas laterales cortas (Pauly y col., 2013). Las hemicelulosas más características de las paredes celulares primarias son el xiloglucano, los arabinoxilanos, (glucurono)arabinoxilanos y glucano mixto (Figura 3; Pauly y col., 2013; Liu y col., 2015; Peña y col., 2016; Pauly y Keegstra, 2016). Tradicionalmente, se ha descrito que estas hemicelulosas se unen mediante puentes de hidrógeno a las microfibrillas de celulosa manteniéndolas en la conformación correcta.

Las pectinas son polisacáridos matriciales ricos en el monosacárido ácido galacturónico (GalU). El contenido de estos polisacáridos en paredes celulares primarias es variable, llegando a representar hasta el 10% del peso seco (Carpita, 1996). Se ha descrito que desempeñan importantes funciones como la absorción y retención de agua, el transporte de iones, la adhesión celular, la determinación del tamaño del poro de la pared celular y actúan como un mecanismo de defensa frente a patógenos, heridas o estreses abióticos (Verhertbruggen y Knox, 2007; Peaucelle y col., 2012; Wolf y Greiner, 2012; Anderson, 2016; Bethke y col., 2016). Las pectinas se pueden clasificar en cuatro dominios diferentes en función de su composición: homogalacturonano, ramnogalacturonano tipo I, ramnogalacturonano tipo II y xilogalacturonano (Figura 4; Scheller y col., 2007; Anderson, 2016). Se considera que estos cuatro dominios están unidos en la pared celular (Fry, 2011) y no forman estructuras independientes, ni polisacáridos únicos, sino que *in muro* forman una estructura continua (Ishii y Matsunaga, 2001; Coenen y col., 2007).

A diferencia de la síntesis de celulosa que se produce en la membrana plasmática, los polisacáridos no celulósicos son sintetizados en las cisternas del aparato de Golgi y, posteriormente, transportados a la pared celular por exocitosis mediada por vesículas (Lerouxel y col., 2006; Driouich y col., 2012; Day y col., 2013; Anderson, 2016). Las enzimas implicadas en la biosíntesis de estos polímeros son diferentes glicosil-transferasas (GTs) que catalizan la formación del esqueleto y de las cadenas laterales del polisacérido; metil-, acetil- y acil-transferasas, que participan en la metilación, acetilación y feruloilación de las moléculas, y, por último, UDP-monosacárido sintetasas, mutasas y epimerasas que sintetizan los UDP-azúcares precursores. La especificidad de estas enzimas determina la secuencia de los restos de azúcares en el polisacárido, así como su patrón de ramificación (Rennie y Scheller, 2014 y referencias incluídas).

1.1.a.2. Compuestos fenólicos

Los compuestos fenólicos, principalmente los hidroxicinamatos o fenilpropanoides, son sintetizados en el citoplasma a partir la fenilalanina a través de la ruta de los fenilpropanoides (Vogt, 2010). Los fenoles más importantes en la pared celular son el ácido ferúlico y el ácido *p*-cumárico, ambos derivados del ácido cinámico (Wallace y Fry, 1994). Una vez sintetizados son transportados al aparato de Golgi y esterificados sobre los polisacáridos gracias a la actividad de la

superfamilia BAHD (familia Pfam PF02458) que codifican CoA-acyl-transferasas (Mitchell y col., 2007; Piston y col., 2010).

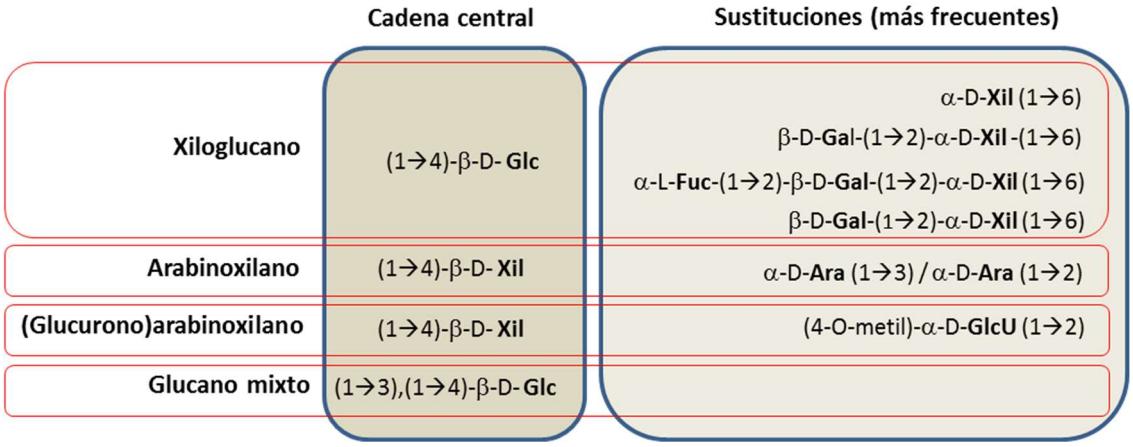


Figura 3. Resumen de las estructuras moleculares, sustituciones y los tipos de enlace más frecuentes de las principales hemicelulosas. Abreviaturas: Xil, xilosa; Glc, glucosa; Gal, galactosa, Fuc, fucosa; GlcU, ácido glucurónico. Basado en Albersheim y col. (2012).

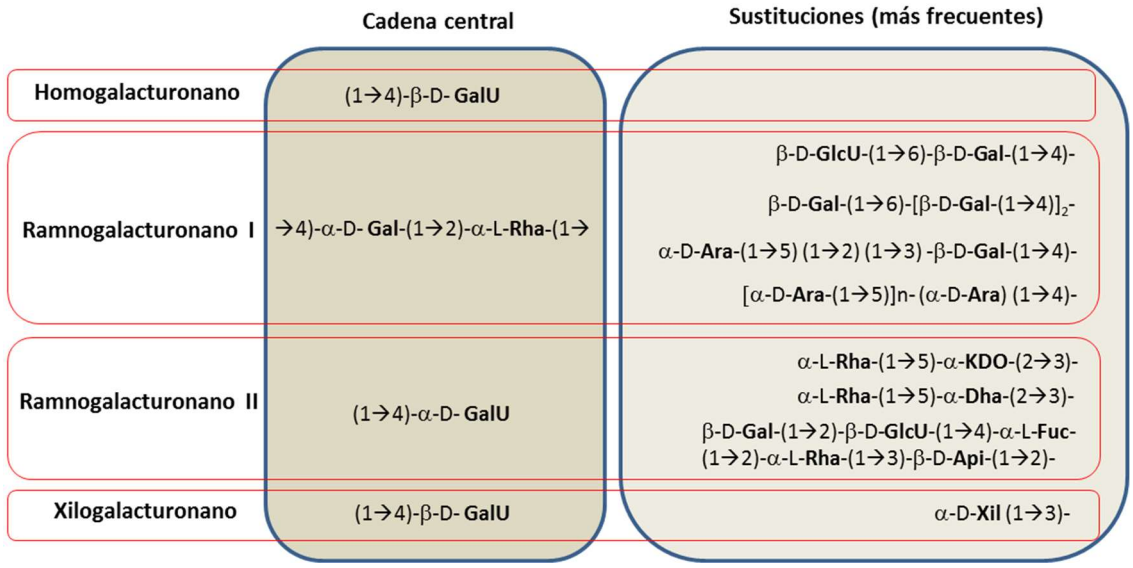


Figura 4. Resumen de las estructuras moleculares, sustituciones y los tipos de enlace más frecuentes de las pectinas. Abreviaturas: Gal, galactosa, Fuc, fucosa; GlcU, ácido glucurónico, Rha, ramnosa; GalU, ácido galacturónico; Api, apiosa; KDO, ácido 3-deoxi-D-mano-2-octulosónico; Dha, ácido 3-deoxi-D-lixo-2-heptulosárico. Basado en Albersheim y col. (2012).

Los compuestos fenólicos son componentes característicos y muy importantes desde el punto de vista funcional de las paredes celulares primarias (Zarra y col., 2012). Los hidroxicinamatos aparecen esterificados con restos α-arabinosil del arabinoxilano (Wende y Fry, 1997), con restos α-xilosil del xiloglucano (Ishii e Hiroi, 1990), con restos α-arabinosil y β-galactosil del ramnogalacturonano I (Harris y Trethewey, 2010) y, probablemente, con glicoproteínas (Obel y col., 2003). Aunque son componentes minoritarios de la pared celular, tienen papeles muy importantes, ya que el ácido ferúlico puede experimentar acoplamiento oxidativo,

en presencia de peróxido de hidrógeno, mediante la acción de peroxidasas (Geissman y Neukom, 1971; Ralph y col., 2004b) dando lugar a los dehidrodímeros (dehidroferulatos, diferulatos o DFAs) (Fry, 2004a; Parker y col., 2005), que permiten el entrecruzamiento de los diferentes polisacáridos a los que los fenoles están esterificados (Ishii, 1997; Saulnier y col., 1999). El entrecruzamiento de los polisacáridos mediados por el acoplamiento fenólico, influye en muchos aspectos de la pared celular, contribuyendo a la rigidez de la célula y el cese del crecimiento, la cohesión tisular y la respuesta frente a estreses bióticos y abióticos limitando su degradabilidad (Buanafina, 2009).

1.1.a.3 Proteínas

Las proteínas de pared celular son un componente minoritario pero esencial, ya que interaccionan con proteínas de la superficie de membrana plasmática, lo que incrementa la cohesión celular, y participan en la modificación *in muro* de los diferentes componentes de la pared celular (Jamet y col., 2006; Uzal y col., 2009; Albenne y col.,2013; Canut y col., 2016). Las proteínas de pared se suelen clasificar en dos grupos básicos: proteínas estructurales que suelen estar inmovilizadas en la pared celular y proteínas solubles las cuales pueden estar ancladas o libres en el apoplasto y la mayoría poseen actividad enzimática (Lee y col., 2004).

Las proteínas estructurales suelen tener secuencias altamente repetitivas de uno o dos aminoácidos y están divididas en 4 grupos principales: proteínas ricas en hidroxiprolina, proteínas ricas en prolina, proteínas ricas en glicina y arabinogalactano proteínas.

Las proteínas solubles, generalmente poseen actividades enzimáticas relacionadas con procesos de extensión de la pared celular, transporte de moléculas, reconocimiento celular y resistencia a patógenos (Rose y col., 2002; Borassi y col., 2015; Marowa y col., 2016). Dentro de este grupo se encuentran las hidroxilasas, transglicosilasas, quinasas y peroxidasas extracelulares. Las expasinas constituyen un grupo amplio y especializado de proteínas solubles de pared que participan en la expansión de la pared celular sin que se haya descrito actividad enzimática asociada (Park y Cosgrove, 2012; Cosgrove, 2015).

La síntesis y ensamblaje de proteínas de pared celular se realiza en el retículo endoplásmico. Muchas de esas proteínas son sintetizadas de manera constitutiva y son reguladas según el estadío de desarrollo o el tejido de la planta. Sin embargo, muchas otras proteínas son sintetizadas exclusivamente cuando las plantas sufren procesos de estrés ambiental y en respuesta a heridas e infecciones, por lo tanto, es un proceso altamente controlado tanto a nivel de síntesis de las proteínas como a nivel de modificación post-traduccional (Albersheim y col., 2011).

1.1.b. Arquitectura de la pared celular primaria

La pared celular primaria está compuesta por dos o tres redes estructurales conectadas entre sí: (i) la red de celulosa-hemicelulosas, (ii) la red de polisacáridos no celulósicos (iii) la red de proteínas (Figura 5; Carpita y McCann, 2000).

Según su composición se pueden diferenciar dos tipos principales de pared celular primaria en angiospermas (Carpita y Gibeaut, 1993). La mayoría de las plantas (dicotiledóneas y monocotiledóneas no commelinoides) poseen pared celular primaria tipo I y las gramíneas y las monocotiledóneas no commelinoides poseen pared celular primaria tipo II (Figura 5; Carpita, 1984). La composición que posea cada tipo de pared celular va a determinar su arquitectura final característica y sus propiedades.

En la pared celular primaria tipo I, la red estructural celulosa-hemicelulosas está formada el entramado celulosa-xiloglucano, componente que se une a las microfibrillas de celulosa determinando su posición (Anderson y col., 2010). Esta red se encuentra embebida en una trama de polisacáridos pécticos, que forma una matriz y determina, entre otras propiedades, el tamaño de poro de la pared celular, o la dirección y extensión del crecimiento de la célula (Kohorn, 2015; Wang y Hong, 2016). La tercera red que compone esta pared celular primaria tipo I es la red de proteínas estructurales. Recientemente, se ha abierto un debate sobre este modelo de redes estructurales, y diversos autores han propuesto un modelo alternativo en el cual indican que las microfibrillas de celulosa se unirían directamente entre ellas y, sólo en algunos casos, mediarían en esta unión las cadenas de xiloglucano. Estos puntos de unión celulosa-xiloglucano (llamados "biomechanical hotspots") podrían ser diana en procesos de relajación de la pared celular, durante el crecimiento de la célula (Figura 6; Park y Cosgrove, 2012; Wang y col., 2013; Cosgrove, 2014; Park y Cosgrove, 2015; Cosgrove, 2016; Xiao y col., 2016; Zhang y col., 2016).

En el caso de pared celular primaria tipo II la hemicelulosa principal es el arabinoxilano, que aparece en mayor o menor medida sustituido por ácido glucurónico formando el (glucurono)arabinoxilano (Pauly y col., 2013). Este tipo de pared posee pocos polisacáridos pécticos y pocas proteínas estructurales en comparación con la pared tipo I (Vogel, 2008). De manera característica es rica en restos de ácido ferúlico y ácido *p*-cumárico esterificados a moléculas de arabinoxilanos. Tal como se indicó anteriormente, los fenilpropanoides de pared sufren dimerización participando en el entrecruzamiento de la red de arabinoxilanos y permitiendo el reforzamiento de la pared celular (Schendel y col., 2016).

Las uniones entre las diferentes redes de ambos tipos de pared celular no se conocen con exactitud hoy en día, pero sí que se sabe que dichas uniones juegan un papel esencial en el reforzamiento y estabilización, en presencia de factores de estrés que afectan a la integridad de la pared celular o a alguno de sus componentes (Voxeur y Höfte, 2016). Por lo tanto, el estudio de esta plasticidad

estructural de la pared celular primaria tiene gran interés para conocer los mecanismos que permiten a las plantas responder ante diferentes tipos de estrés.

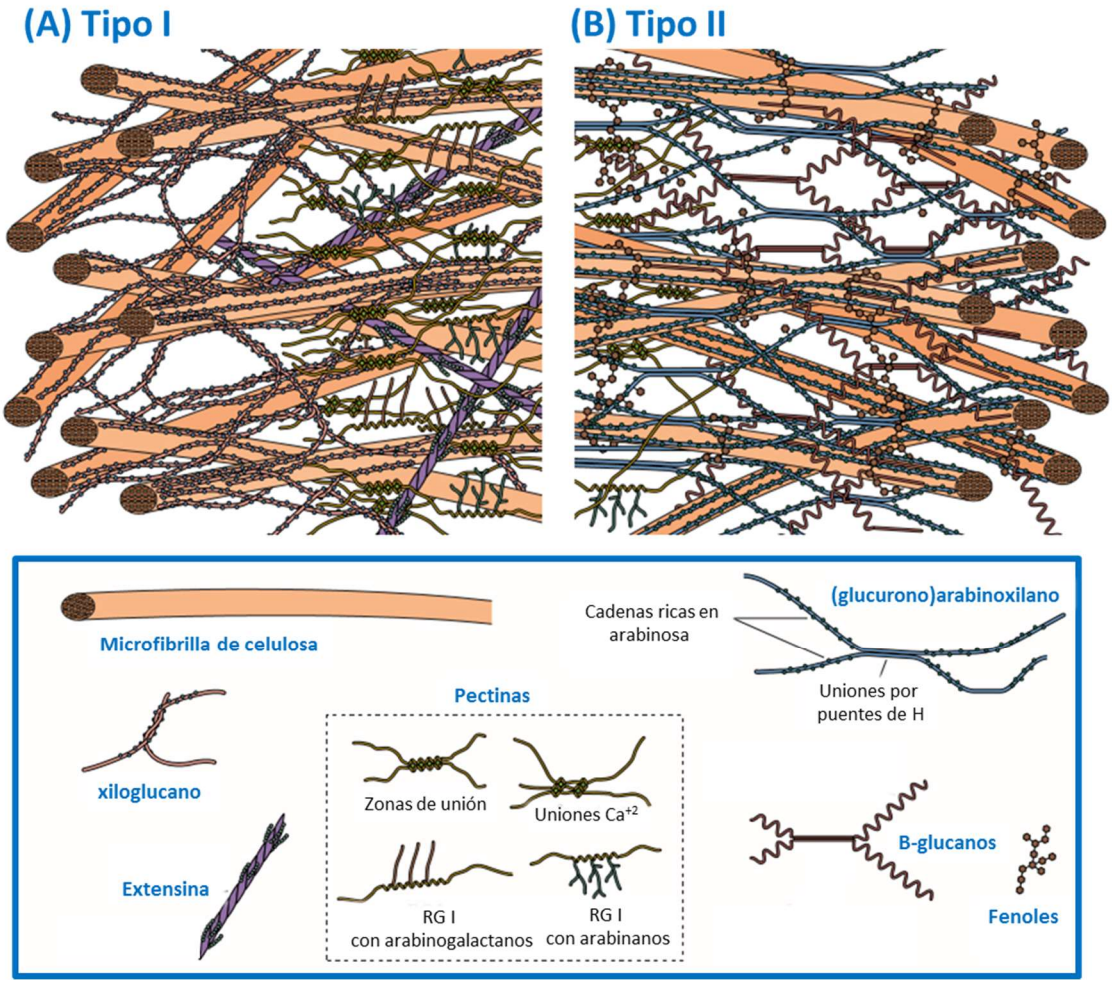


Figura 5. Modelos estructurales de pared celular primaria en plantas. (A) pared celular primaria tipo I y (B) pared celular primaria tipo II. Modificado de Carpita y McCann, 2000. Abreviatura: RG, ramnogalacturonano.

1.2. Plasticidad estructural de la pared celular primaria

La pared celular primaria es un compartimento metabólicamente muy activo y posee una elevada capacidad para acomodarse a nuevas condiciones mediante la modificación de su estructura y/o composición. En los últimos años se han caracterizado paredes celulares con composición alterada debido a modificaciones genéticas y adaptaciones o habituaciones a diferentes tipos de estrés. Gracias a estos estudios se ha avanzado de forma importante en el conocimiento de la composición, estructura y dinámica de la pared celular (Carpita y McCann, 2015).

Una de las metodologías de estudio de esta plasticidad estructural de la pared celular, ha sido el uso de mutantes que afecten a la expresión de genes relacionados con la síntesis de los diferentes componentes de la pared celular o de

genes que regulen estos procesos de biosíntesis (Reiter y col., 1997; McCann y col., 2007: Penning y col., 2009; Largo-Gosens y col., 2014; Carpita y McCann, 2015).

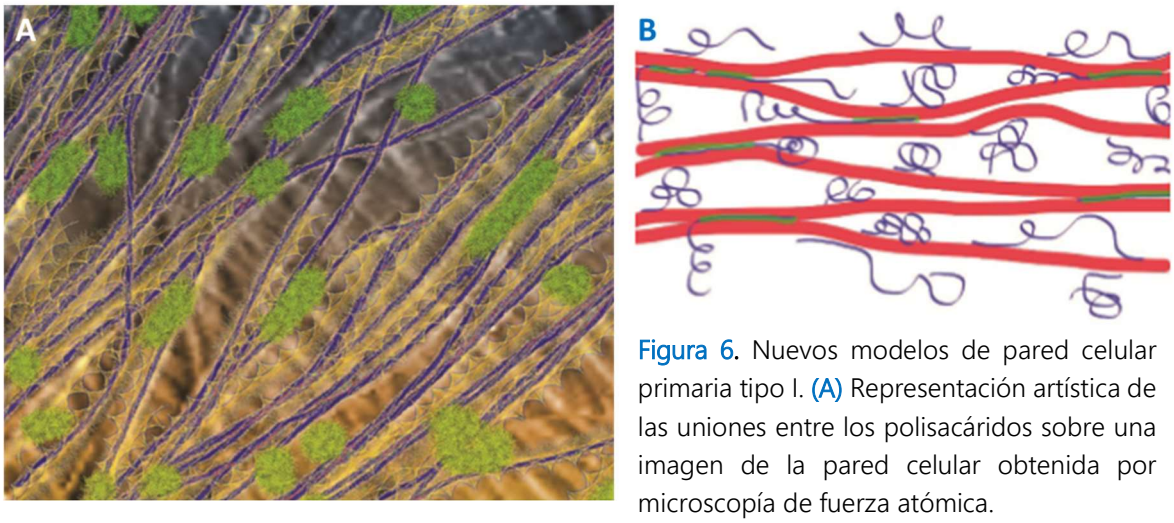


Figura 6. Nuevos modelos de pared celular primaria tipo I. (A) Representación artística de las uniones entre los polisacáridos sobre una imagen de la pared celular obtenida por microscopía de fuerza atómica. Las microfibrillas de celulosa representadas en color azul, donde se observan las uniones potenciales entre estas microfibrillas. El xiloglucano aparece representado en color verde y las pectinas en color amarillo. (B) Nuevo modelo de union de celulosa-xiloglucano ("biochemical hotspots model"). Las microfibrillas de celulosa (rojo) están unidas entre sí por la intermediación del xiloglucano (verdes). El xiloglucano libre está representado en azul. Modificado de Cosgrove (2015, 2016).

En muchos casos la tolerancia a diferentes tipos de estrés ambiental está relacionada con mecanismos que implican modificaciones en la pared celular. El estudio de estas modificaciones sirve para tener conocimiento de los efectos que tiene el estrés en la integridad de la pared celular, y del papel global de esta estructura y de sus componentes en esa tolerancia. Desde hace mucho tiempo se vienen empleando técnicas de cultivo *in vitro* de células, tejidos y órganos vegetales para obtener líneas celulares que sean tolerantes a diferentes tipos de estrés (Sancho y col., 1996; Yamada y col., 2002; Wusheng y Donghua, 2010; Szechyńska-Hebda y col., 2016).

Una de las metodologías que más información ha dado sobre la plasticidad estructural de la pared celular y su relación en la tolerancia a estreses es la habituación de cultivos celulares a unos compuestos relacionados con la inhibición de la biosíntesis de celulosa y que han sido catalogados como inhibidores de la biosíntesis de celulosa o CBIs por sus siglas en inglés (Tabla 1).

1.2.a. Compuestos relacionados con la Inhibición de la biosíntesis de celulosa

Existen una serie de compuestos que, mediante el estudio de modificaciones en la pared celular, han sido relacionados con una inhibición de la biosíntesis de celulosa y, por tanto, han sido incluidos dentro de los CBIs. Este grupo de compuestos afectan el acoplamiento y/o deposición de celulosa en la pared celular de plantas y, por lo tanto, modifican la composición y proporción final de diferentes

componentes de la pared celular y las uniones entre ellos (Tabla 1; Acebes y col., 2010; Álvarez y col., 2012; García-Angulo y col., 2012). La utilización de estos CBIs ha sido muy útil a la hora de estudiar la formación, organización, estructura y plasticidad de la pared celular primaria y secundaria (Satiat-Jeunemaitre y Darzens, 1986; Suzuki y col., 1992; Taylor y col., 1992; Vaughn y Turley, 1999, 2001; Díaz-Cacho y col., 1999; Encina y col., 2001, 2002; Alonso-Simón y col., 2004, 2007, 2011; García-Angulo y col., 2006, 2009; Melida y col., 2009, 2010a, 2010b, 2011; de Castro y col., 2014; 2015), el mecanismo de pérdida de rigidez y la extensión de la pared celular (Hoson y Masuda, 1991; Edelmann y Fry, 1992; Edelmann y Köhler, 1995; Montague, 1995) y para comprender el proceso de formación de la placa celular y la división celular (Vaughn y col., 1996; DeBolt y col, 2007a). Además, el uso de estos inhibidores ha ayudado a entender diferentes procesos de la biosíntesis de celulosa, como el ensamblaje de las diferentes subunidades de celulosa sintasa para la formación de los complejos terminales (Mizuta y Brown, 1992); el acoplamiento de los complejos terminales a los microtúbulos del citoesqueleto que orientan la dirección de la síntesis de celulosa (Fisher y Cyr, 1998; DeBolt y col., 2007b; Brabham y DeBolt, 2012) y la relación entre la síntesis de celulosa y calosa (Delmer, 1987; Delmer y Amor, 1995).

Varios de estos compuestos han sido comercializados como herbicidas para el control de malas hierbas y han sido listados en el grupo L de la "Herbicide Resistance Action Comittee" (HRAC). Este grupo incluye a los herbicidas DCB (diclobenil), clortiamida, isoxabén, taxtomina A y flupoxam (Tabla 1; Menne y Köcher, 2007). Otros compuestos, como la triazofenamida, el CGA 325'615, la aminotriazina, el AE F150944 o el Compuesto 1, están también catalogados como CBI, pero todavía no se han comercializado.

Existen también una serie de compuestos, que han sido agrupados en otras categorías de herbicidas, pero que se han considerado CBIs porque inhiben la síntesis de celulosa como efecto secundario. Este es el caso del quinclorac y del ancimidol, (Acebes y col., 2012). En los últimos años se ha realizado un notable esfuerzo en la identificación de nuevos compuestos químicos que actúen como CBIs. Fruto de este esfuerzo ha sido la identificación de compuestos como la morlina, la cobtorina, el quinoxifeno, el indaziflam, el compuesto CESTRIN ("CESA Trafficking inhibitor") y el acetoxibian (DeBolt y col., 2007b; Yoneda y col., 2007, 2010; Harris y col., 2012; Brabham y col., 2014; Xia y col., 2014; Tateno y col., 2015; Worden y col., 2015).

Recientemente se han agrupado los CBIs por su efecto sobre los complejos terminales o su movilidad (Tabla 1; Brabham y DeBolt, 2012; Tateno y col., 2015). Los autores dividieron los CBIs en tres grupos: (i) inhibidores que disminuyen el número de complejos terminales en la membrana plasmática, donde se incluyen el isoxabén, el quinoxifeno, el CGA 325'615,el AE F150944, la taxtomina A, el CESTRIN y el acetoxibian (Paredez y col., 2006; Bischoff y col., 2009; Crowell y col., 2009; Gutierrez y col., 2009; Harris y col., 2012; Xia y col., 2014; Worden y col., 2015), (ii) inhibidores

que incrementan la cantidad de complejos terminales en la membrana plasmática pero que reducen su movilidad, donde se incluye el DCB y el indaziflam (Herth, 1987; DeBolt y col., 2007a; Brabham y col., 2014) y (iii) inhibidores que modifican la trayectoria de los complejos terminales, donde se incluyen la morlina y la cobtorina (DeBolt y col., 2007b; Yoneda y col., 2007, 2010).

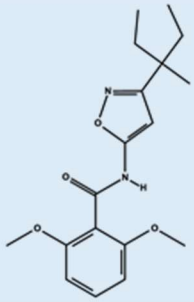
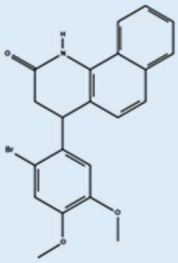
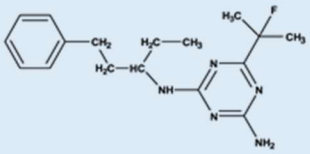
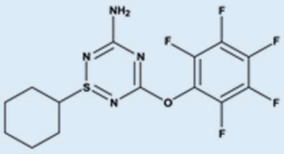
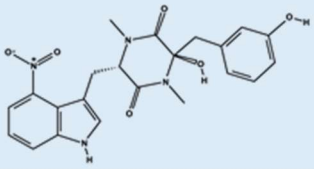
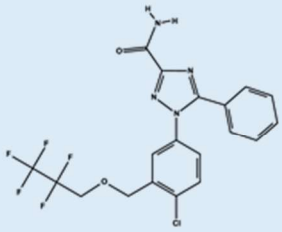
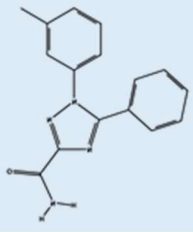
A pesar de que los CBIs son herbicidas altamente específicos y activos a concentraciones relativamente bajas, es posible obtener cultivos celulares habituados a crecer en presencia de estos inhibidores. La habituación de cultivos celulares a CBIs refleja la capacidad que tienen las células de las plantas para sobrevivir con una pared celular modificada y, por tanto, es una herramienta que nos permite estudiar la plasticidad estructural de la pared celular primaria. Hasta el momento se han conseguido cultivos celulares habituados a diferentes CBIs como el DCB (Figura 7; Shedletzky y col., 1990, 1992; Wells y col., 1994; Nakagawa y Sakurai, 1998, 2001; Sabba y col., 1999; Encina y col., 2001, 2002; Alonso-Simón y col., 2004, 2007, 2011; García-Angulo y col., 2006, 2009; Melida y col., 2009, 2010a, 2010b, 2011; de Castro y col., 2014; 2015), isoxabén (Corio-Costet y col., 1999; Sabba y Vaughn, 1999; Diaz-Cacho y col., 1999; Manfield y col., 2004) y taxtomina A (Girard-Martel y col., 2008; Brochu y col., 2010). Cada inhibidor provoca una serie de respuestas específicas, pero todos los cultivos habituados poseen unas características comunes: la disminución en las tasas de crecimiento, células con morfologías irregulares, tendencia a crecer en agregados y paredes celulares con ultraestructura modificada y contenidos reducidos en celulosa que son compensados por otros componentes de la pared.

Los compuestos relacionados con la inhibición de la biosíntesis de celulosa utilizados en la presente tesis doctoral son el DCB (2,6-diclorobenzonitrilo; diclobenil) y quinclorac (Figura 8; ácido 3,7-dicloro-8-quinolinocarboxílico).

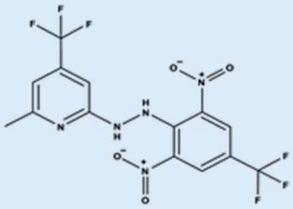
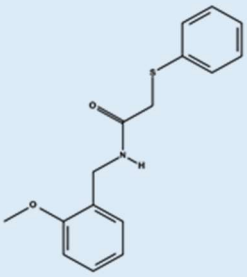
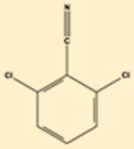
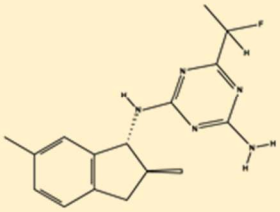
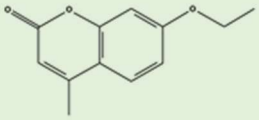
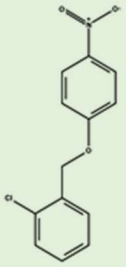
1.2.a.1. DCB

El DCB es el CBI más simple a nivel estructural y ha sido uno de los CBIs más estudiados y caracterizados. Aunque es menos efectivo en monocotiledóneas (Tabla 1; Sabba y Vaughn, 1999), es un compuesto ampliamente usado en la agricultura como herbicida de preemergencia de amplio espectro (Verloop y Nimmo, 1969). El DCB es un herbicida que afecta a la formación de la placa celular, aunque no inhibe la división celular (Galbraith y Shields, 1982) ni la elongación celular (Vaughn y col., 1996; Sabba y col., 1999; Encina y col., 2001, 2002; Vaughn, 2002). El tratamiento con DCB durante la germinación de semillas provoca un engrosamiento de las raíces e hipocótilos y una inhibición del crecimiento y desarrollo de las raíces secundarias (Umetsu y col., 1976; Eisinger y col., 1983; Montague, 1995; Himmelspach y col., 2003; DeBolt y col., 2007b).

Tabla 1 (pag. sig.). Lista de los principales CBIs agrupados por su modo de acción según Tateno y col., 2015.

CBI	Nombre químico	Fórmula química	Referencias
Grupo 1: Reducción del número de complejos terminales en la membrana plasmática			
Isoxabén	N-[3-(1-etil-1-metilpropil)-5-isoxazolil]-2,6-dimetoxibenzamida		Gutierrez y col., 2009
Quinoxifeno	4-(2-bromo-4,5-dimetoxifenil)-3,4-dihidro-1H-benzoquinolin-2-ona		Harris y col., 2009
AE F10944	N2-(1-etil-3-fenilpropil)-6-(1-fluoro-1-metiletil)-1,3,5-triacina-2,4-diamina		Gutierrez y col., 2009
CGA 325'615	1-ciclohexil-5-(2,3,4,5,6-pentafluorfenoxi)-1λ-4,2,6-triacina-3-amina		Crowel y col., 2009
Taxtomina A	Una molécula de 4-nitroindol-3-il con 2,5-dioxopiperacina		Bischoff y col., 2010
Flupoxam	1-[4-cloro-3-(2,2,3,3,3-pentafluoropropoximetil) fenil]-5-fenil-1H-1,2,4-triazol-3-carboxiimida		Kudo y col., 1999
Triazofenamida	1-(3-metilfenil(-5-fenil-1H-1,2,4-triazol-3-carboxiimida		Heim y col., 1998

Continuación Tabla 1 (pág. sig.).

CBI	Nombre químico	Fórmula química	Referencias
Grupo 1: Reducción del número de complejos terminales en la membrana plasmática			
CESTRIN	1-[2,6-dinitro-4-(trifluorometil)fenil]-2-[6-metil-4-(trifluorometil)-piridin-2-il] hidracina		Drakakaki y col., 2011 Worden y col., 2015
Acetobixán	N-(2-metoxibencil)-2-feniltio acetamida		Xia y col.,2015
Grupo 2: Reducción de la movilidad de los complejos terminales en la membrana plasmática			
DCB	2,6-diclorobenzonitrilo		Delmer, 1987 Sabba y col., 1999
Indaziflam	N2-[(1R,2S)-2,3-dihidro-2,6-dimetil-1H-inden-1-il]-6-[(1RS)-1-fluoroetil]-1,3,5-triacina-2,4-diamina		Brahbam y col., 2014
Grupo 3: Modificación de la trayectoria de los complejos terminales en la membrana plasmática			
Morlina	7-epoxi-4-metil cromen-2-ona		DeBolt y col., 2007a
Cobtorina	4-[(2-clorofenil)-metoxi]-1-nitrobenceno		Yoneda y col., 2007

El DCB es un herbicida que inhibe específicamente la biosíntesis de celulosa tanto en algas como en plantas superiores (Brummel y Hall, 1985; Hoson y Masuda, 1991; Corio-Costet y col., 1991; Encina y col., 2002; Alonso-Simón y col., 2004; Orologas y col., 2005; Mélida y col., 2009; García-Angulo y col., 2012). Se ha demostrado que el DCB no afecta a la biosíntesis de otros polisacáridos de pared

celular (Montezinos y Delmer, 1980; Blaschek y col., 1985; Francey y col., 1989), ni a otros procesos como la síntesis de ADN y proteínas, la respiración, la fosforilación oxidativa, el metabolismo lipídico, el metabolismo nucleotídico y el metabolismo de la glucosa (Meyer y Hertz, 1978; Montezinos y Delmer, 1980; Galbraith y Shields, 1982; Delmer, 1987). Hasta la fecha se ha descubierto que este inhibidor afecta a la orientación de las microfibrillas de celulosa (Sugimioto y col., 2001) y a la organización de los microtúbulos (Himmelspach y col., 2003; Peng y col., 2012). Asimismo, se ha demostrado que no disminuye el número de complejos terminales, pero sí disminuye o anula su movilidad (Hertz, 1987; Mizuta y Brown, 1992; DeBolt y col., 2007b; Wightman y col., 2009; Brabham y DeBolt, 2012; Tateno y col., 2015).

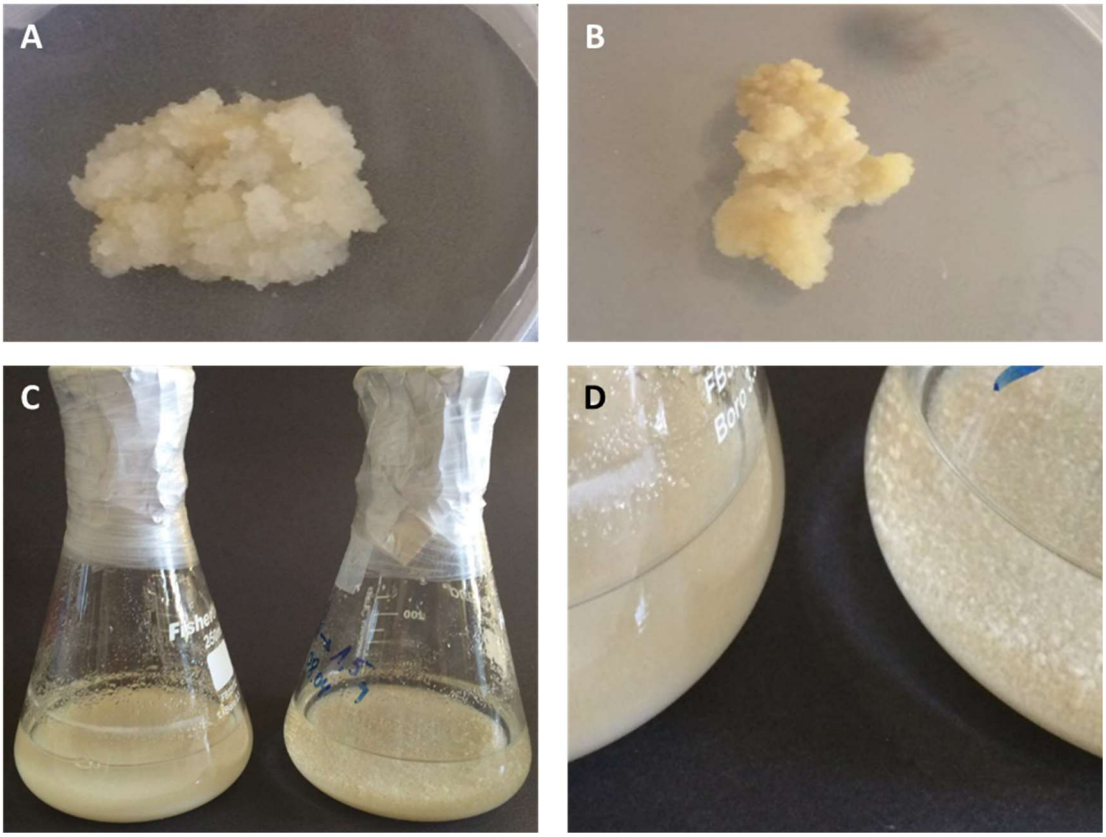


Figura 7. Ejemplo de la habituación de cultivos celulares a CBIs. Callos de maíz no habituado (A) y habituados a DCB (B). Suspensiones celulares de maíz (C) no habituadas (izqda) y habituadas a DCB (drcha). Detalle de los agregados que se forman por la habituación a DCB (dcha) (D).

A pesar de que el DCB ha sido reconocido como CBI desde hace décadas (Hogetsu y col., 1974) todavía no se ha esclarecido completamente su modo de acción. La primera propuesta sobre el modo de acción del DCB consistió en que bloqueaba la iniciación de la biosíntesis de celulosa, a través de la inhibición de la síntesis del sitosterol-β-glucósido, cebador necesario para el inicio de la síntesis de celulosa (Peng y col., 2002). Hasta donde se conoce hoy, no se ha demostrado de forma categórica que la síntesis de celulosa se inicie a partir de sitosterol-β-glucósido, por lo tanto, este modo de acción es cuestionable.

Un segundo modo de acción propuesto sería que el DCB afectara a la cristalización de la celulosa a la hora de depositarse en la pared celular. Esta severa reducción en la síntesis de celulosa se ve acompañada de una alteración en la orientación de las microfibrillas de celulosa, la cual podría ser debida a una desorganización de los microtúbulos (Himmelspach y col., 2013). Además, también se ha descrito que el DCB no afecta al número de complejos terminales, pero sí afecta a la correcta movilidad y función de los mismos (DeBolt y col., 2007b; Peng y col., 2012; Brabham y DeBolt, 2012; Tateno y col., 2015). De acuerdo con este modo de acción está el hecho de que la reducción en celulosa provocada por la habituación de cultivos celulares de alubia (*Phaseolus vulgaris*) a DCB se acompaña de una acumulación de un β-1,4-glucano no cristalino (Encina y col., 2002; García-Angulo y col., 2006).

Se han propuesto tres posibles dianas del DCB. Mediante el uso de un análogo del DCB, la 2,6-diclorofenilacida, Delmer y col. (1987) identificaron a una proteína de unos 12-18 KDa como posible diana del DCB, la cual se descartó que fuera una subunidad CESA, ya que se disociaba fácilmente de la membrana plasmática. Por otro lado, Nakawaga y Sakurai (1998) mediante el uso de anticuerpos frente a la subunidad catalítica CESA1 de tabaco, propusieron que este herbicida se uniría específicamente a esta proteína para estabilizarla y hacerla más resistente a la degradación proteolítica. Esta propuesta de diana del DCB está apoyada por el estudio del mutante *rsw1* (*root swelling1*), que afecta a esta subunidad CESA1, y que presenta una reducción del contenido en celulosa y la aparición de un β-1,4-glucano no cristalino (Arioli y col., 1998). De todas formas, en ningún momento se demostró una unión directa entre el DCB y la subunidad CESA1. La tercera diana propuesta para este inhibidor es la proteína asociada a microtúbulos MAP20. Se ha demostrado que el DCB se unía específicamente a esta proteína MAP20 durante la síntesis de celulosa, sin afectar el acoplamiento de esta proteína con los microtúbulos (Rajangam y col., 2008). Basándose en esas observaciones, los autores propusieron que el papel de la proteína MAP20 en la biosíntesis de celulosa es el ensamblaje de los complejos terminales con los microtúbulos, el cual es afectado por la presencia de DCB (Rajangam y col., 2008). Por último, los autores propusieron que la proteína de 12-18 KDa descrita por Delmer y col. (1987), podría tratarse de esta proteína MAP20 (Rajangam y col., 2008).

Finalmente, mediante el estudio de células de maíz habituadas a DCB nuestro grupo de investigación ha demostrado que la presencia/ausencia de una subunidad específica de los complejos sintasa, la ZmCesA5, es crítica a la hora de determinar la sensibilidad/resistencia de las células al DCB (Mélida y col., 2010a)

Habituación de cultivos celulares a DCB

La obtención de cultivos celulares habituados a DCB se consigue mediante la adición continuada de concentraciones crecientes de este inhibidor a lo largo del cultivo. Mediante la habituación de cultivos celulares a la presencia de un herbicida

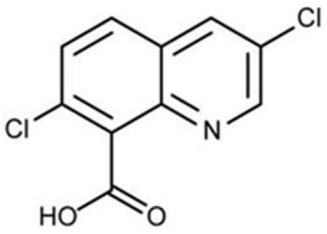
como el DCB, se pueden obtener células capaces de sobrevivir a concentraciones letales del inhibidor. En principio, la habituación a DCB se puede lograr a través de varios mecanismos y modificaciones que se pueden agrupar en dos tipos: dependientes e independientes de la pared celular. En el caso del DCB estos mecanismos van a variar en función del tipo de pared celular primaria, de la especie vegetal, de la concentración y del tiempo de exposición que estén esas células a este inhibidor (Alonso-Simón y col., 2004; de Castro y col., 2014).

La mayoría de los cultivos celulares habituados a DCB presentan paredes tipo I: tomate (Shedletzky y col., 1990), tabaco (Shedletzky y col., 1992; Wells y col., 1994; Nakagawa y Sakurai., 1998, 2001; Sabba y col., 1999) y alubia (Encina y col., 2001, 2002; Alonso-Simón y col., 2004, 2007; García-Angulo y col., 2006; 2009). En términos generales, los mecanismos de habituación dependientes de la pared celular fueron la reducción del contenido en celulosa acompañada por una reducción en hemicelulosas y un incremento en pectinas. Por otro lado, los mecanismos independientes de la pared celular residieron en un incremento en las actividades antioxidantes para paliar el estrés oxidativo provocado por el DCB (García-Angulo y col., 2009).

En el caso de plantas con pared celular primaria tipo II, sólo los cultivos celulares de dos especies vegetales han sido habituados a DCB: cebada (Shedltezky y col., 1992) y maíz (Melida y col., 2009, 2010a, 2010b, 2011; de Castro y col., 2014; 2015). En el caso de las células de maíz, la habituación a concentraciones altas de este inhibidor provocó una serie de modificaciones en la pared celular, que se pueden resumir en un incremento en arabinoxilanos con un mayor grado de entrecruzamiento mediante dímeros de ferúlico en respuesta a una reducción de hasta un 75% en el contenido en celulosa (Mélida y col., 2009; 2010a; 2010b; 2011; de Castro y col., 2014; 2015). Sin embargo, se detectó una reducción en las actividades antioxidantes y una represión de la actividad detoxificadora glutatión-S-transferasa (GST) (Mélida y col., 2010a). Por lo tanto, en base a estos resultados, la habituación de células de maíz a altas concentraciones de DCB parecía residir únicamente en modificaciones en la pared celular.

1.2.a.2 Quinclorac

El quinclorac (Figura 8) es un herbicida ampliamente utilizado en cultivos como los de arroz o cebada para controlar malas hierbas dicotiledóneas (Grossmann y Kwiatkowski, 2000). Este compuesto se ha descrito como un herbicida auxínico (Grossmann y Kwiatkowski, 1995) aunque hay varias respuestas típicas de los herbicidas auxínicos, como la acidificación de la pared celular (Theologis, 1987), el incremento de la respiración o el incremento del contenido en ARN (Koo y



Quinclorac

Figura 8. Estructura química del quinclorac

col., 1991), que el tratamiento con quinclorac no provoca (Grossman y Kwiatkowski, 1995).

Por otro lado, el quinclorac también ha sido catalogado como un inhibidor de la biosíntesis de pared celular en raíces de maíz (Koo y col., 1996). Estos autores demostraron que, en 3 horas de tratamiento con quinclorac, se redujo la incorporación de [^{14}C]glucosa en las paredes celulares de las raíces de plántulas de maíz en un 33 % y, además, esta reducción era mayor a medida que el tiempo de exposición y la concentración de quinclorac incrementaba. En un trabajo posterior con dos especies susceptibles y dos especies tolerantes, concluyeron que la biosíntesis de pared celular fue reducida en un 73 % y 60 % en las raíces de las especies susceptibles y en un 36% y 20% en las raíces de las especies tolerantes (Koo y col., 1997).

Sin embargo, Tresch y Grossmann (2003) demostraron que el tratamiento con quinclorac no producía una inhibición de la biosíntesis de la pared celular en raíces de maíz y de *Echinochloa crus-galli*, ambas especies sensibles a quinclorac. Además, mantuvieron que el quinclorac se comportaba como un herbicida auxínico, ya que provocaba, en las plantas sensibles, una inducción selectiva de la enzima 1-aminociclopropano-1-carboxilato (ACC) sintasa, la cual cataliza un paso clave en la síntesis de etileno (Grossmann y Scheltrup, 1997). Este incremento de la síntesis de etileno provoca una liberación de cianuro de hidrógeno (HCN) hasta unos niveles fisiológicamente tóxicos (Grossmann, 1996, 2000; Abdallah y col., 2006). En línea con estos estudios, y en un intento de confirmar si el quinclorac se comportaba como un CBI, se realizó otro estudio en el que se trataron suspensiones celulares de alubia con una concentración activa de quinclorac durante 20 horas y se comprobó que el herbicida no solo no reducía la incorporación de [^{14}C]glucosa en las paredes celulares, sino que esta incorporación de [^{14}C]glucosa incrementaba ligeramente (García-Angulo y col., 2012).

Por otro lado, mediante el tratamiento de plantas sensibles con quinclorac se ha descubierto que este herbicida provoca un incremento en la producción de especies reactivas de oxigeno (ROS). De hecho, Sunohara y Matsumoto (2004) demostraron que la tolerancia a quinclorac en *Echinochloa oryzicola* estaba relacionada con una elevada capacidad antioxidante que reducía el estrés oxidativo provocado por la acumulación de ROS. Los mismos autores desarrollaron un trabajo posterior donde se comparaban los efectos del quinclorac con otro herbicida auxínico (el ácido 2,4-difenoxiacético o 2,4-D) en raíces de una variedad sensible de maíz (Sunohara y Matsumoto, 2008). Ambos compuestos estimularon la síntesis de etileno y, por tanto, la acumulación de HCN, aunque en el caso del 2,4-D este incremento fue mucho mayor. Sin embargo, el quinclorac provocó una tasa de muerte celular mayor que el tratamiento con 2,4-D, lo que indica que la toxicidad del quinclorac en esa variedad de maíz está relacionada con la producción de ROS. En un experimento parecido, donde trataban con ambos herbicidas a una especie tolerante (*Eleusene indica*) y una sensible (*Digitaria ascendens*), los autores

demostraron que este herbicida provocaba un incremento en la síntesis de etileno y un aumento en el estrés oxidativo de *D. ascendens*, sin embargo, no provocaba ninguno de los dos síntomas en *E. indica* (Sunohara y col., 2010). De hecho, se ha demostrado que especies tolerantes, como el arroz o *E. indica*, y biotipos resistentes al quinclorac de especies sensibles, como *Echinochloa spp*, *Digitaria spp* y *Gallium spp* no incrementan el contenido en etileno, ni incrementan la producción de ROS en respuesta al tratamiento con el herbicida (Grossmann y Kwiatkowski, 1993, 2000; Grossmann, 2000; Van Eerd y col., 2005; Abdallah y col., 2006; Sunohara y col., 2010; Sunohara y col., 2011; Yashuor y col., 2012). Recientemente se ha demostrado que el tratamiento con quinclorac de plantas de arroz (*Orzya sativa*) provocó el incremento de la expresión de genes con actividad detoxificadora, como genes que codificaban para citocromo P450 monoxigenasa, glutatión S-transferasa, UDP-glicosiltransferasas, transportadores ABC y otros transportadores de tóxicos (Xu y col., 2015). Además de estos genes, también se ha demostrado que el quinclorac provoca un incremento general en la expresión de genes de respuesta a auxinas como los genes OsGH3, OsIAA, OsSAUR39 e OsIN2-2 (Xu y col., 2015). Por otro lado, mediante el estudio de biotipos sensibles y resistentes de *E. crus-galli* se ha descubierto recientemente que el gen GH3, integrante de la familia "Gretchen Hagen 3", que regula junto a otros genes la homeostasis de auxinas en plantas, está íntimamente relacionado con la resistencia a este herbicida (Li y col., 2016). Esta conclusión se obtiene del hecho en el que las plantas sensibles tuvieron una gran reducción en la expresión de este gen y las plantas resistentes presentaron un incremento marcado en la expresión de OsGH3 tras el tratamiento con quinclorac (Li y col., 2016). Aun así, los mecanismos de adquisición de esta tolerancia no se han esclarecido todavía.

Habituación de cultivos celulares a quinclorac

Solo hay un estudio hasta la fecha en el que se hayan habituado cultivos celulares a concentraciones letales de quinclorac y la especie utilizada fue la alubia (*Phaseolus vulgaris*) (Alonso-Simón y col., 2008). La habituación de cultivos celulares de alubia a quinclorac se llevó a cabo con el objetivo de comprobar si se comportaba como un CBI típico como el DCB o el isoxabén. Recordemos que la habituación de cultivos celulares a la presencia de estos inhibidores provocaba una reducción drástica del contenido en celulosa y cambios característicos en la composición y estructura de la pared celular (Diaz-Cacho y col., 1999; Encina y col., 2001, 2002; Alonso-Simón y col., 2004, 2007; Manfield y col., 2004; García-Angulo y col., 2006; 2009). El estudio de la composición de las paredes celulares reveló que este herbicida no producía una reducción en el contenido en celulosa en las células habituadas, pero sí producía cambios en la distribución y en la composición en pectinas, sobre todo homogalacturonano y ramnogalacturonano I. Estos cambios se consideraron como un efecto indirecto de la presencia de quinclorac y se concluyó que la habituación de células de alubia a quinclorac no provocaba cambios directos en la biosíntesis de pared celular (Alonso-Simón y col., 2008).

Teniendo en cuenta que estos cultivos poseían tolerancia a quinclorac y que ésta no residía en modificaciones en la pared celular, cabía esperar que en la habituación a quinclorac estuvieran implicados mecanismos independientes de la pared celular, como, por ejemplo, el desarrollo de una mayor defensa antioxidante.

2. Lignina

La lignina es el segundo polímero más abundante de la tierra, llegando a constituir el 25 % de la biomasa vegetal (Higuchi, 1990) y a acumular el 30 % del carbono presente en la biosfera (Amthor y col., 2003; Boerjan y col., 2003). En plantas terrestres la síntesis de lignina se produce principalmente en las paredes celulares secundarias de tipos celulares especializados en el sostén estructural y el transporte de agua y solutos, como los elementos conductores del xilema, fibras del xilema y también las células del esclerénquima y las fibras del floema (Bonawicz y Chapple, 2010). Estas células sufren un proceso de lignificación de la pared celular para reforzar su estructura, proporcionando soporte mecánico e impidiendo la degradación mecánica y biológica de las fibras de celulosa (Grabber y col., 1998). La lignina también actúa como un agente de cohesión entre los polisacáridos de pared haciendo que se compacte y refuerce la pared celular, siendo de esta manera una capa totalmente aislante impidiendo que se produzca el intercambio de agua y minerales con el exterior, lo cual es importante en los tejidos conductores como las células del xilema (Jones y col., 2001).

Además, la lignina se sintetiza como respuesta de defensa frente a infecciones de organismos patógenos o a heridas (Lange y col., 1995; Hawkins y Boudet, 2003; Rogers y Campbell, 2004; Sattler y Funnell-Harris, 2013). En este caso la lignina cumple dos funciones principales: (i) es un compuesto hidrofóbico, pero con una gran capacidad de retener agua, por lo tanto, en caso de herida o infección reduce la deshidratación (Reina y col., 2001) y (ii) como es un polímero difícilmente degradable ayuda a reforzar las paredes de los tejidos dañados siendo una barrera para la entrada de patógenos (Hammond-Kosach y Jones, 1996; Pomar y col., 2004; Gayoso y col., 2010; Miedes y col., 2014). Por otro lado, es un material muy poco digerible, por lo tanto, reduce el valor nutritivo de la planta, lo que la hace menos atractiva para los herbívoros (Buxton y Redfearn, 1997; Moore y Jung, 2001; Pichon y col., 2006; Shadle y col., 2007; Taboada y col., 2010; Novo-Uzal y col., 2011). De la misma forma, la lignina afecta negativamente al procesamiento bioquímico del material lignocelulósico a la hora de obtener fibra alimentaria, pulpa, textiles, papel, químicos y biofueles (Zeng y col., 2014; Ciesielski y col., 2014). Por lo tanto, una modificación del material lignocelulósico, o de la composición de la pared celular puede facilitar el procesamiento bioquímico en la industria para obtener mayor cantidad de fibra, papel o biocombustibles de una forma más sencilla (Sonbol y col., 2009; Fornalé y col., 2010, 2012, 2015; Carpita, 2012; Nookaraju y col., 2013; Yang y col., 2013; Zeng y col., 2014; Ciesielski y col., 2014; Damm y col., 2015).

2.1. Naturaleza y composición de la lignina

La lignina es un heteropolímero tridimensional de naturaleza fenólica resultado de la polimerización oxidativa de tres alcoholes *p*-hidroxicinamílicos (monolignoles), el alcohol *p*-cumarílico, el alcohol coniferílico y el alcohol sinapílico (Figura 9; Ros Barceló, 1997; Vanholme y col., 2010, Liu, 2012). La polimerización de estos tres monolignoles da lugar a las unidades que componen la estructura de la lignina: *p*-hidroxifenilo (H), guayacilo (G) y siringilo (S), las cuales se diferencian exclusivamente en el grado de metoxilación que tiene cada unidad (Ralph y col., 2007). Esta oxidación de los monolignoles va a ser llevada a cabo por peroxidasas de la clase III (CIII-POX) (EC 1.11.1.7), mediante la utilización de H_2O_2 (Fagersteadt y col., 2010; Novo-Uzal y col., 2013; Lin y col., 2016) y/o por lacasas (EC 1.10.3.2) en presencia de O_2 (Berhet y col., 2011; Cesarino y col., 2013; Zhao y col., 2013). Las peroxidasas (POX) implicadas en lignificación muestran especificidad por sustrato distinguiéndose CIII-POX que oxidan p-cumaril y coniferil alcohol (guaiacil POX), de otras que oxidan preferentemente sinapil alcohol (siringil POX) (Gabaldón y col., 2005; Gómez-Ros y col., 2007a; Herrero y col., 2013; Fernández-Pérez y col., 2015a, 2015b, 2015c). Por su parte, las lacasas están especializadas en la oxidación de coniferil alcohol (Berthet y col., 2011). Una vez sintetizados los monolignoles se polimerizan entre sí sin seguir un orden fijado, de hecho, uno de los factores que van a definir la estructura final de la molécula de lignina es la disponibilidad de cada uno de los monolignoles en el citoplasma, generándose una gran variedad de patrones de unión (Ralph y col., 2004a; Vanholme y col., 2010). Por esta razón, la lignina va a ser un polímero fenólico con una alta variabilidad estructural y de enlaces, la cual va a depender de la especie, tejido e, incluso, del tipo de célula (Ralph y col., 2004a; Vanholme y col., 2010).

La lignina presente en gimnospermas está compuesta básicamente por unidades G con una pequeña proporción de unidades H (Vanholme y col., 2010), aunque cabe destacar la excepción de especies como *Ginkgo biloba*, *Pinus radiata* o *Gnetum gnemon*, que también presentan unidades S en su lignina (Novo-Uzal y col., 2009; Nawawi y col., 2015; Wagner y col., 2015). En el caso de angiospermas dicotiledóneas la lignina está compuesta por unidades G y S y, en menor medida, de unidades H (Boerjan y col., 2003). En monocotiledóneas, la lignina tiene una composición mucho más compleja. Además de las unidades típicas G y S, suele presentar mayor contenido en unidades H que en el caso de las dicotiledóneas (Boerjan y col., 2003). Las ligninas de monocotiledóneas presentan también niveles relativamente altos de ácidos hidroxicinámicos, como el ácido ferúlico y el ácido *p*-cumárico (Ralph y col., 2004a, 2004b; Ralph, 2010; Hatfield y Marita, 2010; Sibout y col., 2016), monolignol acetatos (Lu y Ralph, 2008; Martinez y col., 2008; Ralph, 2010; Vanholme y col., 2010; Li y col., 2016), hidroxicinnamil aldehídos, sus respectivos alcoholes y compuestos como el *p*-hydroxibenzoato (Pomar y col., 2002; Ralph, 2010; Li y col., 2016). Recientemente se ha identificado el flavonoide tricina como

nuevo monómero de lignina en monocotiledóneas (Del Rio y col., 2012; Fornalé y col., 2015; Lan y col.2015; Lan y col., 2016; Li y col., 2016).

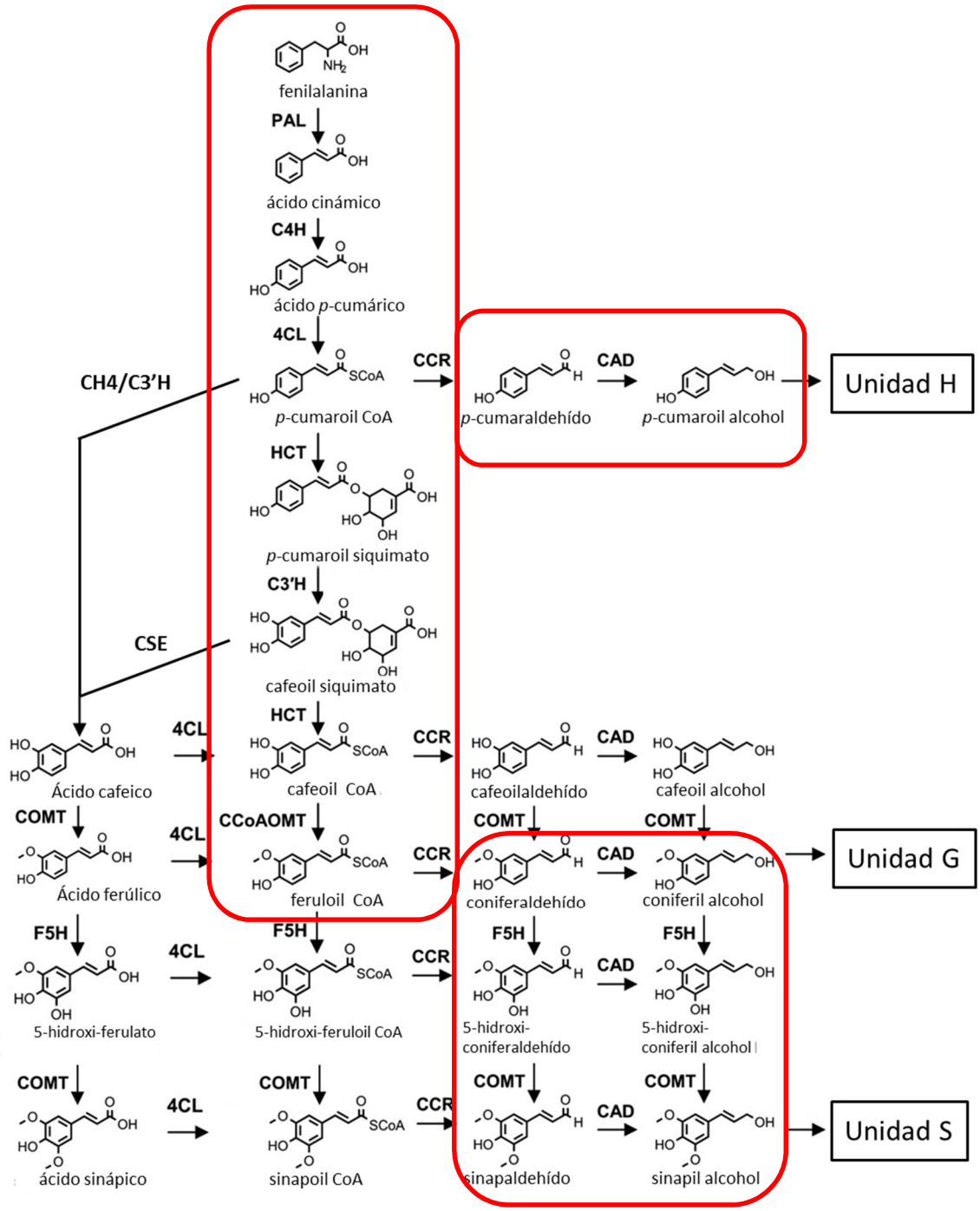


Figura 9. Esquema de la ruta de los fenilpropanoides. Modificado de Li y col., 2010 y Mottiar y col., 2016. PAL: fenilalanina amonio liasa, C4H: cinamato 4-hidroxilasa, HCT: *p*-hidroxicinamoil CoA siquimato/quinato *p*-hidroxicinamoiltransferasa, C3'H: *p*-cumarato 3-hidroxilasa, CSE: cafeoil siquimato esterasa, CCoAOMT: cafeoil CoA-O-metiltransferasa, F5H: ferulato-5-hidroxilasa, COMT: ácido cafeico/ 5-hidroxiconiferilaldehído-O-metiltransferasa, CCR: cinamoil CoA reductasa, CAD: cinamil alcohol deshidrogenasa. En rojo se señala la ruta preferente de síntesis de los monolignoles.

La composición monomérica de este polímero va a determinar su estructura molecular, estableciendo características como las regiones lineares de la lignina, el

nivel de ramificación e incluso el nivel de entrecruzamiento con la parte polisacarídica de la pared celular (Ragauskas y col., 2006; Gómez-Ros y col., 2007b; Neutelings, 2011; Djikanović y col., 2012; Constant y col., 2016). Por tanto, esta composición va a determinar también las características físico-químicas de este polímero, como, por ejemplo, su degradabilidad o su extractabilidad (Ralph y col., 2004a; Fornalé y col., 2010, 2011, 2015; Smith y col., 2015; Mottiar y col., 2016). Así, las ligninas ricas en unidades G (gimnospermas), al no tener metilado su C5, contienen más enlaces resistentes del tipo β-5, 5-5 y 4-O-5. Siguiendo el mismo razonamiento, en las ligninas ricas en unidades S (angiospermas) cuyo C5 está metilado, predominan los enlaces lábiles β-O-4. De ahí que un incremento en unidades S se asocie a una mayor degradabilidad del polímero. (Mottiar y col., 2016). De hecho, el ratio de subunidades S/G ha sido ampliamente utilizado como un aspecto cualitativo para conocer la resistencia a la degradabilidad de la lignina (Neutelings, 2011).

2.2. Biosíntesis de lignina

La ruta de biosíntesis de la lignina es una ruta altamente compleja y ampliamente estudiada. A pesar de haberse investigado en profundidad por diversos autores, en muchos casos, se desconoce el orden de los previsibles pasos y aun no se han caracterizado las enzimas que los catalizarían, aunque se presume que estos factores dependen de la especie e incluso del tejido (Neish, 1968; Whetten y Sederoff, 1995; Humpreys y Chapple, 2002; Barrière y col., 2007; Bonawitz y Chapple, 2010; Hatfield y col., 2010; Vanholme, 2010; Liu, 2012; Mottiar y col., 2016). De todas formas, se cree que la síntesis de lignina es llevada a cabo por complejos multienzimáticos de proteínas localizadas en la membrana del retículo endoplásmico y de plastidios y que cada enzima se encarga de catalizar la reacción de uno o varios sustratos (Achnine y col., 2004; Winkel, 2004; Barrière y col., 2007; Laursen y col., 2015).

La biosíntesis de la lignina comienza con la síntesis del ácido siquímico y continúa con su transformación a L-fenilalanina, el cual es precursor de los hidroxicinamatos de plantas (Herrmann, 1995; Yamada y col., 2008). A partir de este punto comienza la ruta de los fenilpropanoides con desaminación oxidativa de la L-fenilalanina para formar el ácido cinámico por la fenilalanina amonio liasa (PAL, EC 4.3.1.5). Luego actúan las enzimas cinamato 4-hidroxilasa (C4H, EC 1.14.13.11), *p*-cumarato 3-hidroxilasa (C3´H, EC 1.14.13.36), *p*-hidroxicinamoil CoA siquimato/ quinato *p*-hidroxicinamoiltransferasa (HCT, EC 2.3.1.133), cafeoil siquimato esterasa (CSE, EC.1.1.1.-), ácido cafeico/ 5-hidroxiconiferilaldehido-O-metil transferasa (COMT, EC 2.1.1.68), cafeoil CoA-O-metil transferasa (CCoAOMT, EC 2.1.1.104) y la hidroxicinamato CoA ligasa (4CL, EC 6.2.1.12), (Figura 9; Vogt, 2010; Fraser y Chapple, 2011; Vanholme y col., 2013; Mottiar y col., 2016). A través de la ruta de los fenilpropanoides, se sintetizan los compuestos *p*-cumaroil CoA, feruloil-CoA y sinapoil-CoA que entrarán en la ruta específica de biosíntesis de lignina. Cada uno

de estos precursores se reduce doblemente, primero a aldehídos y después a alcoholes, gracias a la actividad de la cinamoil CoA reductasa (CCR, EC1.2.1.449) y la cinamil alcohol deshidrogenasa (CAD, EC 1.1.1.195). El resultado final es la síntesis de los monolignoles: alcohol *p*-cumarílico, alcohol coniferílico y el alcohol sinapílico a partir, respectivamente, de *p*-cumaroil-CoA, feruloil-CoA y sinapoil-CoA (Figura 9; Liu, 2012; Mottiar y col., 2016).

Alternativamente, el alcohol sinapílico se puede sintetizar a partir de coniferilaldehido y coniferilalcohol mediante la actividad de las enzimas ferulato 5-hidrolasa (F5H) y COMT. De hecho, para muchos autores esta es la ruta preferente de síntesis de alcohol sinapílico, resultando que la enzima F5H es la enzima clave para la síntesis de monolignol que da lugar a las unidades S en la lignina (Figura 9; Li y col., 2000; Bonawitz y Chapple, 2010; Weng y col., 2010; Mansfield y col., 2012).

Los monolignoles son sintetizados en el citosol, pero deben ser transportados a la pared celular que es el lugar donde se produce su polimerización en lignina. El transporte hacia la pared se haría en forma de monolignoles libres o glucoconjugados, aunque esta última posibilidad parece no tener mucho apoyo experimental, indicando que los glicosil-monolignoles y glicosil-oligolignoles serían formas de almacenamiento vacuolar (Figura 10; Liu, 2012; Dima y col., 2015). Hasta hace poco se creía que el transporte hacia la pared celular de los monolignoles se realizaba por un proceso de exocitosis a través de vesículas derivadas de ER-Golgi (Samuels y col., 2002). Hoy en día se admite que, además, existe un transporte activo por transportadores de tipo ABC ("ATP-binding cassette") y difusión pasiva mediante interacciones hidrofóbicas a través de la membrana plasmática (Figura 10; Liu y col., 2012; Mottiar y col., 2016). Una vez depositados en la pared celular los monolignoles son oxidados/deshidrogenados por CIII-POX/lacasas dando lugar a la formación de los radicales 4-O-fenoxilo, los cuales son muy reactivos y polimerizan espontánea y aleatoriamente con el polímero de lignina o forman dímeros y oligómeros que se incorporan posteriormente al polímero creciente de lignina (Figura 10; Higuchi, 1990; Hapiot y col., 1994; Liu, 2012).

Tal como se indicó anteriormente la lignina polimeriza en las paredes celulares de determinados tipos celulares. Los datos de los que se disponen hasta el momento indican que el proceso de lignificación de las paredes celulares está fuertemente regulado y asociado a programas de desarrollo, y que la deposición de lignina es un proceso altamente organizado (Liu, 2012). Por ejemplo, en células conductoras del xilema el proceso de lignificación comienza en las esquinas de las células a nivel de la lámina media y región S1 de la pared celular secundaria, extendiéndose hacia el lumen celular (Möller y col., 2006b; Lui, 2012). La caracterización funcional de unas proteínas dirigentes sin actividad enzimática que actuaban como guía para la síntesis de lignanos, hizo que estas proteínas se propusieran también como puntos de iniciación de la polimerización de la lignina (Davin y col., 2008). En el caso de monocotiledóneas y en algunas dicotiledóneas, como *Arabidopsis thaliana*, se ha propuesto el ácido ferúlico como punto de

iniciación de la lignificación, precisamente por su capacidad de formar dímeros o polímeros entre ferúlicos o con otros fenoles de pared entrecruzando polisacáridos adyacentes, lignina o incluso proteínas estructurales (Ralph y col., 2004b; Ralph, 2010; Hatfield y Marita, 2010; Lui, 2012). Recientemente también se ha propuesto el flavonoide tricina descubierto en la lignina de monocotiledóneas como el punto de nucleación de la síntesis de lignina (Lan y col., 2015, 2016).

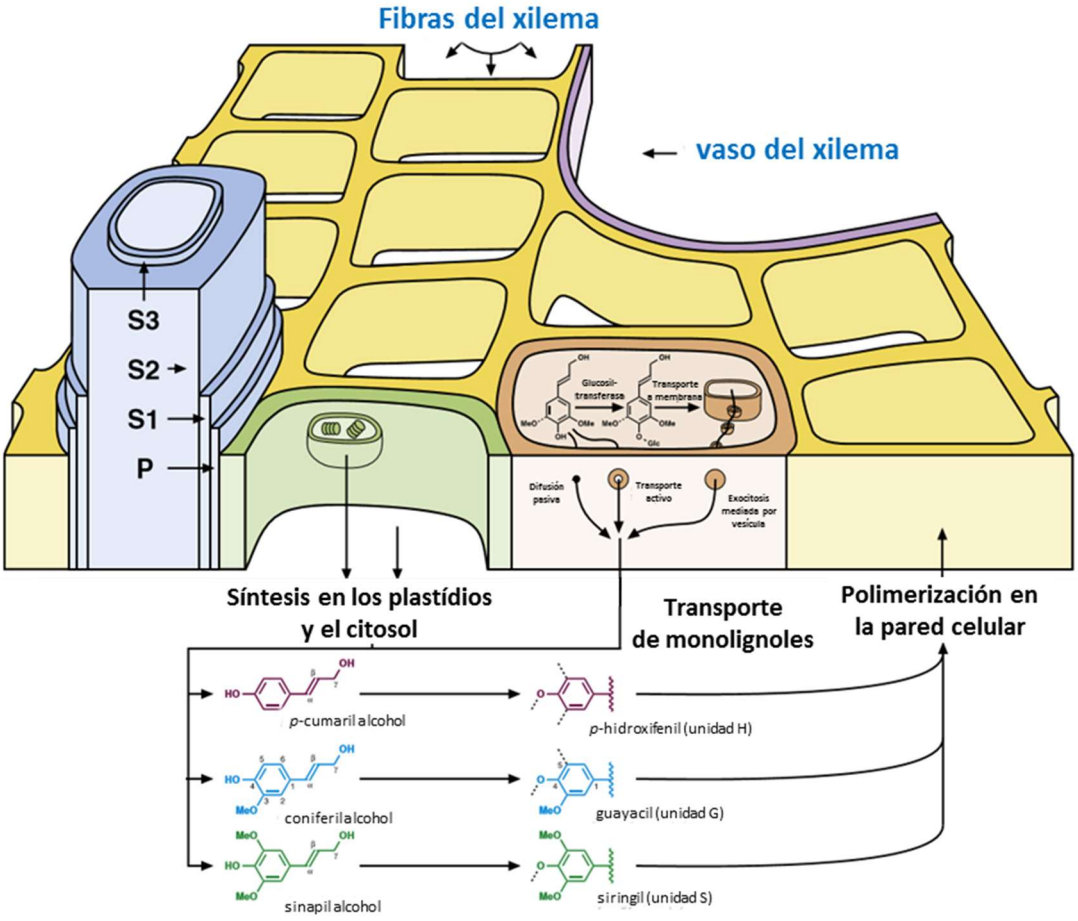


Figura 10. Resumen del proceso de lignificación en plantas. La lignina se deposita principalmente en el xilema. La biosíntesis de los principales monolignoles se produce en el citoplasma (célula verde) y son exportados, mediante los tres modelos propuestos (célula marrón), a la pared celular donde se produce la polimerización (célula amarilla). Modificado de Mottiar y col., 2016.

Dado el coste metabólico que conlleva la polimerización de la lignina y que, además, las células que sufren lignificación de su pared celular pierden su capacidad de elongación y entran en programas de diferenciación, el proceso de lignificación está altamente regulado (Zhao, 2016). En dicho proceso participan factores de transcripción del tipo R2R3-MYB, NAC y WRKY (Fornalé y col., 2006, 2010; Sonbol y col., 2009; Bosch y col., 2011; Du y col., 2012; Cassan-Wang y col., 2013; Vélez-Bermúdez y col., 2015), regulando la expresión de genes que codifican para enzimas de la ruta de biosíntesis de la lignina (Stracke y col., 2001; Du y col., 2012; Cassan-Wang y col., 2013).

2.3. Lignificación ectópica

Como hemos indicado anteriormente, la lignificación, por lo general, se produce en tejidos específicos y en un número limitado de células, pero también se ha demostrado que se puede dar una síntesis de lignina o de compuestos tipo lignina en respuesta a estreses, a heridas o a infecciones por patógenos (Pomar y col., 2004; Moura y col., 2010; Miedes y col., 2014; Vélez-Bermúdez y col., 2015; Yoon y col., 2015). En este caso la lignificación no sólo se da en los tejidos especializados, sino que se puede extender a otros tejidos en un proceso que se denomina lignificación ectópica y que se suele dar de una forma muy rápida para evitar la pérdida de agua, la proliferación del patógeno o incluso la dispersión de tóxicos o enzimas del patógeno en las células de la planta y, de la misma manera, impedir la salida de agua y nutrientes de la planta al patógeno (Smith y col., 2007; Miedes y col., 2012). El proceso de lignificación ectópica se ha descrito en mutantes de arabidopsis que provocan una alteración de la distribución espacial de la lignina, depositándose en tipos celulares que normalmente no lignifican. Por ejemplo, el mutante *de-etiolated3* (*det3*), cuya mutación afecta a la subunidad C de una ATPasa vacuolar (Figura 11; Schumacher y col., 1999), provocaba, entre otros efectos, una reducción de la síntesis de celulosa, que era compensada con una lignina ectópica muy rica en unidades S (Caño-Delgado y col., 2000, 2003; Newman y col., 2004; Rogers y col., 2005). Otros mutantes con este efecto son el *ectopic lignification of the pith* (*elp1*), el *anion altered root morphology* (*arm)* o *pom1-pom1* (*pom1*), mutantes alélicos de una quitinasa (*Chitinase-like1*, AtCTL1), que depositaban lignina en paredes celulares primarias de las células del parénquima (Zhong y col., 2000; 2002; Rogers y col., 2005; Hermans y col., 2010; Sánchez-Rodriguez y col., 2012). La mutación de otro gen de la misma familia *chitinase-like2* (AtCTL2, *atctl2*) provocó también una acumulación de lignina en los hipocótilos de plantas de arabidopsis (Hossain y col., 2010; Sánchez-Rodriguez y col., 2012). Un mutante en el que se ha estudiado mucho este proceso de lignificación es el *ectopic lignification1* (*eli1*) (Caño-Delgado y col., 2000) y su mutante alélico que es el *consitutive expression of VSP1* (*cev1*) (Caño-Delgado y col., 2003). Ambas mutaciones afectaban a la proteína CESA3 y provocaban la acumulación de lignina ectópica en arabidopsis (Figura 11; Caño-Delgado y col., 2000; Ellis y col., 2002; Caño-Delgado y col., 2003).

Además de estos casos, se han descrito otra serie de mutantes que afectaban a la síntesis de celulosa y provocaban la acumulación de una lignina ectópica para reforzar la pared celular. Este es el caso del mutante *root swelling1* (*rsw1*), el cual afecta a la expresión del gen para la proteína CESA1 (Caño-Delgado y col., 2000, 2003), de los mutantes *KOBITO1/ elongation defective1* (*eld1*), que afectan a una proteína extracelular rica en serinas de función desconocida (Pagant y col., 2002; Lertpiriyapong y Sung, 2003), de los mutantes que afectan a la expresión del gen AtCesA6 *procuste1* (*prc1*)/ *THESEUS1* (*THE1*) (Fagard y col., 2000a; Hématy y col., 2007; Denness y col., 2012) y de los dos mutantes *fei* (*fei1* y *fei2*), que afectan a un receptor quinasa con dominios ricos en leucina (Xu y col., 2008) También se ha

descrito un mutante en arroz denominado *ectopic deposition of phenolic components1* (*edp1*) (Sato y col., 2011), en el cual se produce una acumulación de compuestos fenólicos, entre ellos la lignina, en las células parenquimáticas de los internodos de la planta de arroz (Sato y col., 2011).

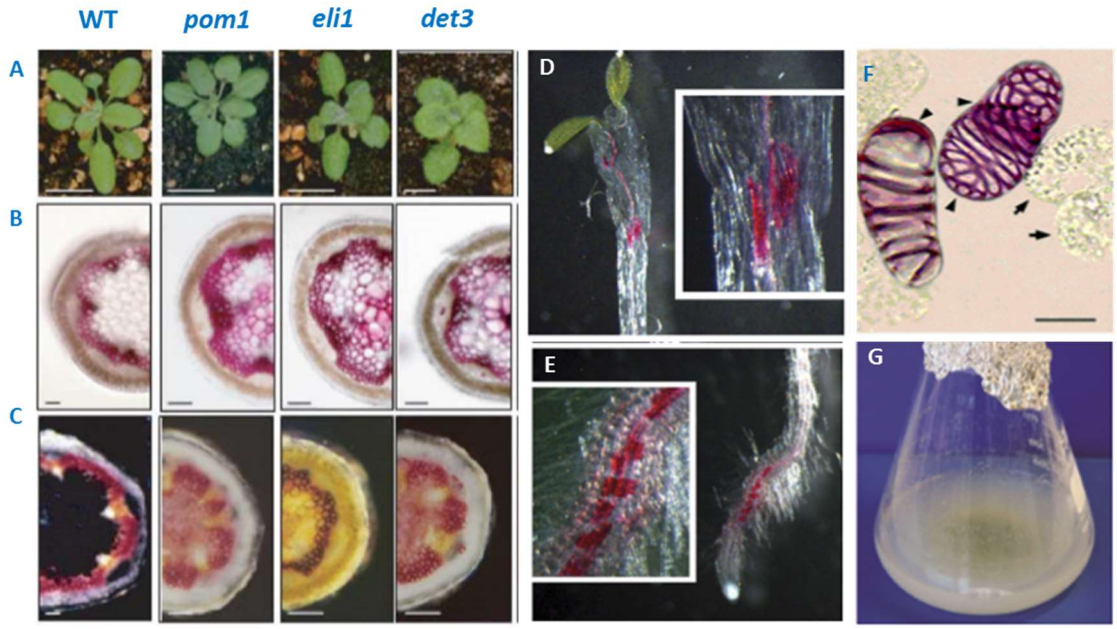


Figura 11. Principales modelos vegetales en los cuales se ha detectado la presencia de una lignificación ectópica. Comparación fenotípica de mutantes de arabidopsis que presentan lignificación ectópica con el fenotipo silvestre: comparación de las rosetas de arabidopsis (A), cortes de tallos de inflorescencias procesados para la detección de lignina mediante la tinción de Wiesner (B) o la tinción de Maüle (C), tomado de Rogers y col., 2005. Presencia de lignificación ectópica por la tinción de Wiesner en raíces de plántulas etioladas de arabidopsis tratadas con taxtomina A (D) e isoxabén (E), tomado de Bischoff y col., 2010. Presencia de elementos traqueales diferenciados en cultivos celulares de *Zinnia elegans*, donde se aprecian el engrosamiento de las paredes celulares y la deposición de lignina (F), tomado de Gomez-Ros y col., 2006. Imagen de una suspensión de células de *Picea abies* en la que se produce la síntesis de una lignina extracelular visible como un depósito de color blanco tras cuatro días de cultivo (G), tomado de Kärkonen y Koutaniemi, 2010.

Al parecer, si se afecta la síntesis de celulosa las plantas responden con lignificación ectópica para reforzar la pared celular. Este hecho también ha sido demostrado mediante el tratamiento de plantas con diferentes CBIs. Mediante el tratamiento con DCB e isoxaben de plántulas de arabidopsis se demostró que se provocaba una síntesis de lignina en la raíz de las plántulas (Desprez y col., 2002). Caño-Delgado y col. (2003) corroboraron que el tratamiento con isoxabén en raíces de plántulas de arabidopsis provocaba una reducción en el contenido en celulosa y una extensiva lignificación ectópica que afecta a la mayoría de las células de la raíz de la plántula. El hecho de que el tratamiento con isoxabén provoca lignificación ectópica en raíces de plantas de arabidopsis ha sido demostrado posteriomente en diversas ocasiones (Figura 11; Bischoff y col., 2009; Hamann y col., 2009; Hamann 2015a, 2015b; Marcos y col., 2015). Con el uso de este inhibidor y mediante la obtención de mutantes que afectan a diversas rutas de señalización de estrés, se

llegó a la conclusión de que los daños y/o la pérdida de la integridad de la pared celular, provocaba una serie de respuestas compensatorias como la lignificación ectópica, las cuales estaban mediadas por una interacción dinámica entre la producción de ROS y de ácido jasmónico (Denness y col., 2011). Sorprendentemente, la habituación de suspensiones celulares de arabidopsis a isoxabén no provocó lignificación ectópica, aunque también cabe destacar que en estos cultivos habituados no se observaron reducciones significativas en el contenido en celulosa y hemicelulosas, típicas de la habituación a CBIs (ver apartado 2.1), sino que, más bien, se observaron cambios en la distribución espacial de dichos polisacáridos (Manfield y col., 2004). El tratamiento de plántulas de arabidopsis con taxtomina A (otro CBI), provocó una reducción de la celulosa cristalina y una lignificación en los hipocótilos de las plántulas tratadas (Bischoff y col., 2009). Otros CBIs con los que se ha obsevado la induccción de la síntesis de una lignina ectópica bajo su tratamiento son el indaziflam, el quinoxifeno y el acetobixan (Harris y col., 2012; Brabham y col., 2014; Xia y col., 2014).

Se ha descrito también un factor de transcripción en arabidopsis, SHINE (AtSHN), que regula la acción coordinada de activación de la síntesis de celulosa y la inhibición de la síntesis de lignina (Ambavaram y col., 2011), con lo que queda patente que la reducción de la síntesis de celulosa y la activación de la síntesis de lignina para reforzar la pared es un proceso compensatorio más que probable.

Por otro lado, en los últimos años se ha documentado ampliamente la relación entre la modificación en la expresión de diferentes factores de transcripción relacionados con la regulación de la deposición de una pared celular secundaria (MYB, NAC y WRKY) y el proceso de lignificación ectópica en células con pared celular primaria (Wang y col., 2010; Cassan-Wang y col., 2013). Así, la mutación del gen MtSTP de *Medicago truncatula*, codificante para un factor de transcripción WRKY que reprime la formación de pared celular secundaria, dio lugar a la deposición de lignina en células que normalmente no lignificaban (Wang y col., 2010). Por otro lado, la sobreexpresión de los factores de transcripción PtMYB4 y AtMYB61, provocó la deposición de lignina ectópica en *Pinus taeda* y *A. thaliana* respectivamente (Patzlaff y col., 2003; Newman y col., 2004). Finalmente, la sobreexpresión de factores de transcripción de la familia NAC (NST1, NST2, SND1/NST3, VDN6 y VDN7) también se asoció a la deposición de lignina ectópica (Grima-Pettenati y col., 2012).

2.4. Lignificación en cultivos celulares

Como ya hemos indicado anteriormente, la lignificación es un proceso asociado a la formación de pared celular secundaria de un número limitado de tipos celulares diferenciados. Desde este punto de vista, no sería esperable que células en cultivo (líquido o sólido) caracterizadas por mantenerse en un estado desdiferenciado y presentar únicamente pared celular primaria, fueran susceptibles de sufrir lignificación. Sin embargo, desde hace años vienen acumulándose

resultados que indican que las células en cultivo pueden sintetizar lignina o polímeros de tipo lignina que incorporan a la pared celular o excretan al medio de cultivo en determinadas condiciones (Novo-Uzal y col., 2009; Kärkönen y Koutaniemi 2010; Shen y col., 2013). En algunos de estos sistemas la lignificación se asocia claramente a la diferenciación de elementos traqueales inducidos mediante tratamiento hormonal (Figura 11.F; Fukuda y Komamine, 1980; Simola y col., 1992; Eberhardt y col., 1993; McCann, 1997; Anterola y col., 2002; Oda y col., 2005; Pauwels y col., 2008; Kärkönen y Koutaniemi, 2010; Yamagishi y col., 2015). En otros casos, la lignificación está asociada a tratamientos con elicitores fúngicos, como es el caso de cultivos celulares de *P. abies* (Messner y Boll, 1993; Lange y col., 1995), o a cambios en la composición del medio de cultivo (Brunow y col., 1990; Kärkönen y col., 2002). En cultivos celulares de diferentes especies de *Populus*, la síntesis de lignina fue activada por medio de un estrés hidríco (Tsutsumi y Sakai, 1993). La sacarosa también ha sido utilizada para inducir la formación de lignina en suspensiones celulares de *P. taeda* (Nose y col., 1995).

El proceso de lignificación en cultivos celulares con elementos traqueales de *Z. elegans* ha sido ampliamente estudiado. Tanto el estrés provocado por los cortes en la planta a la hora de obtener el explanto, como el cultivo con un aporte alto de citoquininas, parecen ser esenciales para poder provocar la diferenciación a elementos traqueales (McCann, 1997). La composición de la lignina que ha sido depositada en la pared celular de estos cultivos celulares no difiere demasiado de la encontrada en los tejidos vasculares de la planta (Stasolla y col., 2003; Möller y col., 2006a). Sin embargo, si esta lignina es secretada al medio extracelular, la composición sí que varía ya que esta lignina extracelular suele poseer más unidades H y uniones más condensadas (de tipo β-β) que la lignina presente en planta (Brunow y col., 1990, 1993; Lange y col., 1995).

Aunque todos estos trabajos han conseguido obtener la síntesis de lignina o material tipo lignina en cultivos celulares, el hecho de que esta lignificación se produzca en paredes celulares primarias ha sido descrita muy pocas veces (Christiernin y col., 2005; Novo-Uzal y col., 2009; Shen y col., 2013).

3. Estrés oxidativo y sistema de defensa antioxidante

Los organismos aeróbicos, incluidas las plantas, utilizan O_2 atmosférico como oxidante en el metabolismo respiratorio. Desde este punto de vista el O_2 es absolutamente necesario para la vida de los organismos aeróbicos, sin embargo, la reducción o excitación de esta molécula puede dar lugar a una serie de compuestos conocidos como ROS que tienen una elevada toxicidad y de los cuales solamente se puede sobrevivir gracias a un elaborado sistema de defensa antioxidante (Halliwell, 2006). La molécula de oxígeno (O_2) es un radical libre necesario en todas las rutas metabólicas aeróbicas. Esta molécula posee dos electrones desapareados y se necesitan 4 electrones para completar su reducción hasta dar lugar a dos moléculas de agua. Cuando una molécula de O_2 acepta electrones se generan unos

intermediarios que son los denominados ROS, donde se incluyen radicales libres como el radical superóxido ($O_2^{\cdot-}$), el radical hidroxilo ($^{\cdot}OH$) y no radicales como el peróxido de hidrógeno (H_2O_2) (Figura 10; Kärkönen y Kuchitsu, 2015; del Río, 2015). Además, la excitación de este O_2 daría lugar al oxígeno singelete ($^1O_2$), molécula que también está reconocido como una ROS no radical (Figura 12; del Rio, 2015). Por lo tanto, estas ROS son compuestos que se producen continuamente en procesos habituales como la fotosíntesis, la respiración y la fotorrespiración, pero que en presencia de un estrés se incrementa su producción (del Rio y col., 2006; Navrot y col., 2007; Gill y Tuteja, 2010; del Río, 2015).

Dado que estos compuestos poseen en su estructura electrones desapareados, poseen una alta reactividad, y un incremento de estas ROS puede provocar la oxidación de compuestos de una manera no controlada, lo que ha sido descrito como estrés oxidativo (Foyer y Noctor, 2005; Demidchik, 2015). Este estrés oxidativo es el resultado de la oxidación macromoléculas como los carbohidratos, proteínas, lípidos y nucleótidos que puede desencadenar la muerte celular (Foyer y Noctor, 2005; Gill y Tuteja, 2010; Demidchik, 2015). A pesar de estos efectos negativos en la célula, las ROS también pueden tener otras funciones como moléculas de señalización de estreses (Baxter y col., 2014; del Rio, 2015; Suzuki, 2015; Mignolet-Spruyt y col., 2016) o una función defensiva frente a organismos patógenos (Lehman y col., 2015; Camejo y col., 2016), por lo tanto, los efectos que desempeñen estas ROS dentro de la célula van a depender de un equilibro delicado entre la síntesis y degradación de estas ROS en el lugar y momento apropiado (del Rio y col., 2015).

3.1. Especies reactivas de oxígeno

El $O_2^{\cdot-}$ es la primera ROS que se genera en una célula mediante la reducción del O_2 con un electrón (Figura 12). Es un compuesto muy inestable, con una vida media de milisegundos, que puede reaccionar con peróxidos lipídicos generados bajo su acción o con aminoácidos específicos tales como la histidina, metionina y triptófano (Dat y col., 2000). En plantas, alrededor del 1-2 % del consumo de O_2 da lugar a la formación de $O_2^{\cdot-}$ en condiciones normales y el lugar donde existe una mayor producción de este compuesto es en el fotosistema I (Gill y Tuteja, 2010). Este compuesto, rápidamente, desencadena la formación del radical libre $^{\cdot}OH$ y $^1O_2$ (Halliwell, 2006) y también puede reducirse a H_2O_2 mediante la acción de la enzima superóxido dismutasa (SOD, Gill y col., 2015).

El H_2O_2 es una ROS generada principalmente mediante la dismutación de la molécula de O_2 por la acción de la enzima SOD (Figura 12; Gill y col., 2015). Este compuesto es sintetizado, sobre todo en los peroxisomas como subproducto derivado de la fotorrespiración y de la oxidación de ácido grasos (Sandalio y col., 2013). El H_2O_2 no es un radical libre, sino que actúa como una molécula que puede oxidar o reducir compuestos en varias rutas metabólicas (del Río, 2015; Kärkönen y Kuchitsu, 2015). Este compuesto es relativamente estable y puede tener un doble

papel: (i) a bajas concentraciones puede actuar como una molécula de señalización celular y (ii) cuando está presente en altas concentraciones provoca un estrés oxidativo, dónde una de sus consecuencias es la inactivación de enzimas mediante la oxidación de sus grupos tiol, lo cual puede desencadenar la muerte de la célula (Quan y col., 2008; Kärkönen y Kuchitsu, 2015). De hecho, el H_2O_2 es uno de los sustratos de un grupo muy amplio de enzimas que son las peroxidasas (POX), las cuales están divididas en múltiples isoformas, cada una con un papel específico en el metabolismo celular, por lo tanto, son las encargadas de controlar muchos de los procesos fisiológicos de la planta (Novo-Uzal y col., 2013; Francoz y col., 2015; Kärkönen y Kuchitsu, 2015).

El $^{\cdot}OH$ es una ROS de las más reactivas, pudiendo reaccionar con moléculas biológicas importantes como el ADN, proteínas, lípidos y polisacáridos (Figura 12; Halliwell y Gutteridge, 2015; Kärkönen y Kuchitsu, 2015). Además, ante la ausencia de una enzima que sea capaz de degradar este radical, el exceso de la concentración de esta molécula suele desencadenar una muerte celular (Vranová y col., 2002; Desikan y col., 2005; del Río, 2015).

El $^1O_2$ es un compuesto que se forma cuando no hay una suficiente disipación de la energía durante el transporte electrónico fotosintético y los pigmentos fotosínteticos reaccionan con el O_2, cediéndole el electrón y formando el $^1O_2$ (Figura 12; Gill y Tuteja, 2010; Telfer, 2014; Laloi y Havaux, 2015). Este compuesto tiene una vida media muy corta debido a su alta reactividad y se le atribuyen uno de los mayores papeles destructivos en el proceso de muerte celular provocada por acumulación de ROS junto con el $^{\cdot}OH$ (Telfer, 2014; del Río, 2015; Laloi y Havaux, 2015)

3.2. Efecto de las especies reactivas de oxígeno en las células de las plantas

En condiciones normales, la producción de ROS en las células de las plantas no tiene un efecto tóxico, ya que estas ROS se metabolizan rápidamente mediante la acción de los sistemas de defensa de la célula. Sin embargo, en situaciones de estrés, se activa la producción de ROS lo que produce un daño en las células y los tejidos donde se produce el estrés (del Río, 2015). Este daño celular viene dado por la oxidación de la mayoría de las biomoléculas como el ADN, las proteínas y los lípidos (Dat y col., 2000).

Uno de los efectos más comunes del estrés oxidativo es la peroxidación lipídica, que es considerada como el proceso más dañino que puede ocurrir en una célula, ya que afecta a la integridad de las membranas lo que fácilmente termina provocando la muerte celular (Farmer y Mueller, 2013; Anjum y col., 2015). La peroxidación lipídica ha sido ampliamente utilizada como indicador de estrés oxidativo, mediante el uso de la técnica "Thiobarbituric Acid Reactive Substances" (TBARS) (Beuge y Aust, 1978).

Otro efecto de la presencia de una alta cantidad de ROS en las células es la oxidación de proteínas (Anjum y col., 2015). Esta oxidación de proteínas es un proceso irreversible, a no ser que sea mediado por aminoácidos con grupos sulfuro, proceso que, en algunos casos, puede ser reversible (Ghezzi y Boneto, 2003; Hancock y col., 2006). La determinación del nivel de carbonilación de proteínas es en un marcador ampliamente usado para cuantificar el daño oxidativo presente en las proteínas celulares (Job y col., 2005; Moller y col., 2007).

La acumulación de ROS puede llegar a dañar el ADN, proceso que se conoce como efecto genotoxico (Tuteja y col., 2009). Este daño en el ADN provoca múltiples cambios en la célula como la activación o represión de la transcripción, la inducción de rutas de señalización, el incremento en los errores producidos durante la replicación o la inestabilidad genómica (Cooke y col., 2003; Ghiani y col., 2014; Halliwell y Gutteridge, 2015). Asimismo, el efecto genotóxico produce cambios fisiológicos en la célula como una reducción de la síntesis de proteínas, la destrucción de las membranas celulares o un daño en las proteínas fotosintéticas que van a afectar al crecimiento y desarrollo de toda la planta (Halliwell y Gutteridge, 2015).

3.3. Sistema de defensa antioxidante

Las ROS se producen de manera habitual en el interior de la célula, por lo tanto, para controlar la producción de ROS, las células poseen unos sistemas de defensa antioxidante que no permiten que se produzcan situaciones prooxidantes (del Río, 2015; Kärkönen y Kuchitsu, 2015). Este sistema de defensa antioxidante se compone de un sistema antioxidante enzimático y un sistema antioxidante no enzimático.

El sistema antioxidante enzimático está compuesto por una serie de proteínas que catalizan la transformación de ROS en moléculas menos dañinas, que están presentes en diferentes orgánulos, por lo general cerca de los lugares donde existe una alta producción de estas ROS (Sharma y col., 2012). Las enzimas que componen este sistema antioxidante son la catalasa (CAT), la SOD, las CIII-POX y las enzimas que componen el ciclo del ascorbato-glutatión, como la ascorbato peroxidasa (APOX), la monodehidroascorbato reductasa (MDHAR), dehidroascorbato reductasa (DHAR), la glutatión reductasa (GR) y la glutatión S-transferasa (GST) (Figura 12).

La CAT (EC 1.11.1.6) es una enzima tetramérica de una masa molecular de 54-59 kDa ampliamente distribuida en todos los organismos aerobios que contiene un grupo hemo en su molécula y que cataliza la reacción de descomposición del H_2O_2 para dar lugar a H_2O y O_2 (Obinger, 2012) (Figura 12; Tabla 2). Esta enzima es altamente eficiente, suele encontrarse con frecuencia en los peroxisomas y glioxisomas y es indispensable para la detoxificación de las ROS bajo condiciones de estrés (Mhamdi y col., 2010; Şen, 2012; Gill y col., 2015; Halliwell y Gutteridge, 2015). Además de catalizar la descomposición del H_2O_2, esta enzima actúa sobre

otros hidroperóxidos, alcoholes, aldehídos y ácidos orgánicos (Gill y Tuteja, 2010; Halliwell y Gutteridge, 2015), lo que la hace una de las enzimas más importantes en el control de ROS.

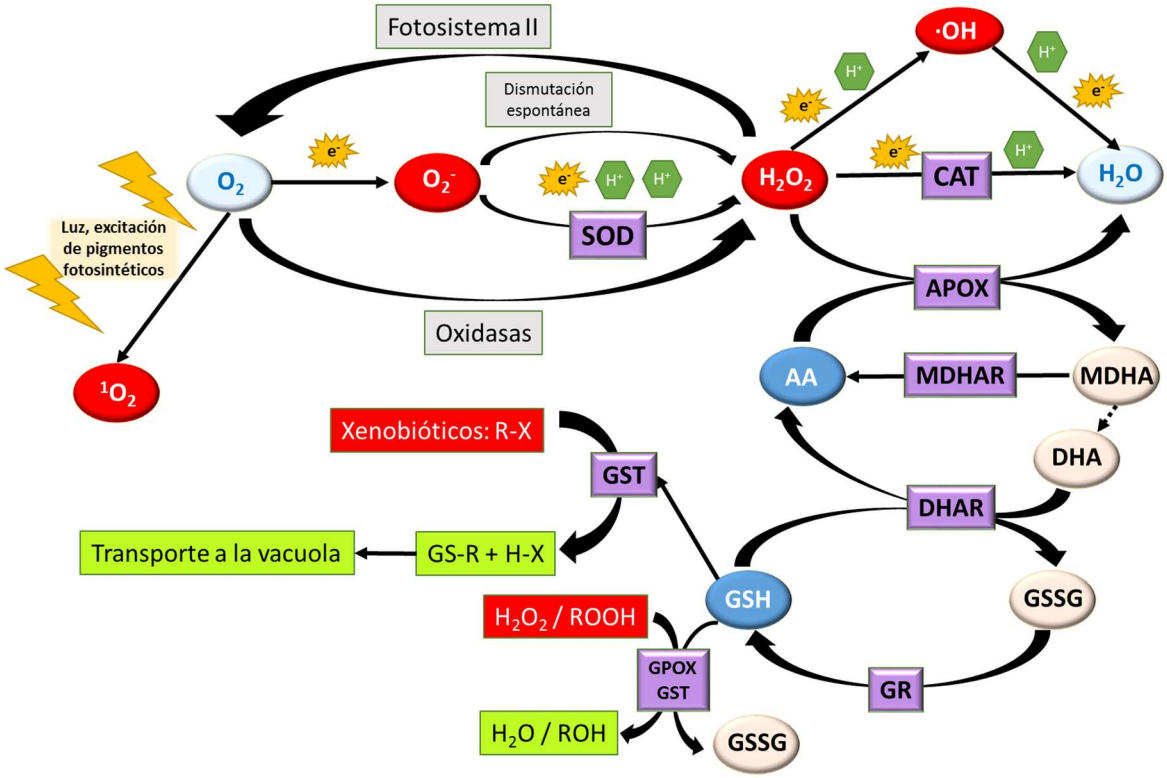


Figura 12. Esquema simplificado de la síntesis de ROS (rojo) y los principales mecanismos de defensa antioxidante (morado). AA, ácido ascórbico; MDHA, monodehidroascorbato; DHA, dehidroascorbato; GSH, glutatión reducido; GSSG, glutatión oxidado SOD, superóxido dismutasa; CAT, catalasa; APOX, ascorbato peroxidasa; MDHAR, monodehidroascorbato reductasa; DHAR, dehidroascorbato reductasa; GR, glutatión reductasa; GST, glutatión S-transferasa; GPOX, glutatión peroxidasa.

La SOD (EC 1.15.1.1) es una enzima presente en todos los organismos aerobios y tiene una distribución amplia a nivel subcelular. Es una metaloenzima que cataliza la dismutación de dos moléculas de O_2^- para dar lugar a una molécula de H_2O_2 y una molécula de O_2 (Figura 12; Tabla2; Gill y col., 2015). Hay tres tipos de SOD en función de su grupo prostético: Fe-SOD, Mn-SOD y Cu/Zn-SOD. La isoenzima Mn-SOD está presente principalmente en mitocondrias y peroxisomas, la isoenzima Cu/Zn-SOD se ha encontrado en el citoplasma, en peroxisomas y en cloroplastos y la isoenzima Fe-SOD, aunque a veces no se detecta en plantas superiores (Ferreira y col., 2002), se ha comprobado su presencia principalmente en cloroplastos (Gill y col., 2015). La principal función de las SODs es la de disminuir los niveles de O_2^-, sobre todo para evitar que no se generen radicales como el $^{\cdot}OH$ ya que este compuesto es mucho más reactivo y dañino (del Río y col., 2015; Gill y col., 2015; Halliwell y Gutteridge, 2015). Además, el control de los niveles de O_2^- es muy

importante, porque esta molécula participa en rutas de señalización celular (del Rio y col., 2006).

Otro grupo de enzimas relacionadas con el control de ROS son las CIII-POX (EC 1.11.1.7). Estas enzimas son de naturaleza glicoproteica y se localizan en vacuola y pared celular (Passardi y col., 2005). Poseen un grupo hemo que catalizan la oxidación monoelectrónica de diversos sustratos, preferentemente aromáticos, utilizando H_2O_2 como agente oxidante (Figura 12; Tabla2) en lo que se denomina ciclo peroxidativo (Morales y Ros-Barceló, 1997; Passardi y col., 2005; Novo-Uzal y col., 2013; Francoz y col., 2015; Kärkönen y Kuchitsu, 2015). Desde este punto de vista, las CIII-POX contribuyen al control del estrés oxidativo por consumo de H_2O_2.

En plantas vasculares las CIII-POX son miembros de una gran familia multigénica. De hecho, en especies como arabidopsis, arroz y maíz se han descrito 73, 138 y 155 genes que codifican para esta clase de enzimas, respectivamente (Luthje y col., 2011; Fawal y col, 2013; Francoz y col., 2015). La mayor parte de estas enzimas son secretadas al apoplasto donde permanecen solubles o ancladas a la membrana plasmática (Luthje y col., 2011). Este alto número de CIII-POX es debido a que son enzimas con cierta inespecificidad de sustrato y que catalizan múltiples reacciones de reforzamiento en la pared celular como la polimerización de la molécula de lignina y suberina (Ros Barceló, 1997; Bernards y col., 2004; Novo-Uzal y col., 2013; Fernández-Pérez y col., 2015a, 2015b; 2015c), el acoplamiento de los residuos de ferúlico de los arabinoxilanos (Ralph y col., 2004b; Encina y Fry, 2005; Lindsay y Fry., 2008), la formación de puentes isoditirosina (y di-isoditirosina) de las proteínas ricas en hidroxiprolina (Fry, 2004b). Asimismo, las CIII-POX están implicadas en la producción de fitoalexinas antifúngicas y antibacterianas y metabolitos secundarios (Almagro y col., 2009). Aunque mayoritariamente las CIII-POX contribuyen a la eliminación de H_2O_2 y el reforzamiento de la pared celular, se ha apuntado que, mediante el denominado ciclo hidroxílico, también pueden generar transitoriamente ROS ($O_2^{\cdot-}$/HOO·), promoviendo así un proceso de relajación de la pared celular cuando hay una inducción de la elongación celular (Passardi y col., 2005; Almagro y col., 2009; Novo-Uzal y col., 2013; Francoz y col., 2015).

La enzima APOX (EC 1.11.1.11) es una proteína hemínica, fundamentalmente monomérica, que lleva a cabo la primera reacción del ciclo ascorbato-glutatión en el que se reduce el H_2O_2, utilizando en ácido ascórbico (AA) como molécula donadora de electrones, generando H_2O y dehidroascorbato (DHA) (Figura 12; Tabla 2). Es la enzima más importante en el control de la producción de ROS, ya que es la enzima que tiene mayor afinidad por el H_2O_2 que la CAT y las CIII-POX (Dat y col., 2000; Sen, 2012). Se han descrito por lo menos 4 isoformas de APOX específica de diferentes compartimentos celulares como en la membrana de los tilacoides (tAPOX) y el estroma (sAPOX) del cloroplasto, en el citosol (cAPOX), y en la membrana de los peroxisomas (mAPOX) (Anjum y col., 2014; Gill y col., 2015).

La GR (EC 1.8.1.7) es una flavoproteína del ciclo del ascorbato-glutatión que juega un papel esencial en el sistema de defensa frente al estrés oxidativo ya que cataliza la reducción de la molécula de glutatión oxidado (GSSG) para mantener los niveles suficientes de glutatión reducido (GSH), utilizando el NAD(P)H como donador de electrones (Figura 12; Tabla 2; Romero-Puertas y col., 2006; Anjum y col. 2012; Gill y col., 2013). Esta enzima está presente en diversos compartimentos celulares en forma de un homodímero de 100-120 kDa (Gill y col., 2013).

Otras enzimas del ciclo del ascorbato-glutatión son la MDHAR y la DHAR. La MDHAR se encarga de catalizar la reducción del monodehidroascorbato (MDHA) a AA usando NAD(P)H como donador de electrones (Figura 12; del Río y col., 2015). Por otro lado, la DHAR cataliza la reducción divalente de DHA a AA utilizando dos moléculas de GSH y por lo tanto generando una molécula de GSSG (Figura 12; Yin y col., 2010).

El grupo de enzimas GST (EC.2.5.1.18) está ampliamente relacionado con respuestas frente a diferentes estreses abióticos (Dixon y col., 2009). Son un grupo muy amplio de enzimas presentes en el citoplasma, de naturaleza dimérica y que poseen multitud de funciones (Figura 12; Dixon y col., 2009). Este grupo de enzimas está dividido en 8 clases dependiendo de su actividad: *tau*, *phi*, *theta*, *zeta*, *lambda*, *DHAR*, *tetraclorohidroquinona dehalogenasa* (*TCHQD*) y *GST microsomales* (Edwards y col., 2010; Rezaei y col., 2013; Csiszár y col., 2014; Yang y col., 2014). Las GST *tau* y *phi* catalizan la adición nucleofílica de grupos sulfhidrilo (-SH) presente en el GSH sobre centros con naturaleza electrofílica de moléculas orgánicas, como xenobióticos, para generar compuestos más hidrosolubles y así favorecer su detoxificación (Dixon y Edwards, 2010a). La clase *theta* posee una notable actividad peroxidasa y se cree que son las enzimas encargadas de reducir los hidroperóxidos lipídicos (Dixon y Edwards, 2010b). La clase *zeta* está relacionada con el catabolismo de la tirosina y la clase lambda se cree que puede tener actividad GR (Dixon y Edwards, 2010b). Además de estas actividades también se ha descrito que tienen un papel en la homeostasis hormonal, la vacuolización de compuestos como la antiocianina y la regulación de la apoptosis (Dixon y col., 2011).

El sistema de antioxidantes no enzimáticos se compone de compuestos como el AA, el GSH, los carotenoides, el α-tocoferol, compuestos fenólicos como los flavonoides, la prolina y otros aminoácidos, compuestos con amonios cuaternarios como la glicina betaína, azúcares y poliaminas, entre otros (Figura 12; Gill y Tuteja, 2010; Gill y col., 2013; Anjum y col.,2014; Noctor y col., 2015).

El AA o vitamina C es el compuesto hidrosoluble más abundante y poderoso en la reducción del daño oxidativo provocado por la acumulación de ROS (Gallie, 2013). La síntesis de este compuesto se da lugar en la membrana mitocondrial interna y se transporta al resto de compartimentos, siendo muy abundante en los cloroplastos de células fotosintéticas y en células meristemáticas (Smirnoff, 2000). Este ASC es un potente antioxidante ubicuo, ya que actúa directamente sobre el $^1O_2$, el $O_2^{\cdot-}$ y el $^{\cdot}OH$ y, además, es el sustrato de la APOX para reducir el contenido

de H_2O_2 (Noctor y col., 2015). Una pequeña parte del ascorbato sintetizado por la célula es transportado al apoplasto, donde se cree que ejerce una primera barrera de defensa frente a oxidantes externos como el ozono, el SO_2 y el NO_2 (Noctor y col., 2015). Además, este ácido ascórbico tiene un papel importante en procesos de señalización celular, modulando el contenido de moléculas como el ácido salicílico, el ácido abscísico, el etileno y las giberelinas (Foyer y Noctor, 2011; Anjum ycol., 2014).

Tabla 2. Esquema de las principales enzimas antioxidantes y la reacción que catalizan.

Actividad antioxidante	Número EC	Reacción que cataliza
Catalasa (CAT)	EC.1.11.1.6	$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$
Superóxido dismutasa (SOD)	EC.1.15.1.1	$2\ O_2^{\cdot-} + 2\ H^+ \rightarrow 2\ H_2O_2 + O_2$
Peroxidasas clase III (CIII-POX)	EC.1.11.1.7	$H_2O_2 + 2RH \rightarrow 2\ H_2O + 2R^{\cdot}$
Ascorbato Peroxidasa (APOX)	EC.1.11.1.11	$H_2O_2 + A \rightarrow 2\ H_2O + DHA$
Glutatión Reductasa (GR)	EC.1.6.4.2	$GSSG + NAD(P)H \rightarrow 2GSH + NAD(P)^+$

La forma reducida del GSH (γ-Gly-Cys-Gly) es un antioxidante no enzimático que está presente de forma abundante en la mayoría de los tejidos y en los diferentes compartimentos celulares (Gill y col., 2013). Este antioxidante reacciona directamente con el $^1O_2$ y el $^{\cdot}OH$, participa en el ciclo del ascorbato-glutatión, es sustrato de POX para regular la concentración de H_2O_2 y protege los grupos tioles de las proteínas, produciendo GSSG (Gill y Tuteja, 2010; Gill y col., 2013). El balance entre el GSH y el GSSG es clave en mantener el estado de oxidación-reducción celular (Foyer y Noctor, 2005; Noctor y col., 2015). Es un compuesto que juega un papel esencial en diversos procesos fisiológicos como la comunicación intercelular, la regulación del transporte de sulfato intra y extracelular, control de rutas de señalización, conjugación de metabolitos, expresión de genes de respuesta a estreses y regulador de la expresión hormonal (Gill y Tuteja, 2010; Halliwell y Gutteridge, 2015). Además, una de sus principales funciones es la de detoxificador de xenobióticos y, al ser un componente de las fitoquelatinas, la quelación de metales pesados para poder ser secuestrados de la vacuola (Foyer y col., 2001). Por último, juega un papel junto con el ascorbato, en el control de procesos de crecimiento y desarrollo como la diferenciación celular, la muerte celular y regulación de la respuesta a patogénesis y el sistema de resistencia adquirido (RSA) en estreses bióticos (Anjum y col., 2014; Noctor y col., 2015).

HIPÓTESIS Y OBJETIVOS

El objetivo general de este trabajo es profundizar en los conocimientos sobre los mecanismos de tolerancia que subyacen al proceso de habituación de cultivos celulares de maíz (*Zea mays*) y alubia (*Phaseolus vulgaris*) a los compuestos relacionados con la inhibición de la biosíntesis de celulosa DCB y quinclorac. Este objetivo global ha sido dividido en los siguientes objetivos parciales:

Hipótesis 1: **La habituación de células de maíz a concentraciones letales de DCB conlleva modificaciones en la estructura y composición de la pared celular primaria tipo II asociada a una disminución del contenido en celulosa**.

Objetivo 1: Estudiar la plasticidad estructural de la pared celular de suspensiones celulares de maíz tratadas y habituadas a DCB. Se realizará una caracterización de las modificaciones a nivel de composición y estructura presentes en la pared celular de suspensiones celulares de maíz tratadas con altas concentraciones de DCB durante un corto periodo de tiempo y en células habituadas a concentraciones bajas y altas de este inhibidor.

Hipótesis 2: **Una respuesta típica al tratamiento de plantas con CBIs es la deposición de una lignina ectópica en respuesta a la deficiencia en celulosa en sus paredes celulares.**

Objetivo 2: Se caracterizará el proceso de lignificación ectópica en paredes celulares primarias con contenido reducido en celulosa mediante el uso de células de maíz tratadas y habituadas a DCB. En el presente objetivo se cuantificará y caracterizará la composición de material tipo lignina y se profundizará en la caracterización del fenómeno de lignificación ectópica mediante el análisis de los niveles de expresión de genes específicos de biosíntesis de lignina. En segundo término, se estudiará la relación entre la deposición del material tipo lignina y procesos de señalización de estrés celular.

Hipótesis 3: **El tratamiento y/o habituación de plantas o cultivos celulares de plantas a compuestos relacionados con la inhibición de la biosíntesis de celulosa está asociado al desarrollo de un sistema de defensa antioxidante.**

Esta hipótesis se desglosa en dos objetivos:

Objetivo 3.1: Se analizará el papel de las estrategias antioxidantes/detoxificadoras como respuestas celulares tempranas al DCB. Para ello se utilizarán células de maíz en fase de habituación incipiente a DCB, así como células de maíz tratadas a tiempos cortos con el inhibidor. Se realizará un estudio de los parámetros de crecimiento y de viabilidad de los cultivos en los tipos celulares indicados. Se cuantificará el nivel de peroxidación lipídica y de peróxido de hidrógeno, como indicadores de estrés oxidativo. Se valorarán actividades enzimáticas antioxidantes y detoxificadoras y se cuantificarán los niveles de antioxidantes no enzimáticos en las células con habituación incipiente y tratadas con DCB.

Objetivo 3.2: Se investigará la implicación del sistema de defensa antioxidante en el tratamiento a tiempos cortos y la habituación de células de alubia a quinclorac. Para ello, se determinarán los parámetros de crecimiento de células habituadas a quinclorac. Se valorará el estado oxidativo y se cuantificarán la actividad de enzimas antioxidantes en células de alubia habituadas a quinclorac. Se estudiará la estabilidad de la respuesta antioxidante mediante su caracterización en células deshabituadas a quinclorac.

MATERIAL Y MÉTODOS

1. Material vegetal y obtención de líneas celulares

El material vegetal utilizado en el presente estudio son cultivos celulares de dos especies vegetales: una dicotiledónea, alubia (*Phaseolus vulgaris* L.), y una monocotiledónea, maíz (*Zea mays* L.).

1.1. Cultivos celulares de maíz (*Zea mays* L.)

En el caso del maíz, se obtuvieron líneas celulares en forma de callo, a partir de embriones inmaduros que fueron cultivados en condiciones asépticas, a 25º C, con un fotoperiodo de 16:8 (3000 luxes ≈ 41 µmoles m^{-2} s^{-2}), durante 30 días y sobre un medio en un medio de cultivo de base Murashige y Skoog (1962), solidificado con agar 8 g L^{-1} y con un aporte de sacarosa 20 g L^{-1} y 2,4-D 9 µM (Lorences y Fry, 1991). Los callos fueron subcultivados de manera rutinaria cada 30 días en un medio de composición idéntica tal y como describe Mélida y col., (2009). Estos callos fueron disgregados en un medio de cultivo líquido con la misma composición e incubados en las mismas condiciones, con una agitación rotatoria a 120 rpm, llegando a obtener suspensiones celulares. Estas suspensiones celulares fueron subcultivadas rutinariamente cada 15 días (Lorences y Fry, 1991).

1.2. Cultivos celulares de alubia (*Phaseolus vulgaris* L.)

En el caso de la alubia, se obtuvieron líneas celulares en forma de callo a partir del primer par de hojas verdaderas, de plántulas crecidas en condiciones de esterilidad, durante 10 días. Las hojas fueron cortadas y depositadas en un medio de cultivo de base Murashige y Skoog (1962), solidificado con agar 8 g L^{-1} y con un suplemento de sacarosa 30 g L^{-1} y de 2,4-D 10 µM. Los explantos foliares fueron incubados en condiciones asépticas, a 25º C y en oscuridad durante 30 días (Encina y col., 2001). Los callos se separaron del explanto y fueron subcultivados de manera rutinaria cada 30 días en un medio de composición idéntica, pero con un aporte de 2,4-D 5 µM (Encina y col., 2001).

2. Proceso de habituación a compuestos relacionados con la inhibición de la biosíntesis de celulosa.

Los cultivos celulares de maíz fueron tratados a tiempos cortos y habituados a DCB y los cultivos celulares de alubia fueron tratados y habituados a quinclorac.

2.1. Tratamiento a tiempo corto y habituación de células de maíz a DCB

Suspensiones celulares de maíz habituadas a crecer en presencia de DCB fueron obtenidas a partir de células no habituadas (SNH) tratadas con 0,3 µM de

DCB durante 4 subcultivos y posteriormente incrementando la concentración del inhibidor hasta 1 µM. De esta forma se obtuvieron células habituadas a bajas concentraciones de DCB, 0,3, 0,5 (I_{50}, de Castro y col., 2014) y 1 µM de DCB utilizadas en este trabajo. Por otro lado, callos previamente habituados a concentraciones muy elevadas de DCB (12 µM, Mélida y col., 2009) fueron disgregados en un medio de cultivo líquido con un suplemento de 6 µM de DCB para obtener suspensiones celulares habituadas a altas concentraciones de este herbicida (Mélida y col., 2011). Estas líneas celulares habituadas a DCB, se denominaron SHx, donde "x" es la concentración del inhibidor en µM. El DCB fue disuelto en DMSO y el rango de concentraciones de este compuesto (0,003-0,01 %; v/v) no afectó al crecimiento de las células de maíz (datos no publicados).

Para investigar los cambios provocados por el tratamiento a corto plazo de DCB, células SNH fueron transferidas a medio de cultivo suplementado con 0,5, 1 y 6 µM de DCB durante 6 días. Estas células fueron denominadas SNH+DCB(x), donde "x" es la concentración del inhibidor en µM.

En resumen, las líneas celulares de maíz utilizadas en este estudio fueron: SNH, SNH+DCB(0.5), SNH+DCB(1), SNH+DCB(6)*, SH0.3, SH0.5, SH1 y SH6.

(*Esta línea celular en el capítulo II y en su correspondiente publicación es denominada como SNH+DCB)

2.2. Tratamiento a tiempo corto y habituación de callos de alubia a quinclorac

Callos de alubia, denominados como NH, fueron subcultivados en medios de cultivo con quinclorac comenzando por la concentración I_{50} (10 µM, Alonso-Simón y col., 2008) e incrementando esta concentración hasta conseguir células capaces de crecer en presencia del inhibidor (15 µM y 30 µM; Alonso-Simón y col., 2008). Estas líneas celulares habituadas fueron nombradas "Qn" donde "n" fue la concentración µM de quinclorac en la que crecieron las células. El quinclorac fue disuelto en dimetilsulfóxido (DMSO), comprobando posteriormente que la concentración más alta de este compuesto (0,3 %, v/v) no afectaba al crecimiento de las células (datos no publicados).

Las células deshabituadas (DH) se obtuvieron a partir de células Q30 cultivadas en un medio sin quinclorac durante 5 subcultivos

Para discernir los efectos de un tratamiento con quinclorac a corto y largo plazo en células de alubia, se realizó, paralelamente, un tratamiento de NH con 10 µM de quinclorac y un tratamiento de Q10 y Q15 con 30 µM de quinclorac durante 15 días. Estas líneas fueron denominadas NH+10, Q10+30 y Q15+30, respectivamente.

En resumen, las líneas celulares utilizadas en este estudio fueron: NH, NH+10, Q10, Q10+30, Q15, Q15+30, Q30 y DH.

3. Crecimiento de los cultivos celulares

3.1. Efecto del DCB en el crecimiento de suspensiones celulares de maíz y estudio de viabilidad

Para evaluar el efecto del DCB en el crecimiento de suspensiones celulares de maíz, se obtuvieron cinéticas de crecimiento de todas las líneas celulares obteniendo los valores de DW de alícuotas de cultivo obtenidas a diferentes tiempos de cultivo. Las curvas de crecimiento se obtuvieron al representar el ln DW frente al tiempo de cultivo en días. La RGR se obtuvo al calcular la pendiente de la porción recta de la curva de crecimiento y el tiempo de duplicación (td) del cultivo se calculó con la siguiente fórmula:

"tiempo de duplicación = ln 2 / RGR".

El estudio de la viabilidad celular fue realizado siguiendo el método de diacetato de fluoreceína (FDA) descrito por Duncan y Windholm (1990). A partir de un stock de FDA (Sigma) 0,2% disuelto en acetona, se preparó la solución de trabajo mediante la adición de 50 µl de este stock en 5 ml de medio de cultivo. Esta solución de trabajo se mezcló a partes iguales con células en suspensión y se incubó a temperatura ambiente y en oscuridad durante 5 minutos. Con un microscopio de epifluorescencia Nikon equipado con un filtro Nikon UV-2A se realizó un contaje de las células, diferenciando a las células vivas porque tuvieron en su interior una fluorescencia brillante verde. El resultado de la viabilidad se expresó en porcentaje de células vivas.

3.2. Efecto del quinclorac en el crecimiento de callos de alubia

Para evaluar el efecto del quinclorac en el crecimiento de callos de alubia, se midió el peso fresco (FW) de callos de las líneas NH, NH+10, Q10 al inicio del cultivo (FWi) y a los 30 días de cultivo (FWf). Con estos datos se calculó la tasa de crecimiento relativa (RGR) mediante el uso de la siguiente fórmula:

$$RGR = [(FWf-FWi)/FWi].$$

Las células después de crecer durante 30 días fueron posteriormente secadas a 60º C hasta obtener un peso constante y así obtener peso seco (DW) y poder calcular el ratio DW/FW.

Los datos de RGR y ratio DW/FW de las líneas celulares Q15 y Q30 fueron tomados de Alonso-Simón y col., (2008) para poder comparar con el resto de líneas celulares.

4. Caracterización de la pared celular

4.1. Extracción de la pared celular

La extracción de pared celular se realizó mediante la metodología descrita por Mélida y col, (2009). Las células de las diferentes líneas fueron recogidas en su fase exponencial de crecimiento, lavadas con agua destilada y congeladas a -20º C inmediatamente. Las células fueron homogeneizadas en motero de porcelana bajo nitrógeno líquido hasta la obtención de un polvo fino. Este material fue tratado con etanol al 70 % (v/v) durante 5 días. Posteriormente las muestras fueron filtradas con un filtro de fibra de vidrio (GF/A, Whatman, GE Healthcare) y los pellets fueron lavados con 6 veces etanol al 70 % (v/v), 6 veces con acetona y se dejaron secar durante unas horas para obtener el residuo insoluble en alcohol (AIR). Este AIR fue resuspendido en DMSO al 90% (v/v) durante 8 horas y esta extracción se repitió 3 veces. Las muestras fueron filtradas y lavadas dos veces con tampón fosfato 0,01 M pH 7,0. En el siguiente paso, los extractos fueron incubados durante 24 horas a 37º C con 2,5 U/ml de la enzima α-amilasa tipo VI-A (Sigma) disuelta en el mismo tampón con el que se realizaron los lavados. Se descartó la suspensión por filtrado y los extractos se lavaron con etanol al 70% (v/v) y acetona y se dejaron secar. Por último, las muestras fueron tratadas con una solución de fenol: ácido acético: agua (2:1:1, v/v/v) durante 8 horas dos veces y los extractos que se obtuvieron una vez filtrados, lavados con etanol al 70% (v/v) y acetona se consideraron como paredes celulares aisladas.

4.2. Fraccionamiento de pared celular

El fraccionamiento de pared celular se realizó tal y como describió Mélida y col., (2009). La primera fracción se obtuvo tras el tratamiento de las paredes celulares con una solución de ácido trans-1,2-diaminociclohexano-N,N,N',N'-tetraacético (CDTA) 0,05 M pH 6,5 durante 8 horas, después las muestras se lavaron con agua destilada y tanto el filtrado del tratamiento como los lavados fueron liofilizados y denominados como fracción CDTA. La muestra fue posteriormente tratada con una solución de KOH 0,1 M con $NaBH_4$ 0,02M durante 2 horas. Este proceso se repite 2 veces, las muestras se filtraron y se lavaron con agua destilada y como en el caso anterior, tanto los extractos como los lavados fueron liofilizados y denominados como fracción KI. El residuo fue tratado con una solución KOH 4M con $NaBH_4$ 0,02M durante 4 horas dos veces y se realizó el lavado de la muestra de la misma manera. Esta fracción fue liofilizada y denominada como fracción KII. Por último, la muestra fue hidrolizada con ácido trifluoroacético (TFA) 2 M, se centrifugó y el sobrenadante se liofilizó y denominó fracción TFA.

4.3 Análisis de pared celular

4.3.a. Monitorización de cambios en la pared celular por FTIR.

Las paredes celulares (2 mg) fueron mezcladas con KBr a una proporción 1:100 (p/p) y fueron compactadas en una prensa "GrasebySpecac" para muestras pequeñas, hasta la obtención de unas tabletas que fueron analizadas, con una resolución de 1 cm^{-1}, en un equipo "Perkin Elmer Spectrum 2000" para poder obtener los espectros FTIR. Del total de los espectros se seleccionó la región entre 800 y 1800 cm^{-1}, ya que esta región es la que contiene información sobre polisacáridos característicos de pared celular (Largo-Gosens y col., 2014). Todos los espectros fueron ajustados en su línea base y normalizados con el programa "Spectrum Software" (v 5.3.1). Los datos fueron exportados a Microsoft Excel 2010 para realizar una normalización del área.

4.3.b. Cuantificación de celulosa

La cuantificación de celulosa se realizó en los extractos de pared celular mediante el método de Updegraff (1969), utilizando las condiciones hidrolíticas para romper los polímeros de pared celular descritos por Saeman y col. (1963). Para ello, de 2 a 3 mg de pared celular se trataron con una solución de ácido acético: ácido nítrico: agua (8:1:2, v/v/v) y se calentaron durante 30 minutos en un baño de agua hirviendo para hidrolizar los polisacáridos no celulósicos. El hidrolizado se centrifugó una vez frio a 3500 rpm durante 15 minutos, se descartó el sobrenadante y el residuo fue lavado con 10 ml de agua destilada y posteriormente 10 ml de acetona. Tras los lavados la muestra fue secada en una estufa a 60º C, se trató con una solución de H_2SO_4 72% (v/v) durante 3 horas a temperatura ambiente y por último se diluyó la muestra hasta tener una concentración final de H_2SO_4 1 M y se volvió a hidrolizar durante 2,5 horas a 110º C. La glucosa liberada se valoró por el método de la antrona descrito por Dische (1962) y los resultados se expresaron como equivalentes de glucosa.

4.3.c Valoración de azúcares presentes en la pared celular

La cuantificación de azúcares totales fue realizada por el método del fenol-sulfúrico descrito por Dubois y col. (1956), utilizando glucosa como estándar y los ácidos urónicos fueron valorados por el método de m-hidroxi-bifenil descrito por Blumenkrantz y Asboe-Hansen, (1973), utilizando ácido galacturónico como estándar.

El contenido en azúcares neutros no celulósicos fue valorado con el método descrito por Albersheim y col. (1967). A todas las fracciones liofilizadas se les añadió 100 µg/ml de mio-inositol como estándar interno y, posteriormente, fueron hidrolizadas con TFA 2 M a 121º C durante 1 hora. Los monosacáridos liberados fueron derivatizados a alditol acetatos y fueron analizados por cromatografía de

gases (GC) en una columna capilar SP-2380 (30m x 0,25 mm I.d.; Supelco) en un equipo "Perkin Elmer Autosystem".

4.3.d Contenido en hidroxicinamatos

Los hidroxicinamatos presentes en pared celular que se valoraron fueron el ácido ferúlico, ácido p-cumárico y diferulatos (DFAs). Para analizar estos componentes de pared celular se utilizaron 50 mg de AIR, los cuales fueron tratados con NaOH 2M durante 4 horas a temperatura ambiente y fueron analizadas por cromatografía líquida de alta resolución (HPLC) con un detector ultravioleta (UV) y mediante el método descrito por Santiago y col. (2006). Para reconocer el compuesto 5,5'-DFA se comparó el tiempo de retención y el espectro UV con un estándar externo de 5,5'-DFA que nos proporcionó amablemente el grupo del Doctor John Ralph (Departamento de Bioquímica, Universidad de Wisconsin, Madison, EEUU). Para el resto de DFAs los espectros de UV se compararon con los anteriormente publicados (Waldron y col., 1996) y se utilizó la absorbancia a 325 nm para realizar la cuantificación de estos restos DFA. El contenido total de restos DFA se calculó mediante la suma de los 3 isómeros encontrados y cuantificados: 8,5'-DFA, 8-O-4-DFA y 5,5'-DFA. En el caso del diferulato 8,5'-DFA la cuantificación se realizó por la suma de las formas cíclica (o benzofurano) y no cíclica (o abierta) detectadas, ya que la forma no cíclica es probable que se detectara debido a la hidrólisis alcalina a la que se sometieron las muestras (Ralph, 1994).

4.4. Detección, cuantificación y caracterización de material tipo lignina

4.4.a Detección: Tinción de Wiesner.

La detección de material tipo lignina se realizó mediante una tinción histoquímica, que detecta la presencia de los cinamil-aldehídos presentes en la lignina, llamada tinción de Wiesner o método del floroglucinol-HCl (Pomar y col., 2002). Para realizar la detección, células de cultivos celulares de maíz fueron lavadas e incubadas en una solución de etanol 70% (v/v) con floroglucinol (1,3,5-trihidroxibenceno) 1% (p/v) durante 5 minutos. La solución de floroglucinol fue retirada y las células fueron acidificadas con HCl 18 % (v/v) hasta la aparición de una tinción de color pardo-rojizo. Las células teñidas fueron observadas en un equipo "Nikon SMZ1500 magnifier" y las fotografías fueron realizadas con una cámara digital "Nikon DXM1200F".

4.4.b Cuantificación: Método gravimétrico de lignina Klason.

El contenido en lignina de las paredes celulares fue calculado mediante el método gravimétrico de lignina Klason con algunas modificaciones. Las paredes celulares fueron hidrolizadas con una solución de H_2SO_4 al 72% (v/v) durante 1 hora a 30º C. Posteriormente la solución fue diluida con agua destilada hasta obtener una concentración final de H_2SO_4 2,5 % (v/v) y se incubó a 115º C durante 1 hora. El

residuo fue filtrado en unos filtros "Durapore polyvinylidene fluoride (PVDF)" con tamaño de poro de 0,45 µm (Millipore, GE Healthcare, Bukinghamshire, UK), fue secado en estufa a 60º C y pesado.

4.4.c Caracterización monomérica: Tioacidólisis

El método de la tioacidólisis, el cual rompe los enlaces β-O-4 presentes en el esqueleto central de la lignina, fue el utilizado para romper la molécula tipo lignina y así poder caracterizar la composición monomérica de este material mediante el uso de cromatografía de gases unida a espectrometría de masas (GC-MS) (Novo-Uzal y col., 2009). Las paredes celulares (15 mg) fueron tratadas con una solución de trifluoruro de boro eterato 0,2 M en una mezcla de dioxano/etanetiol 8,75/1 (v/v) en tubos pirex con tapón de teflón, en ambiente de N_2 gas, y fueron incubadas en un termoblock a 100º C durante 4 horas, agitando las muestras ligeramente cada 30 minutos. Posteriormente las muestras fueron diluidas con 30 ml de agua destilada con el fin de parar la reacción y enfriar la muestra y también se ajustó el pH de la mezcla entre 3,0 y 4,0 con una solución de $NaHCO_3$ 0,4 M. Una vez ajustado el pH se procedió a la extracción, la cual se realizó 3 veces, en un embudo de decantación con 30 ml de diclorometano, los extractos se juntaron y se secaron en un evaporador rotarorio (buchi R-200) a 40º C. El residuo final se resuspendió en 0,2 ml de diclorometano.

Por último, los productos de tioacidólisis se sililaron para poder ser detectables en el GC-MS. Para ello a 10 µl de extracto se añadieron 100 µl de N-O-bis (trimetilsilil) trifluoroacetamida (BSTFA) y 10 µl de piridina (grado GC) y se mantuvieron durante 2-3 horas a temperatura ambiente. Los timetilsililados fueron analizados en un equipo GC-MS "Trace GC, Thermo Finnigan Polaris Q mass Spectrometer" (MA, Whatman, USA) con una columna de GC DB-XLB, J&W (60 m x 0,25 mm l.d.).

4.5 Actividad cinamil alcohol deshidrogenasa

La actividad CAD fue medida mediante el método descrito por Chabannes y col. (2001) modificado por Fornalé y col. (2012). Células de cultivos de maíz fueron homogeneizadas bajo N_2 líquido con un mortero de cerámica hasta la obtención de un polvo fino que fue resuspendido en 5 ml de tampón de extracción (Tris-HCl 100 mM pH 7,5, polietilenglicol 6000 2% (p/v), DTT 5 mM y polivinil polipirrolidona 2 % (p/v)). La suspensión fue centrifugada a 10000 g a 4º C durante 10 minutos y esta operación se repitió 2 o 3 veces hasta que el sobrenadante fuera claro (sin restos celulares). La actividad se analizó en placas de 96 pocillos en las que se añadieron 140 µl de tampón de reacción (Tris-HCl 140 mM pH 8,8), 20 µl de coniferil alcohol 1 mM, 20 µl de $NADP^+$ 200 µM y 20 µl del sobrenadante extraído. Las muestras fueron mezcladas e incubadas durante 10 minutos a 30º C y la absorbancia a 400 nm fue medida en los 10 minutos inmediatamente posteriores, también a 30º C en un lector de placas de 96 pocillos "Synergy HT (Biotech, Winooski, VT, USA)".

Se realizaron varios blancos en los cuales se añadieron 20 µl de tampón de extracción en lugar de la muestra. El contenido total de proteínas siguiendo el método de Bradford (1976).

5. Análisis de la expresión relativa de genes

Para el estudio de expresión de genes las líneas celulares SNH, SH1 y SH6 fueron recogidas durante su fase exponencial de crecimiento. En el caso de las células SNH+DCB(6) se recogieron paralelamente células de esta línea celular y células SNH a los 3 días de cultivo, para poder comparar los niveles de expresión.

En todas estas líneas celulares se procedió a la extracción del ARN total a partir de 1 g de células que fueron homogeneizadas en un mortero de porcelana en N_2 líquido y utilizando el método del reactivo Trizol (Invitriogen, California, EEUU). La pureza e integridad de este ARN total fue medida espectrofotométricamente en un Nanodrop 1000 y en un gel de agarosa al 1% (p/v). Una vez confirmados que la pureza e integridad del ARN son suficientes para realizar el estudio, se procedió a la retrotranscripción del ARN para la obtención del ADN complementario (ADNc) mediante el uso de la enzima "Super Script III First Strand Retro-transcriptase" (Invitrogen) utilizando como cebadores el "oligo $(dT)_{20}$". La medición de la expresión de genes se realizó tanto por la técnica estándar (RT-PCR) como de manera cuantitativa o en tiempo real (qPCR).

5.1. Análisis semicuantitativo por RT-PCR

El análisis semicuantitativo de expresión génica se utilizó para analizar los genes de las rutas de señalización celular mediadas por ácido jasmónico (JA) y ácido salicílico (SA). Los genes que se estudiaron fueron: 12-oxofitodienato reductasa (ZmOPR1, AY921638; ZmOPR2, AY921639 y ZmOPR7, AY921644), NADPH oxidasa (ZmNADPHOX, CK849936), "maize protease inhibitor" (ZmMPI, X78988) y "pathogenesis related protein 1" (ZmPR1, UB2200), "non-expressor of PR1" (ZmNPR1, EU95584). En este caso, como el DCB puede afectar a la actina, se utilizó como gen de referencia la ubiquitina (ZmUBI, U29159; Fornalé y col., 2009).

Las amplificaciones de separaron en un gel de agarosa 1% (p/v) con un aporte de "SYBR Safe DNA gel stain" (Invitrogen) y los geles se fotografiaron en un equipo "Alphaimager HP System" (Protein Simple 3001, San José, California, EEUU). La intensidad de cada banda fue medida por el software "Alpha view v3.4.0.0" (Protein Simple) y esa intensidad se expresó como unidades de intensidad relativa. Todas las bandas fueron normalizadas con el valor de intensidad obtenida en el caso de la ubiquitina y se calcularon los ratios de intensidad relativa: SNH+DCB/SNH, SH1/SNH y SH6/SNH para tener un dato objetivo de incremento o reducción de la expresión génica.

5.2. Análisis cuantitativo por qPCR

La cuantificación de la expresión génica relativa por qPCR fue utilizada para valorar los genes de la ruta de los fenilpropanoides encargados de sintetizar los monolignoles (Vogt, 2010): ferulato 5-hidroxilasa (ZmF5H1, AC210173.4; ZmF5H2, GRMZM2G100158), cinamoil coA reductasa (ZmCCR1, GRMZM2G131205; ZmCCR2, GRMZM2G131836) y cinamil alcohol deshidrogenasa (*ZmCAD1, GRMZM2G118610; *ZmCAD2, Y13733, GRMZM2G844562; ZmCAD3, GRMZM2G046070; ZmCAD4, GRMZM2G700188; ZmCAD5, GRMZM2G443445; ZmCAD6, GRMZM2G090980 y ZmCAD7, GRMZM2G167613) descritos por Guillaumie y col. (2007). Como genes de referencia en maíz, utilizamos dos genes óptimos para estudios de qPCR tal y como describe Manoli y col. (2012): folipoliglutamato sintasa (ZMFPGS, GRMZM2G393334) y "Ubiquitin carrier protein" (ZmUBCP, GRMZM2G102471).

La cuantificación de transcritos mediante qPCR fue llevada a cabo en un equipo "StepOnePlus" (Applied Biosystems, California, EEUU) utilizando el kit de reacción "Power SYBR Green PCR master mix" (Applied Biosystems) y la mezcla de los dos tipos de cebadores a una concentración final de 10 µM. Para realizar la qPCR se utilizaron 2 µl de ADNc, 50 y 100 ng μl^{-1}. Todas las reacciones se realizaron por triplicado. El método comienza con la desnaturalización inicial a 95º C durante 10 minutos seguido de 40 ciclos donde la hibridación se dio a 95º C durante 10 segundos y la elongación a 60º C durante 1 minuto. El cálculo del nivel de transcritos para cada gen se realizó con el software StepOne™ v2.2.2 utilizando el método "δ-δ" (Livak y Schmittigen, 2001). Siempre se realizó en todas las placas, un control negativo en el cual no se añadía ADNc, ni cebadores para reducir el ruido de fondo. Para evitar la formación de dímeros de los cebadores, cada pareja de cebadores fue ensayada con la misma mezcla de reacción sin ADNc para ver si se producía amplificación de ADN y así determinar si se producían dímeros entre cebadores de cada gen y para detectar contaminantes de la reacción.

(*Errata en el capítulo II y en su correspondiente publicación a la hora de indicar los códigos de cada gen ZmCAD1 y ZmCAD2. Los códigos que aparecen en este apartado son los correctos.)

6. Estudio del estrés oxidativo y del sistema de defensa antioxidante

6.1. Extracción de actividades y de metabolitos antioxidantes

Para realizar el estudio del estrés oxidativo y valorar el sistema de defensa antioxidante en las líneas celulares de alubia y maíz, se recogieron células en fase de crecimiento exponencial hasta reunir como mínimo 1 g FW por cada línea celular. Esas células fueron homogeneizadas en presencia de nitrógeno líquido en un mortero de cerámica hasta la obtención de un polvo fino.

Se utilizó el tampón de extracción 1 en la valoración de la lipoxidación lipídica y de las actividades CAT, GR, SOD y GST. El tampón de extracción 1 estuvo

compuesto de Tris-HCl 0,05 M pH 7,5, EDTA 0,1 mM, Triton X-100 0,1 % (v/v), glicerol 10 % (v/v) y ditiotreitol (DTT) 2 mM. Las células homogeneizadas fueron resuspendidas en 5 ml del tampón de extracción y posteriormente se centrifugaron a 15000 g durante 2 minutos a 4º C. El sobrenadante se retiró a un nuevo tubo y se utilizó para realizar las valoraciones de actividades antioxidantes.

En el caso de las valoraciones de actividad POX (CIII-POX y APOX), la extracción se realizó con el tampón de extracción 2. El tampón de extracción 2 estuvo compuesto de Tris-HCl 0,04 M pH 7,2, EDTA-2Na-2H 1 mM y de glicerol 5 % (v/v). De la misma forma al caso anterior, las células homogeneizadas fueron resuspendidas en 5 ml de este tampón y posteriormente fueron centrifugadas a 15000 g durante 2 minutos a 4º C, para poder transferir el sobrenadante a un nuevo tubo y realizar las valoraciones de actividad POX.

Por último, la extracción de metabolitos antioxidantes, GSH y AA, se realizó con el tampón de extracción 3, el cual estuvo compuesto por ácido metafosfórico 5 % (p/v) disuelto en agua. Células de las diferentes líneas celulares (1 g FW) fueron homogeneizadas en N_2 líquido, resuspendidas en 3 ml de este tampón de extracción y, posteriormente, los extractos se dejaron incubar durante 20 minutos en hielo. Los extractos se centrifugaron a 19000 g durante 4 minutos y la valoración de estos metabolitos se realizó inmediatamente después para evitar oxidaciones.

En los extractos en los que se realizó una valoración de actividad enzimática, se cuantificó el contenido total de proteínas siguiendo el método de Bradford (1976).

6.2. Daño oxidativo: niveles de peroxidación lipídica

La peroxidación lipídica fue determinada mediante el método de cuantificación de TBARS descrito por Beuge y Aust (1978), utilizando diferentes concentraciones de malondialdehído (MDA), producto de la peroxidación lipídica, como molécula de referencia. Para calcular los niveles de peroxidación lipídica se mezclaron 20 µl del extracto con 1 ml del tampón de reacción. El tampón de reacción se preparó disolviendo ácido tricloroacético 15 % (p/v), ácido tiobarbitúrico 0,375 % (p/v) e hidroxitolueno butilado 0,01 % (v/v) en una solución ácida de HCl 0,25 M. Esta mezcla se incubó a 100º C durante 15 minutos, posteriormente, las muestras se enfriaron y se centrifugaron a 2500 g durante 15 minutos. La valoración de peroxidación lipídica se realizó midiendo la absorbancia del sobrenadante final a 535 nm.

6.3. Actividad de enzimas antioxidantes

6.3.a Actividad catalasa.

La actividad CAT se calculó según método descrito por Droillard y col., (1987) en el cual se mide la reducción de la absorbancia a 240 nm durante 2 minutos

debido a la ruptura de la molécula de H_2O_2 mediada por esta enzima. La medición se realizó mezclando 0,1 ml de extracto con 3 ml de tampón de reacción (tampón fosfato 50 mM pH 7,0 y H_2O_2 37,5 mM). La actividad fue calculada con el valor de la pendiente de la recta obtenida y utilizando el coeficiente de extinción molar del H_2O_2 a 240 nm: ε_{240} = 39.85 M^{-1} cm^{-1} (Droillard y col., 1987). Se realizó un blanco de reacción añadiendo 0,1 ml de tampón de extracción en lugar de la muestra. También se realizó un blanco de muestra en el que se utilizaron 3 ml de tampón de reacción, 0,1 ml de extracto y se sustituyó el H_2O_2 por H_2O, para poder valorar la actividad CAT debido a la posible presencia de H_2O_2 en el extracto.

6.3.b Actividad glutatión reductasa

La valoración de la actividad GR se realizó mediante el método descrito por Edwards y col. (1990) en el cual se mide la reducción de la absorbancia a 340 nm durante 3 minutos debido a la utilización del NADPH para reducir el GSSG. Para ello se mezclaron 0,1 ml de extracto en 1,35 ml de tampón de reacción (Tris-HCl 100 mM pH 8,0, EDTA 0,1 mM, GSSG 1 mM y $MgCl_2$ 3 mM). Una vez mezclado se añadieron 50 µl de una solución de NADPH 10 mM. Para valorar la actividad GR se utilizó el coeficiente de extinción molar del NADPH a 340 nm, ε_{240}= 6.22 mM^{-1} cm^{-1} (Edwards y col., 1990). Se utilizó en el método un blanco de reacción sustituyendo los 0,1 ml de extracto por 0,1 ml de tampón de extracción. También se realizó un blanco de muestra en el que se mezclaron 1,35 ml de tampón de reacción sin GSSG con 0,05 ml de NADPH 10 mM y 0,1 ml de extracto para valorar la actividad GR debida a la posible presencia de GSSG en el extracto.

6.3.c Actividad superóxido dismutasa

La actividad SOD de los extractos se cuantificó mediante el uso del kit comercial "SOD assay Kit" (Sigma). El kit se basa en la capacidad de la SOD de inhibir la aparición de un compuesto coloreado detectable a 440nm y para la valoración de la actividad se realizó una curva de calibración utilizando diferentes concentraciones de una SOD comercial (de 0,001 U/ml a 2000 U/ml). Para la determinación de esta actividad se utilizaron 20 µl de extracto y de estándar. También se realizó un blanco de reacción en el cual se añadieron 20 µl de tampón de extracción en vez de la muestra.

6.3.d Actividad peroxidasas de la Clase III

En el caso de la actividad CIII-POX se utilizó el método descrito por Adam y col. (1995), en el cual se valora el incremento en la absorbancia a 470 nm durante 2 minutos debido a que las CIII-POX oxidan la molécula de guayacol. Para realizar la cuantificación se mezclaron 3 ml de tampón de reacción que contenía acetato sódico 100 mM pH 5,5 y guayacol 1 mM y 50 µl de sobrenadante. La mezcla se agitó y para comenzar la reacción se añadieron 0,3 ml de H_2O_2 1,3 mM. La actividad se cuantificó con la medida de las pendientes de las rectas obtenidas y utilizando el

coeficiente de extinción molar del guayacol a 470 nm, ε_{470}= 26.6 $mM^{-1}\,cm^{-1}$ (Adam y col., 1995). El blanco de reacción utilizado sustituyó la muestra por 50 µl de tampón de reacción. Se realizó un blanco de muestra mezclando 3 ml de tampón de reacción, 50 µl de sobrenadante y 0,3 ml de H_2O, para valorar la actividad CIII-POX debido a la presencia de H_2O_2 en el extracto.

6.3.e Actividad Ascorbato Peroxidasa

La actividad APOX se midió observando la disminución de la absorbancia a 290 nm durante 4 minutos debido a la oxidación del AA por esta enzima mediante el método descrito por Hossain y Asada (1984). Para ellos se mezclaron 10 µl de muestra en 0,98 ml de tampón HEPES-NaOH 50 mM pH 7,6 con AA 20 mM y se agitó. La reacción comenzó mediante la adición de 10 µl de H_2O_2 1,3 mM. La actividad del extracto se cuantificó utilizando el coeficiente de extinción molar del AA a 290 nm, ε_{290}= 2,8 $mM^{-1}\,cm^{-1}$ (Hossain y Asada, 1984). Se realizaron un blanco de reacción con 10 µl de tampón de extracción y un blanco de muestra añadiendo 0,98 ml de tampón de reacción, 0,01 ml de H_2O y 0,01 ml de muestra.

6.3.f Actividad Glutatión S-transferasa

Para la valoración de la actividad GST se usó el método de Habig y col. (1974) que se basa en el incremento de la absorbancia medida a 340 nm durante un minuto por la formación de un complejo entre el GSH y el compuesto dicloro-2,4-nitrobenceno (DCNB). La reacción se consiguió mezclando 0,93 ml de tampón fosfato 0,1 M pH 7,5 con 20 µl de DCNB 1 mM. Para comenzar la reacción se añadieron 50 µl de muestra y se agitó ligeramente. La actividad GST se calculó utilizando la pendiente de la recta obtenida y el coeficiente de extinción molar del complejo GSH-DCNB, ε_{340}= 9,6 $mM^{-1}\,cm^{-1}$ (Habig y col., 1974). El blanco de reacción se realizó en las mismas condiciones, sustituyendo los 50 µl de extracto por 50 µl de tampón de extracción. Además, se utilizó un blanco de muestra mezclando 0,93 ml de tampón de reacción sin GSH, 0,02 ml de DCNB y 0,05 ml de muestra para valorar la actividad GST debida a la presencia de GSH en la muestra.

6.4. Determinación de isoenzimas de CIII-POX mediante electroforesis en gel de poliacrilamida

Para detectar las isoenzimas de CIII-POX (iso-POX) se utilizaron los mismos sobrenadantes en los que se realizó la cuantificación de actividad CIII-POX. Los sobrenadantes se concentraron utilizando las columnas de concentración por centrifugación Vivaspin 500 (5 KDa, GE Healthcare) y se utilizó una cantidad de 6 µg de proteínas. La separación se llevó a cabo en un gel no desnaturalizante (12% acrilamida), sin SDS, y utilizando un tampón de migración Tris-glicina-SDS (Tris-glicina 25 mM y SDS 0,1 % (p/v)) con un voltaje de 120 V/h. Para detectar las iso-POX se utilizó el método guayacol-H_2O_2 descrito por Mika y col., (2008), en el que los geles fueron lavados repetidas veces con H_2O milli-Q hasta la completa

eliminación del SDS y fueron, posteriormente, incubados con 50 ml de solución de reacción (acetato de sodio 250 mM pH 5,0, guayacol 1 % (v/v) y H_2O_2 0,03 % (v/v)) temperatura ambiente hasta que aparecieran unas manchas de color marrón. Como marcador de peso molecular, se utilizó "EZ-RUN pre-stained protein marker 100" (Fisher Scientific).

6.5. Contenido en metabolitos antioxidantes

6.5.a Medida del contenido en GSH total y GSSG

Para la cuantificación del GSH total (TG) se utilizó el ensayo reciclado con DTNB (ácido 5,5-ditio-bis-2-nitrobenzoico) descrito por Griffith (1980). En este método el DTNB reacciona con grupos sulfhidrilo del GSH dando lugar al compuesto TNB (ácido 5-tio-bis-2-nitrobenzoico), que se puede valorar midiendo la absorbancia a 412 nm. Posteriormente, el GSH se vuelve a recuperar mediante la actividad GR. El ensayo comienza mezclando 700 µl de tampón de reacción (tampón fosfato sódico 0,125 M pH 7,5 y EDTA 6,3 mM), 100 µl de NADPH 2,1 mM, 20 µl de actividad GR a 31,4 U/ml (Gluthatione reductase VI, Sigma) y 100 µl de la muestra. La reacción se comienza añadiendo 100 µl de DTNB 6 mM y se midió la absorbancia a 412 nm. Para calcular el contenido de TG, se realizó una recta patrón con concentraciones de GSH entre 0,05 y 30 µM.

Para la medición del GSSG, se utilizó la misma metodología, pero con un paso previo de bloqueo de los grupos sulfhidrilo. Las muestras se trataron con 100 µl del tampón de reacción y 20 µl de acrilonitrilo comercial estabilizado (Fluka, 99,9%) y se incubaron durante 10 minutos a 25º C. Para calcular el contenido en GSSG se utilizó una recta patrón con concentraciones de GSSG entre 0,05 µM y 3 µM y los estándares también se trataron con acrilonitrilo como se explica anteriormente.

El cálculo del contenido en GSH se realiza restando el contenido en GSSG a la medida de TG. Se calculó el ratio GSH/ GSH total para conocer la proporción del GSH en su forma reducida.

6.5.b Medida del ascorbato total y del dehidroascorbato

Para realizar la medida de AA total (TA) y DHA se utilizó el método descrito por Takahama y Oniki (1992), posteriormente modificado por Kärkönen y Fry (2006). El método comienza con la lectura de la absorbancia a 265 nm de la mezcla de 30 µl de sobrenadante con 1 ml de tampón de reacción compuesto por tampón fosfato 0,09 M pH 6,8 y Na^+-succinato 0,038 M. Posteriormente se añadieron 2 U de la enzima AA oxidasa (Ascorbate oxidase from *Cucurbita sp.*, Sigma) para oxidar el AA a DHA y se midió la reducción de la absorbancia a 265 nm durante 1 minuto. Por otro lado, se realizó un experimento paralelo, en el cual se midió la absorbancia de la mezcla 30 µl de sobrenadante y 1 ml del mismo tampón de reacción y después se añadió DTT a una concentración final de 14,8 mM para reducir el DHA presente

en la muestra a AA y se midió el incremento de absorbancia a 265 nm durante un minuto.

El cálculo del contenido en AA se realizó mediante la resta del TA y el DHA. También se calculó el ratio AA/ TA, para mostrar la proporción relativa de este compuesto en su forma reducida.

6.6 Contenido en ROS. Medida del H_2O_2 apoplástico

La cuantificación de H_2O_2 en el medio de cultivo de las suspensiones celulares de maíz fue realizada mediante el método descrito por Bindschedler y col., (2001), modificado por Cheeseman y col. (2006). El método consiste en añadir 150 µl de medio de cultivo, recogido en diferentes fases del ciclo de cultivo, en 1 ml de tampón de reacción (xylenol orange 100 µM, D-sorbitol 100 µM, $FeSO_4$ 250 µM, $(NH_4)_2SO_4$ 250 µM y etanol al 1% (v/v) en una solución de H_2SO_4 0,025 M). Las muestras se incubaron a temperatura ambiente en constante agitación durante 40 minutos y posteriormente se realizó una medida de la absorbancia a 550 nm. Se realizaron dos blancos: uno en el que se añadieron 150 µl de agua destilada y otro en el que se añadieron 150 µl de medio de cultivo fresco para restar la absorbancia debida a la presencia de H_2O_2 en el medio de culivo.

Para calcular la concentración de H_2O_2 se realizó una recta patrón, utilizando 150 µl de concentraciones de H_2O_2 (de 0,5 a 40 µM, concentración final) utilizando un reactivo comercial al 30% de H_2O_2 (Sigma) y siguiendo el mismo método. Para comprobar la concentración de H_2O_2 en el estándar (ya que es un compuesto inestable) se realizó midiendo la absorbancia a 240 nm y utilizando el coeficiente de extinción molar para el H_2O_2, ε_{240}= 43,6 M^{-1} cm^{-1}.

CAPÍTULO I:

Ectopic lignification in primary cellulose-deficient cell walls of maize cell suspension cultures

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Ectopic lignification in primary cellulose-deficient cell walls of maize cell suspension cultures

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Abstract

Maize (Zea mays L.) suspension-cultured cells with up to 70% less cellulose were obtained by stepwise habituation to dichlobenil (DCB), a cellulose biosynthesis inhibitor. Cellulose deficiency was accompanied by marked changes in cell wall matrix polysaccharides and phenolics as revealed by Fourier transform infrared (FTIR) spectroscopy. Cell wall compositional analysis indicated that the cellulose deficient cell walls showed an enhancement of highly branched and cross-linked arabinoxylans, as well as an increased content in ferulic acid, diferulates and p-coumaric acid, and the presence of a polymer that stained positive for phloroglucinol. In accordance with this, cellulose-deficient cell walls showed a fivefold increase in Klason-type lignin. Thioacidolysis/GC-MS analysis of cellulose-deficient cell walls indicated the presence of a lignin-like polymer with a Syringyl/Guaiacyl ratio of 1.45, which differed from the *sensu stricto* stress-related lignin that arose in response to short-term DCB-treatments. Gene expression analysis of these cells indicated an overexpression of genes specific for the biosynthesis of monolignol units of lignin. A study of stress signaling pathways revealed an overexpression of some of the jasmonate signaling pathway genes, which might trigger ectopic lignification in response to cell wall integrity disruptions. In summary, the structural plasticity of primary cell walls is proven, since a lignification process is possible in response to cellulose impoverishment.

Keywords: Cellulose; DCB; dichlobenil; ectopic lignin; maize.

Abbreviations: Ara, Arabinose; AIR, alcohol insoluble residue; CAD, cinnamyl alcohol dehydrogenase; CCR, cinnamoyl CoA reductase; CDTA, cyclohexane-trans-1,2-diamine-N,N,N′,N′,-tetracetic acid; DCB, dichlobenil or 2,4-dichlorobenzonitrile; DFA, diferulic acid; F5H, ferulate 5-hydroxylase; FTIR, Fourier Transform infrared spectroscopy; Fuc, Fucose; G, guaiacyl lignin unit; Gal, Galactose; GC-MS, gas chromatography- mass spectrometry; Glc, glucose; H, *p*-hidroxyphenyl lignin unit; JA, jasmonic acid; Man, Mannose; PVDF, polyvinilidene fluoride; OPR, 12-oxophytodienato reductase; Rha, Rhamnose; S, sinapyl lignin unit; SA, Salicylic acid SHx, DCB-habituated cells to "x" µM of DCB; SNH, non-habituated maize cells; SNH+DCB, non-habituated cells treated with 6 µM of DCB; UA, uronic acids; Xyl, xylose.

INTRODUCTION

The primary cell wall is a complex structure surrounding the protoplasm of elongating plant cells and it is crucial for shape maintenance and directional growth during cell development (Carpita 1996). Moreover, as the outermost layer of the plant cell, it is an active component in response to biotic and abiotic stresses with the capacity to monitor and maintain its integrity by means of structural and compositional changes (Hamann 2015). As with other grasses, the primary cell wall in maize (type II) is mainly composed of a framework of cellulose microfibrils embedded in a matrix of arabinoxylans. Smaller amounts of xyloglucan, mixed-linked glucans, pectins and glycoproteins can also be found as cell wall matrix components (Carpita 1996).

Cellulose, the main load-bearing structure of plant cell walls, is a polymer of β-1,4 linked glucan chains synthesized by transmembrane protein complexes (Guerriero et al. 2010). Cellulose is deposited in the cell wall in the form of microfibrils probably composed of 18 or 24 chains (Jarvis 2013). Arabinoxylans, the second major component of maize primary cell walls, play a pivotal role since different populations function by tethering adjacent cellulose microfibrils and forming the matrix phase of cell walls (Scheller and Ulvskov 2010). The arabinoxylan backbone is composed of β-1,4-linked xylose residues commonly substituted at C(O)3 and/or C(O)2 with arabinose or (4-O-methyl) glucuronic acid (Fincher 2009). One of the unique features of arabinoxylans from grasses is that the arabinose residues are often esterified at C(O)5 with the hydroxycinnamates ferulic and p-coumaric acids. Due to their high reactivity, polysaccharide-esterified hydroxycinnamates promote arabinoxylan cross-linking, playing a major role in maintaining the integrity of grass cell walls (Buanafina 2009).

Lignin is a complex phenolic heteropolymer predominantly deposited in the secondarily thickened cell walls of specialized plant cell types. Lignin drastically modifies cell wall structure and functions, since after its deposition cell walls acquire hydrophobicity and increase their resistance to mechanical and chemical degradation (Vanholme et al. 2010; Liu, 2012) being a key factor in the evolution of tracheophytes vascular system (Lucas et al. 2013). The main building blocks of lignin are the 4-hydroxycinnamyl alcohols (or monolignols): coniferyl and sinapyl alcohols with lesser amounts of p-coumaryl alcohol (Boerjan et al. 2003). Monolignols are synthesized in the cytosol from phenylalanine by the phenylpropanoid pathway and transported into the cell wall where they are subjected to oxidative cross-linking by cell wall peroxidases, laccases or other phenol oxidases using hydrogen peroxide or oxygen as oxidants (Passardi et al. 2004; Fagerstedt et al. 2010; Kärkönen and Kuchitsu 2014). Once polymerized into lignin, p-coumaryl, coniferyl and sinapyl alcohol give rise to p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively (Vanholme et al. 2010; Liu 2012).

Lignification is a tightly developmentally regulated process commonly associated with the formation of a secondarily thickened cell wall during cell specialization. Besides the developmentally regulated lignin, biotic and abiotic stresses can induce unexpected lignification known as ectopic lignification (Caño-Delgado et al. 2000; Moura et al. 2010; Sattler and Funnell-Harris 2013; Miedes et al. 2014). Although there are very few reports in the literature of this phenomenon in exclusively primary-walled cell cultures, lignin-like polymers have been shown to be produced by in vitro model systems under certain conditions (Novo-Uzal et al. 2009; Kärkönen and Koutaniemi 2010; Shen et al. 2013). In some systems, hormonally triggered cells can differentiate into tracheary elements, in which lignin is deposited in the newly formed secondary cell wall (Fukuda and Komamine 1980; Oda et al. 2005). In other cases, triggered cell cultures (normally by sucrose or elicitor treatments) release extracellular lignin into the culture medium (Simola et al. 1992; Lange et al. 1995; Nose et al. 1995; Kärkönen et al. 2009).

In the last few decades, a series of different approaches using cellulose biosynthesis inhibitors, mutants or transgenic plants have revealed compensatory effects between cellulose and non-cellulosic components of both primary and secondary cell walls. A reduction in cellulose content or an altered pattern of cellulose deposition has been demonstrated to cause changes in matrix polysaccharides and cell wall ectopic lignification (Caño-Delgado et al. 2000, 2003; Desprez et al. 2002; Ellis et al. 2002; Hernández-Blanco et al. 2007; Bischoff et al. 2009; Hamann et al. 2009; Denness et al. 2011; Brabham et al. 2014). Furthermore, lignin-defective transgenic plants respond with qualitative and quantitative changes in the polysaccharide counterpart (Sonbol et al. 2009; Ambavaram et al. 2011; Fornalé et al. 2012).

In previous studies, maize cell lines habituated to otherwise lethal concentrations of DCB (2,6-dichlorobenzonitrile, dichlobenil), a well-known cellulose biosynthesis inhibitor, were obtained by means of incremental exposure over many culturing cycles (Mélida et al. 2009; de Castro et al. 2014). These cell cultures had the capacity to cope with DCB through the acquisition of a modified cell wall in which the cellulosic scaffold was completely or partially replaced by a more extensive network of highly cross-linked arabinoxylans (Mélida et al. 2009, 2010a, 2010b, 2011). Our preliminary data indicated that DCB habituation could also induce ectopic lignification (as cellulose-deficient walls from habituated cells resulted positively for phloroglucinol staining) as a consequence of a reduction in cellulose in maize cells. An in-depth characterization of this phenomenon could further our understanding of the chemical composition of ectopic lignin and the relationship between ectopic lignification and stress responses. In this study, we characterized cell walls from maize suspension-cultured cells habituated to low (1 µM) and high (6 µM) DCB concentrations and from DCB short-term treated cell suspensions (I_{50} value for maize suspension cultured cells is 0.5 µM DCB; de Castro et al. 2014), paying special attention to the putative ectopic lignin/lignin-like

component as well as the expression levels of genes specific for the biosynthesis of monolignol units of lignin and others involved in common stress signaling pathways.

RESULTS

Cell wall fingerprinting indicated increased phenolics-to-polysaccharides ratios due to DCB exposure

FTIR spectra of non-habituated (SNH), DCB short-term treated (SNH+DCB) and habituated to (SH1) 1 and (SH6) 6 µM DCB maize suspension-cultured cells were obtained, normalized and baseline corrected. Averaged difference spectra were obtained by digital subtraction of SNH spectra from each of the DCB-treated/habituated cell lines (Figure I.1). Compared with SNH spectra, those from both short-term treated and habituated cell walls showed negative peaks in the region ranging from 900 to 1200 cm^{-1} where most of the cell wall polysaccharides, including cellulose, absorb (Alonso-Simón et al. 2011; Largo-Gosens et al. 2014). In addition, positive peaks were detected associated with wavenumbers indicative of aromatic rings (1515, 1600 and 1630 cm^{-1}), phenolic rings (1500 cm^{-1}) and phenolic esters (1720 cm^{-1}) (Kačuráková et al. 2000), indicating that both DCB-treated and DCB-habituated cells were enriched in phenolics. In accordance with this, wave number ratios 1540/1160, 1540/1425 and 1540/1740 cm^{-1} normally associated with increased lignin-to-polysaccharides ratios raised in both DCB-treated and DCB-habituated cells (Table I.1).

Table I.1. Fourier transform infrared (FTIR) wavenumber ratios characteristic of lignin and cell wall polysaccharides

FTIR peak height ratio	SNH	SNH+DCB	SH1	SH6
1540/1160 cm^{-1}	0,09	0,14	0,13	0,21
1540/1425 cm^{-1}	0,16	0,21	0,19	0,28
1540/1740 cm^{-1}	0,16	0,18	0,18	0,25

Peak assignment, 1,160 cm^{-1}, C–O–C vibration of the glycosidic link in cellulose, xyloglucan or pectic polysaccharides; 1,425 cm^{-1}, C–H stretching in CH_2 groups of cellulose; 1,540 cm^{-1}, aromatic ring stretching in lignin; 1,740 cm^{-1}, C–O stretch in ester groups. For maize cell line annotations see Figure I.1 legend.

Highly branched and cross-linked arabinoxylans increased in parallel to the DCB habituation process

Cell wall fractionation showed that in both DCB-treated and DCB-habituated cells, most of the non-cellulosic cell wall polysaccharides (70%-80%) corresponded to KOH-extractable hemicelluloses, namely KI and KII fractions (Figure I.S1). Moreover, differences were observed in cell wall fractionation among cell lines. Of

particular note was the increase in strong alkali-extracted hemicelluloses (KII fraction: 29% in SNH vs. 42% in SH6) exclusively associated with habituation to high DCB concentrations (Figure I.S1).

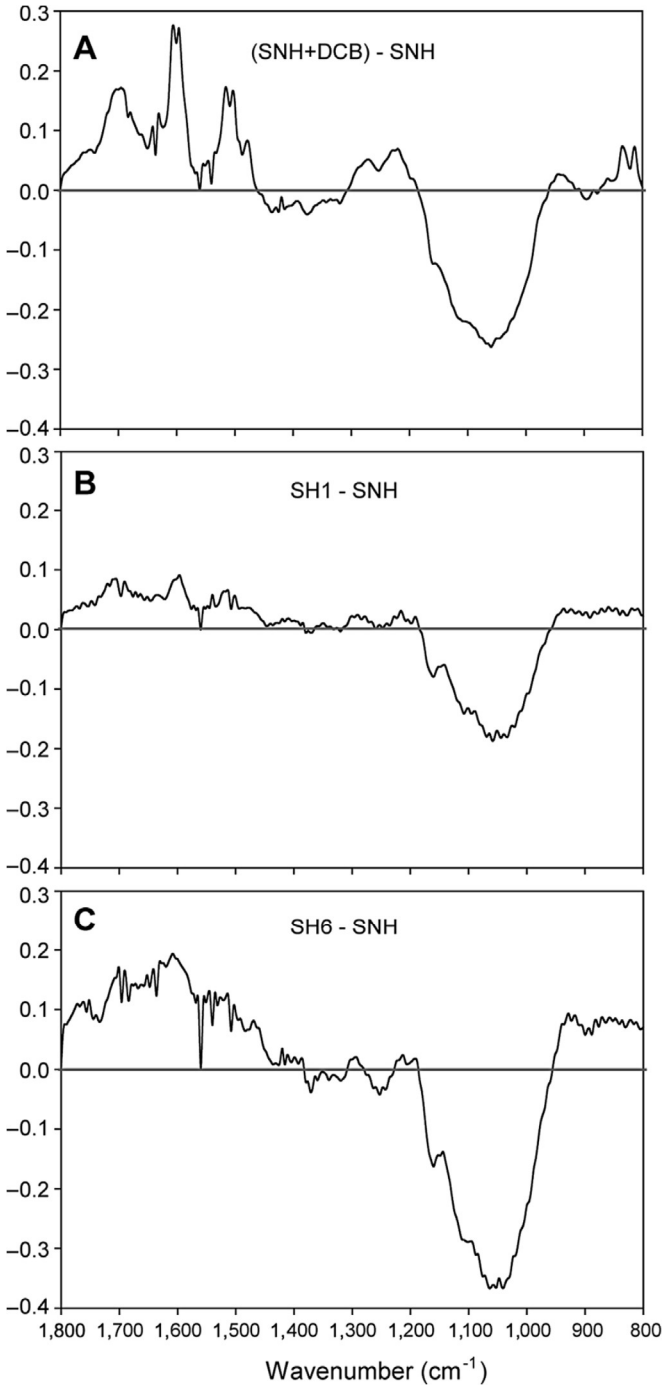

Figure I.1. Fourier transformed infrared (FTIR) spectroscopy analysis of cell walls

Averaged FTIR difference spectra obtained after digital subtraction of the SNH cell wall FTIR spectra from SNH+dichlobenil (DCB), SH1 or SH6 cell wall FTIR spectra. Maize cell lines were annotated as follow: non-habituated (SNH); DCB short-term treated (SNH+DCB), habituated to 1 µM DCB (SH1) or habituated to 6 µM DCB (SH6) maize cell suspension cultured cells.

Table I.2. Arabinose and xylose content in the KII fractions

	(%) Ara+Xyl		
	Cell wall	KII-extracted sugars	Ara:Xyl ratio
SNH	17.9 ± 0.9	59.5 ± 1.7	0.64
SNH+DCB	15.6 ± 0.5	56.7 ± 0.3	0.70
SH1	19.1 ± 1.3	62.4 ± 0.8	0.97
SH6	**30.6 ± 2.9**	**71.5 ± 1.4**	0.83

Mean values ± standard deviation (SD) of three technical replicates per line. For cell line annotation see Figure I.1 legend. Values that are significantly different from SNH are in bold (Student´s t-test, $p<0.05$).

Cellulose reduction is a consequence of habituation to DCB

Cellulose averaged approximately 25% of the cell wall dry weight when assayed in SNH cells (Figure I.3). Cellulose content decreased along DCB-habituated cells in a dose-dependent manner, up to the 50% and 70% reduction found, respectively, in SH1 and SH6 cells when compared with SNH cells (Figure I.3). However, short-term incubations with the cellulose biosynthesis inhibitor did not induce significant reductions in cellulose content. On the other hand, these short-term incubations did increase the amount of a Klason-resistant residue, which could be associated with lignin or a lignin-like phenolic-rich material (Figure I.3). This lignin-like material was also found to be increased in the cell wall of the DCB-habituated cells. Indeed, SH1 and SH6 cells contained approximately 2 to 5 times more of this residue, respectively, when compared with the SNH counterpart (Figure I.3).

Cell wall phenolic profile

Maize primary cell wall typically contains high levels of wall-esterified phenolics, which appear as side-chain decorations of arabinoxylans. *p*-Coumarate, ferulate and their oxidative coupling products, diferulates, increased steeply over the course of the DCB habituation process (Table I.3). In comparison with SNH, SH1 and SH6 cell walls were enriched in the 5,5', 8,5' and specially the 8-O-4' form of diferulates. In all cases, enrichment was more noticeable in SH6 cells.

In comparison to SNH cells, trends similar to those for the DCB-habituated cells were observed when the phenolic profile of DCB short-term treated cells was analyzed (Table I.3). Most notably, there was a marked increase in cell wall esterified *p*-coumarate, with SNH+DCB cells being 132- and 15-fold enriched in *p*-coumarate when compared with SNH and SH6 cells, respectively.

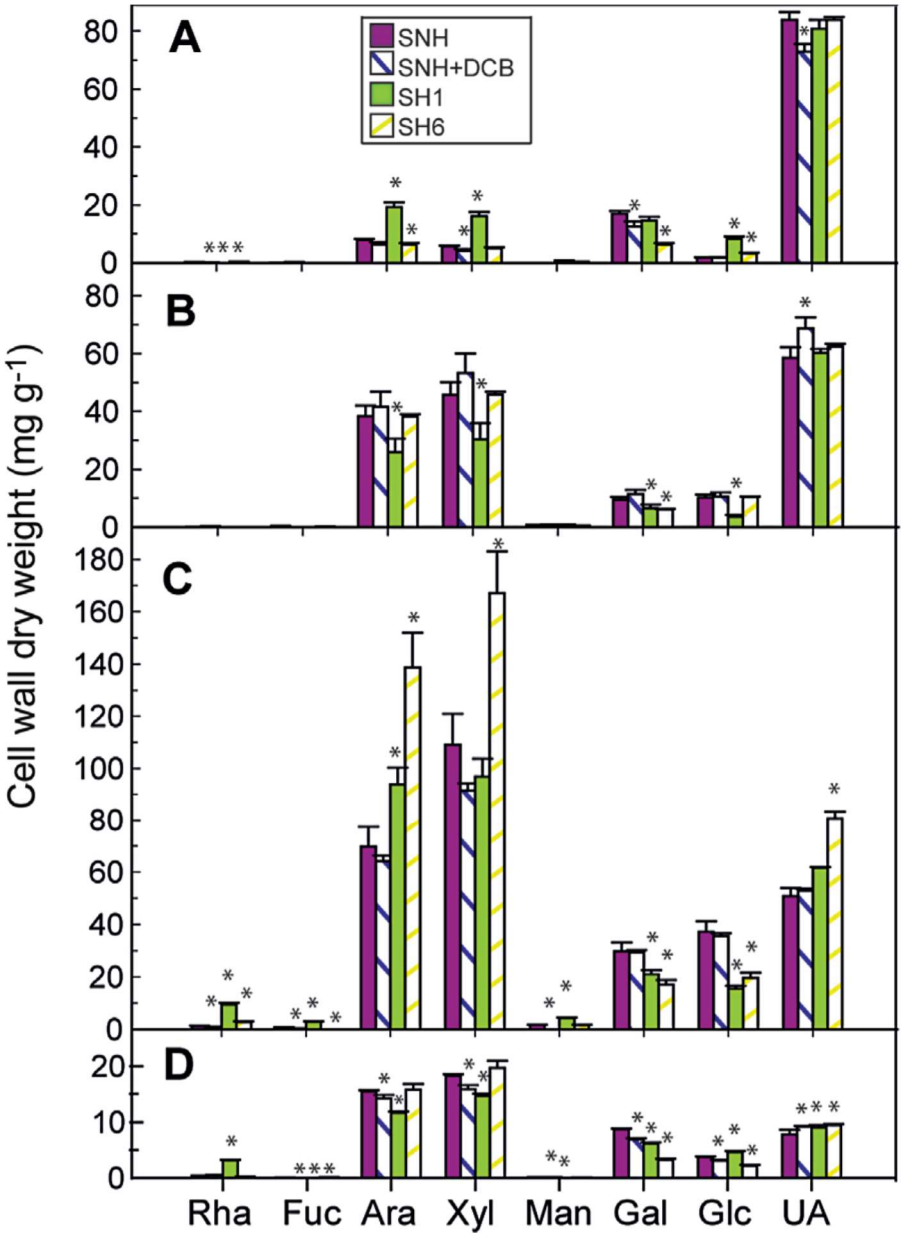

Figure I.2. Cell wall sugar analysis

Sugar composition of (A) CDTA, (B) KI, (C) KII and (D) trifluoroacetic acid (TFA) cell wall fractions obtained from (purple filled) SNH, (blue barred) SNH+DCB, (lime-green filled) SH1 and (yellow barred) SH6 cell lines. For maize cell line annotations see Figure I.1 legend. Ara (arabinose), Fuc (fucose), Gal (galactose), Glc (glucose), Man (mannose), Rha (rhamnose), UA (uronic acids), Xyl (xylose). Data represents the means values ±standard deviation (SD) of three technical replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P<0.05$).

DCB induced the deposition of lignin-like polymers in maize cultured cells

Phloroglucinol-HCl, which specifically stains 4-O-linked hydroxycinnamyl aldehyde residues of lignin (Pomar et al. 2002), was used to preliminarily confirm the presence of a lignin-like phenolic-rich material in the cell walls of DCB short-term treated and habituated cells. This strategy demonstrated that lignin

accumulation depended on the presence of DCB in the culture medium (Figure I.4); maize suspension-cultured cells stained negative for phloroglucinol when cultivated in a medium lacking DCB (Figure I.4A). In the case of maize cells short-term incubated in 6 µM DCB (Figure I.4B) and DCB-habituated cells (Figure I.4C), positive phloroglucinol staining was observed on the surface of cell-aggregates. No evidence of differentiation into tracheary elements were observed in any case (data not shown).

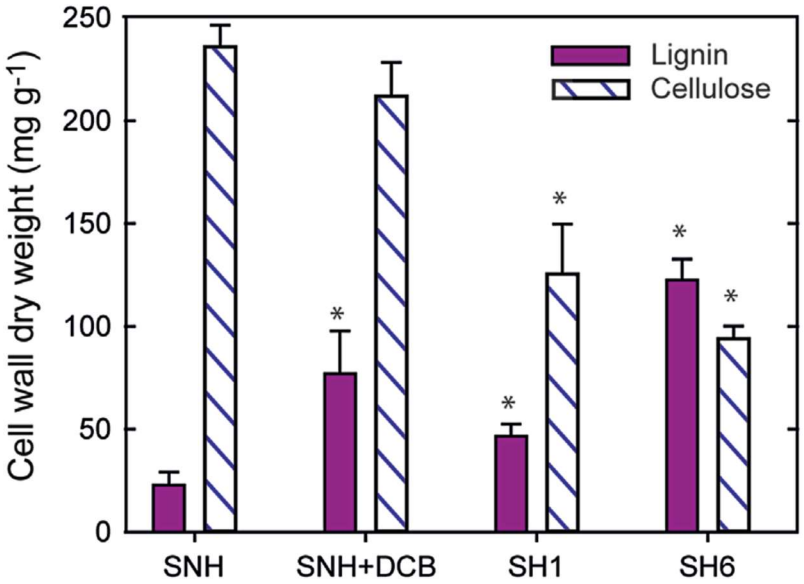

Figure I.3. Comparison of cellulose and lignin content in SNH, SNH+DCB, SH1 and SH6 cell lines

For maize cell line annotations see Figure I.1 legend. Data represents means ± standard deviation (SD) of at least four replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P < 0.05$).

The presence of lignin-like polymers was further confirmed by thioacidolysis followed by gas chromatography coupled to mass spectrometry (GC-MS) of the cleavage products. This analysis also confirmed the presence of trace amounts of sinapyl alcohol (S units) in SNH, SNH+DCB and SH1cell walls (Figure I.5B, D and data not shown). In addition to the S units, measurable amounts of coniferyl alcohol (G units) were detected in SH6 cell walls, but not in the other cell lines (Figure I.5E, F). Indeed, S units were (semi-quantitatively) estimated to be more abundant in SH6 than in any other cell line. Based on thioacidolysis results, an S/G ratio of 1.45 was estimated for the SH6 lignin-like material (Table I.4).

Lignin biosynthesis-specific genes are overexpressed in DCB-habituated cells

In a previous study by our group, we demonstrated that the genes functioning in the initial steps of the phenylpropanoid pathway (Phenylalanine Ammonia-Lyase, Cinnamate 4-Hydroxylase, 4-Coumarate CoA Ligase, Hydroxycinnamoyl-CoA Shikimate/quinate hydroxycinnamoyl Transferase and

Caffeic acid OMethyltransferase) are overexpressed in DCB-habituated cells (Mélida et al. 2010a). The corresponding proteins from such genes are involved in the production of p-coumaroyl-CoA and feruloyl-CoA, the substrates for hydroxycinnamate esterification of arabinoxylans (Lindsay and Fry 2008). Given the evidence of the presence of lignin-like polymers in DCB-habituated cells, quantitative RT-PCR was used to monitor the transcript abundance of cinnamoyl-CoA reductase (CCR), ferulate 5-hydroxylase (F5H) and cinnamyl alcohol dehydrogenase (CAD), involved in the last steps of monolignol synthesis (Figures I.6 and I.7).

Table I.3. Cell wall esterified phenolics composition

			Diferulates			
	p-Coumarate	Ferulate	Total	5,5´-	8-O-4´-	8,5´-[a]
			mg g^{-1}cell wall			
SNH	0.21	0.82	1.83	0.48	0.76	0.59
SNH+DCB	27.80	6.60	2.33	0.56	1.20	0.57
SH1	0.52	11.55	2.29	0.54	1.13	0.62
SH6	1.83	17.71	3.58	1.07	1.58	0.93

Data represented as mg g^{-1} cell wall. Mean values from two independent experiments per cell line. For cell line annotation see Figure I.1 legend. [a]8,5´-diferulate was calculated as the sum of 8-5-open and 8-5 benzofurans forms

A general overexpression of the two *ZmCCR* genes was observed in DCB-habituated cell lines (Figure I.6A, B), whereas short-term treatment with DCB induced only minor changes in *ZmCCR1* and *ZmCCR2* mRNA levels. The expression of *ZmF5H2* was significantly increased in all cell lines when compared with SNH cells, and this enhancement was especially noticeable in DCB-habituated lines (Figure I.6D). In the case of *ZmF5H1*, only SH1 cells showed higher transcript abundance, and indeed this gene was repressed in SNH+DCB and SH6 (Figure I.6C). Both DCB short-term treatment and DCB habituation induced an overexpression of *ZmCAD1*, *ZmCAD5* and *ZmCAD7* genes in comparison with SNH cells (Figure I.7A, D, F). This enhancement was especially marked in the case of *ZmCAD7* transcript levels in SH1 cells, whereas the abundance of *ZmCAD6* transcripts was only significantly increased in habituated cells (Figure I.7E). Moreover, there was a high overexpression (12-fold) of *ZmCAD2* in SH6 cells; however, the transcript levels of this gene were significantly reduced in SH1 cells (Figure I.7B). Surprisingly, there was a significant repression of the transcript levels coding for *ZmCAD3* in DCB-habituated cell lines (Figure I.7C). The *ZmCAD4* transcription levels were too low to be accurately quantified by this procedure. Given the general overexpression of the genes coding for CAD proteins in response to DCB, we measured CAD activity in the different cell lines (Figure I.7G). CAD activity assayed from cell extracts was significantly increased in SNH+DCB and SH6 when compared with SNH cell lines, but unchanged in the case of SH1 cells.

Apoplastic hydrogen peroxide accumulation

Both DCB short-term treated and DCB-habituated cells accumulated significantly more H_2O_2 in the spent medium than SNH cells (Table I.5), although there is no clear relationship between H_2O_2 accumulation and the presence of lignin-like polymers. SH6 cells, which showed the strongest ectopic lignification, did not peak in H_2O_2 content when compared with SH1 or SNH+DCB cells. In fact, SH6 cells accumulated less H_2O_2 during the lag and exponential phases than SH1 or SNH+DCB cells.

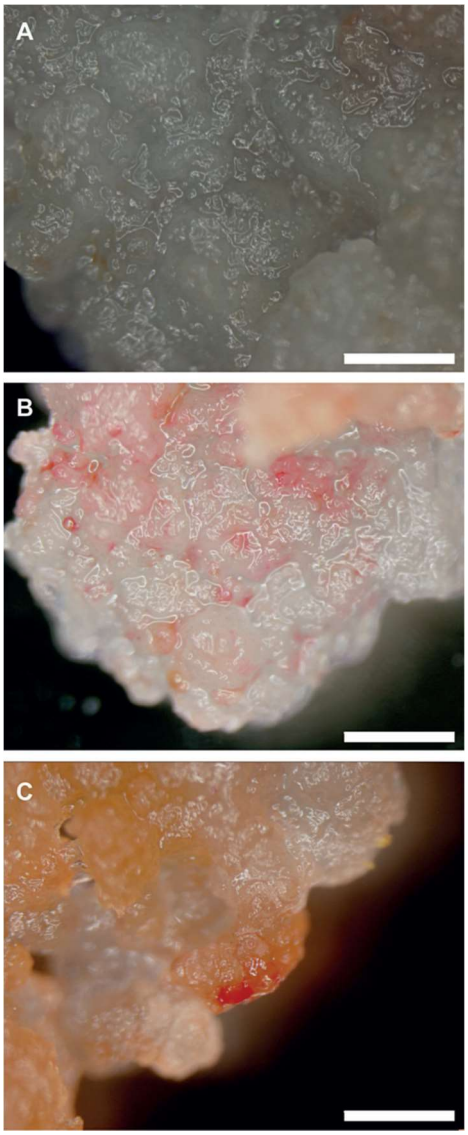

Figure I.4. Phloroglucinol/HCl staining (A) of non-habituated (SNH), (B) SNH+DCB, and (C) SH6 cells

For maize cell line annotations see Figure I.1 legend. Bar = 0.5 mm.

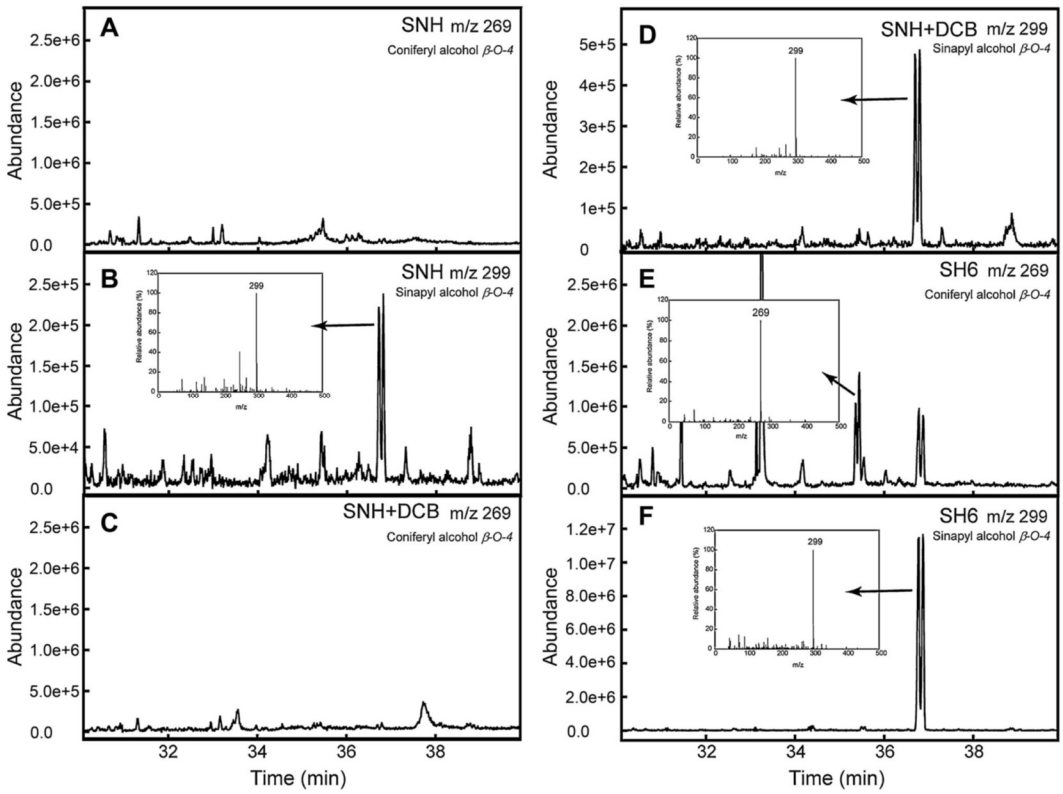

Figure I.5. Lignin monomer composition

Gas chromatography (GC) profiles of the thioethylated monomers (erythro and threo isomers) arising from aryl-glycerol-baryl ether (β-O-4) structures derived from (A, C and E) coniferyl and (B, D and F) sinapyl alcohols from cell walls of (A, B) SNH; (C, D) SNH+DCB; and (E, F) SH6 cell lines. For maize cell line annotations see Figure I.1 legend.

Table I.4. Lignin monomeric composition as revealed by thioacidolysis

	Total Ionic Current (x 10^6) mg^{-1} cell wall		
	Coniferyl alcohol (G units)	Sinapyl alcohol (S units)	S/G ratio
SNH	0	7.1 ± 1.2	-
SNH+DCB	0	**9.7 ± 0.7**	-
SH1	0	**9.5 ± 0.2**	-
SH6	**20.6 ± 1.0**	**29.9 ± 2.5**	1.45

Mean values ± standard deviation (SD) of three replicates per cell line. For cell line annotation see Figure I.1 legend. Values that are significantly different from SNH are in bold (Student's *t*-test, $P < 0.05$).

JA synthetic and JA signalling pathways overexpressed

To determine whether the accumulation of lignin-like material formed part of an abiotic stress response mechanism, RT-PCR was used to monitor the expression levels of several genes from the jasmonic acid (JA) and salicylic acid (SA) stress signaling pathways (Figure I.8). Three 12-oxophytodienoate reductase (OPR) genes, coding for proteins involved in the synthesis of JA, were analyzed. Two of

them (*ZmOPR1* and *ZmOPR2*) were always overexpressed in the presence of DCB, but *ZmOPR7* was only overexpressed in DCB-habituated cell lines, and was slightly repressed by the short-term exposure of SNH cells to DCB (Figure I.8). NADPH oxidase (*NADPHOX*) and maize protease inhibitor (*MPI*) genes are reported to be JA-induced in response to abiotic stresses (Shivaji et al. 2010). The results showed that both genes were overexpressed in the presence of DCB.

For the SA stress signaling pathway, pathogenesis related protein 1 (*PR1*) and non-expressor of PR1 (*NPR1*) genes were studied. The *ZmNPR1* gene was detected, but there were no differences in the expression pattern induced by either DCB exposure or DCB habituation. *ZmPR1* transcripts were not detected in any cell line.

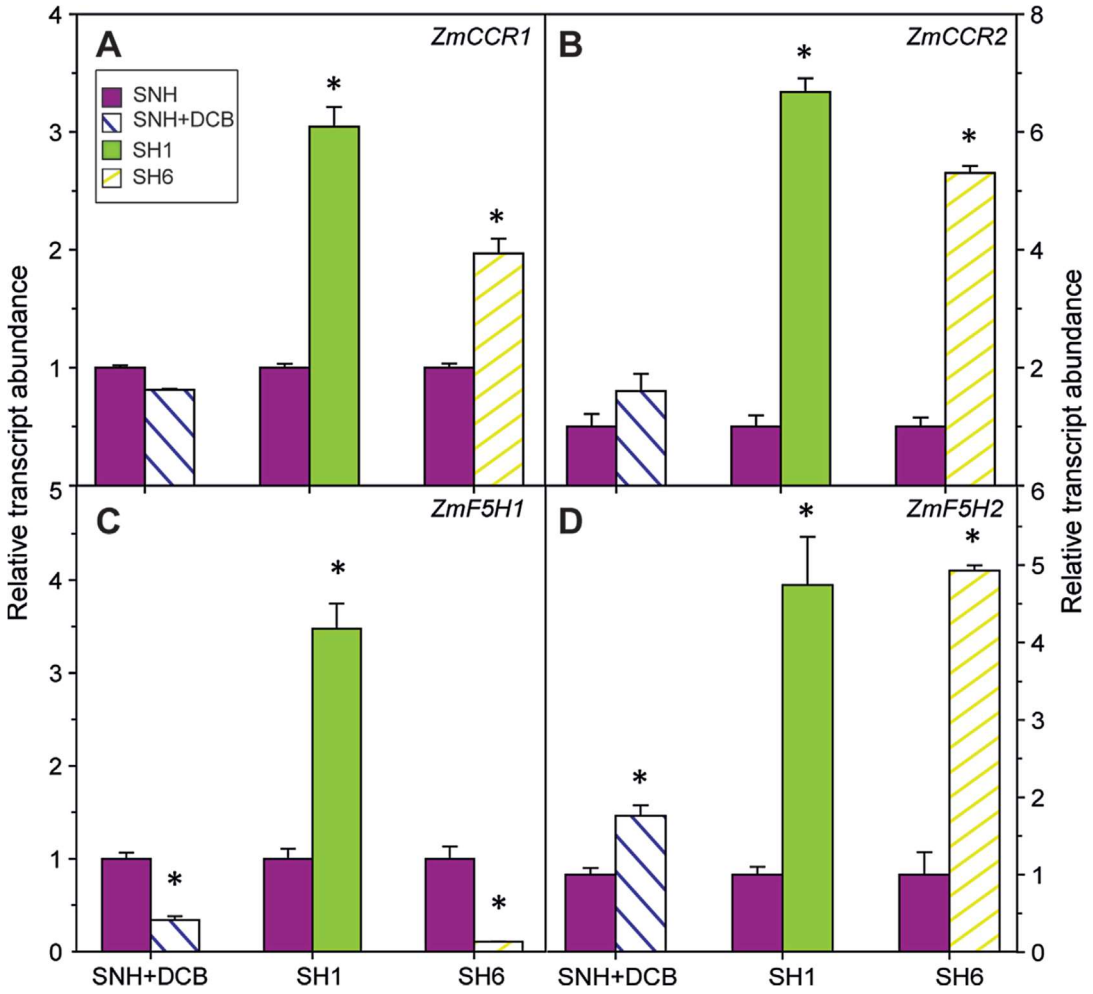

Figure I.6. Quantitative reverse transciption-polymerase chain reaction (qRT-PCR) characterization of (A, B) *ZmCCR* and (C, D) *ZmF5H* genes of SNH, SNH+DCB, SH1 and SH6 cell lines.

The gene expression levels of SNH+DCB, SH1 and SH6 cell lines were always compared against the SNH ones, which are represented as purple bars. For maize cell line annotations see Figure I.1 legend. Data represent relative fold change relative to SNH genes ± standard deviation (SD) of three replicates. Asterisks indicate values that are significantly different from SNH after a Student's t-test ($P<0.05$)

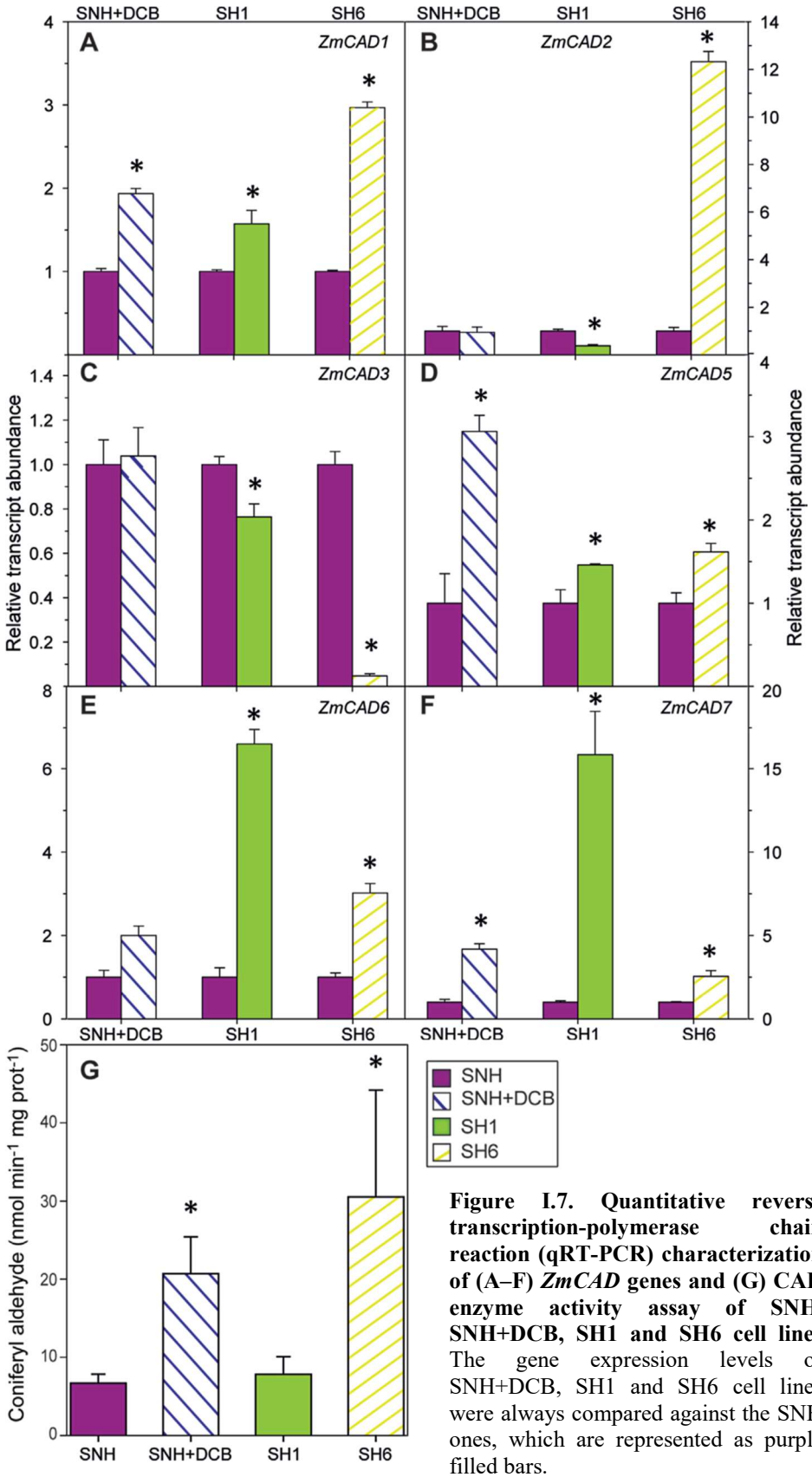

Figure I.7. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) characterization of (A–F) *ZmCAD* genes and (G) CAD enzyme activity assay of SNH, SNH+DCB, SH1 and SH6 cell lines The gene expression levels of SNH+DCB, SH1 and SH6 cell lines were always compared against the SNH ones, which are represented as purple filled bars.

For maize cell line annotations see Figure I.1 legend. For (A–F), data represent relative fold change relative to SNH genes ± standard deviation (SD) of three replicates. For (G), data represents means ± SD of at least nine replicates. Asterisks indicate values that are significantly different from SNH after a Student's t-test ($P<0.05$). *ZmCAD4* mRNA transcripts were not detected.

Table I.5. Apoplastic H_2O_2 concentration measured in the spent medium of the different cell lines

	Lag phase	Exponential phase	Stationary phase
		H_2O_2 (µM)	
SNH	0.4 ± 0.3	0.8 ± 0.1	0.6 ± 0.1
SH1	**1.9 ± 0.3**	**2.4 ± 0.2**	**2.3 ± 0.2**
SH6	**0.8 ± 0.1**	**1.7 ± 0.5**	**2.4 ± 0.2**
Incubation time	1 day	6 days	
SNH+DCB	**1.0 ± 0.1**	**2.0 ± 0.1**	

Mean values ± standard deviation (*SD*) of 3 replicates per line. Values were obtained at the different growth phases for each line. Short-term treated cells (SNH+DCB) were measured 1 day and 6 days after the addition of DCB. Values that are significantly different from SNH are in bold (Student´s t-test, $p<0.05$)

DISCUSSION

In their natural habitats, plant cells must continuously remodel their cell walls in order to grow and to interact with the environment. In order to understand the limits of these interactions, plant cells can be cultivated in fully controlled experimental systems where their capacity to cope with different situations can be better studied. The habituation of plant cell cultures to cellulose biosynthesis inhibitors such as DCB represents a valuable tool to improve our knowledge of the mechanisms involved in plant cell wall structural plasticity (Shedletzky et al. 1992; Encina et al. 2002; Manfield et al. 2004; García-Angulo et al. 2009; Mélida et al. 2009; Brochu et al. 2010; de Castro et al. 2014, 2015).

In previous studies, we have shown that the habituation of maize cells to DCB involves several metabolic modifications (Mélida et al. 2010a; de Castro et al. 2014, 2015). Maize cells habituated to high DCB levels (≥ 30 times higher than DCB I_{50} value) display strong reduction in cellulose and altered expression of several Cellulose Synthase genes (Mélida et al. 2009, 2010a). Although DCB induces oxidative damage (based on lipid peroxidation levels in maize cultured cells; unpublished results), given the level of detoxifying/antioxidant activities measured, it seems that DCB-habituated maize cells do not rely on an antioxidant strategy to cope with this herbicide, which contrasts with the strategy observed in cells of other species, such as bean, in which antioxidant capacity is enhanced when habituated to DCB (García-Angulo et al. 2009; Mélida et al. 2010a). Indeed, the ability of maize cells to grow under high DCB concentrations resides mainly in their capacity to reorganize their cell wall architecture. Through compositional analysis and structural characterization of DCB-habituated cell walls, it has been possible to demonstrate that these cells compensate for cellulose impoverishment with other cell wall components. The mechanism for this accommodation consists of producing a more extensive, cross-linked network of arabinoxylans (Mélida et al. 2009, 2010a, 2010b, 2011). More recently, we have found that some of the cell wall modifications differ according to DCB habituation level (de Castro et al. 2014).

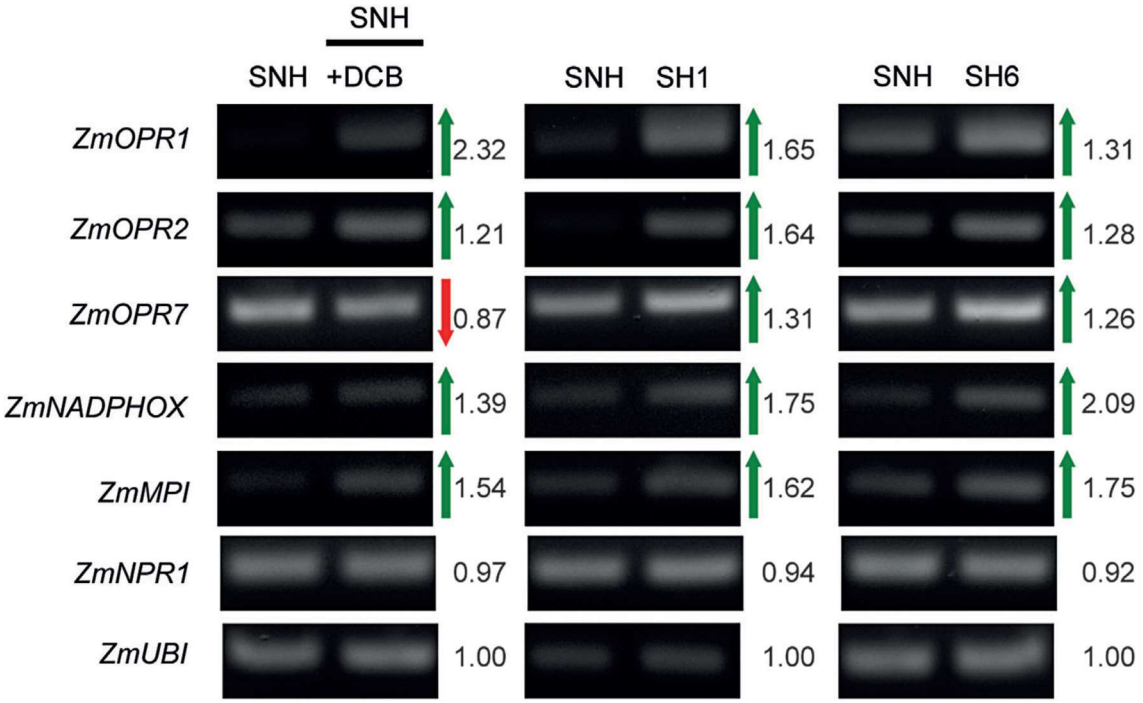

Figure I.8. Relative expression levels of jasmonic acid (JA) and salicylic acid (SA) signaling pathways genes analyzed by reverse transcription-polymerase chain reaction (RT-PCR) of non-habituated (SNH), SNH+DCB, SH1 and SH6 cell lines

For maize cell line annotations see Figure I.1 legend. The up and down arrows indicate less and more mRNA accumulation than SNH cells, respectively. 12-oxophytodienoate reductase (*ZmOPR1*, *ZmOPR2* and *ZmOPR7*), NADPH oxidase (*ZmNADPHOX*), maize protease inhibitor (*ZmMPI*), and non-expressor of pathogenesis related protein 1 (*ZmNPR1*). Pathogenesis related protein 1 (*ZmPR1*) was not detected. Primers can be found in Table I.S1. Numerals indicate the normalised ratios of RT-PCR band intensities calculated by dividing the band intensity of SNH+DCB, SH1 or SH6 by SNH for each gene.

In this study, we used maize cell suspension cultures habituated to low (1 µM DCB, SH1) and high (6 µM DCB, SH6) levels of DCB as well as non-habituated cells treated for a short time with lethal doses of the herbicide (SNH+6 mM DCB). In agreement with previous studies, we have shown that habituated cell lines display dose-dependent reductions in their cellulose content. These cellulose reductions (up to 70% less than in SNH) were compensated by a more extensive network of arabinoxylans, which could only be extracted with strong alkali.

In line with previous results obtained for maize callus cultures habituated to high DCB concentrations (Mélida et al. 2010b; 2011), we found that hydroxycinnamates, the arabinoxylan cross-linkers, experienced quantitative changes that indicated a prominent role of these compounds in a cellulose-deficient cell wall. This is actually one of the singularities of this model system. Most of the cell lines habituated to cellulose biosynthesis inhibitors (or other cell wall stresses) have had type I primary cell walls (i.e. *Arabidopsis*, poplar, bean, tomato), where cellulose reductions were compensated by pectins (Shedletzky et al. 1990; Encina et al. 2002; Manfield et al. 2004; Brochu et al. 2010). In contrast to type I, type II primary

cell walls are characterized by the presence of phenylpropanoids (mainly ferulic and p-coumaric acids), which have an important role in cross-linking hemicelluloses (Wallace and Fry 1994). Ferulate and its dimers increased steeply over the course of the DCB habituation process, but it was the changes in the proportions of esterified p-coumarate, which indicated that something else was happening. Indeed, in this case the changes observed for the short-term treatments were quite striking, as SNH+DCB cells were 132- and 15-fold enriched in p-coumarate when compared with SNH and SH6 cells, respectively. In the case of maize plants, small amounts of p-coumaric acid are esterified to arabinoxylans in primary walls, but later on in wall development, it is found more extensively esterified to lignin (Iiyama et al. 1994; Ralph et al. 1994a). Indeed, p-coumarate incorporation into the cell wall has been positively correlated with lignification (Hatfield and Marita 2010).

These findings suggest the presence of ectopic lignin or lignin-like polymers at least in the case of short-term treated cells, where the induced stress would explain their presence. Surprisingly, not only the short-term treated cells but also the DCB-habituated ones displayed a pink to brownish colour after phloroglucinol staining, indicative of lignin or lignin-like polymers (Pomar et al. 2002).

Our results clearly show the presence of ectopic lignin in maize primary cell walls of both DCB-habituated and short-term treated cells. Therefore, phenolics not only act as hemicellulose cross-linking units in this system, but also constitute monolignol-based polymers similar to lignin that might contribute to stiffening of a cellulose-impoverished wall. Although ectopic lignification has been observed in *Arabidopsis* mutants with reduced cellulose synthesis and in seedlings treated with cellulose biosynthesis inhibitors (Caño-Delgado et al. 2003; Bischoff et al. 2009; Denness et al. 2011), there are few reports of this phenomenon in exclusively primary-walled cell cultures (Ros Barceló 1997). Moreover, transcriptomic approaches using *Arabidopsis* and poplar cell cultures habituated to cellulose biosynthesis inhibitors have shown that several genes specifically involved in lignin synthesis are downregulated (Manfield et al. 2004; Brochu et al. 2010). Lignin-like polymers have been shown to be produced by other in vitro model systems under certain conditions (Kärkönen and Koutaniemi 2010). However, although these systems achieve lignin production in plant cultured cells, ectopic lignin deposition in primary cell wall, the feature of cell suspension cultures presented in this study, has rarely been reported (Christiernin et al. 2005; Novo-Uzal et al. 2009; Shen et al. 2013).

In addition to its roles in cell wall stiffening, lignin deposition has long been implicated as an important defense mechanism against pests and pathogens (Vance 1980; Barros-Rios et al. 2011). Lignin or lignin-like polymers are induced and rapidly deposited in cell walls in response to both biotic and abiotic stresses (Moura et al. 2010; Sattler and Funnell-Harris 2013; Miedes et al. 2014). Two types of lignin can be distinguished: (i) the one normally present in secondarily thickened cell walls with a purely structural role, and (ii) ectopic lignin, unexpectedly deposited in response to

biotic and abiotic stresses. Lignin composition is highly heterogeneous and phylogenetically dependent, but also depends on the role the lignin is expected to play. 'Defense' lignin is often associated with elevated levels of H subunits compared with structural lignin (Ride 1975; Lange et al. 1995; Sattler and Funnell-Harris 2013). Although H units were not present in our system (minor component in monocot lignin; Boerjan et al. 2003), given the phloroglucinol-tonality and compositional differences between SNH+DCB (pink/indicative of a predominance of S units) and the SH6 (brown/S+G units) lignin-like polymers, we propose that these polymers could arise from different stimuli. While short-term DCB-treated cells might produce a *sensu stricto* stress-related lignin, habituated cells might accumulate a structural-related lignin. In accordance with this, S to G ratio estimated for the lignin-like polymer found in DCB-habituated cells (1.45) is close to that of lignin from maize stems (1.4) (Fornalé et al. 2012).

By catalyzing the final hydroxyl-cinnamaldehyde reduction to the corresponding alcohols, CAD is a key enzyme in determining lignin content and composition (Mansell et al. 1974; Fornalé et al. 2012). Although several CAD isoforms (1, 5, 6 and 7) were overexpressed in SH1 cells, CAD activity was found unchanged. Therefore, it could be assumed for this cell line that higher proportions of the cinnamaldehyde moieties are incorporated into the phenolic polymers, as occurs in CAD-transgenic and mutant plants (Ralph et al. 2001; Dauwe et al. 2007; Fornalé et al. 2012). However, CAD activity was found to be approximately three to four times enhanced for SNH+DCB and SH6 cells, respectively, compared to SNH. The increased CAD activity in SNH+DCB cells correlated with the overexpression of several CAD isoforms (1, 5, 6 and 7), and since only S units were found in measurable amounts in their cell walls, these proteins are most probably involved in the sinapaldehyde conversion to sinapyl alcohol. All of these isoforms were also overexpressed in SH6 cells. As a differential result, habituated cells showed a high overexpression of *ZmCAD2*, which could be responsible for the synthesis of coniferyl alcohol from coniferaldehyde. In view of these results, we propose *ZmCAD2* as a candidate for the production of G units, at least in the case of maize cell cultures, as well as a key player in the production of lignin-like polymers in SH6 cells. Interestingly, *ZmCAD2* has been specifically associated with the synthesis of structural lignin in maize plants (Fornalé et al. 2012), which would agree with the synthesis of a structural related lignin in SH6 cells.

Concerning the two steps prior to CAD, different expression patterns were found in each case. CCR isoforms are responsible for the reduction of p-coumaroyl-CoA and feruloyl-CoA to their respective aldehydes. Downregulation of CCR in transgenic poplar has been associated with an up to 50% reduction in lignin content and an increased proportion of cellulose (Leplé et al. 2007). Interestingly, in contrast to these poplar trees, DCB-habituated cells with the opposite situation for the load-bearing polymers (less cellulose and more lignin) showed a significant overexpression of both CCR isoforms. On the other hand, and also in poplar, upregulation of F5H increased the proportion of S units, yielding an S/G ratio of

greater than 35 versus approximately 2 for wild type poplar lignin (Stewart et al. 2009). Both F5H isoforms were overexpressed in SH1 cells, where only S units could be detected, while one of them was highly downregulated for SH6 cells. In summary, rather than a general stress response, a tight regulation of the monolignol biosynthetic pathway was observed in DCB-habituated cells.

Lignin polymerization is preceded by the peroxidase+H_2O_2 (and/or lacasse+O_2) dependent activation of monolignols to free radicals (Fagerstedt et al. 2010). The spent cell culture medium can be regarded as an extension of the apoplast and it can therefore be used as a compartment to monitor changes in the level of cell wall H_2O_2 (Kärkönen and Kuchitsu 2014). The H_2O_2 over-production of SNH+DCB and SH cells may be explained in the context of a reactive oxygen species over-production following cellulose inhibition, as has been previously reported for *Arabidopsis* plants (Dennes et al. 2011) and maize cultured cells habituated to low DCB concentrations (A Largo unpublished data). Given the steep increase in lignin over the course of DCB habituation, a relationship between lignin accumulation and increased apoplastic H_2O_2 contents may be expected (Nose et al. 1995; Kärkönen et al. 2002). However, no differences in apoplastic H_2O_2 were found when SH1 and SH6 cells were compared, indicating that H_2O_2 is not a limiting factor in the ectopic lignification reported in this system. An alternative explanation would be that the lignification is consuming apoplastic H_2O_2 explaining the lower level of apoplastic H_2O_2 measured in SH6 cells when compared with SH1 or SNH+DCB ones. Moreover, a study of class III peroxidase activity did not show differences due to DCB habituation in maize cultured cells (data not shown).

There are several lines of evidence that link ectopic lignification in response to cellulose deficiency with JA signaling. *Constitutive expression of vegetative storage protein 1* (*cev1*) and *ectopic lignin 1* (*eli1-1*) *Arabidopsis* mutants, are defective in the cellulose synthase gene CESA3 involved in cellulose biosynthesis during primary cell wall formation (Ellis and Turner 2001; Ellis et al. 2002; Caño-Delgado et al. 2003). In these mutants, cellulose biosynthesis impairment was compensated by mechanisms such as ectopic lignification, constitutive activation of the JA signaling pathway, and increases in JA and ethylene proportions. In addition, treatments with the cellulose biosynthesis inhibitor isoxaben have been found to phenocopy *eli1-1* lignification in *Arabidopsis* wild type seedlings (Caño-Delgado et al. 2003; Hamann et al. 2009). In JA-insensitive plants, ectopic lignification by isoxaben is reduced, indicating that JA signaling is necessary (Caño-Delgado et al. 2003), a deduction which is further confirmed by the finding that external addition of methyl jasmonate to *Arabidopsis* cell cultures led to increased expression of phenylpropanoid, particularly monolignol biosynthesis (Pauwels et al. 2008). Our results confirm a JA-dependent signaling process in response to cellulose biosynthesis impairment, which led to ectopic lignification. However, according to our RT-PCR results and previous data from proteomic approaches (Melida et al. 2010a; M de Castro unpublished data), stimulation of the lignification mechanism seems to be SA- and ethylene-independent.

In summary, maize suspension-cultured cells with up to 70% less cellulose produced a more extensive and cross-linked network of arabinoxylans together with a polymeric lignin-like material. This modified cell wall architecture is the result of the high structural plasticity of plant primary cell walls in response to a disruption of cell wall integrity. We propose that a JA signaling program might be triggering the observed ectopic lignification, and this model system will be used in future research in order to study the complex networks involved in cell wall integrity maintenance mechanisms.

MATERIALS AND METHODS

Plant material and DCB habituation process

Maize callus-cultured cells (Zea mays L. Black Mexican sweetcorn) were obtained from immature embryos and maintained in Murashige and Skoog media (Murashige and Skoog 1962) supplemented with 9 mM 2,4-D, 20 g L^{-1} sucrose and 8% (p/v) agar at 25°C under photoperiodic conditions (16:8; 3,000 lux ≈ 41µmol m^{-2} s^{-1}). Callus-cultured cells were habituated to grow under originally lethal DCB concentrations, by stepwise transfers to higher DCB levels up to a 12 µM concentration (Mélida et al. 2009). Those cells growing on solid medium were disaggregated and transferred to a liquid medium containing 6 µM DCB (SH6) (Mélida et al. 2011). SH6 cells were maintained at 25°C under light, rotary shaken and routinely subcultured every 15 days. Control cells were designated as non-habituated maize suspension cultured cells (SNH). Cell lines habituated to grow under 1µM DCB (SH1) were obtained from SNH (de Castro et al. 2014). In order to distinguish toxic DCB effects from those owing to the habituation, short-term treatments with high (lethal) DCB concentrations were performed. Maize control cells were grown in a liquid medium containing 6µM DCB for 6 days, ensuring a toxic effect but not giving sufficient time to kill the cells (H Mélida unpublished data). These cells were referred to as SNH+DCB.

Cell wall preparation and fractionation

Cell walls were prepared according to Mélida et al. (2009). Briefly, cells were collected during their exponential growth phase, washed extensively with distilled water and immediately frozen. The cells were disrupted in liquid nitrogen using a mortar and pestle. The resulting fine powders were subjected to extraction in 70% (v/v) ethanol for 5 days. The suspensions were filtered through glass-fiber filters (GF/A, Whatman, GE Healthcare, Buckinghamshire, UK), and the pellets were washed six times with 70% ethanol and six times with acetone and were subsequently air dried, to obtain the alcohol insoluble residue. These were then resuspended in 90% dimethylsulphoxide for 8h three times, filtered as above, washed twice with 0.01M phosphate buffer pH 7.0 and incubated with 2.5 U mL^{-1} of

α-amylase type VI-A dissolved in the same buffer for 24 h at 37° C. The suspensions were filtered again and washed with ethanol and acetone as indicated above. The dry pellets were treated with phenol:acetic acid: water (2:1:1, v/v/v) for two periods of 8 h, then washed and air dried. The final dry pellets were considered the cell wall extracts. Cell wall fractions were obtained by consecutively treating the cell wall residues with KOH solutions according to Mélida et al. (2009). Cell walls were extracted at room temperature with 50 mM trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid (CDTA) at pH 6.5 for 8 h and washed with distilled water. The residue was then incubated with 0.1 M KOH + 20 mM $NaBH_4$ for 2 h (x2) and washed with distilled water. Then 4 M KOH + 20 mM $NaBH_4$ was added to the residue for 4 h (x2), and washed again with distilled water. The extracts were acidified to pH 5.0 with acetic acid, dialyzed and freeze-dried, representing CDTA, KI and KII fractions, respectively. The residue after 4M KOH extraction was hydrolyzed with 2M trifluoroacetic acid (TFA) for 2.5h at 120°C, and after centrifugation, the supernatant was lyophilized and referred to as the TFA fraction.

Cell wall analysis

Tablets for Fourier transform infrared (FTIR) spectroscopy were prepared in a Graseby Specac press from small samples (2mg) of cell walls mixed with KBr (1:100, w/w). Spectra were obtained on a Perkin Elmer Spectrum 2000 instrument at a resolution of 1 cm1. A window between 800 and 1,800 cm1, which contains information of characteristic polysaccharides, was selected in order to monitor cell wall structure modifications. All spectra were normalized and baseline corrected with Spectrum software (v5.3.1). Then, data were exported to Microsoft Excel 2010 and all spectra were area normalized. Cellulose was quantified in crude cell walls by the Updegraff method as described by Encina et al. (2002). Total sugar quantification of cell wall fractions was performed by the phenol-sulphuric acid method (Dubois et al. 1956) and results were expressed as glucose equivalents. The uronic acid sugars were quantified by the *m*-hydroxydiphenyl method described by Blumenkrantz and Asboe-Hansen (1973) using galacturonic acid as reference standard. For the analysis of neutral sugars, freeze-dried cell wall fractions were hydrolyzed with 2 M TFA at 121°C for 1h. *Myo*-inositol was used as an internal standard. The resulting monosaccharides were converted to alditol acetates as described previously (Albersheim et al. 1967) and analyzed by gas chromatography (GC) on a SP-2380 capillary column (30m x 0.25 mm I.d.; Supelco) using a Perkin Elmer Autosystem. Ferulate and *p*-coumarate monomers and ester-bound diferulates were extracted at room temperature from 50 mg of the alcohol-insoluble residues (AIR) using 2M NaOH for 4h and analyzed by high performance liquid chromatography (HPLC) based on a method previously described by Santiago et al. (2006). Retention time and UV spectrum of 5,5'-DFA were compared with freshly prepared external standard solutions of 5,5'-DFA, kindly provided by Dr. John Ralph's group (Department of Biochemistry, University of Wisconsin, Madison, USA). The UV absorption spectra of other DFAs were compared with previously published

spectra (Waldron et al. 1996) and absorbance at 325 nm was used for quantification. Total ester-linked-DFAs concentration was calculated as the sum of three isomers of DFA identified and quantified by this analytical procedure: 8,5'-DFA, 8-O-4'-DFA, and 5,5'-DFA. The 8,5'-DFA concentration was calculated as the sum of the 8,5'-non-cyclic (or open)-DFA and 8,5'-cyclic (or benzofuran)-DFA because the non-cyclic form is most likely formed during alkaline hydrolysis from the native cyclic form (Ralph et al. 1994b). Lignin-like material was quantified by the Klason gravimetric method with minor modifications. Cell wall extracts were hydrolyzed with 72% (w/v) sulfuric acid for 1h at 30°C. Then, the sulfuric acid concentration was diluted to 2.5% (w/v) with water and further incubated at 115° C for 1h. The residues were filtrated through Durapore polyvinylidene fluoride (PVDF) filters (Millipore, GE Healthcare, Buckinghamshire, UK, 0.45 mm), dried and weighed. Thioacidolysis of cell walls, which solubilizes the β-O-4 lignin core, and GC-MS analyses were performed (Novo-Uzal et al. 2009) using a Thermo Finnigan (MA, Waltham, USA) Trace GC gas chromatograph, a Thermo Finnigan Polaris Q mass spectrometer, and a DB-XLB, J&W (60 m x 0.25 mm I.d.) column.

Histochemical staining of cinnamyl-aldehydes Intact filtered maize cells were incubated with 1% (w/v) phloroglucinol in 70% (v/v) ethanol for 5min. Then, the phloroglucinol solution was removed and the cells were further incubated with an 18% (w/v) HCl solution. Stained cells were observed under Nikon SMZ1500 magnifier and photographed using a Nikon Digital Camera DXM1200F.

Relative gene expression analysis

DCB-habituated cells (SH1 and SH6) were collected during their respective exponential growth phases and a set of SNH cells were collected at the same time for comparison. In the case of short-term DCB treatments, SNH+DCB cells and a set of SNH cells were collected on the third day of culture. Total RNA was extracted from homogenized cells of all lines following the procedures established for Trizol reagent (Invitrogen, California, USA). The purity and integrity of the extracted RNA was evaluated spectrophotometrically using a Nanodrop 1000 and running the RNA in 1% agarose gels. RNA (2 mg) was reverse-transcribed with Super Script III First strand retro-transcriptase (Invitrogen) using oligo$(dT)_{20}$ as primer. The synthesized cDNA was used to perform the gene expression analyses by standard and quantitative PCR methods. Semiquantitative expression analysis by RT-PCR was performed for jasmonic (JA) and salicylic acid (SA) signaling pathway genes: 12-oxophytodienoatereductase (*ZmOPR1*, AY921638; *ZmOPR2*, AY921639 and *ZmOPR7*, AY921644), NADPH oxidase (*ZmNADPHOX*, CK849936), maize protease inhibitor (*ZmMPI*, X78988), pathogenesis related protein 1 (*ZmPR1*, UB2200) and non-expressor of PR1 (*ZmNPR1*, EU95584). Primers can be found in Table I.S1. The ubiquitin gene was used as a reference gene for this experiment (*ZmUBI*, U29159) (Fornalé et al. 2006). Reverse transcription-PCR agarose gels were stained with SYBR Safe DNA gel stain (Invitrogen) and gel images acquired with an Alphaimager HP

system (Protein Simple 3001, San Jose, California, USA). The quantification of the bands was performed by using the Alpha view v3.4.0.0. software (ProteinSimple). Band intensity was expressed as relative intensity units. For each individual gene, the band intensity was normalized in relation to ubiquitin and then, the normalized intensity ratios for SNH+DCB/SNH; SH1/SNH and SH6/SNH were calculated. Relative gene expression was determined by qRT-PCR using specific primers for the following genes: ferulate5-hydroxylase (*ZmF5H1* (AC210173.4) and *ZmF5H2* (GRMZM2G100158)), cinnamoyl-CoA reductase (*ZmCCR1* (GRMZM2G131205) and *ZmCCR2* (GRMZM2G131836)) and cinnamyl alcohol dehydrogenase (*ZmCAD1* (Y13733; GRMZM5G844562), *ZmCAD2* (GRMZM2G118610), *ZmCAD3* (GRMZM2G046070), *ZmCAD4* (GRMZM2G700188), *ZmCAD5* (GRMZM2G443445), *ZmCAD6* (GRMZM2G090980) and *ZmCAD7* (GRMZM167613)) as described by Guillaumie et al. (2007). Folylpolyglutamate synthase (*ZmFPGS*; GRMZM2G393334) and the Ubiquitin carrier protein (*ZmUBCP*, GRMZM2G102471) genes were used as reference genes (Manoli et al. 2012). Primers can be found in Supplemental Table I.S1. The qPCR was carried out in a StepOnePlus platform (Applied Biosystems, California, USA) using Power SYBR green PCR master mix (Applied Biosystems), 2 mL of each cDNA concentration (50 and 100 ng mL^{-1}) and a mix of both primers at 10 µM. All samples were run in triplicate with the following temperature profile: initial denaturation at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C for 1 min (annealing and elongation). The relative gene expression was calculated by 'δ–δ' method (Livak and Schmittgen 2001) implemented in the StepOne™ Software v2.2.2. A no-template negative control and a melting curve were performed in each sample set to control the primer dimers and contaminants in the reactions.

CAD enzyme activity assay

CAD enzyme activity was measured by following the method described by Chabannes et al. (2001) modified by Fornalé et al. (2012). Fresh cells were homogenized under liquid nitrogen with a mortar and pestle until a fine powder was obtained, and 5 Ml of extraction buffer (100 mM Tris-HCl pH 7.5, 2% (w/v) PEG 6000, 5 mM DTT and 2% (w/v) PPVP) were added. The suspension was centrifuged at 10,000g for 10 min at 4°C and the supernatant was collected. The centrifugation process was repeated until the supernatant was clear. CAD activity assays were carried out by measuring the absorbance increment at 400 nm when coniferyl alcohol was oxidized to coniferyl aldehyde. The reactions were performed in 96-well plates containing 140 mL of 140 mM Tris-HCl pH 8.8, 20 mL of 1 mM coniferyl alcohol, 20 mL of 200 mM $NADP^+$ and 20 mL of sample. The mixtures were mixed and incubated at 30°C for 10min, and the reactions were measured over the following 10min in a plate reader Synergy HT (Bio-Tech, Winooski, VT, USA) at 30° C. Reaction and sample blanks were routinely used.

Apoplastic H_2O_2 content determination

Apoplastic H_2O_2 content was determined with the xylenol orange method as described by Bindschedler et al. (2001). For the reactions, 150 mL of culture media was mixed with 1 mL of reaction mixture (125 mM xylenol orange, 100 mM D-sorbitol, 25 mM FeSO4, 25 mM $(NH_4)_2SO_4$ and 25 mM H_2SO_4), and absorbance (560 nm) was measured after 40 min of incubation.

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SUPPORTING INFORMATION

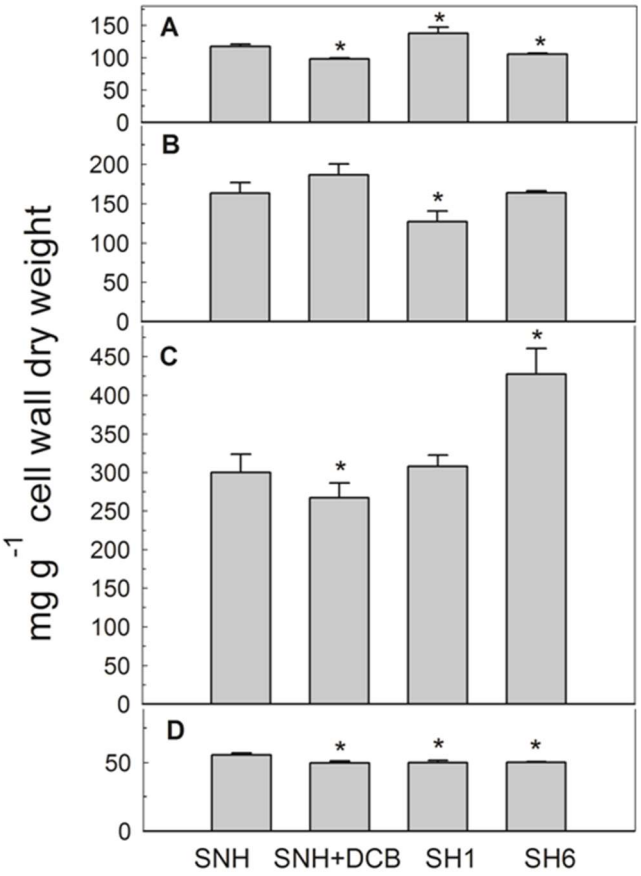

Figure I.S1. Cell wall fractionation Total sugars in (A) CDTA, (B) KI, (C) KII and (D) TFA cell wall fraction obtained from SNH, SNH+DCB, SH1 and SH6 cell lines. For maize cell line annotation see Figure I.1 legend. Data represents the means values ± s.d. of 3 technical replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P<0.05$).

Table I.S1. Primers used in RT-PCR and RT-qPCR experiments

Gene	Primer forward	Primer reverse	Product size
ZmCAD1	CGACTCGCTGGACTACATCA	CTCGTTCACGTACCCCATCT	271
ZmCAD2	CAAGAACGTGACCAGCTTCA	GTGGTACTTCATGGGGCTGT	289
ZmCAD3	GAAGCTATGGAGACGCTTGG	GCAGAGGCCGTGTTCATAAT	107
ZmCAD4	GTCCCCGTTCCATTTCATTA	TGATCGTGACATCGTCGTCT	259
ZmCAD5	CTTAAGGTTGGCGGTGTGAT	GTTAACAAGCCTGGCGAGAG	222
ZmCAD6	TGGCATTGATACAGATGGAACT	TGCTTTACCAAATTTCACAGC	198
ZmCAD7	CCTGCACTTCATCAACAACG	CGGTCGCAGTAGTTCTCCTC	198
ZmCCR1	GGAAGCAGCCGTACAAGTTC	GTCCCTTCTCCTGGAGGTTC	111
ZmCCR2	ATCCAAAGAACGACCACCTG	CTGGCTCGATCATCATCTCA	171
ZmF5H1	GACCGGTTCATCGACAAGAT	CTTGATGACGCACTTGAGGA	387
ZmF5H2	GGAGTTCTCCAAGCTGTTCG	TGCTCGTCGATGATCTTGTC	144
ZmUBCP	CAGGTGGGGTATTCTTGGTG	ATGTTCGGGTGGAAAACCTT	231
ZmFPGS	ATCTCGTTGGGGATGTCTTG	AGCACCGTTCAAATGTCTCC	132
ZmOPR1	ATAGCGGCTTGCTCTACTGC	CATCCAGCTCGAACCTCCTA	244
ZmOPR2	ACCGCTCCATCTTCTACACGCAAG	CCACAATGGCCAATAATGAATG	161
ZmOPR6	AGCAGGCTTTGATGGAGTGGA	TTGGCAAAACGCATCGGAAGG	516
ZmOPR7	CGGCTGTTCATCGCTAATCCCGA	CAATCGCGGCATTACCCAGATGT	248
ZmNADPHOX	ACCAGCGCGTCGGAGTGTTC	TGGCATTTCGATCATTAGTTCTTC	310
ZmMPI	ATGAGCTCCACGGAGTGC	TCAGCCGATGTGGGGCGTC	222
ZmPR1	AGAACCTCTTCTGGGGCAGT	TGGGACAGCAAGAGACACAG	319
ZmNPR1	TCCCTCCAGAAGCAGTTGAT	GCTGTGTACCCACGGCTATT	295

CAPÍTULO II:

Early habituation of maize (*Zea mays*) suspension-cultured cells to 2,6-diclorobenzonitrile is associated with the enhancement of antioxidant status

Largo-Gosens A, Encina A, de Castro M, Mélida H, Acebes JL, García-Angulo P, Álvarez JM. (2016). Early habituation of maize (Zea mays) suspension-cultured cells to 2,6-dichlorobenzonitrile is associated with the enhancement of antioxidant status. Physiologia Plantarum. 157(2): 193-204. Doi: 10.1111/ppl.12411.

Early habituation of maize (*Zea mays*) suspension-cultured cells to 2,6-diclorobenzonitrile is associated with the enhancement of antioxidant status

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Abstract

The cellulose biosynthesis inhibitor 2,6-dichlorobenzonitrile (DCB) has been widely used to gain insights into cell wall composition and architecture. Studies of changes during early habituation to DCB can provide information on mechanisms that allow tolerance/habituation to DCB. In this context, maize-cultured cells with a reduced amount of cellulose (~20%) were obtained by stepwise habituation to low DCB concentrations. The results reported here attempt to elucidate the putative role of an antioxidant strategy during incipient habituation. The short-term exposure to DCB of non-habituated maize-cultured cells induced a substantial increase in oxidative damage. Concomitantly, short-term treated cells presented an increase in class III peroxidase and glutathione S-transferase activities and total glutathione content. Maize cells habituated to 0.3-1 μM DCB (incipient habituation) were characterized by a reduction in the relative cell growth rate, an enhancement of ascorbate peroxidase and class III peroxidase activities, and a net increment in total glutathione content. Moreover, these cell lines showed increased levels of glutathione S-transferase activity. Changes in antioxidant/conjugation status enabled 0.3 and 0.5 μM DCB-habituated cells to control lipid peroxidation levels, but this was not the case of maize cells habituated to 1 μM DCB, which despite showing an increased antioxidant capacity were not capable of reducing the oxidative damage to control levels. The results reported here confirm that exposure and incipient habituation of maize cells to DCB are associated with an enhancement in antioxidant/conjugation activities which could play a role in incipient DCB habituation of maize-cultured cells.

Abbreviations - AA, ascorbate; APOX, ascorbate peroxidase; CAT, catalase; CBI, cellulose biosynthesis inhibitor; CIII-POX, class III peroxidase; DCB, 2,6-dichlorobenzonitrile; DHA, dehydroascorbate; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; FW, fresh weight; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione S-transferase; MDA, malondialdehyde; POX, peroxidase; RGR, relative growth rate; ROS, reactive oxygen species; SHx, suspension-cultured cells habituated to 'x' μM DCB; SNH, non-habituated suspension-cultured cells; SNH+DCB(x), non-habituated suspension-cultured cells treated with 'x' μM DCB; TA, total ascorbate; TBARS, thiobarbituric acid-reactive substances; TG, total glutathione

INTRODUCTION

The compound 2,6-dichlorobenzonitrile (DCB) is a broad-spectrum herbicide used to control a wide range of weed species. Although the mechanism of action of DCB still remains unclear, it has been extensively reported to specifically inhibit the incorporation of [^{14}C]Glc into cellulose in a wide range of systems (Hogetsu et al. 1974, Montezinos and Delmer 1980, Hoson and Masuda 1991, Edelmann and Fry 1992, Shedletzky et al. 1992, García-Angulo et al. 2009) supporting its classification as a cellulose biosynthesis inhibitor (CBI) (Acebes et al. 2010).

One possible strategy to study the effect of CBIs on the composition and/or architecture of plant cell walls is to habituate cell cultures to grow in the presence of high concentrations of these herbicides (long-term habituation to DCB). Several studies have been reported in recent years in which plant-cultured cells with both type I and type II primary walls (typical of dicots and commelinoid monocots, respectively) have been habituated to DCB in this way (Acebes et al. 2010 and references therein, de Castro et al. 2015, Mélida et al. 2015). The cell wall modification depended on the type of primary cell wall (types I or II), the concentration of DCB in the culture medium and the number of subcultures in a given concentration of DCB. As a result of the habituation process, cells develop the capacity to grow and divide with a modified cell wall in which the cellulosic scaffold is replaced by a network of highly cross-linked matrix polysaccharides that differ depending on the cell wall type: pectins in the case of type I cell walls (Shedletzky et al. 1992, Encina et al. 2001, 2002, Alonso-Simón et al. 2004, 2010, García-Angulo et al. 2006, 2009) and feruloylated-arabinoxylans in the case of type II cell walls (Mélida et al. 2009, 2010a, 2010b, 2011, 2015, De Castro et al. 2014, 2015).

However, there is still little information about the cell wall modification associated with a low level of habituation to DCB (incipient or short-term habituation to DCB). To date, only four studies have analyzed the initial stages of DCB habituation in bean (*Phaseolus vulgaris*) cells (type I cell wall) (Alonso-Simón et al. 2004, García-Angulo et al. 2006) and in maize cells (type II cell wall) (de Castro et al. 2014, 2015). Compared with long-term DCB-habituated cells, the reduction in cellulose content was lower (~20% reduction with respect to non-habituated cells), returning to control levels as the time of culture was increased (de Castro et al. 2014). Interestingly, the addition of 0.3–0.5 μM DCB to non-habituated maize cells during one culture cycle (short-term treated cells) induced a ~25% reduction in cellulose content that returned to control levels as the number of subcultures in the presence of the same concentration of herbicide was increased. This reactive behavior was not observed when higher DCB (i.e. 1 μM) concentrations were used (de Castro et al. 2014). These results demonstrate the tight regulatory process governing cell wall metabolism and architecture, but these initial steps have usually been overlooked.

Environmental stresses such as salinity (Hu et al. 2012), temperature (Badiani et al. 1997), nutritional deficiencies (Kováčik et al. 2013), heavy metals (Paradiso et al. 2008, Vuletić et al. 2014), organochlorines (Michalowicz et al. 2009, San Miguel et al. 2012) and herbicides (Geoffroy et al. 2004, Peixoto et al. 2008, García-Angulo et al. 2009, Wu et al. 2010, Karuppanapandian et al. 2011) lead to disruption of the balance between reactive oxygen species (ROS) production and scavenging, which in turn leads to oxidative stress (Apel and Hirt 2004, Gill and Tuteja 2010). Consequently, resistance to oxidative stress is often achieved by developing an antioxidant capacity that comprises enzymatic activities such as class III peroxidase (CIII-POX), ascorbate peroxidase (APOX), catalase (CAT) and glutathione reductase (GR), and antioxidant molecules such as reduced glutathione (GSH), ascorbate (AA) and polyphenols, which scavenge ROS and therefore reduce oxidative damage (Passardi et al. 2005, Ahmad et al. 2008, Gill and Tuteja 2010). In the case of stresses induced by exposure to herbicides, one of the defense responses is catabolism of the molecule in order to reduce the toxic effect. Among other enzymes, glutathione S-transferases (GSTs) detoxify herbicides such as DCB by conjugating the drug with the tripeptide glutathione (GSH), and are considered plant stress molecular markers (Edwards et al. 2000, Gill and Tuteja 2010, Cummins et al. 2011).

The treatment of plant cells with CBIs such as isoxaben or DCB has been related to an increase in antioxidant activities in plant cells (García-Angulo et al. 2009, Mélida et al. 2010a, Denness et al. 2011). In addition, DCB habituation of bean suspension-cultured cells is associated with a stable increment of CIII-POX, which has been hypothesized to increase the antioxidant capacity of cells and possibly contribute to cell wall restructuring (García-Angulo et al. 2009). Interestingly, in the case of maize callus-cultured cells, long-term DCB habituation was not associated with an enhancement of antioxidant activities. In the same study, a severe decrease in GST-conjugation levels was reported for such cell lines, both by activity measurements and by proteomics (Mélida et al. 2010a). Taking these results together, an antioxidant/conjugation strategy may seem unlikely in long-term DCB habituation (Mélida et al. 2010a). However, a clue for the involvement of antioxidants in the early stages of DCB habituation was provided by results indicating that following a short-term treatment with a high concentration of DCB, maize callus-cultured cells presented an increase in CIII-POX, CAT, GR and GST antioxidant activities (Mélida et al. 2010a).

Given the fact that the exposure of maize cells to DCB induces an enhancement of antioxidant and conjugation activities, and that the DCB-dependent decrease in cellulose reverts during the initial stages of habituation, the aim of this study was to gain an insight into the putative role of the antioxidant/conjugation machinery during the initial stages of DCB habituation in maize-cultured cells. To this end, we measured lipid peroxidation and H_2O_2 levels as an indication of oxidative status, followed by the assay of antioxidant (CIII-POX, APOX, GR, CAT) and conjugation (GST) activities as well as GSH and AA contents in maize suspension-cultured cells habituated to 0.3, 0.5 and 1 μM DCB after 11 culture

cycles. In addition, in order to investigate differences in antioxidant/conjugation strategies between DCB habituation and acute DCB effects, the same parameters were assayed in maize suspension-cultured cells after a short-term exposure to 0.5 or 1 μM DCB.

MATERIAL AND METHODS

Plant cell cultures

Maize suspension-cultured cells (Zea mays, Black Mexican sweetcorn) were obtained from rotary shaken (120 rpm) maize callus cultures obtained as described by Lorences and Fry (1991).

Maize suspension-cultured cells (Z. mays, Black Mexican sweetcorn) were routinely grown in Murashige and Skoog (MS) media (Murashige and Skoog 1962) supplemented with 20 g l^{-1} sucrose and 9 μm 2,4-dichlorophenoxiacetic acid at 25°C under photoperiodic conditions (16:8; 3000 lux≈41 μmol m^{-2} s^{-1}), and were rotary shaken (120 rpm) and subcultured fortnightly (Mélida et al. 2011).

Short-term exposure and habituation of maize cells to DCB

In order to determine the effect of short-term DCB treatment, non-habituated suspension-cultured cells (SNH) were transferred to media supplemented with 0.5 or 1 μM DCB for 6 days. These cells were denoted as SNH+DCB (x) where 'x' indicates the DCB concentration (μM) added to the culture media.

Cell cultures habituated to 0.3, 0.5 and 1 μM DCB were obtained from SNH cells after stepwise transfers with gradual increments of DCB in the culture media (de Castro et al. 2014). DCB was dissolved in dimethylsulphoxide, which does not affect maize cell growth at this range of concentrations (0.003–0.01% v/v). For this purpose SNH were treated with 0.3, 0.5 (I_{50}, de Castro et al. 2014) and 1 μM DCB and subcultured in the presence of the herbicide for 10 subcultures (de Castro et al. 2014). Habituated cells were denoted as SHx, where 'x' indicates the DCB concentration (μM) added to the culture media.

Cell growth and viability measurements

Growth curves of all cell lines were obtained at the sixth subculture by measuring the dry weight (DW) gain at different time points in the culture cycle. Relative growth rates (RGRs) were calculated from the slopes of the straight part of the curves after plotting ln DW against time. The doubling time, which is the time that the cell culture takes to double the DW, was calculated as: doubling time=ln 2/RGR.

Viability was measured using the fluorescein diacetate method as described by Duncan and Widholm (1990). Fifty microliter of 0.2% (w/v) fluorescein diacetate (Sigma-Aldrich Quimica SL, Madrid, Spain) stock solution in acetone was diluted with 5 ml of culture medium, and the resulting working solution mixed 1:1 (v/v) with cell suspension on a microscope slide. A Nikon epifluorescence microscope equipped with a Nikon UV-2A filter (330–380 nm excitation, 400 nm dichroic mirror and 435 nm barrier filter) was used for observation of bright green fluorescence emission by viable cells.

Lipid peroxidation levels and enzyme activity assays

Cells from all lines were collected during the exponential growth phase and stored at −80∘C until use. One to five grams fresh weight (FW) of these cells was homogenized under liquid nitrogen with a mortar and a pestle until obtaining a powder.

To quantify CAT (EC. 1.11.1.6), GR (EC 1.8.1.7) and GST (EC 2.5.1.18) enzyme activity and lipid peroxidation, the powdered cells were extracted (1 g FW in 5 ml extraction buffer) using extraction buffer 1: 0.05 M Tris–HCl pH7.5, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) Triton X-100, 10% (v/v) glycerol and 2 mM dithiothreitol, and centrifuged at 15 000 g for 2 min at 4∘C before quantifying the supernatants.

Lipid peroxidation levels were determined by quantification of thiobarbituric acid-reactive substances (TBARS) using malondialdehyde (MDA) as the reference molecule (Buege and Aust 1978). One milliliter of reaction buffer, 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid and 0.01% (v/v) butylated hydroxytoluene in 0.25 M HCl) was mixed with 20 µl of sample and incubated at 100∘C for 15 min. The samples were cooled, centrifuged at 2500 g for 15min and A_{535} was measured in the supernatants. CAT activity was measured as the reduction in A_{240} induced by the catalysis of H_2O_2 for 2 min (Droillard et al. 1987). The activity assay was performed by mixing 3 ml of 50 mM phosphate buffer pH 7.0 with 37.5 mM H_2O_2 and 0.1 ml of sample supernatant. CAT activity was calculated using the molar extinction coefficient for H_2O_2 at 240 nm: ε=39.58 $M^{-1}cm^{-1}$. Quantification of GR activity was performed in accordance with the method described by Edwards et al. (1990), which is based on the reduction in A_{340} due to the oxidation of NADPH for the conversion of glutathione disulfide (GSSG) to its reduced form (GSH). Activity was measured by mixing 0.1ml of sample supernatant with 1.35 ml of reaction buffer (100 mM Tris–HCl pH 8.0, 0.1 mM EDTA, 1 mM GSSG and 3 mM MgCl2) and 0.05 ml of 10 mM NADPH, and calculated using the molar extinction coefficient for NADPH at 340 nm: ε=6.22 mM^{-1} cm^{-1}. GST enzyme activity was determined following the method described by Habig et al. (1974), which is based on an increase in A_{340} due to the formation of a complex between a reduced GSH and the compound chloro-2,4-dinitrobenzene. The reaction was performed by mixing 0.93 ml of 0.1 M potassium phosphate buffer pH 7.5 with 0.02 ml of 0.001 M chloro-2,4-nitrobenzene

and 0.05 ml of sample supernatant, and was measured for 2 min at 30°C. GST activity was calculated using the molar extinction coefficient of the GSH-chloro-2,4-dinitrobenzene complex (ε=9.6 mM^{-1} cm^{-1}).

For peroxidases (CIII-POX; EC 1.11.1.7 and APOX; EC 1.11.1.11), the powdered cells were resuspended in extraction buffer 2 (1 g FW in 5 ml extraction buffer): 0.04 M Tris–HCl pH 7.2, 1 mM EDTA-2Na-2H and 5% (v/v) glycerol, then centrifuged at 15 000 g for 2 min at 4°C before measuring activities in the supernatants. Quantification of CIII-POX activity was performed as described by Adam et al. (1995), based on the increase in A_{470} due to guaiacol oxidation. The reaction was performed with 3 l of reaction buffer (100 mM sodium acetate pH 5.5 and 1 mM guaiacol), 0.3 ml of 1.3 mM H_2O_2 and 0.05 ml of sample supernatant at 25°C for 2 min. Activity was calculated using the molar extinction coefficient for guaiacol at 470 nm: ε=26.6 mM^{-1} cm^{-1}. APOX activity was measured following the method described by Hossain and Asada (1984), in which a reduction in A_{290} due to AA oxidation occurs. Sample supernatants (0.01 ml) were mixed with 0.98 ml of 50 mM HEPES-NaOH buffer pH 7.6 and 20 mM ascorbic acid. The reaction was started by the addition of 0.01 ml of 1.3 mM H_2O_2. Activity was calculated using the molar extinction coefficient for AA at 290 nm: ε=2.8 mM^{-1} cm^{-1}. Protein content was determined by the Bradford method (Bradford 1976).

Total GSH and GSSG measurement

For GSH and GSSG extraction, cells were homogenized under liquid nitrogen with a mortar and pestle. Then, powdered cells (1 g FW) were extracted with 5 ml of 5% (w/v) metaphosphoric acid. Assays were conducted rapidly to avoid oxidation of GSH to GSSG. Total glutathione content (TG=GSH+GSSG) was measured using the DTNB recycling method described by Griffith (1980). GSSG determination was performed using the same method but with a previous treatment with acrylonitrile, a thiol-blocking reagent, following the indications of Matsumoto et al. (1996). GSH content was calculated as the difference between TG and GSSG values.

Total ascorbate and dehydroascorbate measurement

For total AA (TA=AA+DHA) and dehydroascorbate (DHA) extraction, cells in the exponential growth phase were homogenized under liquid nitrogen with a mortar and pestle. Powdered cells (1 g FW) were extracted with 5 ml of 5% (w/v) metaphosphoric acid and kept on ice for 20 min. Extracts were clarified by centrifugation at 19 000 g for 5 min at 4°C and samples from the supernatant were collected for measurements.

AA and DHA were measured spectrophotometrically following the method described by Takahama and Oniki (1992) and modified by Kärkönen and Fry (2006). Extracts (30 µl) were mixed with 1 ml of reaction mix (38 mM Na^+-succinate in 90 mM NaH_2PO_4, pH 6.8) and A_{265} was measured. Furthermore, 2U of AA oxidase from

Cucurbita sp. (Sigma) was added to oxidize AA to DHA and A_{265} was re-measured after 1 min. AA was measured by calculating the reduction in A_{265} upon addition of AA oxidase. In an independent sample, extracts (30 µl) were added to 1 ml of reaction mix (38 mM Na^+-succinate in 90 mM NaH_2PO_4, pH 6.8) and A_{265} was measured. Then, dithiothreitol (freshly prepared, to 14.8 mM) was added to reduce DHA again and A_{265} was re-measured. DHA was determined by measuring the increase in A_{265} upon DHA reduction.

H_2O_2 determination

The H_2O_2 content of spent medium in all cell lines was determined by using the ferrous ammonium sulfate/ xylenol orange method as described by Cheeseman (2006). Aliquots (150 µl) of the spent medium (cell free) were collected during the culture cycle and mixed with 1 ml of reaction buffer (100 µM xylenol orange, 100 µM D-sorbitol, 250 µM $FeSO_4$, 250 µM $(NH_4)_2SO_4$ and 1% ethanol in 25 mM H_2SO_4). A blank reaction was prepared by adding 150 µl of distilled water to 1 ml of reaction buffer. Samples were incubated at room temperature for 40 min with shaking, and A_{550} was measured. Absorbance values obtained for spent medium were corrected by measuring the A_{550} of 150 µl of fresh culture media mixed with 1 ml of reaction buffer and incubated for 40 min as described above.

To obtain the H2O2 concentration, a standard curve with different concentrations of H_2O_2 (from 0.5 to 40 µM) was performed, following the same procedure. Standards were prepared by dilution of reagent grade, 30% H_2O_2 (Sigma). The concentration of H_2O_2 in the reagent was calculated by using absorbance at 240 nm and an extinction coefficient: ε=43.6 M^{-1} cm^{-1}.

Statistical analyses

All results are expressed as the means±SD of at least four replicates. When indicated, differences between means were statistically analyzed by using a Student's *t*-test.

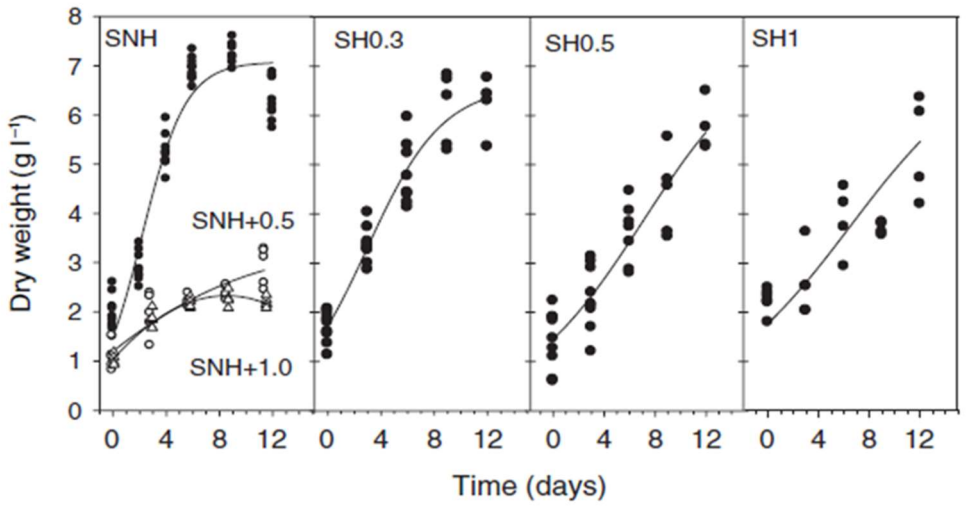
RESULTS

Growth measurements in DCB-habituated cells

SNH cells showed a threefold increase in DW after 7 days of culture in fresh medium (Fig. II.1A). An RGR of 0.3 was calculated for these cell lines. In the stationary phase, SNH-cultured cells maintained high cell viability (91%; Fig. II:1B). The addition of 0.5 or 1.0 µM DCB to the culture medium of SNH cells markedly reduced the accumulation of biomass and cell viability. Consequently, the RGR of DCB short-term treated SNH cells (SNH+DCB(0.5) and SNH+DCB(1)) was reduced by more than 50%. Growth curves of maize cells habituated to low DCB concentrations were

obtained throughout the culture cycle (Fig. II.1A). As the level of DCB habituation increased, cultured cells accumulated less biomass during the stationary phase. DCB-habituated cell lines had higher doubling times, and the RGR decreased as the habituation level rose, in such a way that the RGR in SH0.5 and SH1 cells was almost half the one estimated for SNH cells (Fig. II.1B).

A



B

Cell line	Doubling time	Relative growth rate	% viability at stationary phase
SNH	2.3	0.30	91.0
SNH+DCB(0.5)	5.3	0.12	47.1
SNH+DCB(1.0)	6.0	0.13	39.6
SH0.3	2.9	0.23	68.6
SH0.5	3.9	0.17	75.9
SH1	3.8	0.17	64.4

Fig. II.1. (A) Growth curves of non-habituated -SNH-, DCB short-term treated -SNH+DCB(x)-, and DCB-habituated -SHx- maize suspension-cultured cells. (B) Growth parameters of maize suspension-cultured cell lines. Data represent growth curves for at least four replicates. 'x' indicate the DCB concentration (μM) added to the culture medium.

Oxidative status

In order to determine the oxidative status of SH and SNH cells, lipid peroxidation levels were measured as a function of TBARS formation using MDA as reference molecule (Fig. II.2). Short-term treatment of SNH cells with DCB (0.5 or 1 μM) caused more than a fourfold increase in lipid peroxidation levels when compared with cells cultured in media lacking DCB (SNH). Among DCB-habituated cultured cells, only SH1 cells showed a significant increment in lipid peroxidation levels when compared with SNH cells.

The H_2O_2 accumulated in the spent medium during the cell culture cycle was measured in all cases (Fig. II.3). SNH cells accumulated H_2O_2 in the cell culture medium in a concentration ranging from 0.4 to 0.8 μM. In these control cells, H_2O_2 accumulation peaked in the exponential phase.

Short-term treatments of SNH cells with DCB significantly increased H_2O_2 accumulation during the culture cycle (Fig. II.3). In these cell lines, H_2O_2 began accumulating in the lag phase, reaching a plateau in the exponential growth phase. In the exponential phase (the growth phase selected for lipid peroxidation assays) the H_2O_2 concentration measured in SNH+DCB(0.5) and SNH+DCB(1) spent medium was on average 2.5 to 3-fold higher with respect to SNH cells. In the same way, DCB-habituated cells accumulated a significantly higher concentration of H_2O_2 with respect to SNH cells (Fig. II.3). H_2O_2 accumulation in the exponential phase of SH cells did not markedly differ from that obtained for DCB short-term treated cells. However, differences were found in the kinetics of H_2O_2 accumulation, since SH cells maintained a high H_2O level from the lag phase throughout the cell culture cycle.

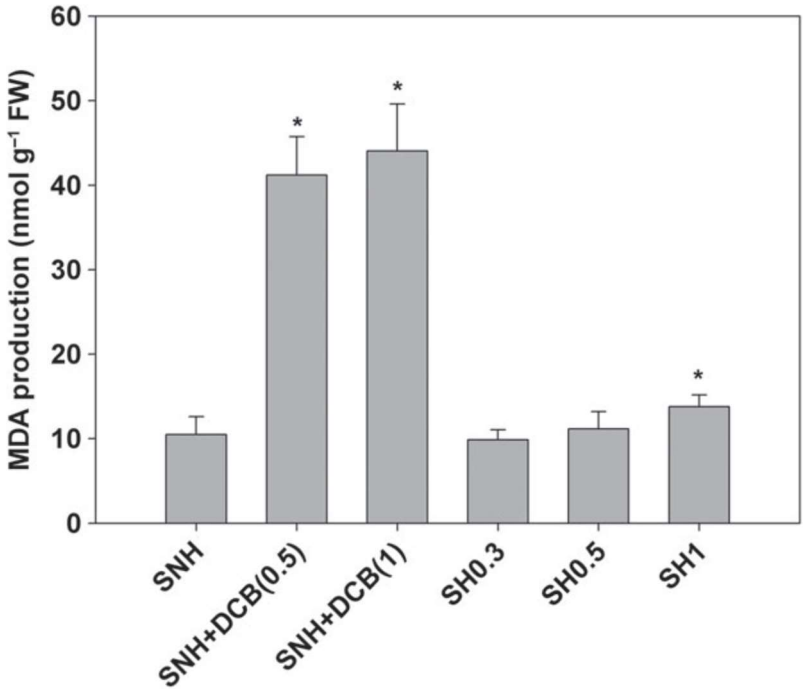


Fig. II.2. Lipid peroxidation levels measured as MDA production in non-habituated -SNH-, DCB short-term treated -SNH+DCB(x)-, and DCB-habituated -SHx- maize suspension-cultured cells. Data represents means±SD of at least four replicates. Asterisks indicate significant differences with respect to SNH cells by Student's *t*-test ($P < 0.05$). 'x' indicates the DCB concentration (μM) added to the culture medium.

Antioxidant and conjugation enzyme activities

In order to gain an insight into the ROS scavenging capacity, the activity of the antioxidant enzymes APOX, CIII-POX, CAT and GR (Fig. II.4) were measured in all the cell lines in their exponential phase.

APOX and CIII-POX activities increased during the DCB habituation process (Fig. II.4A, B). When SNH and SH1 cells were compared, a significant increase (~2.5-fold) in both peroxidase activities was found. In contrast, the habituation process was associated with a gradual decrease in CAT activity (Fig. II.4C). DCB-habituated cells showed similar levels of GR activity to that of control cells (Fig. II.4D).

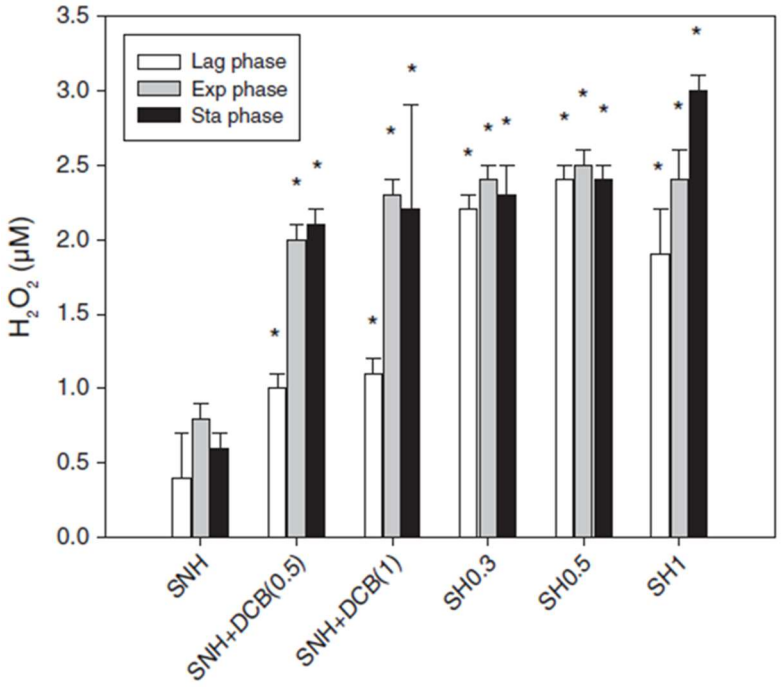


Fig. II.3. Changes in H_2O_2 concentration measured in the spent medium of non-habituated -SNH-, DCB short-term treated -SNH+DCB(x)- and DCB-habituated -SHx- maize suspension-cultured cells at the lag (white), exponential (gray) and stationary (black) phase of the cell culture cycle. Data represent means±SD of three replicates. For each cell culture phase, asterisks indicate significant differences with respect to SNH cells by Student's t-test ($P < 0.05$). 'x' indicates the DCB concentration (µM) added to the culture medium

The short-term treatment of SNH cells with 0.5 µM or 1 µM DCB resulted in a significant enhancement of CIII-POX activity and a slight reduction in APOX activity (Fig. II.4A, B). In the case of CAT activity (Fig. II.4C), no clear trends were found as this activity significantly increased in SNH+DCB(0.5) but did not vary with respect to SNH cells or SNH+DCB(1). As in the case of DCB habituation, GR activity did not vary with respect to control in DCB short-term treated cells (Fig. II.4D).

Besides the enzymatic activities regularly involved in the antioxidant defense mechanism, the involvement of GST activity as a putative DCB detoxifying system was tested (Fig. II.5). The results obtained showed that GST activity significantly increased with respect to control both after a DCB short-term treatment and in DCB-habituated cells.

Glutathione and ascorbate measurements

To study the levels of non-enzymatic antioxidant molecules, TG (GSH+GSSG) and the GSH/TG ratio were quantified in all cell lines in the exponential growth phase (Fig. II.6). The short-term DCB treatment of SNH cells induced a significant increment in TG content (more than 28-fold in both treatments) and an increase in the GSH/TG ratio, indicating that 97–98% of glutathione was in its reduced form. DCB habituation was also characterized by a significant increment In TG content in comparison with SNH cells. The GSH/TG ratio of DCB-habituated cells was only slightly higher than that estimated for SNH cells.

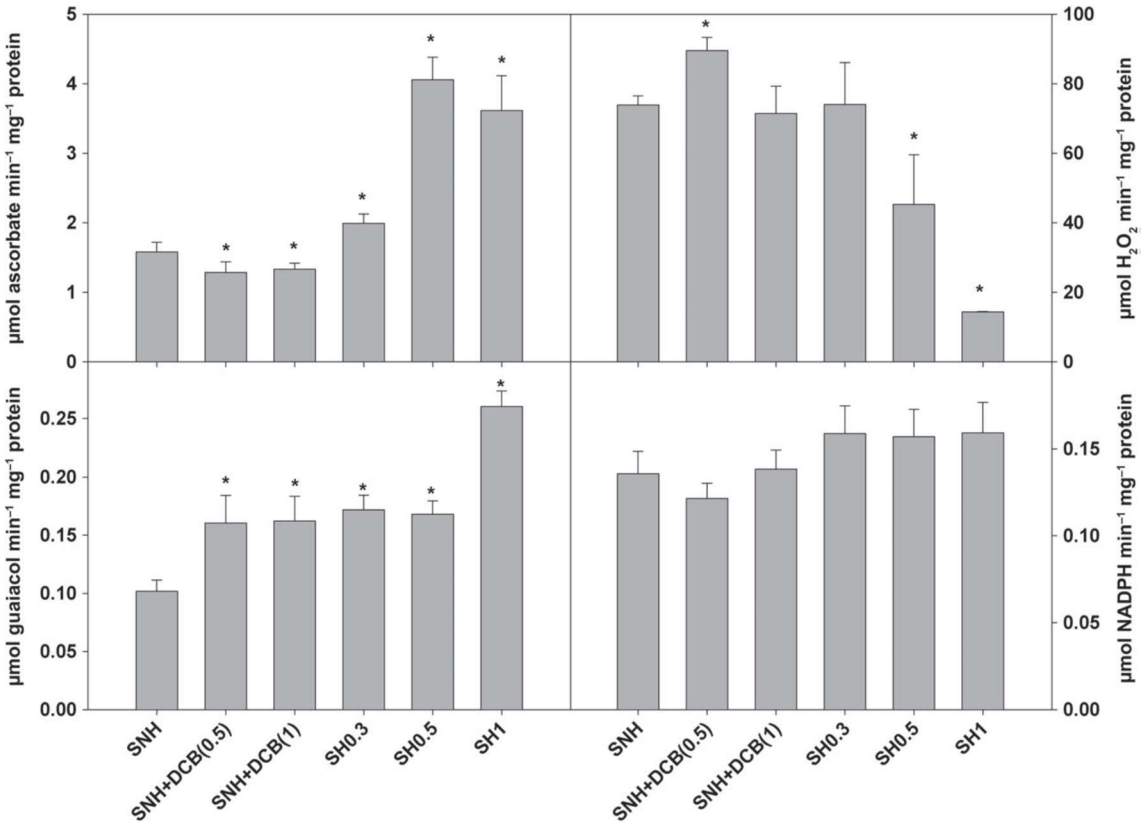


Fig. II.4. Activity of APOX (A), CIII-POX (B), CAT (C) and GR (D) measured in non-habituated -SNH-, DCB short-term treated -SNH+DCB(x)- and DCB-habituated -SHx- maize suspension-cultured cells. Data represent means±SD of at least four replicates. Asterisks indicate significant differences with respect to SNH cells by Student's t-test ($P < 0.05$). 'x' indicates the DCB concentration (µM) added to the culture medium.

TA measured in SNH cells during the exponential phase was on average 1 µmol g−1 FW (Fig. II.7). In SNH cells, ~ 50% of TA was in its reduced form as the average AA/TA ratio was 0.52 (Fig. II.7). Both the short-term DCB treatment and DCB habituation resulted in a reduction in the TA cell content. Moreover, the redox status of AA changed as an increase in DHA (oxidized AA) was detected, with average ratios ranging from 0.29 to 0.17 (Fig. II.7).

DISCUSSION

The habituation of cells to DCB leads to a modified cell wall. This modification depends on the type of cell wall, the concentration of DCB to which cells are habituated and the number of subcultures in a given concentration of DCB. In contrast to cells with a high-level of habituation, FTIR and biochemical analysis revealed that the wall modification of cells with a low level of habituation reverts to that of non-habituated cells if the concentration is maintained under a certain threshold (Alonso-Simón et al. 2004, de Castro et al. 2014). Hence, cell wall changes are not stable during these initial stages of habituation, and therefore the notion of a non-cell wall-related mechanism that allows maize cells to grow under DCB presence is plausible.

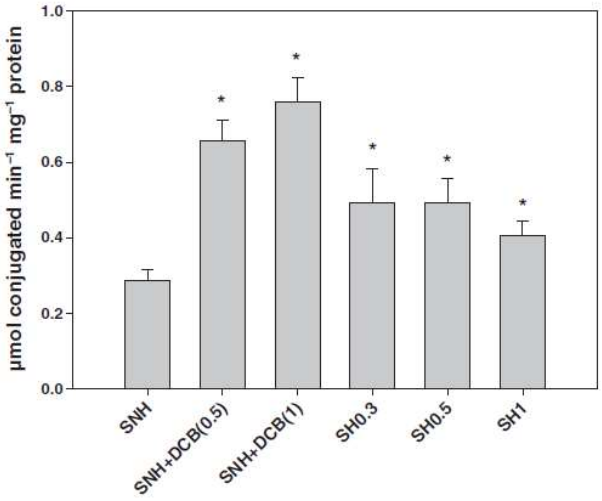


Fig. II.5. GST activity measured in non-habituated -SNH-, DCB short-term treated -SNH+DCB(x)- and DCB-habituated -SHx- maize suspension-cultured cells. Data represent means±SD of at least four replicates. Asterisks indicate significant differences with respect to SNH cells by Student's *t*-test ($P < 0.05$). 'x' indicates the DCB concentration (µM) added to the culture medium.

Early habituation to DCB, as was the case of SH0.3, SH0.5 and SH1 cells reduced cell wall cellulose content by 20% in comparison to SNH cells (de Castro et al. 2014). At this stage, DCB habituation was associated with a dose-dependent enhancement of antioxidant activities, mainly CIII-POX and APOX (Fig. II.4). In light of the results reported here, we suggest that CIII-POX and APOX activities play an active role in the early DCB habituation process of maize-cultured cells by maintaining ROS at a low level. The role of APOXs in the detoxification of H_2O_2 by oxidizing AA as part of the ascorbate–glutathione and water-water cycle has been observed previously (Gill and Tuteja 2010). Physiological and gene expression analysis have widely shown that a common pattern in the response to abiotic stresses is the overexpression of cytosolic APOX isoenzymes and the increase in APOX activity (Mittler 2002, Shigeoka et al. 2002, Gill and Tuteja 2010). Recently it has been demonstrated that nitric oxide positively regulates cytosolic APOX activity

by S-nitrosylation, enhancing the resistance of plants to oxidative stress (Yang et al. 2015). This result opens up the possibility of nitric oxide being a factor contributing to regulate the oxidative stress response of DCB-habituated cells.

The reported increase in the level of CIII-POX activity associated with DCB habituation of maize-cultured cells (Fig. II.4B) is consistent with previous results obtained by our group (García-Angulo et al. 2009). As previously indicated, DCB-habituated bean cells have been shown to feature stable and constitutively high levels of CIII-POX (García-Angulo et al. 2009). In the regular peroxidative cycle, CIII-POX reduces H_2O_2 by oxidation of a variety of co-substrates (Passardi et al. 2005). Furthermore, a CAT-like activity has been reported for type-III POXs, which efficiently detoxifies H_2O_2 (Mika et al. 2004 and references therein). Peroxidase-mediated hydroxylation could also play a role in DCB detoxification as it has been shown that this process occurs by glutathione conjugation of hydroxylated DCB derivatives (Brittebo et al. 1992).

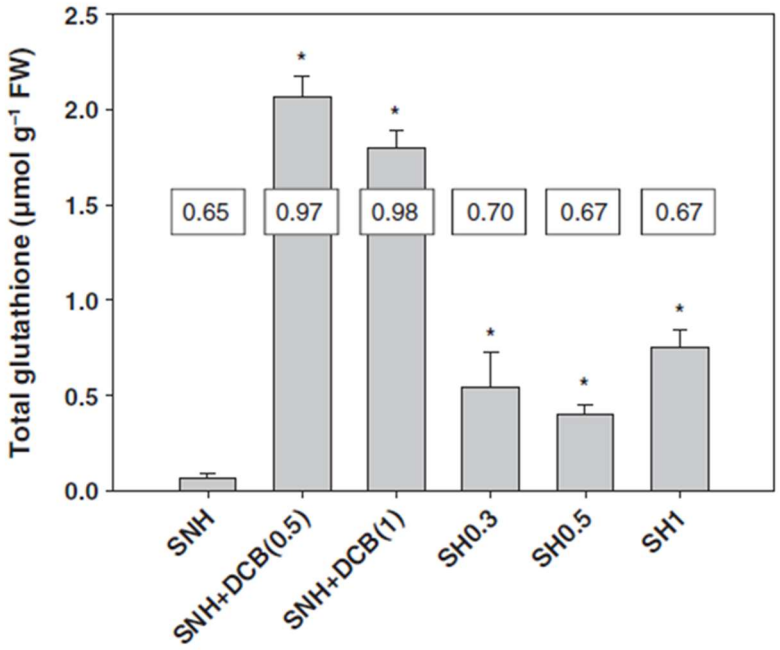


Fig. II.6. Total glutathione (TG) measured in non-habituated -SNH-, DCB short-term treated -SNH+DCB(x)- and DCB-habituated -SHx- maize suspension-cultured cells. Squared values represent the GSH/TG ratio of each cell line. Data represent means±SD of at least six replicates. Asterisks indicate significant differences with respect to SNH cells by Student's *t*-test ($P < 0.05$). 'x' indicates the DCB concentration (µM) added to the culture medium.

In addition, a role for CIII-POXs in maize cell wall remodeling may be proposed as CIII-POXs oxidatively cross-link cell wall hemicelluloses by di-ferulate bonding of arabinoxylans (Fry 2004). In accordance with this, an increased level of di-ferulates has been recorded in both low (de Castro, personal communication) and high levels (Mélida et al. 2009, 2010b, 2011) of DCB habituation. However, in maize cells habituated to high DCB levels, no relationship was found between high

ferulate dimerization and increased peroxidase activity (Mélida et al. 2010a), indicating that cell wall CIII-POX activity is not a limiting factor for ferulate dimerization. In accordance with this, it is likely that changes in CIII-POX activity associated with low levels of DCB habituation do not account for cell wall remodeling. Besides oxidative reinforcement of cell wall, it is possible that CIII-POXs contribute to cell wall loosening by producing hydroxyl radicals (Schopfer 2001).

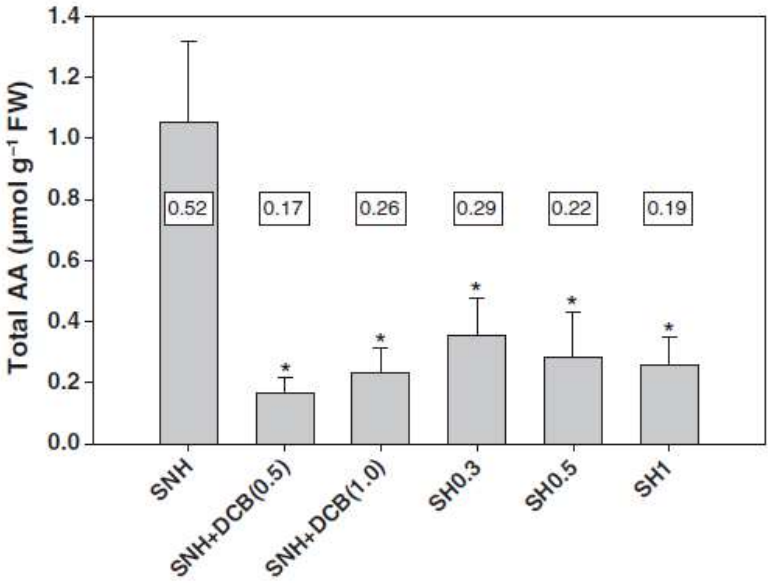


Fig. II.7. Total ascorbate (TA) content measured in non-habituated -SNH-, DCB short-term treated -SNH+DCB(x)- and DCB-habituated -SHx- maize suspension-cultured cells at theexponential phase of the cell culture cycle. Squared values represent the AA/TA ratio of each cell line. Asterisks indicate significant differences with respect to SNH cells by Student's *t*-test ($P < 0.05$). 'x' indicates the DCB concentration (µM) added to the culture medium.

The incipient habituation of maize cells to DCB induced a significant increment in TG content and a slightly higher proportion of its reduced form (GSH) (Fig. II.6), both factors being related to protection against a build-up of stress-induced ROS (Szalai et al. 2009). Several studies have related high GSH/TG ratios to efficient protection of the plant cell against abiotic stresses and the control of cell redox homeostasis through a reduction in ROS content (Szalai et al. 2009, Gill and Tuteja 2010). The increase in GSH observed in abiotic-stressed cells is frequently explained by a higher rate of de novo GSH synthesis (Szalai et al. 2009). This finding would explain why our short-term treated cells showed a high TG content with no significant changes in GR activity (Fig. II.4D). Early DCB habituation of maize cells was also associated with an increase in GST activity (Fig. II.5), pointing to high TG contents and further GSH conjugation of DCB as mechanisms implicated in the habituation of maize cells to low DCB concentrations (<1 µM). High GSH availability and GST activity would be consistent with high rates of DCB-conjugation and would partially explain the recovery in cellulose content reported as the number of subcultures in presence of low concentrations of DCB increases (de Castro et al.

2014). Intriguingly, maize cells habituated to high DCB levels (> 6 μM) did not show increased levels of GST activity (Mélida et al. 2010a), prompting us to speculate that GST activity is solely implicated in early habituation to DCB.

SH0.5 and SH1 cells showed a reduction in CAT activity (Fig. II.4C). Although in a context of increased antioxidant protection a reduction in CAT activity might appear contradictory, it seems a consistent result as this same effect has already been reported for maize cells habituated to high DCB levels (Mélida et al. 2010a). Interestingly, it has been widely reported the association between reduced CAT activity, H_2O_2 accumulation and GSH biosynthesis as it may occur in our experiment (Smith 1985, Queval et al. 2009, Noctor et al. 2012). In the same way, DCB-habituation is associated with a reduction in TA content and a relative increase in its reduced form (Fig. II.7), contrary to what might be expected of an antioxidant strategy. However, in some systems it has been shown that abiotic stress reduces AA content (Gill and Tuteja 2010).

The enhancement of antioxidant machinery in SH0.3 and SH0.5 cells was apparently sufficient to cope with the oxidative stress provoked by the herbicide because their lipid peroxidation levels did not significantly differ from that of SNH cells (Fig. II.2), although SH cells accumulated a significantly higher level of H_2O_2 in the culture medium (Fig. II.3). In the case of SH1 cells, DCB habituation induced activation of the antioxidant system, but this defense response was apparently insufficient to control the putative formation of ROS, leading to a slight but significant increase in oxidative damage when compared with control cells (Fig. II.2). The oxidative damage that SH1 cells putatively sustained could explain their lower RGR, which was reduced by almost half compared with SNH cells (Fig. II.1B). An alternative explanation for the reduced RGR of SH1 cells could be a more effective inhibition of cell wall expansion through DCB inhibition of cellulose biosynthesis (de Castro et al 2014). It should be borne in mind that unlike SH0.3 and SH0.5 cells, cellulose content of SH1 cells did not revert to control levels as the number of subcultures in the presence of DCB increased (de Castro et al. 2014).

The response of SNH cells to a short-term treatment with DCB was characterized by cell growth impairment expressed both as DW gain and cell viability and a significant rise in oxidative stress measured as lipoperoxidation (increased by more than fourfold in comparison with SNH cells), indicating that DCB induces ROS formation as indicated by the build-up of H_2O_2 measured in the spent medium. It can be speculated that short-term treated maize cells, even when not controlling oxidative damage, putatively display an antioxidant/conjugation strategy as has previously been found (Mélida et al. 2010a). The antioxidant response is supported by an enhancement in CIII-POX and GST activities, high TG levels and GSH/TG ratios. In summary, our results indicate that in an attempt to cope with oxidative stress, short-term DCB-treated cells responded with substantial de novo GSH biosynthesis and an enhancement of CIII-POX and GST activities. GSH content and GST activity were reduced during the subsequent subcultures, although

it nevertheless remained at high levels in comparison with SNH cell levels, and CIII-POX and APOX activities increased. In contrast to a long-term DCB habituation, where DCB cannot be efficiently detoxified and cells cope with the herbicide by a cell wall remodeling strategy, during incipient habituation the antioxidant–conjugation machinery seems to be good enough to revert the initial stress situation and successfully cope with the herbicide. Results reported here show that depending on the level of DCB-induced stress maize cells develop alternative coping strategies.

Author contributions - A. L. G. designed research, performed research, analyzed data, wrote manuscript. A. E. provided research opportunity, designed research, performed research, analyzed data, wrote manuscript. M, d. C. performed research, revised manuscript. H. M. designed research, assisted in performing the research, revised manuscript. J. L. A. provided research opportunity, designed research, assisted in performing the research, revised manuscript. P. G. A. designed research, assisted in performing the research, revised manuscript. J. M. A. provided research opportunity, supervised project, designed research; wrote manuscript.

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CAPÍTULO III:

Quinclorac-habituation of bean (*Phaseolus vulgaris*) cultured cells is related to an increase in their antioxidant capacity

Largo-Gosens A, de Castro M, Alonso-Simón A, García-Angulo P, Acebes JL, Encina A, Álvarez JM. (2016). Quinclorac-habituation of bean (Phaseolus vulgaris) cultured cells is related to an increase in their antioxidant capacity. Plant Physiology and Biochemistry. Aceptado, "In press". Doi: 10.1016/j.plaphy.2016.06.011.

Quinclorac-habituation of bean (*Phaseolus vulgaris*) cultured cells is related to an increase in their antioxidant capacity

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Abstract

The habituation of bean cells to quinclorac did not rely on cell wall modifications, contrary to what it was previously observed for the well-known cellulose biosynthesis inhibitors dichlobenil or isoxaben. The aim of the present study was to investigate whether or not the bean cells habituation to quinclorac is related to an enhancement of antioxidant activities involved in the scavenging capacity of reactive oxygen species. Treating non-habituated bean calluses with 10 μM quinclorac reduced the relative growth rate and induced a two-fold increase in lipid peroxidation. However, the exposition of quinclorac-habituated cells to a concentration of quinclorac up to 30 μM neither affected their growth rate nor increased their lipid peroxidation levels. Quinclorac-habituated calluses had significantly higher constitutive levels of three antioxidant activities (class-III peroxidase, glutathione reductase, and superoxide dismutase) than those observed in non-habituated calluses, and the treatment of habituated calluses with 30 μM quinclorac significantly increased the level of class III-peroxidase and superoxide dismutase. The results reported here indicate that the process of habituation to quinclorac in bean callus-cultured cells is related, at least partially, to the development of a stable antioxidant capacity that enables them to cope with the oxidative stress caused by quinclorac. Class-III peroxidase and superoxide dismutase activities could play a major role in the quinclorac-habituation. Changes in the antioxidant status of bean cells were stable, since the increase in the antioxidant activities were maintained in quinclorac-dehabituated cells.

Keywords: Antioxidant activities; cell culture habituation; herbicide; oxidative damage; *Phaseolus vulgaris*; quinclorac.

Highlights

- A short exposure with 10 μM quinclorac increases lipid peroxidation of bean calluses.
- Quinclorac-habituation is associated with a low level of lipid peroxidation in comparison to non-habituated bean cells treated with quinclorac.
- Quinclorac-habituated cells have an enhanced constitutive antioxidant status.
- The antioxidant status of quinclorac-habituated cells was maintained after removing the herbicide.
- Class-III peroxidase and superoxide dismutase activities increased in quinclorac-habituated cells and could play a major role in the quinclorac-habituation.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; CIII-POX, class III peroxidase; CAT, catalase; CBI, cellulose biosynthesis inhibitor; DTT, dithiothreitol; DH, quinclorac-dehabituated cells; DMSO, dimethylsulphoxide; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; FW,

fresh weight; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; IsoPOX, peroxidase isoforms; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate; NH, non-habituated cells; PAGE, polyacrylamide gel electrophoresis; POX, peroxidase, Qn, quinclorac-habituated cells to "n" μM quinclorac; RGR, relative growth rate; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; SOD, superoxide dismutase; TBARS, thiobarbituric acid reacting substances.

INTRODUCTION

Quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) is a highly selective auxin-type herbicide mainly used to control broad-leaved weeds and harmful grass weeds in rice crops and lawns (Grossmann 2000; 2010).

It was previously reported that quinclorac inhibits the incorporation of glucose into cellulose in a dose and time-dependent manner (Koo et al. 1996; 1997), being regarded as a cellulose biosynthesis inhibitor (CBI) (Vaughn 2002). However, other works challenged the correlation of cellulose inhibition effect and quinclorac mechanism of action (Tresch and Grossmann 2003). In an attempt to elucidate whether quinclorac directly inhibited cellulose biosynthesis, our group proceeded to habituate bean callus-cultured cells to grow in otherwise lethal concentrations of the herbicide. In addition, modifications in cell wall composition due to the habituation process were analysed (Alonso-Simón et al. 2008). The results obtained showed that the mechanism of bean cells habituation to quinclorac differed from that reported for well-known CBIs such as dichlobenil (Encina et al. 2001, 2002) or isoxaben (Díaz-Cacho et al. 1999). In the dichlobenil and isoxaben-habituation processes, bean cells developed the capacity to divide and expand, with a modified cell wall in which the xyloglucan-cellulose network had been partially replaced by pectins. Quinclorac habituated cells did not show a decrease in the cellulose content, and the minor changes observed in the distribution and post-depositional modifications of homogalacturonan and rhamnogalacturonan I during the habituation process seemed to be due to a side-effect of quinclorac presence (Alonso-Simón et al. 2008). Moreover, short-term treatment of bean suspension-cultured cells with quinclorac concentrations that significantly reduced their dry weight gain (10 μM) did not decrease the incorporation of [14C]glucose to cell wall polysaccharides; in fact, the glucose incorporation increased (García-Angulo et al. 2012). Therefore, the mechanism of quinclorac-habituation did not seem to rely on a modification of cell wall structure and/or composition.

In some species, habituation of cell cultures to CBIs leads to an increase in antioxidant capacity. This is the case of bean cell cultures where habituation to dichlobenil is associated with high class III-peroxidase (CIII-POX) activity (García-Angulo et al. 2009). In the case of maize cells, an increased antioxidant capacity seems to take part in changes associated to the incipient dichlobenil-habituation process (Largo-Gosens et al. 2016), however, antioxidant activities are not implicated in the long-term habituation to high dichlobenil concentrations (Mélida et al. 2010).

In sensitive species, quinclorac induces the activity of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which increases the level of ACC (Grossmann and Scheltrup 1997). The subsequent oxidation of this compound to ethylene leads to cyanide accumulation which can interrupt electron flow in chloroplast and mitochondria leading to reactive oxygen species (ROS) overproduction (Navrot et al. 2007) and is considered to be responsible for the phytotoxic effects of quinclorac (Grossmann and Kwiatkowski 2000; Abdallah et al. 2006). In susceptible dicots, the response to quinclorac is related to increased abscisic acid biosynthesis, which also leads to overproduction of ROS (Van Eerd et al. 2005; Grossmann 2010). By transcriptomic analysis, it has been recently demonstrated that quinclorac treatment of rice plants (*Orzya sativa*), provoked the enhancement of several groups of genes related with drug detoxification (Xu et al. 2015). Additionally, the induction of the expression of the gene *EcGH3.1*, that belongs to Gretchen Hagen 3 gene family and regulates the auxin homeostasis has been demonstrated to play a key role in *Echinochloa crus-galli* resistance to quinclorac (Li et al. 2016).

Quinclorac has been reported to induce the overproduction of ROS causing oxidative injury in several sensitive species. Sunohara and Matsumoto (2004) demonstrated the relationship between antioxidant capacity and quinclorac tolerance in several monocots. Later, same authors suggested that the cell death of a quinclorac-sensitive variety of maize may be caused by the overproduction of ROS, but not by ethylene or cyanide action (Sunohara and Matsumoto 2008). However, tolerant species (*Oryza sativa* and *Eleusine indica*) and resistant biotypes of susceptible species (*Echinochloa* spp.; *Digitaria* spp. and *Galium* spp.) neither increase ethylene and cyanide production, nor overproduce ROS in response to quinclorac treatment (Grossmann and Kwiatkowski 1993; 2000; Grossmann 2000; Van Eerd et al. 2005; Abdallah et al. 2006; Sunohara et al. 2010, 2011; Yasuor et al. 2012).

Given that i) bean cells habituation to quinclorac does not seem to rely on cell wall modifications; ii) bean cells habituation to other herbicide such as dichlobenil is associated with high CIII-POX activity and iii) quinclorac treatment in sensitive species provokes an overproduction of ROS, the aim of the present study was to investigate whether or not the habituation of bean cells to quinclorac is related to an enhancement of antioxidant activities involved in the scavenging capacity of reactive oxygen species. Moreover, the stability of a putative antioxidant capacity was further investigated by using quinclorac-habituated cells transferred for several subcultures in a medium lacking quinclorac (dehabituated cells).

To our knowledge, this is the first time that a quinclorac-habituated cell line has been used to investigate the role of the antioxidant machinery connected to the tolerance to quinclorac. For this purpose, CIII-POX, glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) activities, as well as lipid peroxidation as an indicator of oxidative damage, were measured in a set of cell

lines grown on solid medium: non-habituated, habituated to different quinclorac concentrations (ranging from 10 to 30 μM), and dehabituated, as well as non-habituated cells cultured in the presence of 10 μM quinclorac and quinclorac-habituated cells treated with 30 μM quinclorac. Lastly, polyacrylamide gel electrophoresis (PAGE) to separate the peroxidase isoforms (isoPOX) of all cell lines was performed.

MATERIAL AND METHODS

Plant material and quinclorac habituation

Bean (*Phaseolus vulgaris* L.) cell lines were obtained and subcultured as described by Encina et al. (2001) on Murashige and Skoog (1962) solid basal medium supplemented with sucrose (30 g L^{-1}), 10 μM 2,4-D (2,4-dichlorophenoxyacetic acid) and agar (8 g L^{-1}).

Quinclorac was dissolved in dimethylsulphoxide (DMSO). Non-habituated bean cell lines (NH) were habituated by adding stepwise increments in the concentration of quinclorac to the culture medium, beginning at the I_{50} value for quinclorac (10 μM) and continuing until obtaining bean calluses that were capable of growing under otherwise lethal concentrations of the herbicide (Alonso-Simón et al. 2008). In order to account for DMSO effects, during the habituation process NH cells were supplemented with DMSO ranging from 0.1% to 0.3% (v/v). The highest DMSO concentration used in this experiment, 0.3% (v/v), did not affect the parameters determined in this study (data not shown). Habituated cells were denoted as Qn, where n indicates the quinclorac concentration in μM. In summary, NH, Q10, Q15 and Q30 cell lines were used in this study. Q30 cells were transferred to a medium lacking quinclorac for five subcultures, obtaining dehabituated (DH) cells. All different cell lines were regularly subcultured every 30 days.

A set of NH calluses was subcultured in the presence of 10 μM quinclorac for 30 days and denominated as NH+10, while sets of Q10 and Q15 calluses were subcultured in the presence of 30 μM quinclorac for 30 days, and were denominated Q10+30 and Q15+30 respectively.

Effect of quinclorac on bean callus growth

To evaluate the effect of quinclorac on callus cell growth, fresh weight (FW) gain was measured in NH, NH+10 and Q10. The relative growth rate (RGR) was determined as follows:

$$RGR = [(FWf-FWi)/FWi] 174$$

where FWi and FWf indicate the fresh weight of calluses at 0 and 30 days respectively. To determine the dry weight (DW), calluses were dried at 60ºC for 72h

and were weighed. Data for RGR and DW/FW ratio of Q15 and Q30 were taken from Alonso-Simón et al. (2008) for comparison.

Activity assays of antioxidant enzymes and lipid peroxidation

In order to measure GR (EC 1.8.1.7), SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) activities, as well as lipid peroxidation levels, cells of all lines were collected at their exponential growth phase and stored at -80ºC until use. Calluses (1 g FW) were homogenized in liquid nitrogen using a mortar and pestle and 5 mL 0.05 M Tris-HCl pH 7.5, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol and 2 mM DTT were added. These extracts were centrifuged at 15000 g for 2 min and the supernatants were used for the measurements.

GR activity was determined as described by Klapheck et al. (1990) measuring the decrease in A_{334} caused by NADPH oxidation during 3 min for the conversion of GSSG to GSH (ε_{334}= 6.2 mM^{-1} cm^{-1}). The reaction was performed at 25ºC with 0.1 mL of supernatant, 1.35 mL reaction buffer (100 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM GSSG and 3 mM $MgCl_2$) and 0.05 mL 10 mM NADPH.

SOD activity was evaluated using a SOD assay kit (Sigma, Alcobendas, Spain). Briefly, SOD activity was measured as the inhibition of the formation of a colored compound which can be detected at 440 nm and activity was determined from the inhibitory curve made with different concentrations of standards of a commercial SOD (from 0.001 U/mL to 2000 U/mL). For the determination of SOD activity, 0.02 mL of sample supernatant or standard was used.

CAT activity was measured by Droillard's method (Droillard et al. 1987), based on absorbance reduction at 240 nm due to H_2O_2 reduction to water (ε_{240}= 39.58 M^{-1} cm^{-1}). Measurements were performed during 2 min at 25ºC by adding 0.1 mL of supernatant extract to 3 mL reaction buffer (50 mM phosphate buffer pH 7.0 and 37.5 mM H_2O_2).

Lipid peroxidation levels were determined by measuring the concentration of thiobarbituric acid reacting substances (TBARS), using malondialdehyde (MDA), an end product of lipid peroxidation, as the reference molecule (Buege and Aust 1978). Aliquots of supernatant (20 µL) were mixed with 1 mL 15% (w/v) trichloroacetic acid, 0.375% thiobarbituric acid and 0.01% butylated hydroxytoluene in 0.25 M HCl. The mixture was incubated at 100ºC for 15 min, then cooled to room temperature and centrifuged at 2500 g for 15 min. Absorbance at 535 nm was measured in the supernatants. Blank reactions (sample blank) were used. Lipid peroxidation levels were expressed as µM MDA per g FW calculated on the basis of a calibration curve obtained with MDA dimethyl acetal (0-10 µM, Merck, Hohenbrunn, Germany).

For CIII-POX (EC 1.11.1.7) assay, 1 g FW of bean cells from all cell lines was homogenized in liquid nitrogen using a mortar and pestle and 5 mL 0.04 M Tris-HCl pH 7.2, 1 mM EDTA-2Na-2H and 5% (v/v) glycerol were added. The homogenate was centrifuged at 15000 g for 2 min and the supernatant was used to

perform the assay. CIII-POX was measured following the method described by Adam et al. (1995), based on the increase in absorbance at 470 nm due to guaiacol oxidation (ε_{470}= 26.6 mM^{-1} cm^{-1}). The reaction was performed with 3 mL reaction buffer (100 mM sodium acetate pH 5.5 and 1 mM guaiacol), 0.3 mL 1.3 mM H_2O_2 and 0.05 mL supernatant.

Protein content was determined following the method described by Bradford (1976).

Polyacrylamide gel electrophoresis (PAGE)

To detect peroxidase isoforms (iso-POX), supernatant of extracts obtained as indicated for the measurement of CIII-POX activities (see paragraph above) were used. Samples were concentrated using Vivaspin 500 (cutoff 5KDa; GE Healthcare) and a final amount of 6 μg of proteins were loaded into polyacrylamide gel. Proteins were separated using a non-denaturing gel (12% acrylamide) and running denaturing conditions with Tris-glycine SDS buffer (25 mM Tris-glycine, pH 8.8, and 0.1% SDS (w/v)) at 120 V/h. Peroxidase protein spots were detected by the guaiacol-H_2O_2 procedure as described by Mika et al (2008); gels were washed with water during 5 min (x3) to remove the SDS, and were incubated with 50 mL of guaiacol-H_2O_2 solution (1.0% guaiacol (v/v) and 0.03% H_2O_2 (v/v) in 250 mM sodium acetate buffer pH 5.0) for 30 min until brown spots were visible. For molecular weight determination, EZ-RUN pre-stained protein marker 100 was used as external marker (Fisher Scientific, Spain).

Statistical analyses

All results are represented as the means ± s.d. of at least 3 replicates. Differences between means were statistically analysed using a Student's *t*-test ($p<0.05$). When indicated, a one-way ANOVA analysis followed by the Tukey-test was used to compare treatments ($p<0.05$). Statistics were performed with Statistica software after the data had been tested for normality.

RESULTS

Effect of quinclorac on bean callus growth

A RGR of 1.65 was calculated for NH bean callus-cultured cells. The presence of 10 μM quinclorac (I_{50} concentration, Alonso-Simón, et al. 2008) in the culture medium for 30 days was found to reduce the growth of NH by half (Table III.1). Quinclorac-habituated (Q) cells did also show an impaired growth capacity. In average, a 64% to 72% reduction of RGR was measured in Q cells when compared with NH controls (Table III.1). The culture of Q30 cells in a medium lacking quinclorac (DH cells) for five subcultures did not rendered an increment in the RGR. No clear trends were observed when DW/FW ratios were compared between cell lines (Table III.1).

Table III.1. Relative growth rate (RGR) and final DW/FW ratio of non-habituated 260 calluses (NH), non-habituated calluses treated with 10 μM quinclorac (NH+10), 261 calluses habituated to 10, 15 or 30 μM quinclorac (Q10, Q15 and Q30), and 262 dehabituated calluses (DH), Data represent average values of at least 6 263 replicates. Different letters indicate significant differences between cell lines by 264 one-way ANOVA ($p<0.05$).

Cell Line	RGR	DW/FW ratio
NH	1.65 ± 0.84^{a}	$0.054\pm0.015^{a,b,c}$
NH+10	0.81 ± 0.34^{b}	0.064 ± 0.003^{b}
Q10	0.52 ± 0.20^{b}	$0.055\pm0.018^{a,b,c}$
Q15*	0.59 ± 0.10^{b}	$0,044\pm0.001^{a}$
Q30*	0.47 ± 0.15^{b}	0.050 ± 0.003^{c}
DH	0.58 ± 0.49^{b}	$0.055\pm0.021^{a,b,c}$

* data from Alonso-Simón et al. (2008), included for comparison.

Effect of quinclorac on oxidative status and antioxidant activities

As an indicator of oxidative status, the membrane lipid peroxidation level was quantified in all cell lines by MDA formation (Fig. III.1). A Short-term treatment of NH cells with 10 μM quinclorac (NH+10) induced a significant increase in TBARS levels by more than three-fold. Quinclorac habituation was associated to a steep reduction in the oxidative damage to such an extent that the level of lipid peroxidation measured in Q30 cells did not significantly differ from that in NH cell lines. A short-term treatment of quinclorac habituated cells with 30 μM quinclorac (Q10+30 and Q15+30) did not increase their lipid peroxidation level, but reduced it significantly. In the case of DH cells the lipoperoxidation level measured was slightly higher than that found in NH cells.

To determine the antioxidant capacity of quinclorac-habituated cells, GR, SOD, CAT and CIII-POX activities were determined in crude cell extracts. A short-term treatment of NH cells with 10 μM quinclorac (NH+10) induced an enhancement of CIII-POX activity. No changes in GR and SOD activities were detected (Fig. III.2).

All quinclorac-habituated cells showed a significantly increased GR activity in comparison with NH cells. Moreover, GR activity steeply increased over the course of the habituation process (Fig. III.2.A). The short-term exposure of Q10 and Q15 to 30 μM quinclorac did not induce changes in GR activity when compared with their respective untreated cell lines (Fig. III.2.A).

The pattern of changes in SOD and CIII-POX activities over the course of quinclorac-habituation differed from those reported for GR activity. SOD and CIII-POX antioxidant activities only differed from control cells when measured at the highest habituation level (Q30). However, Q10 and Q15 cells significantly increased both antioxidant activities (Fig. III.2.B and C) upon 30 μM quinclorac treatment (Q10+30 and Q15+30). CAT activity was not detected in any cell line tested.

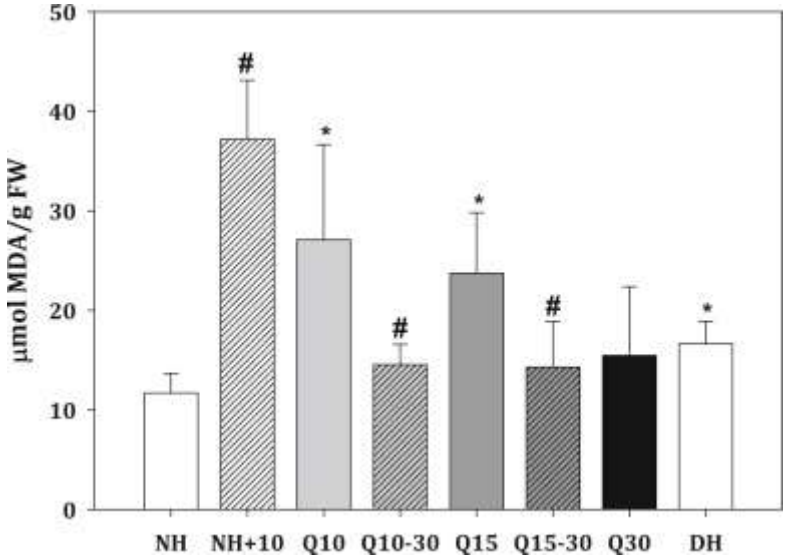

Figure III.1. Lipid peroxidation levels of: non-habituated cells (NH); non-habituated cells treated with 10 µM quinclorac (NH+10); cells habituated to 10 µM (Q10), 15 µM (Q15) and 30 µM quinclorac (Q30); Q10 and Q15 cells transferred to 30 µM quinclorac during 30 days (Q10+30 and Q15+30) and dehabituated cells (DH). Data represent the average values ± s.d. of at least 3 replicates. Asterisks indicate significant differences with respect to NH cells by using Student's t-test. Hashtag indicate the significant differences using Student's t-test between treated and the corresponding untreated cell line.

Quinclorac-dehabituated cells showed no significant differences in lipid peroxidation levels (Fig.III.1), and exhibited increased levels of GR, SOD and CIII-POX activities when compared with those of NH cells (Fig. III.2).

Peroxidase isoforms

To obtain further information about isoPOX, a semi-native PAGE of cell extracts was performed, followed by H_2O_2-guaiacol staining (Fig. III.3).

Two isoPOX (I-II) were detected in NH calluses (Fig III.3). IsoPOX II was present in all cell lines analysed. Contrary, isoPOX I was not detected in Q10 and Q15 cells and it was again detected in Q30. Interestingly this same IsoPOX was detected when Q10 and Q15 cells were treated with 30 µM quinclorac (Q10+30 and Q15+30).

The staining intensity of the isoPOX I and II markedly increased in Q30, Q10+30 and Q15+30 when compared with NH cells.

Quinclorac-dehabituation is associated with the maintenance of a high staining intensity for isoPOX I and II.

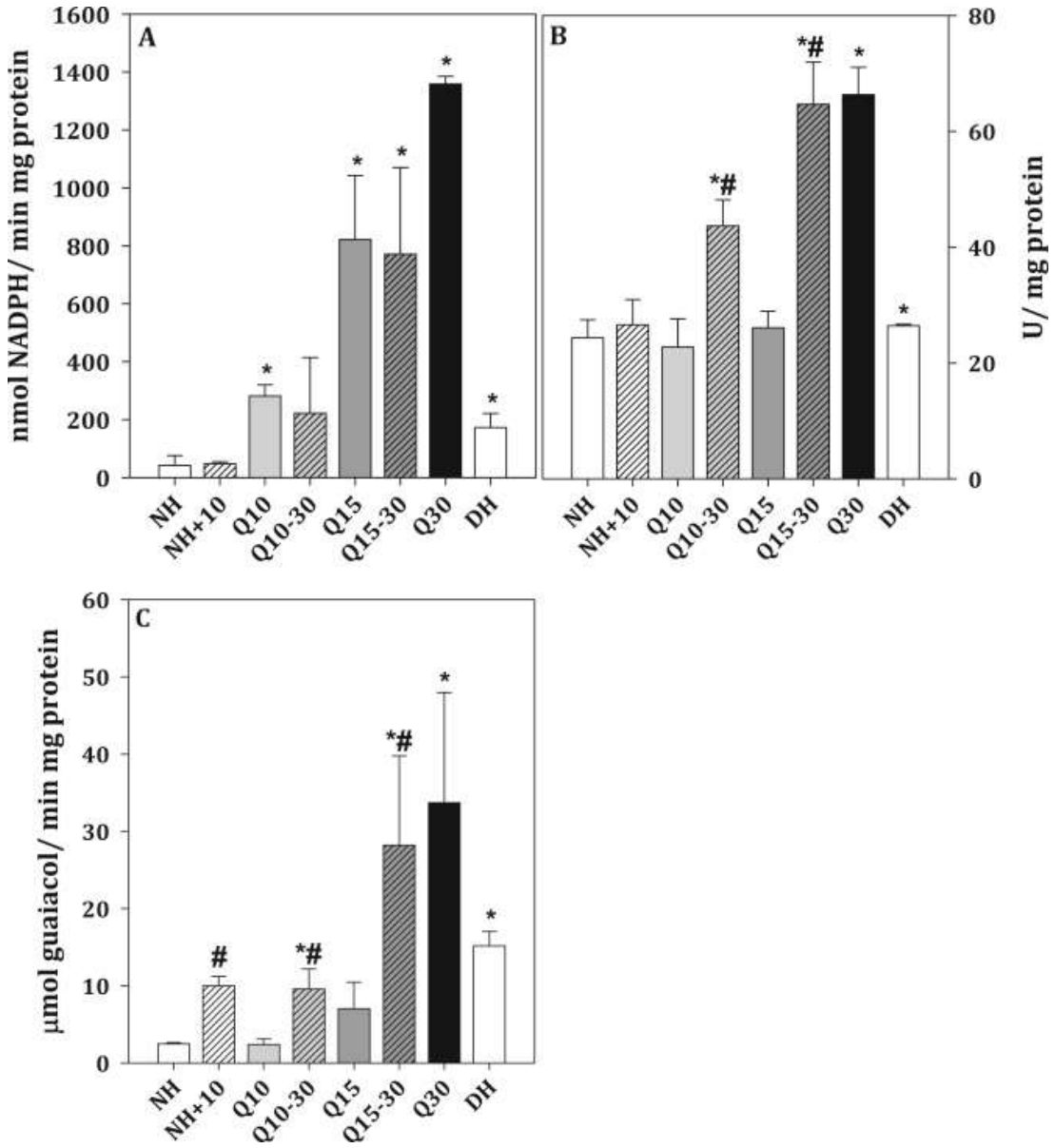

Figure III.2. Activity of GR (A), SOD (B) and CIII-POX (C) in non-habituated, quinclorac-habituated and quinclorac-dehabituated cells. Data represent means ± s.d. of at least 3 replicates. One SOD unit (U) will inhibit the rate of reduction of cytochrome c by 50% in a coupled system, using xanthine and xanthine oxidase, at pH 7.8 at 25ºC in a 3 ml reaction mixture (Sigma). Cell lines, asterisks and hashtag as in Fig. III.1.

DISCUSSION

The exposure of plant cells to a variety of abiotic stresses such as heavy metals (Paradiso et al. 2008), organochlorines (Michalowicz and Duda 2009; Michalowicz et al. 2009; San Miguel et al. 2012) and herbicides (Geoffroy et al. 2004; García-Angulo et al. 2009) often unbalances ROS production and scavenging, leading to oxidative stress (Apel and Hirt 2004; Gill and Tuteja 2010). Excessive ROS production can damage cellular components such as proteins, lipids, DNA and polysaccharides, which interrupts normal metabolism (Dat et al. 2000). The

triggering of these oxygen intermediates leads to the activation of defence mechanisms in plant cells, which consist of antioxidants, such as ascorbate and glutathione, and enzymatic antioxidant activities such as CIII-POX, CAT, SOD and the ascorbate–glutathione cycle enzymes, ascorbate peroxidase (APOX) and GR (Apel and Hirt 2004; Passardi et al. 2005; Gill and Tuteja 2010). Additionally, stress-induced ROS can act as signaling molecules specifically inducing several cell and molecular responses (Miller et al. 2009; 2010; Kärkönen & Kuchitsu 2015).

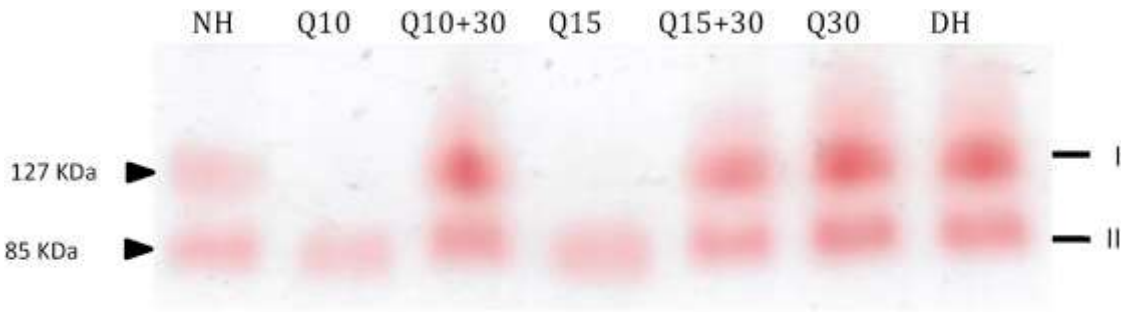

Figure III.3. Semi-native PAGE of peroxidase isoforms in non-habituated, quinclorac-habituated and quinclorac-dehabitutated cells. Cell lines indicated as in Fig. III.1. Different isoPOX were indicated with italic numbers. Molecular weights of external markers are indicated at left.

It has been postulated that the mode of action of quinclorac differs between grasses and broad-leaved weeds (Koo et al. 1991). However, quinclorac toxicity assays on FW gain in bean callus-cultured cells (Alonso-Simón et al. 2008; García-Angulo et al. 2012) indicated that quinclorac is active in the range of concentrations previously reported for maize seedlings, regarded as a model for quinclorac-susceptible grasses (Sunohara and Matsumoto 2008). Our study suggests that in the same way as a series of other sensitive species (Sunohara and Matsumoto 2004; Sunohara and Matsumoto 2008), bean calluses suffer quinclorac-induced ROS formation, causing oxidative harm, since treatment of NH cells with quinclorac (NH+10) increased lipid peroxidation above the level observed in the other cell lines analysed (Fig. III.1).

The habituation of bean calluses to quinclorac is associated with an increased constitutive level of antioxidant activities (Fig. III.2). In fact, the level of the activities was higher as the level of quinclorac in the culture medium rose. Thus Q30, the cell line habituated to the highest concentration of quinclorac, also had the highest level of GR, SOD and CIII-POX activities (Fig. III.2). These results were also confirmed by a higher staining intensity in both of the isoPOX separated by PAGE (Fig. III.3).

This enhancement of antioxidant capacity is associated with a steep reduction in lipid peroxidation levels to the extent that Q30 and NH cells had similar levels of oxidative damage. Results reported here point to bean cells habituated to quinclorac having developed an antioxidant strategy that allows them to cope with quinclorac-induced oxidative stress.

Despite of enhanced controlling the quinclorac-induced oxidative stress, Q cells maintain a lower growth capacity in comparison to NH cells as previously reported by our group (Alonso-Simón et al. 2008). These results point to quinclorac affecting growth of habituated cell lines by mechanisms related neither to cell wall modification nor oxidative damage (Grossmann 2010).

During the habituation process, bean cultured cells have to cope with stepwise increments of quinclorac concentration added to the culture medium. In a way that mirrored the habituation procedure, Q10 and Q15 calluses where subcultured in a medium with 30 μM quinclorac for a short period of time. The results showed that Q10 and Q15 cells responded to this treatment by significantly increasing CIII-POX and SOD activities but not GR activity. In addition, upon 30 μM quinclorac short-time treatment, Q10 and Q15 cells showed a higher staining intensity for IsoPOX II, and the recovery of IsoPOX I. These findings suggest that CIII-POX and SOD antioxidant activities play a primary role in quinclorac habituation process. Interestingly, an association between increased lipid peroxidation levels and low CIII-POX and SOD activities was found in Q10 and Q15 cells.

A short treatment of NH cells with 10 μM quinclorac (NH+10) provoked an enhancement of CIII-POX activity, but GR and SOD activities did not experience any changes (Fig. III.2). These results strengthen the hypothesis that CIII-POX activity is especially responsive to quinclorac-induced stress. Interestingly, same results have already been reported for bean cultured cells habituated to diclobenil (García-Angulo et al. 2009). According to our results, the increment in CIII-POX activity measured in NH+10 cells did not seemed to have the capacity to cope with quinclorac-induced oxidative stress, since NH+10 cells had the highest lipid peroxidation levels (Fig. III.1). Moreover, NH+10 cells had almost half RGR by comparison with NH cells (Table III.1), indicating that quinclorac treatment provokes a reduction in NH cell growth that could be closely related to oxidative stress.

Quinclorac-dehabituated bean cells (DH) were obtained by subculturing Q30 cells in a medium lacking quinclorac for five subcultures. The most remarkable characteristics of DH calluses were: i) a growth rate similar to quinclorac-habituated cells, therefore lower to that found in NH cells (Table III.1), ii) slightly higher lipid peroxidation levels than NH cells (Fig. III.1), iii) higher antioxidant activities than NH calluses but much lower than quinclorac-habituated calluses (Fig. III.2) and iv) the upkeep of the enhanced staining of the two isoPOX (Fig. III.3). These results allow us to suggest that the increase in antioxidant activities that putatively confers habituated cells with the capacity to cope with quinclorac is stable and independent of the presence of quinclorac in the culture medium. Interestingly, an acquired high POX activity has also been found in dichlobenil-dehabituated bean calluses grown in a medium lacking the inhibitor (García-Angulo et al. 2009).

The undetectable levels of CAT activity would indicate that this enzyme does not play a role in quinclorac habituation. This finding is consistent with previous results in which no CAT activity was detected in non-habituated and dichlobenil-

habituated bean cells (García-Angulo et al. 2009). One possible explanation could be the plant material used, as heterotrophic calluses, grown under dark conditions have been reported to have a lower CAT activity than plant tissues (Kim et al. 2004).

We have previously observed that bean calluses can be habituated to herbicides following one or more mechanisms. The habituation of bean calluses to dichlobenil was associated with a high scavenging capacity of ROS, mainly by CIII-POX activity (García-Angulo et al. 2009) and also with the capacity of the cells to divide and expand with a modified cell wall in which the xyloglucan-cellulose network had been partially replaced by pectins (Encina et al. 2001; 2002). However, quinclorac-habituated bean cells have a non-modified cell wall (Alonso-Simón et al. 2008) and, as was observed in this study, this habituation seemed to be related to a high antioxidant capacity. Other herbicides have also provoked oxidative stress in plant materials other than bean cultured cells. This is case for fluroxypyr, where increasing concentrations of this herbicide caused accumulation of various ROS and at the same time reduced the shoot growth of *Oryza sativa*. Fluroxypyr-induced oxidative stress significantly changed SOD, CAT, APOX and POX activities. With the exception of POX activity, the rest of the antioxidant activities showed a general increase at low herbicide concentrations and a decrease at high fluroxypyr levels (Wu et al. 2010).

In sum, based on the observations of the present study, it can be concluded that quinclorac exerts oxidative stress on bean calluses. The acquisition of a prominent antioxidant capacity against quinclorac-induced oxidative stress seems to be an important factor in habituation to quinclorac. Lastly, this adaptative antioxidant response to quinclorac remains stable in the absence of the herbicide.

CONCLUSIONS

The habituation of bean calluses to high quinclorac concentrations (30 μM) was associated with increased constitutive levels of class III-peroxidase, glutathione reductase, and superoxide dismutase activities. These findings correlated with a reduction in the lipid peroxidation level in habituated cell lines, which was always significantly lower than that found in non-habituated cells following short-term treatment with 10 μM quinclorac. Changes in the antioxidant status of bean cells were maintained when quinclorac-habituated cells were cultured in a medium lacking quinclorac.

Author's contribution - ALG: designed research; performed research; analysed data; wrote manuscript; MdC: performed research; revised manuscript; AAS: writing and editing work; PGA: designed research; assisted in performing the research, revised manuscript; JLA: designed research; assisted in performing the research; revised manuscript; AE: provided research opportunity, supervised project, designed research; performed research; analysed data; wrote manuscript; JMA: provided research opportunity, supervised project, designed research, wrote manuscript.

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DISCUSIÓN

Este trabajo se enmarca en la línea de investigación principal del Área de Fisiología Vegetal de la Universidad de León sobre el estudio de compuestos relacionados con la inhibición de la biosíntesis de celulosa y de la plasticidad estructural de la pared celular. De especial importancia ha sido la obtención de cultivos celulares habituados a concentraciones elevadas de estos inhibidores. Como cabría esperar, el mecanismo de habituación depende del tipo de inhibidor y de la especie habituada. De forma general, la tolerancia a estos compuestos radicaba en una serie de procesos metabólicos que se pueden reunir en dos grandes grupos: aquellos que afectan a la pared celular y aquellos otros que atenúan el estrés oxidativo que desencadena su aplicación. El estudio de los primeros refleja la gran plasticidad estructural de la pared celular primaria y ha permitido profundizar en el estudio de los mecanismos moleculares responsables de esa plasticidad. Por otro lado, mediante el estudio de los segundos se puede concluir que existen diversas estrategias de defensa antioxidante que van a depender de la especie y del tipo de inhibidor utilizado, de su concentración y del tiempo de exposición al inhibidor (Encina y col., 2001, 2002; Alonso-Simón y col., 2004, 2007, 2011; García-Angulo y col., 2006, 2009; Melida y col., 2009, 2010a, 2010b, 2011; de Castro y col., 2014; 2015).

La pared celular es una estructura que rodea el protoplasto de las células de las plantas que y posee una alta plasticidad estructural. De esta forma, la pared celular es clave en procesos como la orientación y el crecimiento celular y la diferenciación celular a lo largo de la morfogénesis de la planta. Por tanto, la pared celular regula el crecimiento de la planta y las interacciones de esta con el medio que le rodea. De hecho, las propiedades físicas y el comportamiento mecánico de las plantas frente a cualquier factor externo van a estar determinados por la composición y estructura de su pared celular (Bidhendi y Geitmann, 2015; Wang y col., 2016). De lo expuesto se entiende que la pared celular es una estructura plástica, capaz de modificar su composición y/o arquitectura, lo que va a permitir el crecimiento, supervivencia y adaptación de la planta al ambiente.

Durante las últimas décadas se ha avanzado enormemente en el estudio de la plasticidad estructural de la pared celular, debido al gran interés que tiene este componente celular desde un punto de vista de la biología de las células de las plantas, así como a través de sus aplicaciones en las industrias alimentaria, textil, papelera, energética y de la construcción (Cosgrove y col., 2009; Yang y col., 2013; Burton y Fincher, 2014). Una de las herramientas más utilizadas para el estudio de esta capacidad de remodelación de la pared celular ha sido el uso de un grupo de compuestos catalogados como CBIs (para una revisión Acebes y col., 2011; Álvarez y col., 2012; Tateno y col., 2015). Una consecuencia repetitiva del tratamiento con compuestos como DCB, el isoxabén o la taxtomina A (algunos de los CBIs más utilizados) es un descenso notable en el contenido en celulosa y una remodelación de la arquitectura de la pared celular como respuesta a esa reducción en celulosa (Caño-Delgado y col., 2000, 2003; Bischoff y col., 2009; Gutierrez y col., 2009; Brahbam y Debolt, 2012; García-Angulo y col., 2012; Tateno y col., 2015). Para

ampliar el conocimiento de cómo actúan este tipo de inhibidores y conocer más a fondo qué cambios se provocan en la célula, se ha desarrollado una metodología que consiste en la habituación de cultivos celulares a CBIs, que se consigue mediante el subcultivo repetitivo de células en concentraciones crecientes de los CBIs (Díaz-Cacho y col., 1999; Encina y col., 2001, 2002; Manfield y col., 2004; García-Angulo y col., 2006, 2009; Alonso-Simón y col., 2008; Mélida y col., 2009; Brochu y col., 2010). Con el uso de esta estrategia se puede determinar en mayor medida qué serie de modificaciones tienen lugar en células habituadas a concentraciones letales de estos inhibidores las cuales permiten la supervivencia de estas células en condiciones tan desfavorables. Las modificaciones celulares debidas a la habituación a CBIs pueden ser divididas en dos tipos: (i) aquellas que dependen de la pared celular, que residen en cambios en la estructura y composición de esta estructura y (ii) aquellas independientes de la pared celular, generalmente relacionadas con la limitación de la toxicidad del herbicida. Estas modificaciones van a depender directamente del tipo de pared celular primaria que posean los cultivos, del inhibidor al que se habitúen y de factores como la concentración de este inhibidor y el tiempo de exposición en el que las células van a estar en contacto con el CBI (Shedletzky y col., 1990, 1992; Encina y col., 2001, 2002; Alonso-Simón y col., 2004; de Castro y col., 2014)

La gran mayoría de los estudios de habituación a este tipo de inhibidores se han centrado en el estudio de las modificaciones de la pared celular y fueron realizados principalmente en cultivos de especies que poseen una pared celular primaria tipo I como son el tomate (Shedletzky y col., 1990; Wells y col., 1994), tabaco (Nakagawa y Sakurai, 1998; Sabba y col., 1999), arabidopsis (Manfield y col., 2004), chopo (Brochu y col., 2010) y alubia (Díaz-Cacho y col., 1999; Encina y col., 2001, 2002; Alonso-Simón y col., 2004, 2008; García-Angulo y col., 2006, 2009). En términos generales los cambios en la pared celular que provoca la habituación a estos herbicidas se resumen en la sustitución de la red celulosa-xiloglucano por una red de pectinas más entrecruzadas (Shedletzky y col., 1990; Sabba y Vaughn, 1999; Encina y col., 2001, 2002; Manfield y col., 2004; Alonso-Simón y col., 2004, 2008; García-Angulo y col., 2006, 2009). En el caso concreto de la habituación de cultivos de alubia a DCB, han sido descritas otras modificaciones como la presencia de un β-(1,4)-glucano no cristalino íntimamente asociado a la celulosa, acumulación de aposiciones en la pared celular enriquecidas en pectinas, incremento del grado de entrecruzamiento entre pectinas y xiloglucano, aumento de la actividad xiloglucano endotransglicosidasa, reducción del nivel de fucosilación del xiloglucano, reducción en los niveles de arabinogalactano proteínas, cambios en los niveles de extensina y modificación de la estructura del xiloglucano (Shedletzky y col., 1992; Encina y col., 2002; García-Angulo y col., 2006, Alonso-Simón y col., 2007, Alonso-Simón, y col. 2010)

De entre todos estos trabajos, sólo un par de ellos estudiaron cambios en el metabolismo celular que pudieran estar relacionado con un incremento en la tolerancia al herbicida. García-Angulo y col. (2009) demostraron que la habituación

de células de alubia al herbicida DCB provocaba una reducción en la capacidad de biosíntesis de celulosa y un incremento estable en la actividad CIII-POX que además se mantenía una vez que se retiraba el DCB del medio de cultivo. Esta característica de los cultivos se asoció a la adquisición de una tolerancia al DCB, ya que tanto las células habituadas como las que no tenían DCB en el medio (células deshabituadas) eran más tolerantes a este herbicida. Por otro lado, Brochu y col. (2010) demostraron que células de chopo habituadas y deshabituadas al CBI taxtomina A eran más tolerantes a otros CBIs como el DCB y el isoxaben. Mediante estudios transcriptómicos, estos autores llegaron a la conclusión que las células habituadas y deshabituadas a taxtomina A modificaban de la expresión de genes relacionados con la síntesis de componentes de pared celular y de genes que codificaban enzimas de modificación del DNA y la cromatina, lo que sugiere que la habituación a ese inhibidor provocaba cambios epigenéticos estables, que se mantenían en el proceso de deshabituación y que podrían estar relacionados con esta tolerancia a CBIs (Brochu y col., 2010).

Únicamente se han habituado cultivos celulares de dos especies que poseen una pared celular primaria tipo II, y a un solo CBI, que en este caso es el DCB. Los cultivos celulares que se utilizaron son de cebada (Shedletzky y col., 1992) y de maíz (Mélida y col., 2009, 2010a, 2010b; de Castro y col., 2014, 2015). La habituación de callos de maíz a DCB implicó una serie cambios metabólicos como un descenso en el crecimiento celular, una reducción en el contenido en celulosa y un incremento en el contenido de polisacáridos hemicelulósicos (Mélida y col., 2009). Estas modificaciones han sido también observadas en suspensiones celulares de maíz habituadas a DCB en la presente tesis doctoral. Tanto el tratamiento a tiempos cortos como la habituación a DCB provocaron una reducción de la tasa de crecimiento y una reducción notable en la viabilidad de las células (Figura II.1). La habituación a DCB redujo el contenido en celulosa en pared celular (hasta un 70 % en suspensiones habituadas a DCB -SH6-) en comparación con SNH (Figura I.1; I.3 y Tabla I.1) y esta pared celular fue reforzada mediante el desarrollo de una red extensa de arabinoxilanos más entrecruzados (Figura I.2.C). En línea con resultados obtenidos anteriormente en callos de maíz (Mélida y col., 2010b, 2011), la habituación a DCB también provocó un incremento gradual en el contenido en hidroxicinamatos como el ácido *p*-cumárico, el ácido ferúlico y los diferulatos esterificados a estos arabinoxilanos, lo que explicaría su mayor entrecruzamiento (Tabla I.3). Un dato que destaca frente al resto, es que el tratamiento a tiempos cortos con DCB (SNH+DCB) se asoció a un incremento significativo en la concentración de ácido *p*-cumárico (132 y 15 veces más que las células SNH y SH6, respectivamente; Tabla I.3). La concentración de ácido *p*-cumárico en las células SNH+DCB fue incluso 3 veces mayor en comparación con el contenido en ácido ferúlico y diferulatos (Tabla I.3), compuestos que son mucho más habituales en las paredes celulares primarias (Hatfield y Marita, 2010).

La función del ácido *p*-cumárico no es clara todavía, pero sí que se sabe que no suele estar presente en altas concentraciones en la pared celular, y que no actúa

como molécula de entrecruzamiento fenólico (Hatfield y Marita, 2010). En el caso concreto del maíz, puede aparecer esterificado en la pared celular, pero a lo largo del desarrollo de la planta suele encontrarse en mayor medida asociado a polímeros fenólicos tipo lignina y además se ha propuesto como un cofactor a la hora de polimerizar este tipo de moléculas (Ralph y col., 1994b; Hatfield y col., 2008; Hatfield y Marita, 2010). De la misma forma, se ha comprobado que la presencia de altas concentraciones de ácido *p*-cumárico y ácido ferúlico en pared celular estuvo correlacionada con procesos de lignificación (Zanardo y col., 2009; Hatfield y Marita, 2010). Por lo tanto, una de las posibles explicaciones podría ser que estas paredes celulares con deficiencia en celulosa fueran reforzadas con la acumulación de polímeros fenólicos tipo lignina. Con el objetivo de responder a esta pregunta nos propusimos un análisis en profundidad del componente fenólico de las paredes celulares de líneas tratadas a tiempos cortos con una concentración alta (6 µM, la I_{50} para suspensiones celulares de maíz fue de 0,5 µM de DCB, de Castro y col., 2014) de DCB (SNH+DCB) y líneas habituadas a dos niveles: bajos (1µM de DCB; SH1) y altos (6 µM de DCB; SH6).

Para conseguir una detección de la deposición de este material tipo lignina en las paredes celulares de nuestras líneas celulares se utilizó un marcaje mediante la tinción de Wiesner. Con el uso de esta técnica se demostró aparición de una coloración de tonos rosados y pardos que indicaban la presencia de polímeros tipo lignina en líneas celulares tratadas y habituadas a DCB (Figura I.4; Pomar y col. 2002). Además, cabe destacar que en el proceso de habituación a medida que se redujo el contenido en celulosa, la cantidad de lignina Klason incrementó (Figura I.3), lo que apuntaba al hecho de que las redes de celulosa y lignina están reguladas de manera coordinada, es decir, que deficiencias en uno de los componentes hace que se active la síntesis del otro componente (Denness y col., 2011; Gray y col., 2012). Poniendo atención en el análisis composicional de la lignina en las diferentes líneas celulares podemos concluir que la presencia y proporción de cada monolignol va a depender directamente de la concentración y del tiempo de exposición a DCB (Tabla I.3). Así, el tratamiento de células no habituadas con DCB durante cortos periodos de tiempo (SNH+DCB) se asoció a la formación de un material tipo lignina con una predominancia de unidades S (Tabla I.3). Esto avalaría la hipótesis de que la presencia de ácido *p*-cumárico ayuda a la incorporación de unidades S en la lignina actuando como agente oxidante, ya que las peroxidasas de maíz son ineficientes en la oxidación del sinapil alcohol (Hatfield y col., 2008; Hatfield y Marita, 2010). Por otro lado, las células SH6 son las únicas que poseen un material tipo lignina con unidades S y G con proporción (ratio S/G 1,45, Tabla I.3) similar a la descrita en tallos de maíz (1,4; Fornalé y col., 2012). Por lo tanto, en base a estos resultados, hipotetizamos que el material tipo lignina presente en las células SNH+DCB es una lignina ectópica sintetizada en respuesta a estrés o una "lignina de defensa" y, sin embargo, en las líneas SH6 se trataría de una "lignina estructural" que se sintetiza en las paredes celulares como parte de una respuesta de reforzamiento en una pared celular con bajos niveles de celulosa.

Para profundizar en los cambios metabólicos que dan lugar a la síntesis de este material tipo lignina se realizó un estudio de la expresión de los genes que codifican las enzimas encargadas de los últimos pasos de la ruta de los fenilpropanoides: ferulato 5-hidroxilasa (F5H), cinamoil coA reductasa (CCR) y cinamil alcohol deshidrogenasa (CAD) (Figura 13; Vogt, 2010; Fornalé y col., 2012). La enzima F5H es la encargada de hidroxilar el coniferilaldehído para dar lugar al sinapilaldehído previa metilación (Vogt, 2010; Mansfield y col., 2011). Las dos isoformas de ZmF5H1 y ZmF5H2 fueron sobreexpresadas en la línea SH1 que únicamente poseía unidades S en su material tipo lignina. Sin embargo, la expresión de isoforma ZmF5H1 estuvo muy reprimida en células SNH+DCB y SH6. Por esta razón sugerimos que esta isoforma no estaría implicada directamente en la formación de unidades S ya que esta unidad está presente en el material tipo lignina de todas las líneas tratadas y habituadas a DCB (SNH+DCB, SH1 y SH6; Figura I.6 C y D). En el caso del gen ZmCCR, gen que codifica la enzima que cataliza la conversión de ácido *p*-cumárico y de feruloil CoA en sus respectivos aldehídos, se observó un incremento en la expresión de los dos genes descritos en todas las líneas celulares al compararse con las células no habituadas (ZmCCR1 y ZmCCR2; Figura I.6.A y I.6.B). Por último, la enzima CAD cataliza el paso de los tres hidroxicinamaldehídos a sus correspondientes alcoholes (Vogt, 2010; Fornalé y col., 2012). En este caso se midieron los niveles de expresión de los 7 genes ZmCAD1-7, así como la actividad CAD en todas las líneas celulares (Figura I.7). Atendiendo a los niveles de expresión de los genes ZmCAD, destacó que la sobreexpresión de los genes ZmCAD1 y ZmCAD5 es común en todas las líneas celulares, al igual que la presencia de unidades S en su material tipo lignina (Tabla I.3). Por lo tanto, es probable que estos dos genes, ZmCAD1 y ZmCAD5, estén implicados en la transformación del sinapiladehído en sinapil alcohol. La línea celular SH6 fue la única que presentó unidades G en su material tipo lignina, y, también, fue la única en la que se observó un incremento de la expresión del gen ZmCAD2 (Figura I.7), por lo que sugerimos que este gen sería el responsable de la formación del coniferil alcohol. Además, el gen ZmCAD2 ha sido asociado a la síntesis constitutiva de lignina en maíz (Fornalé y col., 2012) lo que refuerza la hipótesis de que, en esta línea celular, se esté sintetizando un material tipo lignina de carácter estructural. Cabe destacar que en las células SH1 se observó una sobreexpresión de varios genes CAD (ZmCAD1, ZmCAD5, ZmCAD6 y ZmCAD7), pero, sin embargo, no se detectó una mayor actividad CAD (Figura I.7). En consecuencia, se puede asumir que la lignina ectópica presente en paredes de células SH1 incorporara una mayor cantidad de cinamaldehídos y otros componentes fenólicos en este material tipo lignina como ocurre en plantas con la expresión de CAD modificada (Ralph y col. 2001; Dauwe y col., 2007; Fornalé y col., 2012)

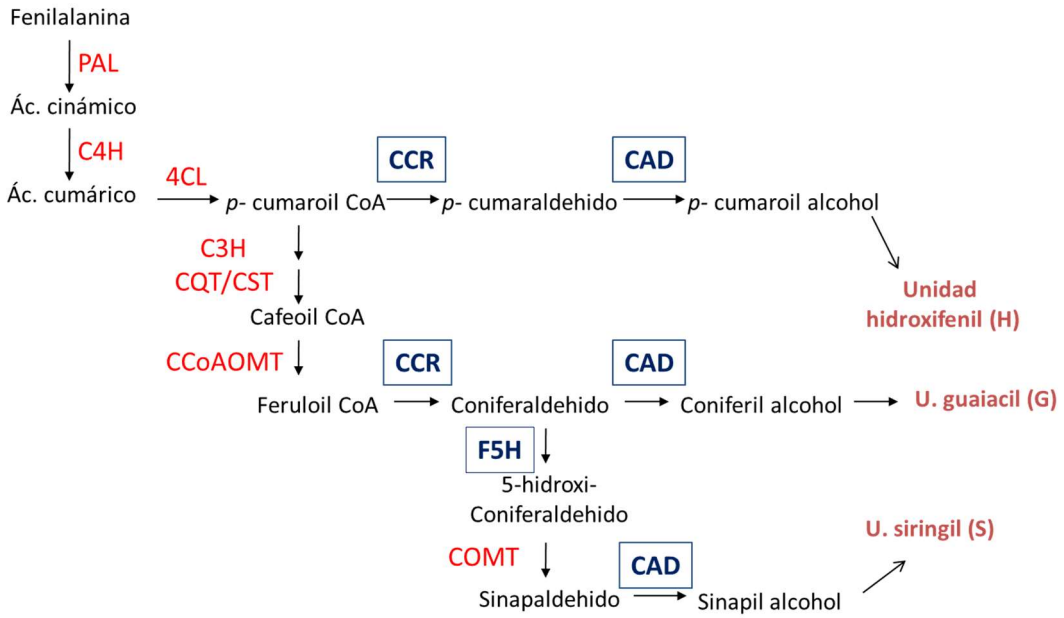

Figura 13. Esquema de la ruta de los fenilpropanoides. Modificado de Li y col., 2010 y Mottiar y col., 2016. Descripción de las abreviaturas de las enzimas que participan en la ruta están presentes en la Figura 7.

La acumulación de lignina en tipos celulares que no desarrollan pared celular secundaria como las células en cultivo, también denominada lignificación ectópica, había sido descrita en mutantes de *Arabidopsis* (*cev1* y *eli1*) con contenido reducido en celulosa y, además, demostraron que este proceso de lignificación ectópica estaba acompañada por una activación constitutiva de la ruta de señalización de estrés mediadas por ácido jasmónico (JA) y una acumulación de JA y etileno (Ellis y Turner, 2001; Ellis y col., 2002; Caño-Delgado y col., 2003). De la misma forma, el tratamiento de plántulas de *Arabidopsis* con isoxabén provocaba respuestas muy similares a la descritas en el mutante *eli1* (Caño-Delgado y col., 2003; Hamann y col., 2009). Además, en estudios posteriores, se ha demostrado que este proceso de lignificación ectópica está regulado por especies reactivas de oxígeno (ROS) y JA en dos etapas (Hamann y col., 2009; Denness y col., 2011). Uno de los factores necesarios para que se desencadene la lignificación ectópica es una explosión oxidativa ("oxidative burst") generada por la enzima NADPH oxidasa *Respiratory Burst Oxidase Homolog D* (RBOHD) (Hamann y col., 2009; Dennes y col., 2012).

En nuestras líneas celulares, el proceso de lignificación ectópica estuvo regulado por la ruta de señalización mediada por JA, ya que se observó un incremento de la expresión de genes de síntesis (ácido 12-oxofitodienoico reductasa; OPR) de esta hormona (ZmOPR1, ZmOPR2 y ZmOPR7) y de genes de respuesta a la acumulación de JA (ZmMPI), tanto en las líneas tratadas con DCB como en las habituadas al inhibidor (Figura I.8). Además, se detectó una sobreexpresión, en SNH+DCB, SH1 y SH6, de un gen que codifica para una NADPH oxidasa (ZmNADPHOX), enzima que ha sido relacionada con respuestas a estrés (Figura I.8; Shivaji y col., 2010; Kärkönen y Kuchitsu). La secuencia amplificada en el

presente trabajo (CK849936) pertenece a un EST de un gen de maíz (Unigene, LOC100136880 (umc1051)) actualmente descrito como una NADPH oxidasa *Respiratory Burst Oxidase-like protein D* (RBOHD; NM_001164287.1). Así pues, tanto el tratamiento, como la habituación de células de maíz a DCB provocaron la sobreexpresión de una NADPH oxidasa (RBOHD) que tendría una implicación en la modulación de la deposición de lignina ectópica (Figura I.8; Hamann y col., 2009; Denness y col., 2012).

Por otro lado, también se ha propuesto el NADPH como donador de electrones (vía NADPH oxidasas de la familia RBOH) para el oxígeno molecular (O_2) presente en el apoplasto, dando lugar a la formación de anión superóxido (O_2^-), el cual será posteriormente dismutado, espontáneamente o mediado por la enzima superóxido dismutasa (SOD), en peróxido de hidrógeno (H_2O_2) y O_2 (Kärkönen y Kuchitsu, 2015). Por tanto, el incremento de la expresión del gen ZmNADPHOX (RBOHD) podría estar también implicado en el incremento del H_2O_2 en el apoplasto que se observó en todas las líneas celulares (Tabla I.5). El aumento de la concentración de H_2O_2 apoplástico indica que esta molécula podría estar siendo utilizada por CIII-POX para polimerizar el material tipo lignina (Fagerstedt y col., 2010; Novo-Uzal y col., 2013; Kärkönen y Kuchitsu, 2015; Francoz y col., 2015) o para incrementar el entrecruzamiento de los arabinoxilanos mediante el acoplamiento oxidativo de los restos ferúlico presentes en estas moléculas (Ralph y col., 2004b; Encina y Fry, 2005; Lindsay y Fry., 2008). Cabe destacar que las células SH1 son las que mayor contenido en H_2O_2 apoplástico poseen (Tabla I.5) y, sin embargo, no es la línea celular con mayor contenido en lignina Klason (Figura I.3), apuntando a que la concentración de H_2O_2 no sería un factor limitante en la síntesis del material tipo lignina.

Como ya hemos indicado anteriormente el proceso de habituación a DCB y los cambios a nivel metabólico provocados por ella van a depender directamente de la concentración de DCB presente en el medio y del tiempo en el que las células van a estar presentes a este inhibidor (Alonso-Simón y col., 2004; de Castro y col., 2014). Una característica especial que tienen los cultivos durante los primeros pasos de la habituación a DCB es que, a diferencia de lo que ocurre con células habituadas a altos niveles de DCB, la reducción del contenido en celulosa revierte a lo largo de los subcultivos hasta llegar a tener unos niveles similares a las células no habituadas (Alonso-Simón y col., 2004; de Castro y col., 2014). Por lo tanto, las modificaciones de la pared celular durante los primeros pasos de habituación a DCB no son estables, lo que lleva a pensar que es probable que exista un mecanismo independiente de las modificaciones en la pared que permita a las células crecer bajo la presencia de DCB.

Esta hipótesis se refuerza cuando se observan las cinéticas de crecimiento de células de maíz tratadas con, y habituadas a bajos niveles de DCB (Figura II.1). El tratamiento de células de maíz con 0,5 y 1 µM de DCB provocó una reducción drástica, tanto de la tasa de crecimiento relativa como de la viabilidad celular. Sin

embargo, después de 6 subcultivos bajo la misma concentración de DCB en el medio de cultivo, estas células recuperaron parcialmente su tasa relativa de crecimiento y también incrementaron considerablemente su viabilidad (Figura II.1).

En un intento de esclarecer los mecanismos asociados a la habituación a altos niveles de DCB y teniendo en cuenta que la habituación de cultivos de alubia a DCB provocó un incremento estable en la actividad CIII-POX (García-Angulo y col., 2009), Melida y col. (2010a) investigaron la posibilidad de que el desarrollo de un sistema de defensa antioxidante fuera uno de los mecanismos que participaran en la habituación a DCB. Sin embargo, la habituación de cultivos de maíz a altos niveles de DBC no provocaba un incremento en las actividades antioxidantes estudiadas (Mélida y col., 2010a). Por otro lado, es interesante que el tratamiento de células de maíz no habituadas con altas concentraciones de DCB sí que provocaba un incremento de las actividades antioxidantes/detoxificadoras estudiadas (Mélida y col., 2010a).

Teniendo en cuenta estas premisas y la posibilidad de que la presencia de ROS sea necesaria para que en células tratadas y habituadas a DCB se produzca una lignificación ectópica, se procedió a realizar una medida del contenido en H_2O_2 en el medio de cultivo de células de maíz tratadas con 0,5 y 1 µM de DCB y habituadas a 0,3, 0,5 y 1 µM de DCB (Figura II.3). Los resultados demostraron que en todas las líneas celulares se produce un incremento significativo del contenido en H_2O_2 en todas las etapas de crecimiento celular (Figura II.3). Por otro lado, el estudio de los niveles de peroxidación lipídica como marcador del daño oxidativo reveló que sólo se producía un daño oxidativo en los tratamientos con DCB a tiempos cortos y en la línea SH1 (Figura II.2), lo que nos indica la existencia de un mecanismo de control del daño oxidativo relacionado con el proceso de habituación a bajas concentraciones de DCB.

El estudio de tal mecanismo durante la habituación incipiente de células de maíz a DCB permitió detectar un aumento de la actividad de enzimas antioxidantes, principalmente APOX y CIII-POX, que dependió directamente de la concentración de este inhibidor (Figura II.4). Estos resultados indican que ambas actividades antioxidantes, podrían ser las encargadas de mantener los niveles de ROS lo suficientemente bajos para controlar el daño oxidativo inducido por el DCB (Figura II.2). Como hemos indicado anteriormente, la actividad CIII-POX está relacionada con procesos de remodelación de la pared celular (Kärkönen y Kuchitsu, 2015; Francoz y col., 2015). Sin embargo, en la habituación a altos niveles de DCB no se observó un incremento de esta actividad (Mélida y col., 2010a). Por lo tanto, sugerimos que la actividad CIII-POX en habituación incipiente a DCB no estaría relacionada con la remodelación de la pared celular, sino que participaría en el control del balance de ROS. En apoyo de esta hipótesis está el hecho de que en las células tratadas con DCB es la única actividad antioxidante que incrementa significativamente (Figura II.4B).

Tanto las células tratadas con DCB, como la habituación a bajos niveles de DCB, provocaron un incremento en el contenido en el TG y un aumento del ratio GSH/TG (Figura II.6). Teniendo en cuenta que la actividad glutatión reductasa (GR, responsable del incremento de GSH por reducción de GSSG) no experimenta ningún cambio significativo (Figura II.4D), y que, además, estas líneas celulares presentan mayor actividad GST (Figura II.5) la explicación más plausible es la síntesis *de novo* de GSH, proceso que ha sido ampliamente relacionada con procesos de estrés abiótico (Szalai y col., 2009). El incremento en la concentración de GSH, y un aumento marcado en la actividad GST, sobre todo en las células tratadas a tiempos cortos (SNH+DCB(0,5) y SNH+DCB(1)), indican que durante la habituación incipiente a DCB, se pueda estar dando un proceso de detoxificación mediante la conjugación del DCB con GSH. De hecho, proponemos que la detoxificación del DCB podría explicar parcialmente la reversión del contenido en celulosa que se produce en los primeros pasos de habituación a DCB (de Castro y col., 2014).

Tanto los tratamientos como la habituación a bajas concentraciones de DCB provocaron fuertes reducciones en la actividad CAT, como ya se había descrito para la habituación a altos niveles de DCB (Mélida y col., 2010a), y en el contenido en AA (Figura II.4C, Figura II.7). La reducción de la actividad CAT ha sido ampliamente relacionada con una acumulación de H_2O_2 y una acumulación de GSH (Smith, 1985; Queval y col., 2009; Noctor y col., 2012).

A modo de resumen, proponemos que las células de maíz tratadas con DCB (SNH+DCB) presentan un incremento en la actividad GST, como respuesta a la presencia de DCB en el medio de cultivo. La estrategia de detoxificación no es lo suficientemente potente como para eliminar completamente el inhibidor y se produce una inhibición en la biosíntesis de celulosa dependiente de DCB. Esta pérdida en la integridad de la pared celular desencadena una seria de respuestas como un estallido oxidativo mediado por una NADPH oxidasa (RBOHD) que hace que se incremente el contenido en H_2O_2 en el apoplasto. A partir de aquí, se desencadena una respuesta antioxidante que no es capaz de controlar la formación de ROS, ya que provoca un daño oxidativo y, consecuentemente, una reducción significativa de la tasa de crecimiento y viabilidad celular. Todo este proceso estaría acompañado por la síntesis de un material tipo lignina principalmente compuesto por unidades S, una composición atípica, por lo que proponemos que está lignificación ectópica se está produciendo como una respuesta de defensa a la pérdida de integridad de pared celular.

En el caso de las células habituadas a concentraciones bajas de DCB el incremento de la actividad GST parece contribuir a la detoxificación eficiente del DCB. De esta manera se podría explicar que las reducciones del contenido en celulosa revirtieran hasta niveles presentes en células no habituadas. Aun así, la presencia de este inhibidor provoca un incremento en H_2O_2 que es eficazmente controlado por el incremento de actividades antioxidantes, ya que estas células poseen niveles de peroxidación lipídica similares a las células no habituadas. Un

caso especial es el de la línea celular SH1, ya que parece ser que, a este nivel, la enzima GST no detoxifica eficientemente el DCB y, por lo tanto, las células no son capaces de revertir esta disminución en el contenido en celulosa (de Castro y col., 2015). También se provoca un incremento en el contenido en H_2O_2 (probablemente mediado por la NADPH (RBOHD)) y un posterior incremento de las actividades antioxidantes el cual no es suficiente para controlar esta formación de ROS ya que se provoca daño oxidativo. Este incremento en H_2O_2 apoplástico probablemente esté siendo sustrato de peroxidasas en el apoplasto para dar lugar a un reforzamiento de la pared celular deficiente en celulosa mediante el entrecruzamiento de los arabinoxilanos vía formación de diferulatos o la síntesis de un material tipo lignina también de composición atípica.

Por último, en el caso de células habituadas a altas concentraciones de DCB la reducción del contenido en celulosa llegó a ser hasta del 70 %. Se produce un incremento en la concentración de H_2O_2 y no hay un incremento en la estrategia antioxidante, lo que que este H_2O_2 presente en el apoplasto es un compuesto que está siendo utilizado para remodelar su pared celular y para la síntesis de un material tipo lignina con unas características más similares a una lignina estructural.

El quinclorac es un herbicida auxínico muy selectivo y ampliamente usado para el control de malas hierbas dicotiledóneas (Grossmann, 2000, 2010). El modo de acción de este herbicida no está completamente esclarecido y ha sido objeto de debate en los últimos años. Koo y col. (1996, 1997) propusieron que el quinclorac provocaba una reducción de la incorporación de [^{14}C]glucosa en paredes celulares de diferentes especies y, por lo tanto, que alteraba la formación de la pared celular. Por este motivo este herbicida fue considerado como CBI (Vaughn, 2002). Sin embargo, otros autores indicaron que el quinclorac no producía una inhibición en la síntesis de celulosa y que todos los cambios que provocaba en la pared celular eran debidos, en mayor medida, a un efecto colateral (Tresch y Grossmann, 2003). En un intento de demostrar si el quinclorac inhibía la síntesis de celulosa, nuestro grupo trató suspensiones celulares de alubia con concentraciones de quinclorac que reducían en un 50 % su crecimiento (I_{50}, 10 µM) y se comprobó que no solo no disminuía la incorporación de [^{14}C]glucosa en la pared celular, sino que esta incorporación aumentaba (García-Angulo y col., 2012). Para ahondar en el estudio de las modificaciones que provocaba en la pared celular, se procedió a habituar callos de alubia a concentraciones crecientes del este herbicida (Alonso-Simón y col., 2008). El estudio de la habituación a quinclorac reveló que no se producía un descenso en la concentración de celulosa en paredes celulares y que los cambios en la distribución y estructura del homogalacturonano y del ramnogalacturonano I podían atribuirse a un efecto secundario a la presencia de quinclorac (Alonso-Simón, 2008). En resumen, la habituación de cultivos de alubia a quinclorac no parecía estar relacionada con modificaciones en la estructura o composición de la pared celular.

Se ha descrito que en plantas sensibles a quinclorac, el tratamiento con dicho compuesto provoca un incremento en la producción de ROS dependiente de auxinas, lo cual podría estar relacionado con el efecto fitotóxico de este herbicida (Grossman y Scheltrup, 1997; Grossmann y Kwiatkwoski, 2000; Grossmann, 2001; Abdallah y col., 2006; Navrot y col., 2007; Yashuor y col., 2012). Mediante el estudio de especies o biotipos resistentes, se demostró que la resistencia a quinclorac no residía en cambios en su absorción, translocación o metabolismo, sino en el control de la producción de ROS y por tanto en la reducción del daño oxidativo (Van Eerd y col., 2005; Sunohara y Matsumoto, 2004; 2008; Abdallah y col., 2006; Sunohara y col., 2010, 2011; Yashuor y col., 2012).

El tratamiento a tiempos cortos de callos de alubia no habituados con 10 µM de quinclorac (I_{50}, Alonso-Simón y col., 2008) provocó una reducción del 50% en la tasa de crecimiento relativo celular (Tabla III.1). Esta reducción puede ser debida al incremento en la producción de ROS, ya que estas células presentan un incremento significativo en los niveles de peroxidación lipídica (Figura III.1). Así pues, atendiendo al estudio de las actividades antioxidantes, el tratamiento a tiempos cortos con quiclorac provocaría un incremento en la actividad CIII-POX (Figura III.2) incapaz de controlar el daño oxidativo.

Sin embargo, durante el proceso de habituación de células de alubia a concentraciones crecientes del herbicida se observó un aumento gradual de la respuesta antioxidante, siendo la línea Q30 la que poseía mayor actividad en las tres enzimas antioxidantes estudiadas, CIII-POX, APOX y GR (Figura III.2). Además, se observaron cambios cualitativos y cuantitativos en el perfil de isoformas de peroxidasas: aparición de la iso-POX I en la línea celular Q30 (ausente en Q10 y en Q15) y un incremento en la intensidad de la tinción en las dos bandas isoPOX encontradas en esa línea celular (Figura III.3). Este incremento en la respuesta antioxidante asociado a la habituación a concentraciones crecientes de quinclorac redujo también de forma gradual el daño oxidativo (Figura III.1). Teniendo en cuenta estos resultados, nosotros proponemos que las células de alubia habituadas a quinclorac han desarrollado una estrategia antioxidante que les permite hacer frente al estrés oxidativo inducido por quinclorac.

Aunque el incremento de las actividades antioxidantes provocó una reducción del daño oxidativo en las células habituadas al quinclorac, este sistema de defensa no fue suficiente para obtener una recuperación de la tasa de crecimiento celular (Tabla III.1). Interpretamos, por tanto, que el quinclorac reduce el crecimiento de las líneas celulares habituadas a través de un mecanismo independiente de modificaciones en la pared celular o del daño oxidativo (Grossmann, 2010).

Para intentar entender mejor cómo responden las actividades antioxidantes durante el proceso de habituación a concentraciones crecientes de quinclorac, evaluamos el cambio en la capacidad antioxidante de las células cuando las líneas celulares Q10 y Q15 eran tratadas con quinclorac 30 µM. Los resultados indicaron

que tras este tratamiento se inducía un incremento en las actividades CIII-POX y SOD (Figura III.2). Además, el tratamiento también se asoció a la aparición de la isoPOX I (no presente en las líneas Q10 y Q15) y a un incremento en la intensidad de la tinción de las dos isoPOX (Figura III.3). Curiosamente, la peroxidación lipídica presente en estas células es similar a la encontrada en células no habituadas (Figura III.1), lo que pone de manifiesto la implicación de estas dos enzimas en el proceso de habituación a quinclorac.

Por último, la retirada del quinclorac en células deshabituadas (DH) reflejó un descenso notable en las tres actividades antioxidantes, pero, aun así, los niveles de actividad fueron significativamente mayores que las células NH (Figura III.2). Un dato interesante es que las células DH mantuvieron una alta intensidad de la tinción de las dos isoPOX (Figura III.3). Este menor desarrollo de una estrategia antioxidante provocó un ligero incremento en el daño oxidativo en células DH (Figura III.1). Sorprendentemente, las células deshabituadas incrementaron su tasa de crecimiento, en comparación con la medida en las células habituadas, pero no llegaron a tener una reversión de la tasa de crecimiento al nivel de las células NH (Tabla III.1). Estos resultados nos vuelven a indicar que debe existir otro mecanismo de acción del quinclorac independiente del desarrollo de un sistema de defensa antioxidante, ya que las células deshabituadas sufren mayor daño oxidativo el cual no afecta a su tasa de crecimiento.

Un dato remarcable es que en todos los tratamientos que suponían un incremento en la concentración de quinclorac al que se exponían las células (tanto NH+10, como Q10+30 y Q15+30) se observó un incremento en la actividad CIII-POX (Figura III.2C), lo que parece apuntar a que esta enzima está íntimamente ligada al proceso de habituación a quinclorac. De igual manera, la actividad CIII-POX parece ser clave en el proceso de habituación al DCB (Figura II.4; García-Angulo y col., 2009).

Los resultados obtenidos en el presente trabajo confirman que los mecanismos de tolerancia de células de maíz a DCB van a depender de la concentración y del tiempo de exposición a este inhibidor. Durante el proceso de habituación incipiente de maíz a DCB las células la adaptación reside en el desarrollo de una estrategia antioxidante y detoxificadora. Cuando la concentración y/o el tiempo de exposición de las células a DCB aumenta, se provoca una reducción del contenido en celulosa que desencadena una serie de mecanismos compensatorios los cuales se pueden resumir en el incremento de una red de arabinoxilanos más entrecruzados y la deposición de un material tipo lignina. Aunque no sea la primera vez que se caracterice lignina presente en células con pared celular primaria, sí que es la primera vez que se caracteriza la composición y síntesis de lignina ectópica en respuesta a deficiencia en celulosa en paredes celulares. Por otro lado, se ha demostrado la implicación del sistema de defensa antioxidante en la habituación de células de alubia a quinclorac.

CONCLUSIONES

1. La habituación de suspensiones celulares de maíz a DCB provocó la reducción de hasta un 70 % en el contenido en celulosa en sus paredes celulares. En condiciones de deficiencia en celulosa, las células habituadas a DCB presentaron una pared celular modificada en la que destacaba el incremento en el contenido en arabinoxilanos. El estudio del perfil fenólico de las paredes de células habituadas reveló un aumento en la concentración de fenóles monoméricos (ácido *p*-cumárico y ácido ferúlico) y dímeros de ácido ferúlico que aparecieron esterificados a la red de arabinoxilanos, incrementando su entrecruzamiento. El incremento de hasta 132 veces en la concentración de ácido *p*-cumárico en células con un tratamiento a tiempo corto con DCB indica que este compuesto fenólico puede tener una implicación en el proceso de habituación de células de maíz a DCB.

2. Tanto el tratamiento a tiempo corto como la habituación de células de maíz a DCB, provocaron la deposición de lignina ectópica en respuesta a una deficiencia en celulosa. El material tipo lignina presente en células tratadas a tiempo corto y en habituación incipiente a DCB estuvo compuesta únicamente por unidades S, mientras que el material estudiado en células habituadas a altos niveles de DCB presentó una composición monomérica con un ratio S/G de 1,45, similar a la lignina estructural descrita en paredes secundarias de plantas de maíz. En base a estos resultados se concluye en la existencia de dos tipos de lignificación en nuestro sistema experimental: lignificación de defensa en células con habituación incipiente (y células tratadas con DCB) y lignificación estructural en células habituadas a largo plazo.

3. El estudio de los niveles de expresión de genes de síntesis de monolignoles (ferulato 5-hidroxilasa, cinamoil coA reductasa y cinamil alcohol deshidrogenasa) fue un indicio de la existencia de una alta regulación a nivel transcripcional de estos genes durante el proceso de habituación a DCB. La sobreexpresión de genes de síntesis de ácido jasmónico y de un gen NADPH oxidasa de la familia *RBOHD* indicaron la implicación de las rutas de señalización por especies reactivas de oxígeno y ácido jasmónico en la regulación del proceso de lignificación ectópica en respuesta a la deficiencia en celulosa provocado por la habituación de células de maíz a DCB.

4. El tratamiento a tiempos cortos de células de maíz con DCB provocó una reducción del crecimiento y de la viabilidad celular como consecuencia de un incremento significativo del nivel de estrés oxidativo reflejado en el aumento de los niveles de peroxidación lipídica y del incremento en el contenido de H_2O_2 apoplástico (presente en el medio de cultivo).

5. Las células tratadas con DCB presentaron una estrategia antioxidante y detoxificadora mediada por un incremento en la actividad peroxidasas de la clase III, glutatión S-transferasa y la presencia de altos niveles de glutatión total. Las células con habituación incipiente, mantuvieron un incremento en el contenido en

glutatión total y en la actividad glutatión S-transferasa y se observó un aumento en las actividades peroxidasas de la clase III y ascorbato peroxidasa. A partir de los resultados obtenidos en el presente trabajo se puede concluir que el desarrollo de una estrategia antioxidante y detoxificadora resultaría un factor determinante en la habituación incipiente al DCB.

6. El proceso de habituación al quinclorac en cultivos celulares de alubia reside, al menos parcialmente, en el desarrollo de una estrategia antioxidante que permite a las células controlar el estrés oxidativo asociado al tratamiento con quinclorac. Proponemos que las actividades peroxidasa de la clase III y superóxido dismutasa tienen un papel central en la habituación a quinclorac. La estrategia antioxidante presente en células habituadas a quinclorac es estable ya que se conservó una vez retirado el inhibidor del medio de cultivo.

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Fourier transform mid infrared spectroscopy applications for monitoring the structural plasticity of plant cell walls

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Fourier transform mid-infrared (FT-MIR) spectroscopy has been extensively used as a potent, fast and non-destructive procedure for analyzing cell wall architectures, with the capacity to provide abundant information about their polymers, functional groups, and *in muro* entanglement. In conjunction with multivariate analyses, this method has proved to be a valuable tool for tracking alterations in cell walls. The present review examines recent progress in the use of FT-MIR spectroscopy to monitor cell wall changes occurring *in muro* as a result of various factors, such as growth and development processes, genetic modifications, exposition or habituation to cellulose biosynthesis inhibitors and responses to other abiotic or biotic stresses, as well as its biotechnological applications.

Keywords: cell wall, stress, mutants, development, FT-MIR spectroscopy

INTRODUCTION

The plant cell wall is a very complex structure, mainly formed by carbohydrates and proteins, which surrounds the plant's protoplasts. This structure not only determines a cell's size and shape, but also provides protection against stresses and biological damage (Seifert and Blaukopf, 2010).

The importance of plant cell wall is twofold: on the one hand, it has many applications for human society, providing raw material which can be processed to make textiles, paper, wood, livestock feed, dietary fiber, fuel, etc. (Harris et al., 2010). On the other hand, its structure is incredibly resistant to both physical and chemical stresses while at the same time being extremely plastic, endowing cells with the capacity to adapt to different situations by modifying its composition and structure (Cheung and Wu, 2011). Therefore, knowledge of the mechanisms by which such adaptations occur could facilitate the design of cell walls with compositions adapted to different specific uses.

However, its complex structure and composition, as well as its resistance, render the plant cell wall a difficult target to study. There are many different molecules in its structure which may be linked with different kind of bonds, from weaker hydrogen bonds to stronger ester o even ether bonds. Moreover, all these molecules are arranged in different network which are not totally independent from each other (Fry, 2011). Thus, the main scaffold of plant cell wall is formed by cellulose microfibrils, linked to hemicelluloses by hydrogen bonds. This cellulose-hemicellulose network is embedded in a gel of matrix pectins, including simple and more complex homogalacturonans, rhamnogalacturonans, arabinans, galactans, and arabinogalactans. Several structural proteins and a set of enzymes also form part of the plant cell wall (Carpita and Gibeaut, 1993).

As a result of its structural complexity, research into the plant cell wall is extremely challenging, and even more so given how time-consuming it is to isolate and subsequently extract and fractionate its different components. These procedures require large amounts of sample and involve the use of contaminant solvents and harsh conditions, which may alter the native structure of plant cell wall components during isolation. Thus, fourier transform mid-infrared (FT-MIR) spectroscopy (also known as FTIR spectroscopy) has been extensively used to analyze plant cell walls (Dokken et al., 2005; Alonso-Simón et al., 2011). In contrast with other analytical methods, FT-MIR analysis is a fast procedure that provides information of polysaccharides *in muro*, without the need to extract or solubilize- and therefore alter- any cell wall component (**Figure 1**). The chemical specificity of mid-infrared region allows identification of certain peaks related to cell wall components (Smith-Moritz et al., 2011; **Table 1**). Moreover, carbohydrates show high absorbance in the 1,200–950 cm^{-1} region, known as the fingerprint region, where the position and intensity of the bands is specific for each polysaccharide (Oliveira et al., 2009). In addition, this technique only requires a small amount of sample, and may even be combined to optical microscopy (FT-MIR microspectroscopy) to analyze small areas of the plant cell wall. In this case, a microscope accessory is attached to the FTIR device in such a way that the infrared beam from the spectrometer is diverted to pass through a sample placed in the microscope stage. Thus, the sample can be mapped, moving it under computer control such that different areas of the sample are measured in turn, generating an array of spectra. These spectra can be correlated with visual images of the sample, so that optically observed features can be associated with functional groups (McCann et al., 2001).

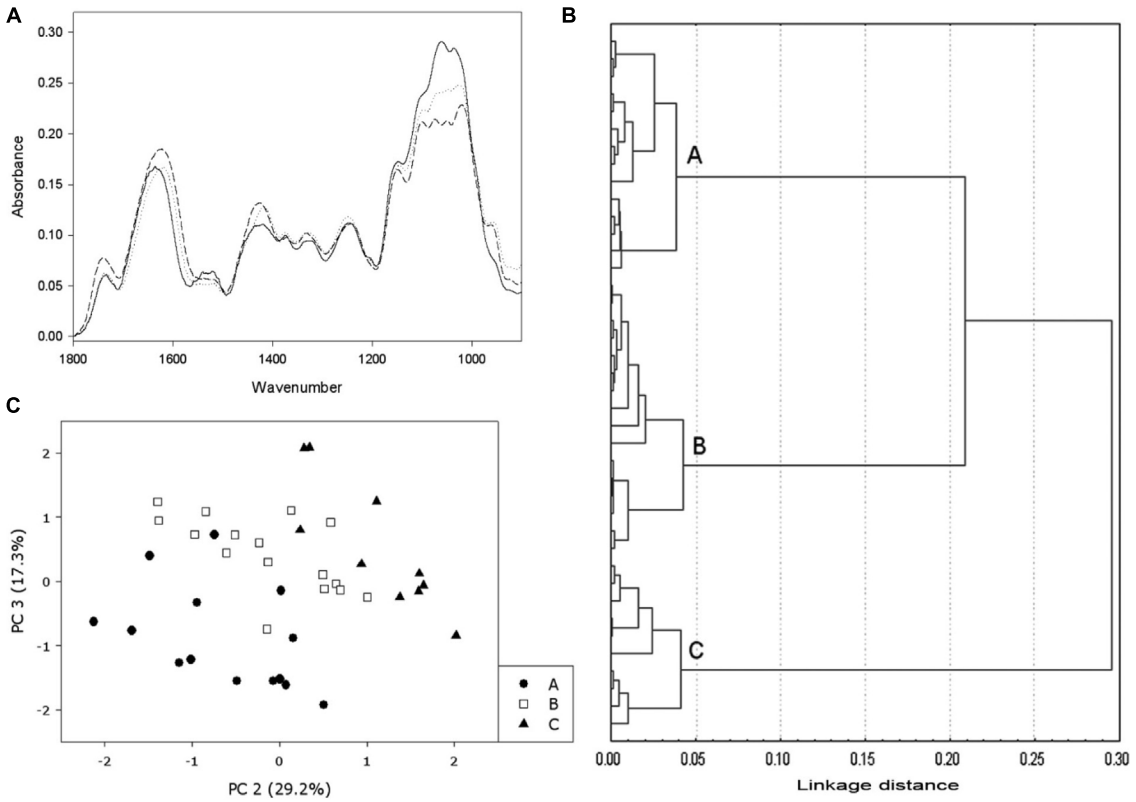

FIGURE 1 | Example of FT-MIR use in the monitorization of DCB-habituated cells. (A) FT-MIR spectra from different types of cells: non-habituated cells (solid line), low level of habituation (dotted line) and high level of habituation (dashed line). **(B)** Cluster analysis performed on several spectra from non-habituated cells and DCB-habituated cells, growing on concentrations ranging from 0.5 to 12 μM; the analysis made three different groups: non-habituated (group A), low level of habituation (group B) and high level of habituation to DCB (group C). **(C)** Principal Component Analysis (PCA) performed on the same bulk of spectra. The same groups of cluster analysis can be observed. (Data from Alonso-Simón et al., 2004).

Besides FT-MIR, which encompasses the wavelength region over the range of 400–4000 cm^{-1} (25000–2500 nm), FT-NIR, which includes the 4000–12000 cm^{-1} wavelength region (700–2500 nm), has also been applied in order to identify plant cell wall variations associated with specific monosaccharide dissimilarities and thus identify cell wall mutants from a potential mutant population (Smith-Moritz et al., 2011).

Despite recent significant improvements, the application of FT-MIR analysis may also present certain limitations due to the huge amount of data included in a single spectrum, and also because of overlapping bands corresponding to different plant cell wall components. The combined use of FT-MIR with statistical tools, mainly multivariate analysis, has improved interpretation of the obtained results, especially when analyzing a large number of spectra (**Figure 1**). Thus, the combination of features such as rapidity, low amount of sample required and the huge amount of data obtained from this analysis, renders FT-MIR in conjunction with multivariate analysis a valuable tool for tracking or monitoring alterations in plant cell walls in response to different situations. In this review, we examine recent use of FT-MIR spectroscopy to monitor plant cell wall modifications occurred not only during processes such as growth and development, but also as a consequence of mutation or overexpression of cell wall-related genes and in response to different kinds of biotic and abiotic stress, paying special attention to exposure or habituation to cellulose biosynthesis inhibitors (CBIs).

THE USE OF FT-MIR SPECTROSCOPY TO STUDY CHANGES IN CELL WALL COMPOSITION AND ARCHITECTURE RELATED TO GROWTH AND DEVELOPMENT

Over the last two decades, a considerable amount of research has been conducted on the involvement of the cell wall in plant growth and development. FT-MIR has proved to be a useful tool for monitoring and/or corroborating cell wall changes related to diverse stages of plant development.

Fourier transform mid-infrared spectroscopy has been used successfully to characterize growth-related modifications in the composition and structure of a wide range of cell types and cultures. Using this approach, growth throughout the culture cycle of *Arabidopsis* suspension-cultured cells has been elucidated, indicating that the mechanical properties of the cell wall correlate with its composition during cell growth. The main modifications revealed by FT-MIR are a loss of lignin (revealed by a decrease in the band between 1530 and 1540 cm^{-1}; **Table 1**) and a predominance of polysaccharides (denoted by increases in the bands at 1040, 1060,

Table 1 | A summary of assignment of wave numbers obtained by FT-MIR spectroscopy to main cell wall components.

Assigned cell wall component	Wavenumber (cm^{-1})	Reference
Protein	1550, 1650	Séné et al. (1994), Wang et al. (2009b)
Pectins	952, 1014, 1097, 1104, 1146, 1243	Coimbra et al. (1999), Kacuráková et al. (2000)
De-esterified pectins (carboxylic acid).	1420, 1600–1630	Mouille et al. (2003)
Esterified uronic acid	1740	McCann et al. (1992)
Pectin's acetylester	1017, 1047, 1100	Gou et al. (2008)
Lignin	1510, 1530–1540, 1595	Rodrigues et al. (1998)
Syringyl units of lignin	1330	Faix (1992)
Guaiacyl units of lignin	1270	Faix (1992)
Phenolic compounds	1430	Séné et al. (1994)
Phenolic ring	1515, 1630	Séné et al. (1994), Carpita et al. (2001)
Phenolic ester	1720	Séné et al. (1994), Carpita et al. (2001)
Cellulose	900, 990, 1040, 1060, 1109, 1160, 1320, 1367	Carpita et al. (2001), Brown et al. (2005), Rubio-Díaz et al. (2012)
Xyloglucan	1041, 1078, 1120, 1317, 1371	Coimbra et al. (1999), Kacuráková et al. (2000)
Arabinogalactan	1043, 1078, 1139	Barbosa et al. (2013)
Glycerolipids and wax hydrocarbons	1470, 2850, 2920	Kanter et al. (2013)
Wax- or suberin-like aliphatic compounds	1318, 1372, 1734–1745	Zeier and Schreiber (1999), Wang et al. (2009a)

1160, and 1245 cm^{-1}) as cell growth progresses (Radotić et al., 2012).

In terms of specific stages of plant cell growth and development, FT-MIR spectroscopy has contributed to characterize changes in cell walls during seed development and germination. More specifically, cell wall differences between hard and soft wheat (*Triticum aestivum*) endosperm textures, as well as cell wall modifications during wheat grain development, have been studied by means of FT-MIR microspectroscopy and subsequent statistical and imaging analysis (Barron et al., 2005). These techniques have demonstrated that the main difference between the two endosperm textures is the presence of higher amounts of a water-extractable arabinoxylan in the peripheral endosperm of soft grains. Furthermore, this technique has revealed marked differences in the ratio of β-1,3-1,4 glucans / arabinoxylans, and in the arabinoxylan structure, depending both on cell position within the endosperm grain and the stage of development (Saulnier et al., 2009).

Similarly, the use of FT-MIR microspectroscopy and statistical analysis to study the involvement of cell wall components in *Arabidopsis* embryo germination has confirmed that inactivation of arabinogalactan proteins using the Yariv reagent induces changes in cell wall composition (an enrichment in cellulose - revealed by peaks at 900 and 1320 cm^{-1} - and an impoverishment in pectins - denoted by a decrease in the peaks at 1014, 1094, 1152, 1238, and 1741 cm^{-1} - leading to cessation of embryo germination and abnormal cotyledon embryos formation (Zhong et al., 2011).

Regarding seedling growth, FT-MIR spectroscopy has been used in order to study the role of the cell wall and its components throughout this process. Since plant coleoptiles have a simple and homogeneous structure, they have frequently been selected to perform this kind of study. Maize (*Zea mays*) coleoptile growth has been investigated with FT-MIR microspectroscopy using a chemical imaging tool and neural networks to classify infrared spectra (Carpita et al., 2001; McCann et al., 2001, 2007). This approach revealed dynamic cell wall changes among tissues of this growing organ, and has determined the importance, from among other cell wall components, of β-1,3-1,4 glucan, and glucomannans during cell elongation (Carpita et al., 2001).

Cell wall modifications during root growth have also been analyzed by means of FT-MIR spectroscopy. The roots of *Cicer arietinum*, *Clivia miniata,* and *Iris germanica* have been used to describe three developmental stages in endodermal cell walls during root growth, related with the deposition of suberin, lignin, cell wall proteins, and carbohydrates in these cells (Zeier and Schreiber, 1999).

In a further study aimed at determining the involvement of cell wall components in relation to salt resistance in developing casparian strips, three cultivars of rice (*Oryza sativa*) with different salt resistance were selected and FT-MIR spectroscopy was used to monitor the accumulation of suberin, lignin, proteins, and polysaccharides during the development of the casparian strips (Cai et al., 2011). The difference FT-MIR spectra revealed that the content of suberin as well as other cell wall component was higher in the resistant rice cultivar than in the two susceptible cultivars (Cai et al., 2011).

Concerning the study of cell wall changes related to the stem development, FT-MIR spectroscopy has been applied to *Brachypodium distachyon* during three developmental stages: elongation, inflorescence emergence, and senescence, revealing that as the stem develops, an accumulation of lignin, crystalline cellulose, and xylan occurs in the cell walls (Matos et al., 2013).

Recently, a combination of FT-MIR microspectroscopy and a focal plane array (FPA) detector, which increments spatial resolution and the data handling by orthogonal projections to latent structures discriminant analysis (OPLS-DA), has enabled the extraction of spectra from single cell types, the characterization of different chemotypes by using the full spectra information rather than only discrete characteristic bands, and the acquisition of chemical landscapes by multivariate analysis (Gorzsás et al., 2011). This technique has been applied to characterize the chemotypes of the different secondary xylem cell types (vessels, fibers, and rays) across the annual wood ring of aspen (*Populus tremula*) and to monitor changes in these cell walls. In fiber cells, lignin was observed to predominate in earlywood and hemicelluloses/cellulose in latewood, which could be explained by the development of an S2 layer of secondary cell wall during the growing season. Similarly, xylem ray cells were found to contain more aromatic compounds (lignin and monolignols) in earlywood and more pectins and/or hemicelluloses in latewood. These findings could be due to changes in the pectin-rich protective layer typical of ray cells. Vessel elements were more uniform throughout the annual ring, probably due to its rapid development (Gorzsás et al., 2011). In another study, FT-MIR microspectroscopy has been useful to monitor the postmortem lignification of tracheary elements of *Zinnia elegans*, revealing the presence of lignin-characteristic bands, as 1510 and 1595 cm^{-1}, in the FT-MIR spectra of developing tracheary elements (Pesquet et al., 2013). The exposure of these cells to piperonylic acid, an inhibitor of lignin monomer biosynthesis, induced a reduction in the absorbance on these bands. Moreover, a supply of coniferyl alcohol, or both coniferyl and synapyl alcohols, to the piperonylic acid-treated tracheary elements induced FT-MIR spectra similar to those of non-treated cells (Pesquet et al., 2013).

Pollen tube growth has frequently been studied by means of FT-MIR spectroscopy. Thus, treatment with different toxins that stop pollen tube growth and subsequent analysis of cell wall infrared spectra has revealed that the changes in esterified and acidic pectic distributions and their relative contents are associated with the cessation of pollen tube growth in *Picea wilsonii* (Kong et al., 2006; Sheng et al., 2006; Chen et al., 2008), *Picea meyeri* (Chen et al., 2007), and *Pinus bungeana* (Wang et al., 2009c). The formation of multiple pollen tubes from a single pollen grain has been studied in *Luffa cylindrica*, where FT-MIR microspectroscopy has confirmed that lower amounts of pectins and abnormal cell wall components are deposited in the multiple pollen tubes without nuclei walls (Jiang et al., 2009). These abnormalities were related to lignin, pectin, cellulose, callose and overall cell wall carbohydrate content. Likewise, a study of *Arabidopsis* root hair growth cessation induced by treatment with $CdCl_2$ has revealed that cadmium provokes a disruption in vesicle trafficking that affects cell wall deposition and tip growth. The main modifications measured by FT-MIR and immunolabeling of the tip cell wall included a reduction in esterified pectins and an increment in de-esterified pectins, other polysaccharides and proteins (Fan et al., 2011).

Lastly, changes in cell wall composition during fruit development and ripening have been analyzed by FT-MIR spectroscopy to determine the influence of cell wall on fruit development. This is the case of the study of a guaiacyl-syringyl-lignin, which is important during pear (*Pyrus bretschneideri* cv. Dangshan Su) fruit ripening; the use of FT-MIR spectroscopy has made it possible to determine that this lignin has more guaiacyl than syringyl groups in its structure (Cai et al., 2010). FT-MIR spectroscopy has also been used to study the contribution of pectin composition in the ripening of strawberry (*Fragaria x ananassa*) fruits (Posé et al., 2012), and to monitor changes in cell wall composition and cellulose content through the development of cotton (*Gossypium hirsutum*) fibers (Abidi et al., 2014). The main cell wall changes during cotton fiber development consist of a reduction in proteins and pectins, the de-esterification of these pectins, and a huge increment in the cellulose content due to the synthesis of a secondary cell wall (Abidi et al., 2014). Furthermore, FT-MIR spectroscopy has contributed to an analysis of the presence of phenolic compounds (mainly phenolic esters) bound to the cell wall of mature cotton fibers (Fan et al., 2009). FTIR and synchrotron infrared imaging have been used to monitor the acetyl esterification of cell walls of black cotton-wood (*Populus trichocarpa*), showing that *p*-coumarate accumulates in young leaves and declines in mature leaves, while ferulate and acetate are predominantly found in stems. Over the course of stem development, the amount of ferulate increases, whereas the initial amount of *p*-coumarate diminishes (Gou et al., 2008).

Other studies on changes throughout growth and development processes have focused on genetic control of the expression of different kinds of genes, or on the effect of different types of stress on normal plant development, and these will be discussed in the following sections.

MUTATION AND OVEREXPRESSION OF CELL WALL-RELATED GENES

Fourier transform mid infrared spectroscopy has been used to analyze modifications in cell wall structure and composition due to the mutation or overexpression of genes that presumably have a function in plant cell wall biosynthesis or in its modification. Studies on control of the expression of cell wall-related genes have yielded information about cell wall biosynthesis and turnover of the different cell wall components, thus providing further insights into the function of these genes.

CELLULOSE BIOSYNTHESIS-RELATED GENES

Cellulose is the major scaffolding polysaccharide in the primary and secondary cell walls of plants, and it is synthesized by the cellulose synthase complexes located at the plasma membrane. These complexes are constituted by six subunits forming a hexagonal rosette. Each subunit is composed of six cellulose synthase (CESA) proteins which synthesize the 36 β-(1,4)-glucan chains that form the cellulose microfibril. However, this model has now been questioned, and 12-36 glucan chains for the microfibril are currently being considered (see McFarlane et al., 2014 for a review). *CesA* genes can be divided in two groups, one of which is related to cellulose biosynthesis of the primary cell wall while the other is related to cellulose biosynthesis of the secondary cell wall (Hamann et al., 2004; McFarlane et al., 2014).

Fourier transform mid infrared spectroscopyhas been applied to discriminate *Arabidopsis* cellulose mutants from a set of characterized and uncharacterized cell wall mutants. For this end, infrared spectra from the cell wall of mutants, and wild type plants treated with the CBI isoxaben were obtained, and a set of statistical tools were used, with the separation and classification of these mutants being visualized by means of a dendrogram (Robin et al., 2003). It was observed that alleles of the same loci were clustered, as were wild type plants treated with low concentrations of isoxaben, whereas the other clustered mutations were those which affected cellulose biosynthesis-related genes as well as the plants treated with high concentrations of isoxaben, which both showed a reduction in cellulose content. The same authors performed a similar study which confirmed the high capacity of these techniques to discriminate mutants with cellulose defects from other cell wall mutants, irrespective of whether they had been characterized or not (Mouille et al., 2003).

Besides this application of FT-MIR, the technique has been used to ascertain the role of different *CesA* genes in cellulose biosynthesis. Thus, to determine the role of CesA6 in cellulose biosynthesis in *Arabidopsis*, non-polarized deuteration-FT-MIR spectroscopy was applied to the *procuste* mutation (*prc-1*), which affects CesA6 expression. This mutant showed a reduction of hypocotyl elongation when it was grown in dark conditions (Fagard et al., 2000; MacKinnon et al., 2006). FT-MIR spectroscopy of *prc-1* cell walls revealed a small reduction in cellulose content, which was accompanied by a relative increase in pectin and altered pectin esterification. Using polarized FT-MIR with FE-SEM-emission-scanning electron microscopy to analyze the orientation of the cellulose microfibrils, it was observed that *prc-1* cellulose microfibrils in elongated cells were similar to wild type cell walls, although less uniform, whereas in short cells of the mutant, the orientation was random, ranging between 0 and 180° to the cell axes (MacKinnon et al., 2006).

In addition, in order to ascertain the role of *AtCesA2* or *AtCesA5* genes and their relation with AtCesA6, FT-MIR microspectroscopy was applied to cell walls from dark-grown hypocotyls of mutants *cesa2* and *cesa5*, and double mutants *cesa2 cesa5*, *cesa2 cesa6* and *cesa5 cesa6* (Desprez et al., 2007). Spectra of cell walls from *cesa2*, *cesa5* and even *cesa2 cesa5* clustered with those of wild type controls, whereas those of double mutants *cesa2 cesa6* and *cesa5 cesa6* were grouped with *prc 1-1* and other cellulose deficient mutants. These results, combined with others, suggest that CESA2, CESA5, and CESA6 proteins are partially redundant in the cellulose complex (Desprez et al., 2007).

The *thanatos* mutant (*than*) is affected in the secondary structure of the catalytic cytosolic domain of AtCESA3 (Daras et al., 2009). This mutation provokes several phenotypic changes, such as a reduction in plant growth, especially when the mutation is homozygous. FT-MIR analysis of *than* mutant cell walls showed a reduction in cellulose that was corroborated by a strong decline in [^{14}C]glucose incorporation into cellulose. Moreover, the cell wall FT-MIR spectra revealed a reduction in saturated ester groups and an increment in proteins and phenolic compounds, indicating ectopic lignin deposition in the mutant (Daras et al., 2009).

Regarding the characterization of cellulose biosynthesis in the secondary cell wall, FT-MIR spectroscopy has also been applied to a set of 16 genes putatively related to secondary cell wall formation (Brown et al., 2005). These were selected by using profiling techniques, and some examples are *irx1* (irregular xylem), *irx3*, and *irx5* mutants, which are caused by defects in the CesA gene family (CesA8, CesA7, and CesA4, respectively), and their proteins form part of the complex involved in cellulose synthesis in secondary cell wall [for a revision see McFarlane et al. (2014)]. FT-MIR and subsequent PCA revealed that these mutants exhibit an important reduction in cellulose content (Brown et al., 2005). Previously, cell walls of *irx3* had been studied using solid-state NMR spectroscopy and FT-MIR microscopy, showing that sclerenchyma tissues of hypocotyls of this mutant contained reduced amounts of crystalline cellulose that did not seem to be replaced by amorphous glucans (Ha et al., 2002).

FT-MIR has also been useful in the characterization of three *exigua* mutants, which are impaired in secondary cellulose biosynthesis: they showed different cell wall changes in the vascular tissue, but all presented a reduced cellulose content concomitant with an increase in lignin (Rubio-Díaz et al., 2012).

In another study, FT-MIR was applied to the ammonium oxalate-extracted mucilage of seeds of the triple *Arabidopsis* mutant *cesa2*/*cesa5*/*cesa9*, revealing differences not only in xyloglucan and pectin composition but also in cellulose, thus indicating not only that cellulose is present in mucilage, but also that cellulose mutants have mucilage with altered composition (Mendu et al., 2011).

In addition to *Arabidopsis*, other species, such as potato (*Solanum tuberosum*) have been used to study *CesA* homolog genes (Oomen et al., 2004). In this case, up- and downregulation of four *CesA* genes was studied with FT-MIR to analyze changes in cellulose as well as overall polysaccharide content in tuber sections, showing different reductions in the cellulose levels of transgenic lines wirh respect to wild type samples.

Besides to *CesA*, other genes have been implicated in cellulose biosynthesis. A point mutation in the KORRIGAN (KOR) gene that encodes a β,1-4 endoglucanase, impairs a reduction in cellulose deposition in secondary cell wall, causing the *Arabidopsis irregular xylem 2* (*irx2*) mutant phenotype (Szyjanowicz et al., 2004). The FT-MIR spectra of deuterated primary cell walls of *irx-2-1* showed that there were apparently no changes either in the crystalline structure of cellulose or in the non-cellulosic polysaccharide composition, compared to wild type cell walls. These results complement other findings on KOR expression and protein immunolocalization, and suggest that KOR might be involved in the processing of growing microfibrils during later stages of secondary cell wall formation or in the release of cellulose synthase complexes (Szyjanowicz et al., 2004).

To conclude this section, another gene whose characterization has been associated with cellulose biosynthesis mutants and to which FT-MIR has been applied, is a plasma-membrane bound receptor-like kinase, THESEUS (THE-1). FT-MIR microspectroscopy and PCA applied to *prc1-1* and the double mutant *the-1/prc1-1* grouped both mutants together, and in the same cluster as other cellulose mutants, indicating that they are cellulose deficient.

This finding concurs with other related results, suggesting that the THE1 protein may operate as a cell wall integrity sensor, mediating the response of growing plant cells to perturbations in cellulose synthesis (Hématy et al., 2007).

HEMICELLULOSE AND PECTIN BIOSYNTHESIS GENES

FT-MIR has also been successfully used to study the biosynthesis or modifications of other cell wall polysaccharides, namely hemicelluloses and pectins.

Xylans are the most abundant hemicelluloses in secondary cell walls, and FT-MIR spectroscopy has proved to be an effective means of characterizing and grouping secondary cell wall mutants. The application of FT-MIR spectroscopy and multivariate analyses to a set of irregular xylem mutants, has enabled characterization of *irx7*, *irx8* and *irx9*, which showed important reductions in xylose content in the inflorescence stem, whereas their cellulose content did not seem to be affected, indicating that the alteration in cell wall composition of these mutants is consistent with a lack of β-1,4-linked xylosyl residues associated with xylan (Brown et al., 2005). Subsequently, FT-MIR was applied to a set of xylan-affected and other secondary cell-wall mutants: *irx14*, *parvus-3*, *irx1*, *irx3*, *irx5*, *irx7*, *irx8* and *irx9* (Brown et al., 2007). A principal components analysis enabled discrimination between cellulose-affected mutants and those affected in xylans: *parvus-3*, *irx7*, *irx8* and *irx9* formed a group, whereas *irx14* formed a single cluster. These data, together with polysaccharide fractionation, cell wall sugar and PACE analyses, show that *irx7*, *irx8* and *irx9* are xylan-deficient mutants (Brown et al., 2007).

FT-MIR was applied in another study to analyze the *irx8* mutant. The results showed that it had potential alterations in non-cellulosic polymers, helping to demonstrate that its dwarf phenotype could not be attributed to a cellulose deficiency. Moreover, sugar analysis and cellulose quantification revealed that it had glucuronoxylan and homogalacturonan deficiencies (Persson et al., 2007). In addition, an extensive analysis using FT-MIR microspectroscopy combined with OPLS-DA demonstrated that the *fra8* (*irx7*) gene in *Arabidopsis* causes a decrease in the proportion of xylan and lignin, and that it is involved in xylan biosynthesis in lignified as well as in non-lignified fibers of the secondary xylem of hypocotyl (Gorzsás et al., 2011).

Further studies have described new mutants affected in xylan composition, and have led to the conclusion that *irx 7–9* as well as *parvus* mutants are affected in different glycosyltransferases involved in xylan synthesis (for a review see Pauly et al., 2013 and Jensen et al., 2013).

FT-MIR spectroscopy has also been used to ascertain some of the genes involved in xyloglucan biosynthesis, another of the main hemicellulosic polysaccharides. FT-MIR analysis of the *xxt5* mutant showed that it displays comparable cell wall changes to *xxt1 xxt2* mutant plants (Zabotina et al., 2008), XXT1 and XXT2 having previously being implicated in xyloglucan biosynthesis. Nowadays, the three genes are known to codify xyloxyltransferases involved in xyloglucan biosynthesis (Pauly et al., 2013).

Besides biosynthesis, modification of xyloglucan has also been elucidated using FT-MIR spectroscopy. Xyloglucan endotransglycosylase/hydrolase (XTH) enzymes have been implicated in modification of the xyloglucan structure, and as a consequence, in cell wall reorganization. In tomato plants overexpressing XTH1, FT-MIR cell wall spectra from the apical hypocotyls of these transgenic plants showed that the linkages between pectic polysaccharides, and between xyloglucan and cellulose differ with respect to the wild type, and that this structure could be related to altered cell wall extensibility in their hypocotyls (Miedes et al., 2011).

Regarding pectin biosynthesis and modification, FT-MIR analyses of transgenic plants overexpressing AtRHM1, a gene putatively implied in rhamnose biosynthesis, have revealed that surplus rhamnose upon overexpression is used in the synthesis of rhamnogalacturonan (Wang et al., 2009a). In another study, overexpression of an inhibitor of pectin methylesterase (PMEI4) delayed growth acceleration in *Arabidopsis* hypocotyls. FT-MIR spectroscopy also showed an increase in ester bonds in these transgenic lines, concomitant with reduced pectin methylesterase activity, thus demonstrating a role of pectin methyl esterification in growth control (Pelletier et al., 2010). Related to this, pectin acetylesterase has been shown to play a role in cell wall properties and cell extensibility, controlling the degree of pectin acetylation. FT-MIR has helped to confirm that transgenic black cotton-wood plants overexpressing pectin acetyl esterase 1 (PtPAE1) have a decreased ratio of ester peaks in relation to polysaccharides in style and filament tissues compared with wild type, which is related to decreased cellular elongation in these organs (Gou et al., 2012).

LIGNIN BIOSYNTHESIS-RELATED GENES

The genetic control of lignin biosynthesis has usually been elucidated by modifying the expression of phenylpropanoid pathway genes, thus disrupting the normal formation of several phenolic compounds. This pathway terminates with the formation of the three monolignols (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) which act as a source to synthesize the lignin leading to the three lignin units: the *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units.

One of the relevant steps of the phenylpropanoid pathway is carried out by a group of enzymes that are classified as *O*-methyltrasferases (OMTs). This group is composed of two principal enzymes: caffeic acid 5-*O*-methyltrasferase (COMT) and caffeoyl coenzyme A 3-*O*-methyltransferase (CCoAOMT), both of which are responsible for the two methylation steps necessary for biosynthesis of the monolignols previous to their incorporation into lignocellulose. By obtaining an *Arabidopsis* CCoAOMT 1 mutant (*ccomt1*), a gene that is only expressed in lignified tissues, and a double mutant with the other OMTs gene, COMT 1 (*ccomt1 comt1*), it has been demonstrated that these enzymes have a redundant function in the synthesis of lignin, flavonoids, and synapoyl malate (Do et al., 2007). FT-MIR microspectroscopy has been used to corroborate the reduction in lignin and the increment in S units caused by the *ccomt1* mutation (Do et al., 2007). However, in woody poplar (*Populus tremula x Populus alba*) the CCoAOMT gene is essential for normal lignin biosynthesis (Zhong et al., 2000). By using an antisense approach against CCoAOMT, a reduction in the expression of this gene induced a decrease in Klason lignin content. The use of diffuse reflectance infrared Fourier transformed spectroscopy (DRIFTS) has corroborated this

reduction in lignin, which was less condensed and cross-linked in the transgenic poplar wood (Zhong et al., 2000). Another study of OMTs involvement in lignin biosynthesis was carried out on tobacco (*Nicotiana tabacum*), using antisense transformation to suppress the caffeic/5-hydroxyl-ferulic acid O-methyltransferase, an enzyme involved in synapyl alcohol formation for lignin (Blaschke et al., 2004). Neither analytical measurements nor FT-MIR spectroscopy data revealed any changes in lignin content as a result of this reduced OMT expression. However, FT-MIR spectroscopy of extracted lignin showed that the stem lignin presented a relative increase in G units compared with S units (whose characteristic IR bands are 1270 cm^{-1} and 1330 cm^{-1}, respectively) in antisense-OMT tobacco, and that this change in lignin composition was more pronounced under CO_2 treatment (Blaschke et al., 2004).

Cinnamoyl-coenzyme A reductase (CCR) is the first enzyme in the monolignol biosynthesis branch of the phenylpropanoid pathway. This enzyme catalyzes the conversion of cinnamoyl coA esters to the corresponding cinnamaldehydes. FT-MIR spectroscopy has been used to determine or corroborate changes in the cell wall and lignin composition of plants with a modified CCR expression. Knock-out of the AtCCR1 gene, which controls the constitutive biosynthesis of lignin, yielded dwarfed *Arabidopsis* plants with delayed senescence (Derikvand et al., 2008). FT-MIR microspectroscopy has also been used to study the changes provoked by mutation in the xylem and stem fibers of these plants, confirming the significant reduction in lignin content measured by several analytical techniques. FT-MIR spectra revealed that the fiber region presented a significant reduction in lignin and enrichment in hemicelluloses. There was no detection of lignin reduction in the xylem region, but it showed the same enrichment in hemicelluloses (Derikvand et al., 2008). In the case of woody poplar, downregulation of CCR by antisense transformation induced a reduction in lignin content and the appearance of an orange-brown coloration in the outer xylem (Leplé et al., 2007). This reduction in lignin content was corroborated by both FT-MIR spectroscopy and analytical studies. FT-MIR spectroscopy supported the notion that this mutation increases the breakdown and remodeling of non-cellulosic cell wall polymers and decreased biosynthesis, mainly of the hemicelluloses. Furthermore, an analysis of the spectra obtained revealed that ferulic acid had been incorporated into the cell wall (Leplé et al., 2007).

The last step of monolignols biosynthesis, prior to polymerization into the lignocellulose molecule, is catalyzed by cinnamyl alcohol dehydrogenase (CAD), converting the cinnamaldehydes into their corresponding alcohols. In *Arabidopsis* floral stems, *CAD-C* and *CAD-D* genes are the primary genes involved in lignin biosynthesis. A FT-MIR spectroscopy study of the double mutant *cad-c cad-d* revealed that the repercussions on xylem and fiber cell wall composition were different. The mutation provoked a reduction in lignin content and a higher amount of G units in their structure, especially in xylem fibers. Furthermore, FT-MIR analysis corroborated an enhancement of cinnamaldehydes incorporated into the cell wall of both cell types (Sibout et al., 2005). Similarly, an antisense transformation of full length cDNA from the CAD gene in the woody plant *Eucalyptus camaldulensis* did not induce any change in lignin content observable by FT-MIR, nor in lignin composition by pirolysis. Moreover, no significant differences in monosaccharide content were detected when studied by FT-MIR using reference standards (Valério et al., 2003).

These studies provide evidence of the existence of direct genetic control of the expression of phenylpropanoid pathway genes. In addition, control of the expression of peroxidases, enzymes necessary for oxidative coupling of the monolignols to synthesize the lignin molecule, has also yielded information about this lignin polymerization process. A study of the *Arabidopsis AtPrx72* knock-out mutant, a gene which is homologous to the *Zinnia elegans ZePrx* gene that has been related to lignin biosynthesis, revealed a decrease in lignin content and in the S moieties of this molecule that has been confirmed by FT-MIR spectroscopy (Herrero et al., 2013). In addition, another strategy is the analysis of a transcription factor that regulates the expression of different genes of this pathway and therefore also regulates lignin biosynthesis. One group of transcription factors is the MYB family. Within this group, *Arabidopsis* MYB103 transcription factor indirectly regulates lignin biosynthesis (Öhman et al., 2013). MYB103 is a member of a transcriptional network that regulates secondary cell wall biosynthesis in the xylem of *Arabidopsis* and it is related to cellulose biosynthesis. Suppression by means of t-DNA insertion of this gene induced a huge reduction in the expression of the ferulate 5-hydroxylase gene (F5H, a member of the phenylpropanoid pathway). Interestingly, lignin content was not affected, and FT-MIR microspectroscopy study of the vessel elements, xylem fibers and inter-fascicular fibers of the inflorescence stem revealed a higher proportion of G units in the lignin of all cell types of the mutant plants (Öhman et al., 2013).

OTHER MUTANTS WITH MODIFIED CELL WALLS

Lastly, the cell walls of a wide range of mutants or gene-overexpressing plants have been shown to be affected. The exact gene affected is frequently unknown, but a clear quantitative or qualitative modification in their cell wall composition has been found. In some of these plants FT-MIR has been used to characterize their cell walls. Some examples include the rolling leaf mutant (*rlm*) of rice, where FT-MIR spectra from foliar cell walls showed lower protein and polysaccharide contents (Bai et al., 2008); the root-specific α-expansin gene of rice, OsExpa8, where FT-MIR of cell walls of plants overexpressing this gene displayed increased ratios of polysaccharide/lignin content, therefore supporting a role for expansins in cell elongation and plant growth (Ma et al., 2013); or the *shaven3* (*shv3*) mutant of *Arabidopsis*, whose encoded protein SHV3 has two tandem repeating glycerophosphoryl diester phosphodiesterase-like domains and a glycosylphosphatidylinositol anchor, where FT-MIR of cell walls from the double mutant *shv3* and the paralog *svl1* revealed an alteration in cellulose content and modification in pectins (Hayashi et al., 2008).

ANALYSIS OF EFFECTS OF BIOTIC AND ABIOTIC STRESS ON THE CELL WALL BY MEANS OF FT-MIR

In nature, plants are exposed to diverse environmental stress factors, and the responses of plants to these stresses involve changes at

physiological, biochemical and molecular levels. Abiotic stress factors, such as heat, cold, drought, salinity, presence of heavy metals, or poor nutrition, have a worldwide impact on agriculture, since they present a serious threat to crop production. Besides these abiotic factors, biotic stress factors such as fungi, bacteria, virus, nematodes, and herbivores also compromise plant survival (Wang et al., 2003; Atkinson and Urwin, 2012). As the cell wall is normally the first line of defense at cellular level against all of these stresses, technologies such as FT-MIR spectroscopy have frequently been used in order to monitor changes in this structure due to stress factors.

Among the diverse sources of stress, drought is the most important factor limiting plant growth, reproductive development and, ultimately, survival (Arbona et al., 2013). Desiccation tolerance in poikilohydric plants is related to changes in the structure and composition of the cell wall (Vicré et al., 2004; Moore et al., 2006). The dried state of desiccation-tolerant tissues limits the type of technique that can be applied to study conformation and stability of biomolecules. One of the few suitable techniques for dried tissue analysis is FT-MIR spectroscopy, because it can be used irrespective of the hydration state of the tissue (Wolkers and Hoekstra, 2003). Species such as *Boea hygrometrica* – a desiccation-tolerant angiosperm (or resurrection plant) – have been selected as models to investigate changes in gene expression and cell wall adaptation during extreme dehydration, and morphological changes such as smooth cell wall folding have been observed in cell wall architecture during the process of drying and re-watering. With regard to the chemical composition of the leaf cell wall, the FT-MIR spectra indicated that protein levels rose upon desiccation and remained at those levels after re-watering (Wang et al., 2009b), whereas an increase of both esterified and de-esterified pectins was observed in rehydrated leaves. The absorbance levels at wavenumbers assigned to phenolics showed no differences in lignin or monolignols between hydrated and dehydrated leaves. Lastly, the intensity of peaks corresponding to octadecyl octadecanoate indicated that wax or suberin-like aliphatic compounds increased in rehydrated leaves (Wang et al., 2009a).

Regarding nutrient deficiency studies, callus-cultured cells of vine (*Vitis vinifera*) were subject to nitrogen, phosphorous and sulfur impairment, and changes in their cell walls were analyzed with FT-MIR spectroscopy (Fernandes et al., 2013). This study showed differences in cellulose content when comparing control and sulfur deficient (with nitrogen) and phosphorous deficient calluses. Nitrogen deficient calluses exhibited a lower amount of cellulose and proteins, and a higher amount of pectins.

Heat stress also causes alterations in cell wall components (Hasanuzzaman et al., 2013). When leaves of coffee plants were grown at 37°C, important changes in their cell walls were identified: the pectic content was decreased by almost 50% in the cell wall of heat stressed leaves (Barbosa et al., 2013). Based upon the FT-MIR spectra of water-soluble polysaccharides, temperatures of 37°C induce a higher content of arabinose and galactose and a reduced content of mannose, glucose, uronic acid, rhamnose, and fucose. In the hemicellulosic fractions, the main components were arabinoxylans and xyloglucans; and the xylose content was decreased under heat stress (Barbosa et al., 2013).

Fruit is usually stored at low temperatures in order to delay ripening; however, tropical fruits are susceptible to chilling injury and this is the cause of extensive post-harvest losses. Chilling injury damage involves alteration in the properties of the cell walls. In one study, mango (*Mangifera indica* L. cv. "Red 6") fruits were subjected to low-temperature stress and then analyzed by histochemical and scanning electron microscopy together with FT-MIR. The results showed that chilling injury symptoms (sunken lesions, alterations in the outer pericarp, and changes in cuticular waxes and cell walls) were limited in fruit treated with methyl salicylate as compared to the 5°C control (Han et al., 2006), demonstrating the positive effects of this compound as regards conferring tolerance to low-temperature stress (Fung et al., 2004). A FT-MIR spectrometric analysis indicated high proportions of linear long-chain aliphatic, phenolic rings and pectic polysaccharides in cell wall extracts but lower amounts of cellulose, in mango fruit stored at 5°C with methyl salicylate (Han et al., 2006).

Air pollution in general and ozone in particular affects plants, altering their growth and pollen production intensity (Booker et al., 2009). Furthermore, ozone seems to affect both the morphology of pollen (size, shape, surface structure) and the abundance of allergenic protein (Kanter et al., 2013). FT-MIR has shown that pollen samples exposed to elevated ozone levels exhibit a reduction in phenolic compounds. The reduction in absorbance of the FT-MIR peaks corresponding to acetyl ester of pectin in fumigated pollen was consistent with expressed sequence tags data. At the same time, an increased pectic content was observed, hence indicating that de-esterification could be an effect of ozone treatment (Kanter et al., 2013).

Plant responses to biotic stresses involve physiological and biochemical changes, including activation of the expression of defense-related genes, whose function is to protect the plant against these kinds of stresses (Hamann, 2012). FT-MIR analysis of *pmr5* (powdery mildew resistant) *Arabidopsis* plants, which possess a mutation of unknown function that nevertheless confers protection against powdery mildew infection, showed an increment in pectin in their cell walls, and that these pectins had a lower degree of methyl esterification or O-acetylation (Vogel et al., 2004), basically coinciding with the FT-MIR spectra of the mutant *pmr6*, affected in a glycosylphosphatidylinositol-anchored pectate lyase-like gene (Vogel et al., 2002). Other mutants affected in different subunits of the heterotrimeric G-proteins, such as *agb1* and the double mutant *agg1 agg2*, exhibited similar FT-MIR spectra to each other, but different from those of wild type, suggesting that G-protein subunits play a role in the control of cell wall composition and in the immune response of *Arabidopsis* plants (Delgado-Cerezo et al., 2012).

By means of FT-MIR spectroscopy it has been possible to monitor the changes in wood elm (*Ulmus minor*) biochemistry as a consequence of fungi infection by *Ophiostoma novo*-ulmi (Martín et al., 2005). Lower levels of polysaccharides and higher levels of phenolic and aliphatic compounds in xylem tissues of inoculated elms were found, suggesting that cell wall degradation occurred as a result of fungal enzyme activity. The high levels of aliphatic compounds could be related to the presence of tyloses and to the

suberization of parenchyma cell walls. A prominent negative peak at 983 cm^{-1} was also found, suggesting a higher starch content in resistant elm trees; thus, a reduction in starch should be associated with tree susceptibility to infection (Martín et al., 2005). FT-MIR combined with PCA has therefore proved to be a valid means of monitoring plant-pathogen interaction and identifying resistant genotypes, a function that could be applied to other species.

FT-MIR spectroscopy has also been used to detect cell wall changes provoked by infection with *Verticillium longisporum*: the main difference found between non-infected and infected cells occurred in the degree of pectin esterification (Floerl et al., 2012). When the effects of a combination of biotic and abiotic stresses (infection with *Phaeomoniella chlamydospora* and cell exposure to NaCl) were monitored in stems, leaves and roots of grapevine using FT-MIR spectroscopy, major changes were observed in cells exposed to both stresses (Oliveira et al., 2009). The spectra of leaves subjected to both stresses were characterized by higher amounts of pectic polysaccharides, xylose-rich polysaccharides and cellulose.

FT-MIR MONITORING OF CELL WALL CHANGES ASSOCIATED WITH CBIs

CBIs are a heterogeneous group of compounds with the capacity to interfere with cellulose biosynthesis (for a review, see Acebes et al., 2010). FT-MIR spectroscopy has been applied in conjunction with multivariate analyses to monitor cell wall changes associated to CBIs, related either to (i) short-term exposure of plant cell cultures or complete plants to CBIs, or (ii) habituation/dehabituation of plant cell cultures to these kinds of compound.

Some studies have applied FT-MIR spectroscopy in order to characterize the effect of short-term exposure to CBIs on cell wall structure and composition, such as the study carried out by Peng et al. (2013) on *Arabidopsis* seedlings treated with 2,6-dichlorobenzonitrile (DCB), a well-known CBI. The authors used FT-MIR spectroscopy to confirm the reduction in cellulose and pectin content in the cell wall previously observed with S4B (a specific fluorescence dye for cellulose) staining, or JIM5 (a monoclonal antibody that recognizes relatively unesterified pectic epitopes) immunolabeling. Moreover, FT-MIR spectra also showed a huge increase in protein in the cell wall of treated seedlings (Peng et al., 2013).

Another set of experiments related to a short-term exposure to CBIs was conducted in a recent study of the role of cellulose biosynthesis in the polarized growth of *Pinus bungeana* pollen tubes (Hao et al., 2013). Inhibition of cellulose biosynthesis by means of a DCB treatment induced a dose-dependent change in the growth rate and morphology of pollen tubes by altering the chemical composition of the tube wall. These cell wall modifications, initially characterized with fluorochromes and monoclonal antibodies, consisted of the accumulation of callose and pectins in the tips of the pollen tube and a decreased content of cellulose. A comparison between the FT-MIR spectra of control pollen tubes and the spectra of pollen tubes treated with DCB revealed changes in absorbance intensity and location of specific peaks, further confirmed by chemical and immunolabeling analyses. The difference spectra generated by digital subtraction of the control spectra from those of DCB-treated pollen tubes revealed that the saturated ester peak and the amide stretches increased, while cellulose content distinctly decreased with increasing DCB concentrations.

FT-MIR has also been used to analyze the cell wall composition of the *Arabidopsis* mutant *aegeus* (*cesa1*^{aegeus}), which shows resistance to the CBI quinoxyphen (Harris et al., 2012). In this case, the *aegeus* mutant displayed semi-dominant inheritance similar to that observed for the isoxaben resistance mutant *cesa3*$^{ixr1-2}$. It has been suggested that quinoxyphen and isoxaben share a common mechanism of action on cellulose biosynthesis, and further analysis of quinoxyphen-exposed seedlings has revealed a hyper-accumulation of callose and ectopic lignin production, modifications that have also been observed with other CBIs (Harris et al., 2012).

FT-MIR microspectroscopy and PCA were applied to screen *Arabidopsis* mutants deficient and non-deficient in cellulose, and to compare them with the wild type treated with a variety of CBIs, such as DCB, isoxaben, thaxtomin A and flupoxam (Mouille et al., 2003). Two main branches were distinguished in the resulting dendrogram: one branch grouped cellulose deficient mutants and wild type treated with a high concentration of CBIs, while the other branch contained untreated wild type, or wild type treated with a low concentration of CBI, together with the mutants not affected in cellulose content. The alignment of the spectra corresponding to the wild type treated with thaxtomin A was one of the first pieces of evidence supporting the idea that thaxtomin A inhibits the synthesis of cellulose, which has also been confirmed by chemical analysis (Scheible et al., 2003).

The second group of experiments included habituating cell cultures to grow in the presence of CBIs. Initially, tomato cultured cells (with a type I cell wall) were habituated to DCB and were characterized by biochemical or immunolabeling techniques and later compared with data from FT-MIR spectroscopy (Shedletzky et al., 1990; Wells et al., 1994). These analyses of DCB-habituated cells showed a unique cell wall with drastically reduced levels of cellulose, where the major load-bearing network of the cell wall was formed by calcium-linked pectins. Immunogold labeling with the JIM5 antibody showed that pectins from the habituated cell walls were mainly unesterified. Further characterization of the cells walls by FT-MIR spectroscopy yielded additional evidence that supported the previous biochemical and immunogold-labeling studies (Wells et al., 1994).

Bean (*Phaseolus vulgaris*) calluses (also with cells surrounded by type I cell wall) were habituated to grow in a relatively high DCB concentration (12 μM) and then characterized (Encina et al., 2001). Cell wall isolation and fractionation and the subsequent chemical analyses showed that the xyloglucan–cellulose network of non-habituated cell walls was partly replaced in DCB-habituated cell walls by a pectin-rich network mainly formed of cross-linked polyuronides with a large proportion of homogalacturonan. These modifications were comparable to those described for bean calluses habituated to isoxaben (Díaz-Cacho et al., 1999), suggesting a common mechanism of habituation to both CBIs. A comparison of the FT-MIR spectra obtained from the cell walls of DCB-habituated and non-habituated calluses showed that the former had higher peaks associated with ester linkages and an increase

in carboxylic acid stretching. Accordingly, the difference spectrum (DCB-habituated cells minus non-habituated cells) showed peaks for ester linkages, free carboxylic groups and uronic acids. Globally, these FT-MIR results confirmed data obtained with chemical and immunological analyses of the entire or fractionated cell walls of DCB-habituated cell lines, showing an increase in both esterified and non-esterified pectins.

Multivariate analysis applied to the FT-MIR spectra data has been a useful technique to monitor further cell wall changes during the habituation of bean calluses to DCB (Alonso-Simón et al., 2004). A dendrogram obtained by cluster analysis of the spectra showed three main branches corresponding to different levels of habituation to DCB: (i) non-habituated calluses and low level habituated calluses (calluses habituated to up to 0.5 μM DCB and calluses with a low number of subcultures in a low concentration of DCB), (ii) medium level habituated calluses (calluses habituated from 0.5 to 4 μM DCB with more than 13 subcultures), and (iii) high level habituated calluses (calluses grown in the highest concentrations of DCB). Principal components revealed that the separation of the above three groups of spectra was associated with peaks related to cellulose and pectins.

In a further study, bean cell suspensions instead of calluses were progressively habituated to DCB, and their cell wall modifications were monitored using a combination of techniques, including FT-MIR spectroscopy. The FT-MIR spectra showed that cell suspensions underwent an increasing enrichment in pectins as the level of habituation to DCB became greater (Encina et al., 2002). When DCB-habituated cells were returned to a medium lacking the herbicide (dehabituation), the observed changes in the FT-MIR spectra were partially reversed, and by the eighth subculture in absence of DCB, spectra resembled those of non-habituated cells (Encina et al., 2002). Long-term dehabituated cells (more than a hundred subcultures in absence of DCB) showed that habituated cells restored cellulose and xyloglucan levels to the cell wall and decreased their pectin content, and that only subtle differences regarding non-habituated cells remained (García-Angulo et al., 2009a). A supplementary study involving FT-MIR spectroscopy proved that some of the cell wall changes induced in bean suspension-cultured cells during habituation were different from those induced in cells during dehabituation, and therefore that habituation and dehabituation follow diverse pathways: PCA indicated that dehabituated cells had more pectins and that these displayed a lower degree of methyl esterification than those of habituated ones (García-Angulo et al., 2009b).

FT-MIR spectroscopy has been also applied in conjunction with PCA to monitor changes in the type II cell wall during habituation of maize cultures to DCB (Mélida et al., 2009). The FT-MIR spectra of walls of DCB-habituated cell lines showed differences in wavenumbers attributed to cellulose, phenolic components, arabinose, and proteins, with respect to non-habituated cells.

More recently, FT-MIR spectroscopy was applied to monitor early changes in cell walls from maize suspension-cultured cells, associated with the first stages of DCB-habituation (de Castro et al., 2014). The difference spectra obtained for each low-habituated cell line compared with the non-habituated cell line showed decreased peaks in wavenumbers attributed to cellulose, together with changes in other peaks associated with phenols and pectins. The changes associated with this incipient habituation tended to revert and this reversion depended on the concentration of the inhibitor and the length of period in contact with it (de Castro et al., 2014).

ASSESSING BIOTECHNOLOGICAL APPLICATIONS OF CELL WALL MODIFICATIONS

The plant cell wall is the most abundant renewable resource on the planet (Pauly and Keegstra, 2008) and many studies over the last decade have focused on cell wall polymers because they are a source of reduced carbon, which can be used to produce "green fuels." This material can be obtained from plant wastes that are produced in large amounts by sectors such as the forestry, pulp and paper industries, and can be used as a substrate for ethanol production (Pauly and Keegstra, 2010; Bhatia et al., 2012). Despite the considerable economic interest of the cell walls of plant wastes, only about 2% of this resource is currently used by humans (Pauly and Keegstra, 2008). Cell walls of plant wastes have been analyzed by several techniques, including FT-MIR spectroscopy (Lupoi et al., 2014 and references therein).

The process of transforming lignocellulosic biomass into ethanol requires pretreatment in order to release the mono- or oligosaccharides which can then be fermented into ethanol from larger molecules, such as cellulose or hemicelluloses. Using FT-MIR spectroscopy, glucose, mannose, xylose, and acetic acid has been rapidly quantified in liquors from dilute-acid-pretreated soft-wood and hard-wood slurries in the batch reactor during optimization of pretreatment conditions (Tucker et al., 2000).

In order to transform plant cell walls, it is necessary to use saccharification technology to break down the recalcitrant bonds and release the fermentable sugars. Lignocellulose is the main component that must be processed throughout chemical and enzymatic hydrolysis. To analyze its composition, FT-MIR spectroscopy has been used in combination with PLS (partial least squares) regression to predict sugar production from enzymatic hydrolysis. Six different lignocellulosic raw biomasses pretreated with several levels of NaOH have been analyzed, and FT-MIR spectroscopy has made it possible to describe the solubilization of biomass components (glucose, xylose, lignin) in the fingerprint region of 800–1800 cm^{-1} (Sills and Gossett, 2012).

Regarding ethanol production, a screening strategy has been developed based on stalk geometry and PLS predictive models of FT-MIR spectra collected from soluble sugars and cell wall fractions in *Sorghum bicolor* bagasse. Feedstocks were assayed for total fermentable sugar yields including stalk biomass, soluble sugar concentrations and cell wall saccharification. This method enables prediction of enzymatic cell wall digestibility, and it has been incorporated into a holistic high-throughput screen in a biofuels context (Martin et al., 2013).

Other waste types, such as sugarcane bagasse and coconut fiber, have been used to characterize their main constituents and their behavior in thermal degradation. In both cases, the infrared absorption spectra were related to the presence of lignin,

hemicellulose and cellulose, characteristic of natural fibers. Both thermoanalytical and FT-MIR techniques have shown that sugarcane bagasse has a higher thermal stability than coconut fiber (Mothé and de Miranda, 2009).

Similarly, studies have been conducted on the intra-specific variability of lignin and energy contents in extractive-free wood of hybrid poplar progenies (*Populus trichocarpa* × *deltoides*), to determine whether the range is sufficient for the development of quantitative prediction models based on FT-MIR (Zhou et al., 2011). In addition, some timber species such as *Dipterocarpus kerrii*, *Hopea plagata*, *Parashorea malaanoman*, *Shorea almon*, and *Shorea contorta* have been compared, focusing on their durability and potential applications. The observed peaks in wood represent major cell wall components such as cellulose, hemicelluloses and lignin. FT-MIR spectroscopic assays of wood and isolated lignin from *D. kerrii* and *H. plagata* have revealed differences with respect to *P. malaanoman* and *Shorea* sp., species with a short service life. FT-MIR spectra identified lignin composition and ligno-protein content as the principal sources of variation (Rana et al., 2010).

Considering the importance of cell walls in agricultural research, it is surprising that relatively few crop species have benefited from the tools available to rapidly profile their wall polysaccharides (Nguema-Ona et al., 2012). However, FT-MIR has recently been applied to optimize and implement a rapid analysis of the cell wall composition and structure of grapevine leaves. A combination of high-throughput techniques were used, including monosaccharide compositional analysis and FT-MIR spectroscopy, thereby obtaining a rapid profile of their wall polysaccharides (Moore et al., 2014). Similarly, variations in cell wall composition have been monitored by FT-MIR spectroscopy in stems and leaves of a series of *Miscanthus* species and genotypes, at different stages of development, in order to determine their possible application in programs aimed at improving this bioenergy feedstock (da Costa et al., 2014). Variations in cell wall composition reside mainly in the contribution of secondary walls in the stages of peak biomass and after senescence, in comparison to actively growing organs.

FT-MIR analysis of polysaccharide residues from the cell walls of fruits and vegetables such as tomato, potato, pumpkin, carrot and celery root has been used to evaluate differences among cell wall residues and among species, showing that discrimination between the cell wall residues of fruits and vegetables is feasible with the use of FT-MIR spectra in the regions 1800–1200 cm^{-1} and 1200–850 cm^{-1}, combined with PCA (Szymanska-Chargot and Zdunek, 2013).

To sum up, FT-MIR spectroscopy has been applied for discrimination of wood from several species, determination of chemical wood composition as well as estimation of ethanol production from renewable agro waste.

CONCLUDING REMARKS

FT-MIR spectroscopy combined with multivariate analysis is a rapid, non-destructive and easy technique that only requires small amounts of sample and provides abundant information about the "in muro" organization of cell wall polymers and functional groups.

Although FT-MIR has many advantages over traditional analyses of plant cell walls, it is not usually employed in isolation to analyze cell walls, but rather to confirm the results obtained with other chemical or labeling analyses of entire or fractionated cell walls. This is mainly because the information it provides is often incomplete, with limitations derived from the complexity of spectra with overlapping peaks and the vibrational coupling of chemical bonds from different cell wall polymers. Therefore, FT-MIR spectroscopy has been used to monitor cell wall modifications, and to confirm or to complement the data obtained with traditional techniques, such as chemical analyses, fluorescence or immnunolabeling of the entire or fractionated cell walls.

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Ectopic lignification in primary cellulose-deficient cell walls of maize cell suspension cultures

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Research Article

Abstract Maize (*Zea mays* L.) suspension-cultured cells with up to 70% less cellulose were obtained by stepwise habituation to dichlobenil (DCB), a cellulose biosynthesis inhibitor. Cellulose deficiency was accompanied by marked changes in cell wall matrix polysaccharides and phenolics as revealed by Fourier transform infrared (FTIR) spectroscopy. Cell wall compositional analysis indicated that the cellulose-deficient cell walls showed an enhancement of highly branched and cross-linked arabinoxylans, as well as an increased content in ferulic acid, diferulates and *p*-coumaric acid, and the presence of a polymer that stained positive for phloroglucinol. In accordance with this, cellulose-deficient cell walls showed a fivefold increase in Klason-type lignin. Thioacidolysis/GC-MS analysis of cellulose-deficient cell walls indicated the presence of a lignin-like polymer with a Syringyl/Guaiacyl ratio of 1.45, which differed from the *sensu stricto* stress-related lignin that arose in response to short-term DCB-treatments. Gene expression analysis of these cells indicated an overexpression of genes specific for the biosynthesis of monolignol units of lignin. A study of stress signaling pathways revealed an overexpression of some of the jasmonate signaling pathway genes, which might trigger ectopic lignification in response to cell wall integrity disruptions. In summary, the structural plasticity of primary cell walls is proven, since a lignification process is possible in response to cellulose impoverishment.

Keywords: Cellulose; DCB; dichlobenil; ectopic lignin; maize
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INTRODUCTION

The primary cell wall is a complex structure surrounding the protoplasm of elongating plant cells and it is crucial for shape maintenance and directional growth during cell development (Carpita 1996). Moreover, as the outermost layer of the plant cell, it is an active component in response to biotic and abiotic stresses with the capacity to monitor and maintain its integrity by means of structural and compositional changes (Hamann 2014). As with other grasses, the primary cell wall in maize (type II) is mainly composed of a framework of cellulose microfibrils embedded in a matrix of arabinoxylans. Smaller amounts of xyloglucan, mixed-linked glucans, pectins and glycoproteins can also be found as cell wall matrix components (Carpita 1996).

Cellulose, the main load-bearing structure of plant cell walls, is a polymer of β-1,4 linked glucan chains synthesized by transmembrane protein complexes (Guerriero et al. 2010). Cellulose is deposited in the cell wall in the form of microfibrils probably composed of 18 or 24 chains (Jarvis 2013). Arabinoxylans, the second major component of maize primary cell walls, play a pivotal role since different populations function by tethering adjacent cellulose microfibrils and forming the matrix phase of cell walls (Scheller and Ulvskov 2010). The arabinoxylan backbone is composed of β-1,4-linked xylose residues commonly substituted at C(O)3 and/or C(O)2 with arabinose or (4-O-methyl) glucuronic acid (Fincher 2009). One of the unique features of arabinoxylans from grasses is that the arabinose residues are often esterified at C(O)5 with the hydroxycinnamates ferulic and *p*-coumaric acid. Due to their high reactivity, polysaccharide-esterified hydroxycinnamates promote arabinoxylan cross-linking, playing a major role in maintaining the integrity of grass cell walls (Buanafina 2009).

Lignin is a complex phenolic heteropolymer predominantly deposited in the secondarily thickened cell walls of specialized plant cell types. Lignin drastically modifies cell wall structure and functions, since after its deposition cell walls acquire hydrophobicity and increase their resistance to mechanical and chemical degradation (Vanholme et al. 2010; Liu 2012) being a key factor in the evolution of tracheophytes vascular system (Lucas et al. 2013). The main building blocks of

lignin are the 4-hydroxycinnamyl alcohols (or monolignols): coniferyl and sinapyl alcohols with lesser amounts of *p*-coumaryl alcohol (Boerjan et al. 2003). Monolignols are synthesized in the cytosol from phenylalanine by the phenylpropanoid pathway and transported into the cell wall where they are subjected to oxidative cross-linking by cell wall peroxidases, laccases or other phenol oxidases using hydrogen peroxide or oxygen as oxidants (Passardi et al. 2004; Fagerstedt et al. 2010; Kärkönen and Kuchitsu 2014). Once polymerized into lignin, *p*-coumaryl, coniferyl and sinapyl alcohol give rise to *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively (Vanholme et al. 2010; Liu 2012).

Lignification is a tightly developmentally regulated process commonly associated with the formation of a secondarily thickened cell wall during cell specialization. Besides the developmentally regulated lignin, biotic and abiotic stresses can induce unexpected lignification known as ectopic lignification (Caño-Delgado et al. 2000; Moura et al. 2010; Sattler and Funnell-Harris 2013; Miedes et al. 2014). Although there are very few reports in the literature of this phenomenon in exclusively primary-walled cell cultures, lignin-like polymers have been shown to be produced by *in vitro* model systems under certain conditions (Novo-Uzal et al. 2009; Kärkönen and Koutaniemi 2010; Shen et al. 2013). In some systems, hormonally triggered cells can differentiate into tracheary elements, in which lignin is deposited in the newly formed secondary cell wall (Fukuda and Komamine 1980; Oda et al. 2005). In other cases, triggered cell cultures (normally by sucrose or elicitor treatments) release extracellular lignin into the culture medium (Simola et al. 1992; Lange et al. 1995; Nose et al. 1995; Kärkönen et al. 2009).

In the last few decades, a series of different approaches using cellulose biosynthesis inhibitors, mutants or transgenic plants have revealed compensatory effects between cellulose and non-cellulosic components of both primary and secondary cell walls. A reduction in cellulose content or an altered pattern of cellulose deposition has been demonstrated to cause changes in matrix polysaccharides and cell wall ectopic lignification (Caño-Delgado et al. 2000, 2003; Desprez et al. 2002; Ellis et al. 2002; Hernández-Blanco et al. 2007; Bischoff et al. 2009; Hamann et al. 2009; Denness et al. 2011; Brabham et al. 2014). Furthermore, lignin-defective transgenic plants respond with qualitative and quantitative changes in the polysaccharide counterpart (Sonbol et al. 2009; Ambavaram et al. 2011; Fornalé et al. 2012).

In previous studies, maize cell lines habituated to otherwise lethal concentrations of DCB (2,6-dichlorobenzonitrile, dichlobenil), a well-known cellulose biosynthesis inhibitor, were obtained by means of incremental exposure over many culturing cycles (Mélida et al. 2009; de Castro et al. 2014). These cell cultures had the capacity to cope with DCB through the acquisition of a modified cell wall in which the cellulosic scaffold was completely or partially replaced by a more extensive network of highly cross-linked arabinoxylans (Mélida et al. 2009, 2010a, 2010b, 2011). Our preliminary data indicated that DCB habituation could also induce ectopic lignification (as cellulose-deficient walls from habituated cells resulted positively for phloroglucinol staining) as a consequence of a reduction in cellulose in maize cells. An in-depth characterization of this phenomenon could further our understanding of the chemical composition of ectopic lignin and the relationship between ectopic lignification and stress responses. In this study, we characterized cell walls from maize suspension-cultured cells habituated to low (1 μM) and high (6 μM) DCB concentrations and from DCB short-term treated cell suspensions (I_{50} value for maize suspension cultured cells is 0.5 μM DCB; de Castro et al. 2014), paying special attention to the putative ectopic lignin/lignin-like component as well as the expression levels of genes specific for the biosynthesis of monolignol units of lignin and others involved in common stress signaling pathways.

RESULTS

Cell wall fingerprinting indicated increased phenolics-to-polysaccharides ratios due to DCB exposure

FTIR spectra of non-habituated (SNH), DCB short-term treated (SNH+DCB) and habituated to (SH1) 1 and (SH6) 6 μM DCB maize suspension-cultured cells were obtained, normalized and baseline corrected. Averaged difference spectra were obtained by digital subtraction of SNH spectra from each of the DCB-treated/habituated cell lines (Figure 1). Compared with SNH spectra, those from both short-term treated and habituated cell walls showed negative peaks in the region ranging from 900 to 1,200 cm^{-1} where most of the cell wall polysaccharides, including cellulose, absorb (Alonso-Simón et al. 2011; Largo-Gosens et al. 2014). In addition, positive peaks were detected associated with wavenumbers indicative of aromatic rings (1,515, 1,600 and 1,630 cm^{-1}), phenolic rings (1,500 cm^{-1}) and phenolic esters (1,720 cm^{-1}) (Kačuráková et al. 2000), indicating that both DCB-treated and DCB-habituated cells were enriched in phenolics. In accordance with this, wave number ratios 1,540/1,160, 1,540/1,425 and 1,540/1,740 cm^{-1} normally associated with increased lignin-to-polysaccharides ratios raised in both DCB-treated and DCB-habituated cells (Table 1).

Highly branched and cross-linked arabinoxylans increased in parallel to the DCB habituation process

Cell wall fractionation showed that in both DCB-treated and DCB-habituated cells, most of the non-cellulosic cell wall polysaccharides (70%-80%) corresponded to KOH-extractable hemicelluloses, namely KI and KII fractions (Figure S1). Moreover, differences were observed in cell wall fractionation among cell lines. Of particular note was the increase in strong alkali-extracted hemicelluloses (KII fraction: 29% in SNH vs. 42% in SH6) exclusively associated with habituation to high DCB concentrations (Figure S1).

The monosaccharide composition of each of the fractions was determined by gas chromatography and spectrophotometric methods (Figure 2). The CDTA-pectic fraction was enriched in uronic acids, and minor amounts of the neutral sugars Ara, Xyl, Gal and Glc were also detected (Figure 2A). The abundance of uronic acids compared to neutral sugars indicated the presence in the CDTA fraction of homopolymers based on the acidic sugars (i.e. homogalacturonan). However, only minor differences in the CDTA fraction were found between SNH and the rest of the lines. KOH-extracted hemicelluloses were mainly composed of Ara, Xyl and uronic acids, indicative of (glucurono-) arabinoxylans and/or acidic pectins associated to arabinoxylans (de Castro et al. 2014),

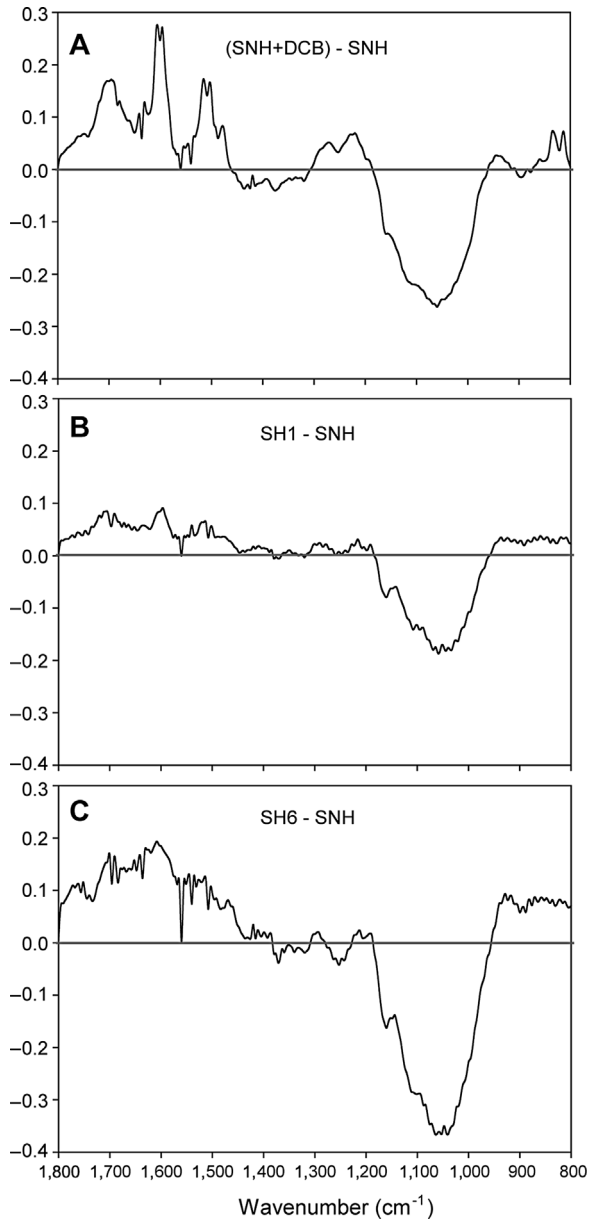

Figure 1. Fourier transformed infrared (FTIR) spectroscopy analysis of cell walls
Averaged FTIR difference spectra obtained after digital subtraction of the SNH cell wall FTIR spectra from SNH+dichlobenil (DCB), SH1 or SH6 cell wall FTIR spectra. Maize cell lines were annotated as follow: non-habituated (SNH); DCB short-term treated (SNH + DCB), habituated to 1 μM DCB (SH1) or habituated to 6 μM DCB (SH6) maize cell suspension-cultured cells.

Table 1. Fourier transform infrared (FTIR) wavenumber ratios characteristic of lignin and cell wall polysaccharides

FTIR peak height ratio	SNH	SNH + DCB	SH1	SH6
1540/1160 cm^{-1}	0.09	0.14	0.13	0.21
1540/1425 cm^{-1}	0.16	0.21	0.19	0.28
1540/1740 cm^{-1}	0.16	0.18	0.18	0.25

Peak assignment, 1,160 cm^{-1}, C–O–C vibration of the glycosidic link in cellulose, xyloglucan or pectic polysaccharides; 1,425 cm^{-1}, C–H stretching in CH_2 groups of cellulose; 1,540 cm^{-1}, aromatic ring stretching in lignin; 1,740 cm^{-1}, C–O stretch in ester groups. For maize cell line annotations see Figure 1 legend.

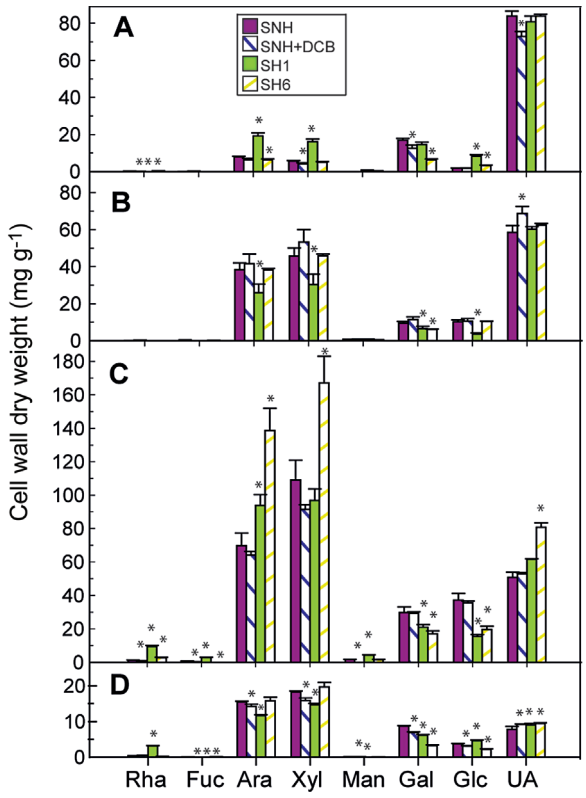

Figure 2. Cell wall sugar analysis
Sugar composition of (**A**) CDTA, (**B**) KI, (**C**) KII and (**D**) trifluoroacetic acid (TFA) cell wall fractions obtained from (purple filled) SNH, (blue barred) SNH+DCB, (lime-green filled) SH1 and (yellow barred) SH6 cell lines. For maize cell line annotations see Figure 1 legend. Ara (arabinose), Fuc (fucose), Gal (galactose), Glc (glucose), Man (mannose), Rha (rhamnose), UA (uronic acids), Xyl (xylose). Data represents the means values ± standard deviation (SD) of three technical replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P < 0.05$).

followed by minor amounts of Gal and Glc (Figure 2B, C). Quantitatively, KII represented the main fraction, and a monosaccharide analysis revealed an increase in the Ara and Xyl proportions associated with habituation to DCB (especially with high concentrations), but not with short-term exposures (Figure 2C; Table 2). The observed increase in the Ara-to-Xyl

ratio detected in SH1 and SH6 when compared with SNH, indicated not only a quantitative increase in heteroxylans but also the presence of highly substituted xylan populations (Table 2). The final residues after CDTA and alkali extractions were TFA-hydrolyzed. Gas chromatography analysis of the TFA fraction resembled that from KI fraction with lesser proportions of the acidic sugars, suggestive of alkali-resistant heteroxylans (Figure 2D). No differences among cell lines were found for this fraction.

Table 2. Arabinose and xylose content in the KII fractions

	(%) Ara + Xyl		Ara:Xyl ratio
	Cell wall	KII-extracted sugars	
SNH	17.9 ± 0.9	59.5 ± 1.7	0.64
SNH+DCB	15.6 ± 0.5	56.7 ± 0.3	0.70
SH1	19.1 ± 1.3	62.4 ± 0.8	0.97
SH6	**30.6 ± 2.9**	**71.5 ± 1.4**	0.83

Mean values ± standard deviation (SD) of three technical replicates per line. For cell line annotation see Figure 1 legend. Values that are significantly different from SNH after a Student's *t*-test ($P < 0.05$) are highlighted in bold.

Cellulose reduction is a consequence of habituation to DCB

Cellulose averaged approximately 25% of the cell wall dry weight when assayed in SNH cells (Figure 3). Cellulose content decreased along DCB-habituated cells in a dose-dependent manner, up to the 50% and 70% reduction found, respectively, in SH1 and SH6 cells when compared with SNH cells (Figure 3). However, short-term incubations with the cellulose biosynthesis inhibitor did not induce significant reductions in cellulose content. On the other hand, these short-term incubations did increase the amount of a Klason-resistant residue, which could be associated with lignin or a lignin-like phenolic-rich material (Figure 3). This lignin-like material was also found to be increased in the cell wall of the DCB-habituated cells. Indeed, SH1 and SH6 cells contained approximately 2 to 5 times more of this residue, respectively, when compared with the SNH counterpart (Figure 3).

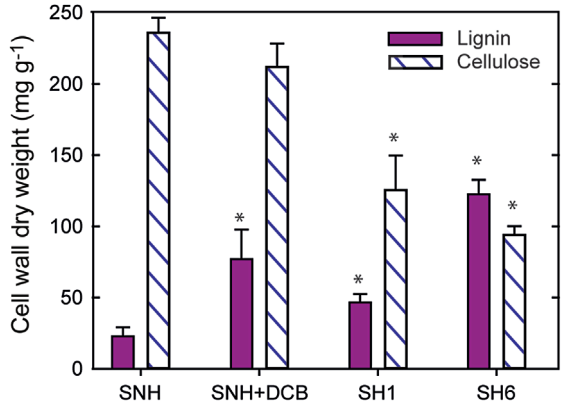

Figure 3. Comparison of cellulose and lignin content in SNH, SNH+DCB, SH1 and SH6 cell lines

For maize cell line annotations see Figure 1 legend. Data represents means ± standard deviation (SD) of at least four replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P < 0.05$).

Cell wall phenolic profile

Maize primary cell wall typically contains high levels of wall-esterified phenolics, which appear as side-chain decorations of arabinoxylans. *p*-Coumarate, ferulate and their oxidative coupling products, diferulates, increased steeply over the course of the DCB habituation process (Table 3). In comparison with SNH, SH1 and SH6 cell walls were enriched in the 5,5′, 8,5′ and specially the 8-*O*-4′ form of diferulates. In all cases, enrichment was more noticeable in SH6 cells.

In comparison to SNH cells, trends similar to those for the DCB-habituated cells were observed when the phenolic profile of DCB short-term treated cells was analyzed (Table 3). Most notably, there was a marked increase in cell wall esterified *p*-coumarate, with SNH+DCB cells being 132- and 15-fold enriched in *p*-coumarate when compared with SNH and SH6 cells, respectively.

DCB induced the deposition of lignin-like polymers in maize cultured cells

Phloroglucinol-HCl, which specifically stains 4-O-linked hydroxycinnamyl aldehyde residues of lignin (Pomar et al. 2002), was used to preliminarily confirm the presence of a lignin-like phenolic-rich material in the cell walls of DCB short-term treated and habituated cells. This strategy demonstrated that lignin accumulation depended on the presence of DCB in the culture medium (Figure 4); maize suspension-cultured cells stained negative for phloroglucinol when cultivated in a medium lacking DCB (Figure 4A). In the case of maize cells short-term incubated in 6 μM DCB (Figure 4B) and DCB-habituated cells (Figure 4C), positive phloroglucinol staining was observed on the surface of cell-aggregates. No evidence of differentiation into tracheary elements were observed in any case (data not shown).

The presence of lignin-like polymers was further confirmed by thioacidolysis followed by gas chromatography coupled to mass spectrometry (GC-MS) of the cleavage products. This analysis also confirmed the presence of trace amounts of sinapyl alcohol (S units) in SNH, SNH+DCB and SH1

Table 3. Cell wall esterified phenolics composition

	p-Coumarate	Ferulate	Diferulates			
			Total	5,5′-	8-O-4′-	8,5′-[a]
	mg g^{-1} cell wall					
SNH	0.21	0.82	1.83	0.48	0.76	0.59
SNH+DCB	27.80	6.60	2.33	0.56	1.20	0.57
SH1	0.52	11.55	2.29	0.54	1.13	0.62
SH6	1.83	17.71	3.58	1.07	1.58	0.93

Mean values from two independent experiments per cell line. For cell line annotation see Figure 1 legend. [a] 8,5′-diferulate was calculated as the sum of 8-5-open and 8-5 benzofurans forms.

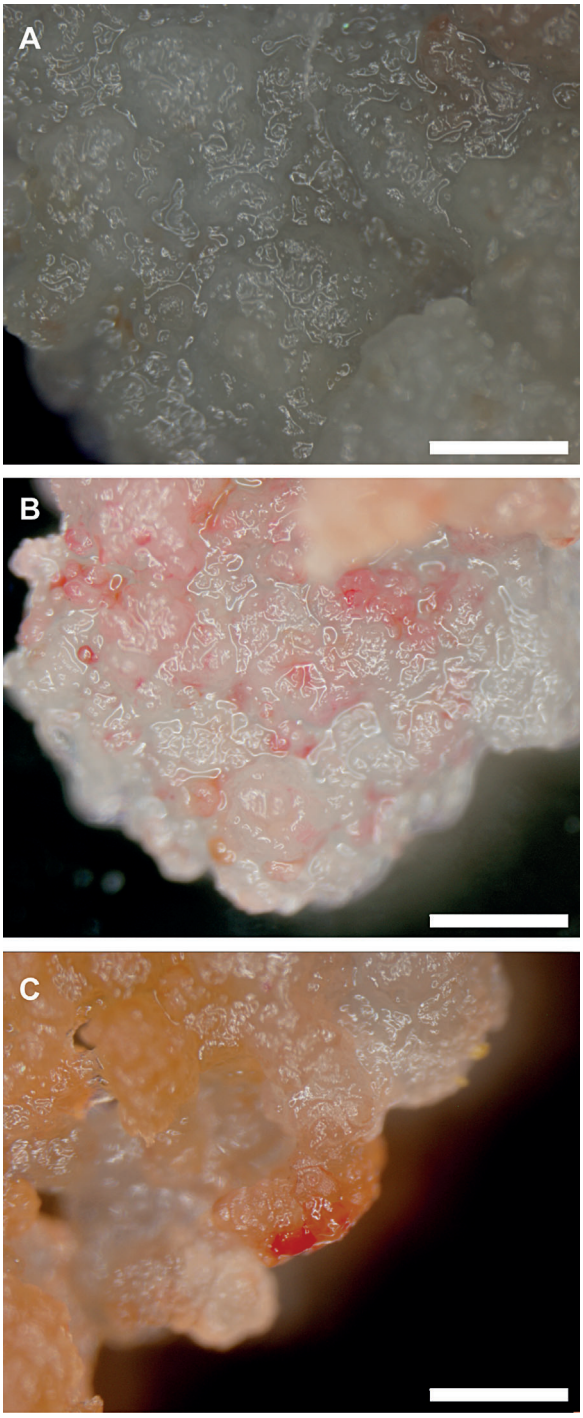

Figure 4. Phloroglucinol/HCl staining (A) of non-habituated (SNH), (B) SNH + (DCB), and (C) SH6 cells
For maize cell line annotations see Figure 1 legend. Bar = 0.5 mm.

cell walls (Figure 5B, D and data not shown). In addition to the S units, measurable amounts of coniferyl alcohol (G units) were detected in SH6 cell walls, but not in the other cell lines (Figure 5E, F). Indeed, S units were (semi-quantitatively) estimated to be more abundant in SH6 than in any other cell line. Based on thioacidolysis results, an S/G ratio of 1.45 was estimated for the SH6 lignin-like material (Table 4).

Lignin biosynthesis-specific genes are overexpressed in DCB-habituated cells

In a previous study by our group, we demonstrated that the genes functioning in the initial steps of the phenylpropanoid pathway (*Phenylalanine Ammonia-Lyase*, *Cinnamate 4-Hydroxylase*, *4-Coumarate CoA Ligase*, *Hydroxycinnamoyl-CoA Shikimate/quinate hydroxycinnamoyl Transferase* and *Caffeic acid O-Methyltransferase*) are overexpressed in DCB-habituated cells (Mélida et al. 2010a). The corresponding proteins from such genes are involved in the production of *p*-coumaroyl-CoA and feruloyl-CoA, the substrates for hydroxycinnamate esterification of arabinoxylans (Lindsay and Fry 2008). Given the evidence of the presence of lignin-like polymers in DCB-habituated cells, quantitative RT-PCR was used to monitor the transcript abundance of *cinnamoyl-CoA reductase* (*CCR*), *ferulate 5-hydroxylase* (*F5H*) and *cinnamyl alcohol dehydrogenase* (*CAD*), involved in the last steps of monolignol synthesis (Figures, 7 6).

A general overexpression of the two *ZmCCR* genes was observed in DCB-habituated cell lines (Figure 6A, B), whereas short-term treatment with DCB induced only minor changes in *ZmCCR1* and *ZmCCR2* mRNA levels. The expression of *ZmF5H2* was significantly increased in all cell lines when compared with SNH cells, and this enhancement was especially noticeable in DCB-habituated lines (Figure 6D). In the case of *ZmF5H1*, only SH1 cells showed higher transcript abundance, and indeed this gene was repressed in SNH+DCB and SH6 (Figure 6C). Both DCB short-term treatment and DCB habituation induced an overexpression of *ZmCAD1*, *ZmCAD5* and *ZmCAD7* genes in comparison with SNH cells (Figure 7A, D, F). This enhancement was especially marked in the case of *ZmCAD7* transcript levels in SH1 cells, whereas the abundance of *ZmCAD6* transcripts was only significantly increased in habituated cells (Figure 7E). Moreover, there was a high overexpression (12-fold) of *ZmCAD2* in SH6 cells; however, the transcript levels of this gene were significantly reduced in SH1 cells (Figure 7B). Surprisingly, there was a significant repression of the transcript levels coding for ZmCAD3 in DCB-habituated cell lines (Figure 7C). The *ZmCAD4* transcription levels were too low to be accurately quantified by this procedure. Given the general overexpression of the genes coding for CAD proteins in response to DCB, we measured CAD activity in the different cell lines (Figure 7G). CAD activity assayed from cell extracts was significantly increased in SNH+DCB and SH6 when compared with SNH cell lines, but unchanged in the case of SH1 cells.

Apoplastic hydrogen peroxide accumulation

Both DCB short-term treated and DCB-habituated cells accumulated significantly more H_2O_2 in the spent medium than SNH cells (Table 5), although there is no clear relationship between H_2O_2 accumulation and the presence of lignin-like polymers. SH6 cells, which showed the strongest ectopic lignification, did not peak in H_2O_2 content when compared with SH1 or SNH+DCB cells. In fact, SH6 cells accumulated less H_2O_2 during the lag and exponential phases than SH1 or SNH+DCB cells.

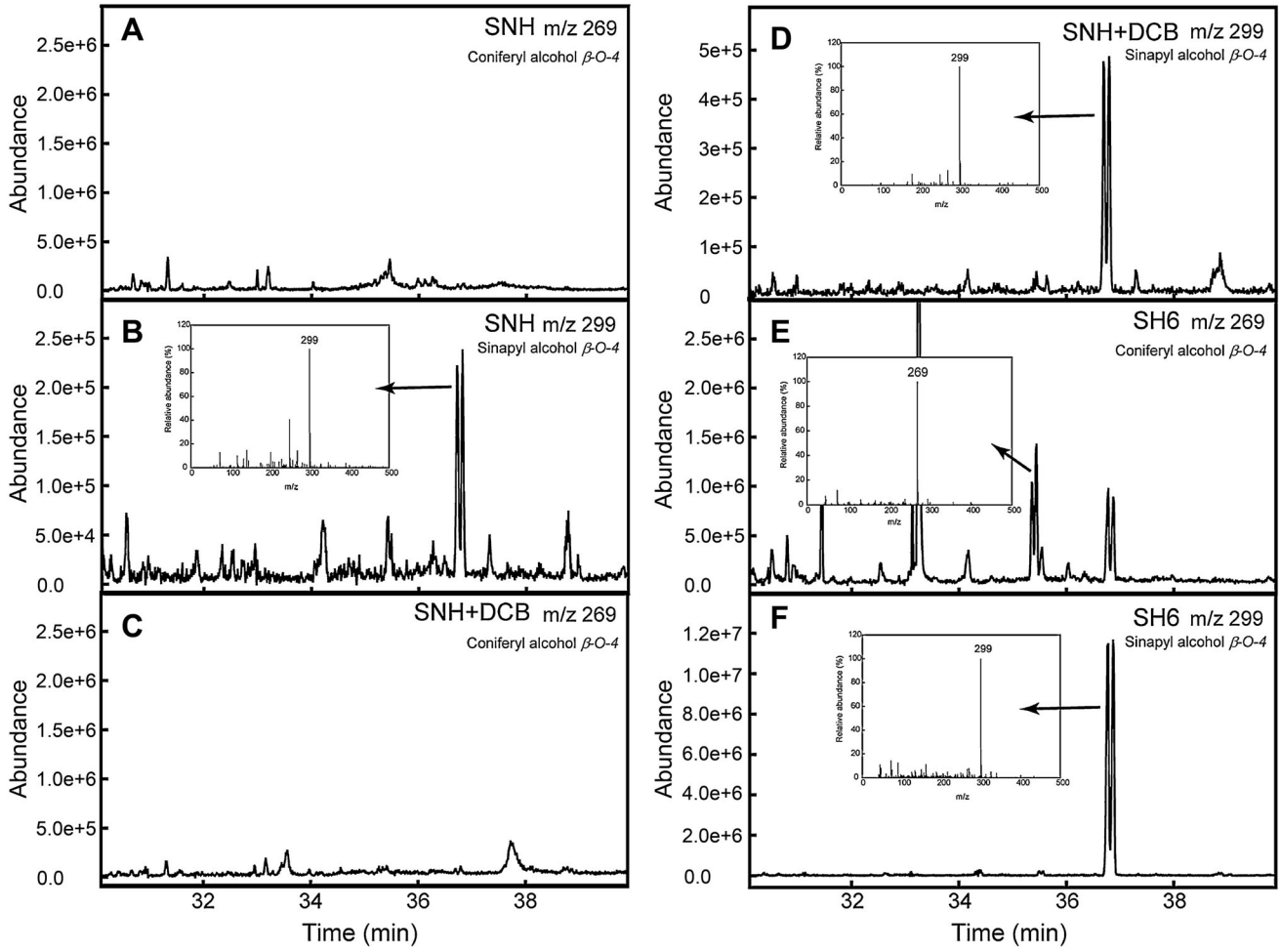

Figure 5. Lignin monomer composition

Gas chromatography (GC) profiles of the thioethylated monomers (erythro and threo isomers) arising from aryl-glycerol-b-aryl ether (β-*O*-4) structures derived from **(A, C** and **E)** coniferyl and **(B, D** and **F)** sinapyl alcohols from cell walls of **(A, B)** SNH; **(C, D)** SNH+DCB; and **(E, F)** SH6 cell lines**.** For maize cell line annotations see Figure 1 legend.

JA synthetic and JA signalling pathways overexpressed

To determine whether the accumulation of lignin-like material formed part of an abiotic stress response mechanism, RT-PCR was used to monitor the expression levels of several genes from the jasmonic acid (JA) and salicylic acid (SA) stress signaling pathways (Figure 8). Three *12-oxophytodienoate reductase* (*OPR*) genes, coding for proteins involved in the synthesis of JA, were analyzed. Two of them (*ZmOPR1* and *ZmOPR2*) were always overexpressed in the presence of DCB, but *ZmOPR7* was only overexpressed in DCB-habituated cell lines, and was slightly repressed by the short-term exposure of SNH cells to DCB (Figure 8). *NADPH oxidase* (*NADPHOX*) and *maize protease inhibitor* (*MPI*) genes are reported to be JA-induced in response to abiotic stresses (Shivaji et al. 2010). The results showed that both genes were overexpressed in the presence of DCB.

For the SA stress signaling pathway, *pathogenesis related protein 1* (*PR1*) and *non-expressor of PR1* (*NPR1*) genes were studied. The *ZmNPR1* gene was detected, but there were no differences in the expression pattern induced by either DCB exposure or DCB habituation. *ZmPR1* transcripts were not detected in any cell line.

Table 4. Lignin monomeric composition as revealed by thioacidolysis

	Total ionic current ($\times 10^6$) mg^{-1} cell wall		
	Coniferyl alcohol (G units)	Sinapyl alcohol (S units)	S/G ratio
SNH	0	7.1 ± 1.2	–
SNH+DCB	0	**9.7 ± 0.7**	–
SH1	0	**9.5 ± 0.2**	–
SH6	**20.6 ± 1.0**	**29.9 ± 2.5**	1.45

Mean values ± standard deviation (SD) of three replicates per cell line. For cell line annotation see Figure 1 legend. Values that are significantly different from SNH are in bold (Student's *t*-test, $P < 0.05$).

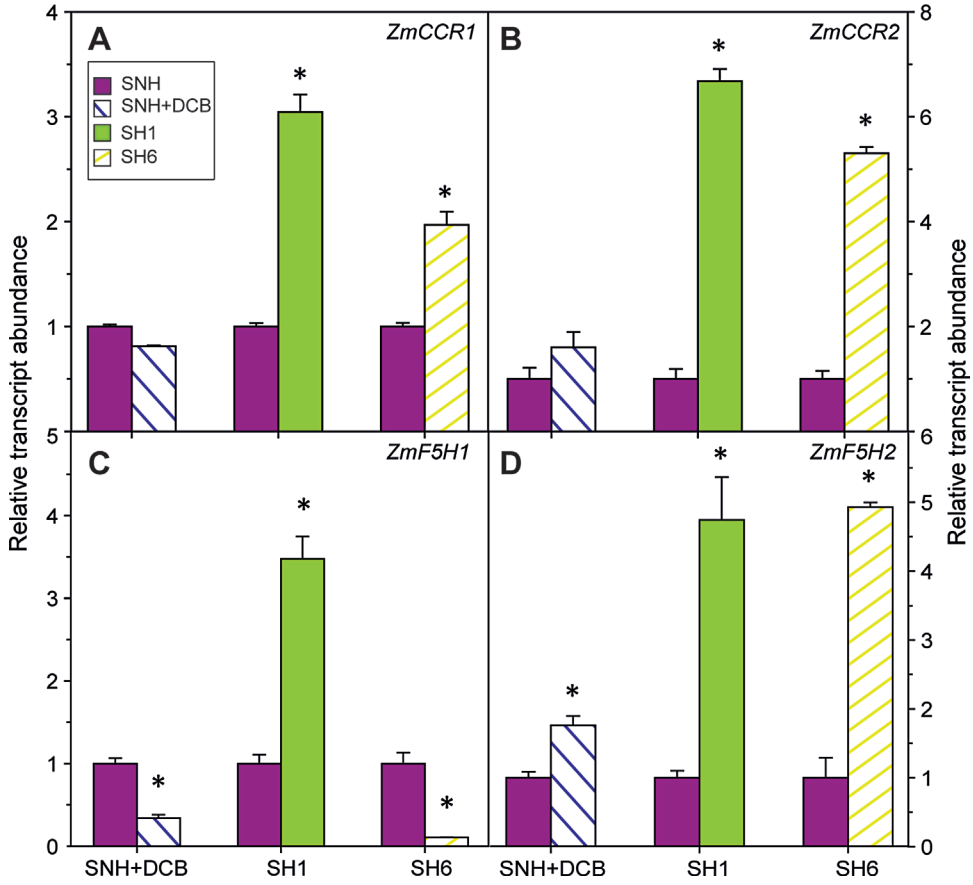

Figure 6. Quantitative reverse transciption-polymerase chain reaction (qRT-PCR) characterization of (A, B) *ZmCCR* and (C, D) *ZmF5H* genes of SNH, SNH+DCB, SH1 and SH6 cell lines

The gene expression levels of SNH+DCB, SH1 and SH6 cell lines were always compared against the SNH ones, which are represented as purple bars. For maize cell line annotations see Figure 1 legend. Data represent relative fold change relative to SNH genes ± standard deviation (SD) of three replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P < 0.05$).

DISCUSSION

In their natural habitats, plant cells must continuously remodel their cell walls in order to grow and to interact with the environment. In order to understand the limits of these interactions, plant cells can be cultivated in fully controlled experimental systems where their capacity to cope with different situations can be better studied. The habituation of plant cell cultures to cellulose biosynthesis inhibitors such as DCB represents a valuable tool to improve our knowledge of the mechanisms involved in plant cell wall structural plasticity (Shedletzky et al. 1992; Encina et al. 2002; Manfield et al. 2004; García-Angulo et al. 2009; Mélida et al. 2009; Brochu et al. 2010; de Castro et al. 2014, 2015).

In previous studies, we have shown that the habituation of maize cells to DCB involves several metabolic modifications (Mélida et al. 2010a; de Castro et al. 2014, 2015). Maize cells habituated to high DCB levels (≥ 30 times higher than DCB I_{50} value) display strong reduction in cellulose and altered expression of several *Cellulose Synthase* genes (Mélida et al. 2009, 2010a). Although DCB induces oxidative damage (based on lipid peroxidation levels in maize cultured cells; unpublished results), given the level of detoxifying/antioxidant activities measured, it seems that DCB-habituated maize cells do not rely on an antioxidant strategy to cope with this herbicide, which contrasts with the strategy observed in cells of other species, such as bean, in which antioxidant capacity is enhanced when habituated to DCB (García-Angulo et al. 2009; Mélida et al. 2010a). Indeed, the ability of maize cells to grow under high DCB concentrations resides mainly in their capacity to reorganize their cell wall architecture. Through compositional analysis and structural characterization of DCB-habituated cell walls, it has been possible to demonstrate that these cells compensate for cellulose impoverishment with other cell wall components. The mechanism for this accommodation consists of producing a more extensive, cross-linked network of arabinoxylans (Mélida et al. 2009, 2010a, 2010b, 2011). More recently, we have found that some of the cell wall modifications differ according to DCB habituation level (de Castro et al. 2014).

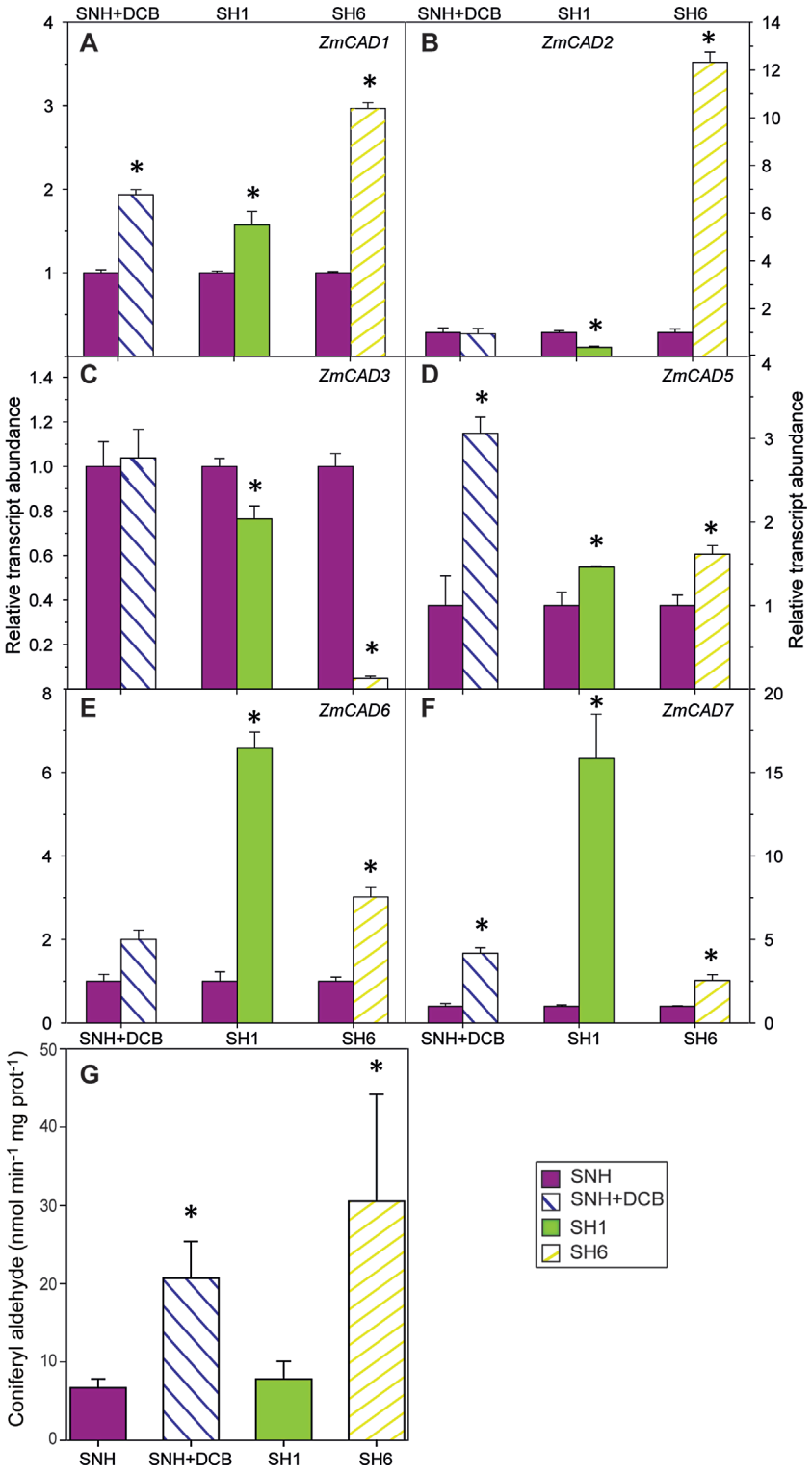

Figure 7. Quantitative reverse transcription-polymerase chain reaction(qRT-PCR) characterization of (A–F) *ZmCAD* genes and (G) CAD enzyme activity assay of SNH, SNH+DCB, SH1 and SH6 cell lines

The gene expression levels of SNH+DCB, SH1 and SH6 cell lines were always compared against the SNH ones, which are represented as purple filled bars. For maize cell line annotations see Figure 1 legend. For (**A–F**), data represent relative fold change relative to SNH genes ± standard deviation (*SD*) of three replicates. For (**G**), data represents means ± SD of at least nine replicates. Asterisks indicate values that are significantly different from SNH after a Student's *t*-test ($P < 0.05$). ZmCAD4 mRNA transcripts were not detected.

Table 5. Apoplastic H_2O_2 concentration measured in the spent medium of the different cell lines

	Lag phase	Exponential phase	Stationary phase
	H_2O_2 (μM)		
SNH	0.4 ± 0.3	0.8 ± 0.1	0.6 ± 0.1
SH1	**1.9 ± 0.3**	**2.4 ± 0.2**	**2.3 ± 0.2**
SH6	**0.8 ± 0.1**	**1.7 ± 0.5**	**2.4 ± 0.2**
Incubation time	1 day	6 days	
SNH+DCB	**1.0 ± 0.1**	**2.0 ± 0.1**	

Mean values ± standard deviation (*SD*) of three replicates per line. Values were obtained at the different growth phases for each line. Short-term treated cells (SNH + DCB) were measured 1 day and 6 days after the addition of DCB. Values that are significantly different from SNH are in bold (Student's *t*-test, $P < 0.05$).

In this study, we used maize cell suspension cultures habituated to low (1 μM DCB, SH1) and high (6 μM DCB, SH6) levels of DCB as well as non-habituated cells treated for a short time with lethal doses of the herbicide (SNH+6 μM DCB). In agreement with previous studies, we have shown that habituated cell lines display dose-dependent reductions in their cellulose content. These cellulose reductions (up to 70% less than in SNH) were compensated by a more extensive network of arabinoxylans, which could only be extracted with strong alkali.

In line with previous results obtained for maize callus cultures habituated to high DCB concentrations (Mélida et al. 2010b; 2011), we found that hydroxycinnamates, the arabinoxylan cross-linkers, experienced quantitative changes that indicated a prominent role of these compounds in a cellulose-deficient cell wall. This is actually one of the singularities of this model system. Most of the cell lines habituated to cellulose biosynthesis inhibitors (or other cell wall stresses) have had type I primary cell walls (i.e. *Arabidopsis*, poplar, bean, tomato), where cellulose reductions were compensated by pectins (Shedletzky et al. 1990; Encina et al. 2002; Manfield et al. 2004; Brochu et al. 2010). In contrast to type I, type II primary cell walls are characterized by the presence of phenylpropanoids (mainly ferulic and *p*-coumaric acids), which have an important role in cross-linking hemicelluloses (Wallace and Fry 1994). Ferulate and its dimers increased steeply over the course of the DCB habituation process, but it was the changes in the proportions of esterified *p*-coumarate, which indicated that something else was happening. Indeed, in this case the changes observed for the short-term treatments were quite striking, as SNH+DCB cells were 132- and 15-fold enriched in *p*-coumarate when compared with SNH and SH6 cells, respectively. In the case of maize plants, small amounts of *p*-coumaric acid are esterified to arabinoxylans in primary walls, but later on in wall development, it is found more extensively esterified to lignin (Iiyama et al. 1994; Ralph et al. 1994a). Indeed *p*-coumarate incorporation into the cell wall has been positively correlated with lignification (Hatfield and Marita 2010).

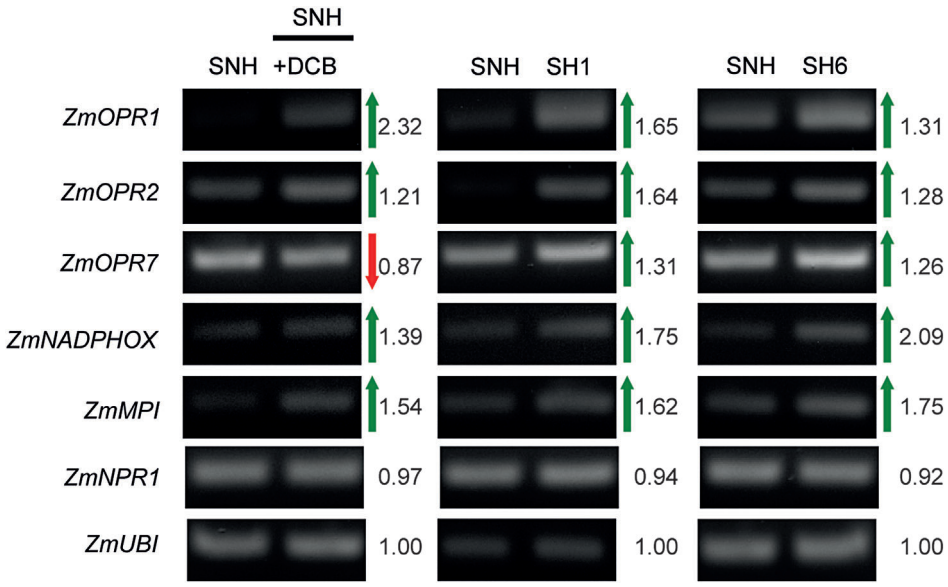

Figure 8. Relative expression levels of jasmonic acid (JA) and salicylic acid (SA) signaling pathways genes analyzed by reverse transcription-polymerase chain reaction (RT-PCR) of non-habituated (SNH), SNH + (DCB), SH1 and SH6 cell lines
For maize cell line annotations see Figure 1 legend. The up and down arrows indicate less and more mRNA accumulation than SNH cells, respectively. 12-oxophytodienoate reductase (*ZmOPR1*, *ZmOPR2* and *ZmOPR7*), NADPH oxidase (*ZmNADPHOX*), maize protease inhibitor (*ZmMPI*), and nonexpressor of pathogenesis related protein 1 (*ZmNPR1*). Pathogenesis related protein 1 (*ZmPR1*) was not detected. Primers can be found in Table S1. Numerals indicate the normalised ratios of RT-PCR band intensities calculated by dividing the band intensity of SNH+DCB, SH1 or SH6 by SNH for each gene.

These findings suggest the presence of ectopic lignin or lignin-like polymers at least in the case of short-term treated cells, where the induced stress would explain their presence. Surprisingly, not only the short-term treated cells but also the DCB-habituated ones displayed a pink to brownish colour after phloroglucinol staining, indicative of lignin or lignin-like polymers (Pomar et al. 2002).

Our results clearly show the presence of ectopic lignin in maize primary cell walls of both DCB-habituated and short-term treated cells. Therefore, phenolics not only act as hemicellulose cross-linking units in this system, but also constitute monolignol-based polymers similar to lignin that might contribute to stiffening of a cellulose-impoverished wall. Although ectopic lignification has been observed in *Arabidopsis* mutants with reduced cellulose synthesis and in seedlings treated with cellulose biosynthesis inhibitors (Caño-Delgado et al. 2003; Bischoff et al. 2009; Denness et al. 2011), there are few reports of this phenomenon in exclusively primary-walled cell cultures (Ros Barceló 1997). Moreover, transcriptomic approaches using *Arabidopsis* and poplar cell cultures habituated to cellulose biosynthesis inhibitors have shown that several genes specifically involved in lignin synthesis are downregulated (Manfield et al. 2004; Brochu et al. 2010). Lignin-like polymers have been shown to be produced by other *in vitro* model systems under certain conditions (Kärkönen and Koutaniemi 2010). However, although these systems achieve lignin production in plant cultured cells, ectopic lignin deposition in primary cell wall, the feature of cell suspension cultures presented in this study, has rarely been reported (Christiernin et al. 2005; Novo-Uzal et al. 2009; Shen et al. 2013).

In addition to its roles in cell wall stiffening, lignin deposition has long been implicated as an important defense mechanism against pests and pathogens (Vance 1980; Barros-Rios et al. 2011). Lignin or lignin-like polymers are induced and rapidly deposited in cell walls in response to both biotic and abiotic stresses (Moura et al. 2010; Sattler and Funnell-Harris 2013; Miedes et al. 2014). Two types of lignin can be distinguished: (i) the one normally present in secondarily thickened cell walls with a purely structural role, and (ii) ectopic lignin, unexpectedly deposited in response to biotic and abiotic stresses. Lignin composition is highly heterogeneous and phylogenetically dependent, but also depends on the role the lignin is expected to play. 'Defense' lignin is often associated with elevated levels of H subunits compared with structural lignin (Ride 1975; Lange et al. 1995; Sattler and Funnell-Harris 2013). Although H units were not present in our system (minor component in monocot lignin; Boerjan et al. 2003), given the phloroglucinol-tonality and compositional differences between SNH+DCB (pink/indicative of a predominance of S units) and the SH6 (brown/S+G units) lignin-like polymers, we propose that these polymers could arise from different stimuli. While short-term DCB-treated cells might produce a *sensu stricto* stress-related lignin, habituated cells might accumulate a structural-related lignin. In accordance with this, S to G ratio estimated for the lignin-like polymer found in DCB-habituated cells (1.5) is close to that of lignin from maize stems (1.4) (Fornalé et al. 2012).

By catalyzing the final hydroxyl-cinnamaldehyde reduction to the corresponding alcohols, CAD is a key enzyme in determining lignin content and composition (Mansell et al. 1974; Fornalé et al. 2012). Although several *CAD* isoforms (*1*, *5*, *6* and *7*) were overexpressed in SH1 cells, CAD activity was found unchanged. Therefore, it could be assumed for this cell line that higher proportions of the cinnamaldehyde moieties are incorporated into the phenolic polymers, as occurs in CAD-transgenic and mutant plants (Ralph et al. 2001; Dauwe et al. 2007; Fornalé et al. 2012). However, CAD activity was found to be approximately three to four times enhanced for SNH+DCB and SH6 cells, respectively, compared to SNH. The increased CAD activity in SNH+DCB cells correlated with the overexpression of several *CAD* isoforms (*1*, *5*, *6* and *7*), and since only S units were found in measurable amounts in their cell walls, these proteins are most probably involved in the sinapaldehyde conversion to sinapyl alcohol. All of these isoforms were also overexpressed in SH6 cells. As a differential result, habituated cells showed a high overexpression of *CAD2*, which could be responsible for the synthesis of coniferyl alcohol from coniferaldehyde. In view of these results, we propose ZmCAD2 as a candidate for the production of G units, at least in the case of maize cell cultures, as well as a key player in the production of lignin-like polymers in SH6 cells. Interestingly, *CAD2* has been specifically associated with the synthesis of structural lignin in maize plants (Fornalé et al. 2012), which would agree with the synthesis of a structural related lignin in SH6 cells.

Concerning the two steps prior to CAD, different expression patterns were found in each case. CCR isoforms are responsible for the reduction of *p*-coumaroyl-CoA and feruloyl-CoA to their respective aldehydes. Downregulation of *CCR* in transgenic poplar has been associated with an up to 50% reduction in lignin content and an increased proportion of cellulose (Leplé et al. 2007). Interestingly, in contrast to these poplar trees, DCB-habituated cells with the opposite situation for the load-bearing polymers (less cellulose and more lignin) showed a significant overexpression of both *CCR* isoforms. On the other hand, and also in poplar, upregulation of *F5H* increased the proportion of S units, yielding an S/G ratio of greater than 35 versus approximately 2 for wild type poplar lignin (Stewart et al. 2009). Both *F5H* isoforms were overexpressed in SH1 cells, where only S units could be detected, while one of them was highly downregulated for SH6 cells. In summary, rather than a general stress response, a tight regulation of the monolignol biosynthetic pathway was observed in DCB-habituated cells.

Lignin polymerization is preceded by the peroxidase+H_2O_2 (and/or lacasse+O_2) dependent activation of monolignols to free radicals (Fagerstedt et al. 2010). The spent cell culture medium can be regarded as an extension of the apoplast and it can therefore be used as a compartment to monitor changes in the level of cell wall H_2O_2 (Kärkönen and Kuchitsu 2014). The H_2O_2 over-production of SNH+DCB and SH cells may be explained in the context of a reactive oxygen species over-production following cellulose inhibition, as has been previously reported for *Arabidopsis* plants (Dennes et al. 2011) and maize cultured cells habituated to low DCB concentrations (A Largo unpublished data). Given the steep increase in lignin over the course of DCB habituation, a relationship between lignin accumulation and increased apoplastic H_2O_2 contents may be expected (Nose et al. 1995; Kärkönen et al. 2002). However, no differences in

apoplastic H_2O_2 were found when SH1 and SH6 cells were compared, indicating that H_2O_2 is not a limiting factor in the ectopic lignification reported in this system. An alternative explanation would be that the lignification is consuming apoplastic H_2O_2 explaining the lower level of apoplastic H_2O_2 measured in SH6 cells when compared with SH1 or SNH+DCB ones. Moreover, a study of class III peroxidase activity did not show differences due to DCB habituation in maize cultured cells (data not shown).

There are several lines of evidence that link ectopic lignification in response to cellulose deficiency with JA signaling. *Constitutive expression of vegetative storage protein 1* (*cev1*) and *ectopic lignin 1* (*eli1-1*) *Arabidopsis* mutants, are defective in the cellulose synthase gene *CESA3* involved in cellulose biosynthesis during primary cell wall formation (Ellis and Turner 2001; Ellis et al. 2002; Caño-Delgado et al. 2003). In these mutants, cellulose biosynthesis impairment was compensated by mechanisms such as ectopic lignification, constitutive activation of the JA signaling pathway, and increases in JA and ethylene proportions. In addition, treatments with the cellulose biosynthesis inhibitor isoxaben have been found to phenocopy *eli1-1* lignification in *Arabidopsis* wild type seedlings (Caño-Delgado et al. 2003; Hamann et al. 2009). In JA-insensitive plants, ectopic lignification by isoxaben is reduced, indicating that JA signaling is necessary (Caño-Delgado et al. 2003), a deduction which is further confirmed by the finding that external addition of methyl jasmonate to *Arabidopsis* cell cultures led to increased expression of phenylpropanoid, particularly monolignol biosynthesis (Pauwels et al. 2008). Our results confirm a JA-dependent signaling process in response to cellulose biosynthesis impairment, which led to ectopic lignification. However, according to our RT-PCR results and previous data from proteomic approaches (Mélida et al. 2010a; M de Castro unpublished data), stimulation of the lignification mechanism seems to be SA- and ethylene-independent.

In summary, maize suspension-cultured cells with up to 70% less cellulose produced a more extensive and cross-linked network of arabinoxylans together with a polymeric lignin-like material. This modified cell wall architecture is the result of the high structural plasticity of plant primary cell walls in response to a disruption of cell wall integrity. We propose that a JA signaling program might be triggering the observed ectopic lignification, and this model system will be used in future research in order to study the complex networks involved in cell wall integrity maintenance mechanisms.

MATERIALS AND METHODS

Plant material and DCB habituation process

Maize callus-cultured cells (*Zea mays* L. Black Mexican sweetcorn) were obtained from immature embryos and maintained in Murashige and Skoog media (Murashige and Skoog 1962) supplemented with 9 μM 2,4-D, 20 g L^{-1} sucrose and 8% agar at 25 °C under photoperiodic conditions (16:8; 3,000 lux ≈ 41 μmol m^{-2} s^{-1}). Callus-cultured cells were habituated to grow under originally lethal DCB concentrations, by stepwise transfers to higher DCB levels up to a 12 μM concentration (Mélida et al. 2009). Those cells growing on solid medium were disaggregated and transferred to a liquid medium containing 6 μM DCB (SH6) (Mélida et al. 2011). SH6 cells were maintained at 25 °C under light, rotary shaken and routinely subcultured every 15 days. Control cells were designated as non-habituated maize suspension-cultured cells (SNH). Cell lines habituated to grow under 1 μM DCB (SH1) were obtained from SNH (de Castro et al. 2014).

In order to distinguish toxic DCB effects from those owing to the habituation, short-term treatments with high (lethal) DCB concentrations were performed. Maize control cells were grown in a liquid medium containing 6 μM DCB for 6 days, ensuring a toxic effect but not giving sufficient time to kill the cells (H Mélida unpublished data). These cells were referred to as SNH+DCB.

Cell wall preparation and fractionation

Cell walls were prepared according to Mélida et al. (2009). Briefly, cells were collected during their exponential growth phase, washed extensively with distilled water and immediately frozen. The cells were disrupted in liquid nitrogen using a mortar and pestle. The resulting fine powders were subjected to extraction in 70% (v/v) ethanol for 5 days. The suspensions were filtered through glass-fiber filters (GF/A, Whatman, GE Healthcare, Buckinghamshire, UK), and the pellets were washed six times with 70% ethanol and six times with acetone and were subsequently air dried, to obtain the alcohol insoluble residue. These were then resuspended in 90% dimethylsulphoxide for 8 h three times, filtered as above, washed twice with 0.01 M phosphate buffer pH 7.0 and incubated with 2.5 U mL^{-1} of α-amylase type VI-A dissolved in the same buffer for 24 h at 37 °C. The suspensions were filtered again and washed with ethanol and acetone as indicated above. The dry pellets were treated with phenol:acetic acid: water (2:1:1, v/v/v) for two periods of 8 h, then washed and air dried. The final dry pellets were considered the cell wall extracts.

Cell wall fractions were obtained by consecutively treating the cell wall residues with KOH solutions according to Mélida et al. (2009). Cell walls were extracted at room temperature with 50 mM *trans*-1,2-Diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (CDTA) at pH 6.5 for 8 h and washed with distilled water. The residue was then incubated with 0.1 M KOH + 20 mM $NaBH_4$ for 2 h (×2) and washed with distilled water. Then 4 M KOH + 20 mM $NaBH_4$ was added to the residue for 4 h (×2), and washed again with distilled water. The extracts were acidified to pH 5.0 with acetic acid, dialyzed and freeze-dried, representing CDTA, KI and KII fractions, respectively. The residue after 4 M KOH extraction was hydrolyzed with 2 M trifluoroacetic acid (TFA) for 2.5 h at 120 °C, and after centrifugation, the supernatant was lyophilized and referred to as the TFA fraction.

Cell wall analysis

Tablets for Fourier transform infrared (FTIR) spectroscopy were prepared in a GrasebySpecac press from small samples (2 mg) of cell walls mixed with KBr (1:100, w/w). Spectra were obtained on a Perkin Elmer Spectrum 2000 instrument at a resolution of 1 cm^{-1}. A window between 800 and 1,800 cm^{-1}, which contains information of characteristic polysaccharides, was selected in order to monitor cell wall structure modifications. All spectra were normalized and baseline

corrected with Spectrum software (v5.3.1). Then, data were exported to Microsoft Excel 2010 and all spectra were area-normalized.

Cellulose was quantified in crude cell walls by the Updegraff method as described by Encina et al. (2002). Total sugar quantification of cell wall fractions was performed by the phenol-sulphuric acid method (Dubois et al. 1956) and results were expressed as glucose equivalents. The uronic acid sugars were quantified by the *m*-hydroxydiphenyl method described by Blumenkrantz and Asboe-Hansen (1973) using galacturonic acid as reference standard.

For the analysis of neutral sugars, freeze-dried cell wall fractions were hydrolyzed with 2 M TFA at 121 °C for 1 h. *Myo*-inositol was used as an internal standard. The resulting monosaccharides were converted to alditol acetates as described previously (Albersheim et al. 1967) and analyzed by gas chromatography (GC) on a SP-2380 capillary column (30 m × 0.25 mm i.d.; Supelco) using a Perkin Elmer Autosystem.

Ferulate and *p*-coumarate monomers and ester-bound diferulates were extracted at room temperature from 50 mg of the alcohol-insoluble residues (AIR) using 2 M NaOH for 4 h and analyzed by high performance liquid chromatography (HPLC) based on a method previously described by Santiago et al. (2006). Retention time and UV spectrum of 5,5′-DFA were compared with freshly prepared external standard solutions of 5,5′-DFA, kindly provided by Dr. John Ralph's group (Department of Biochemistry, University of Wisconsin, Madison, USA). The UV absorption spectra of other DFAs were compared with previously published spectra (Waldron et al. 1996) and absorbance at 325 nm was used for quantification. Total ester-linked-DFAs concentration was calculated as the sum of three isomers of DFA identified and quantified by this analytical procedure: 8,5′-DFA, 8-*O*-4′-DFA, and 5,5′-DFA. The 8,5′-DFA concentration was calculated as the sum of the 8,5′-non-cyclic (or open)-DFA and 8,5′-cyclic (or benzofuran)-DFA because the non-cyclic form is most likely formed during alkaline hydrolysis from the native cyclic form (Ralph et al. 1994b).

Lignin-like material was quantified by the Klason gravimetric method with minor modifications. Cell wall extracts were hydrolyzed with 72% (w/v) sulfuric acid for 1 h at 30 °C. Then, the sulfuric acid concentration was diluted to 2.5% (w/v) with water and further incubated at 115 °C for 1 h. The residues were filtrated through Durapore polyvinylidene fluoride (PVDF) filters (Millipore, GE Healthcare, Buckinghamshire, UK, 0.45 μm), dried and weighed.

Thioacidolysis of cell walls, which solubilizes the β-*O*-4 lignin core, and GC-MS analyses were performed (Novo-Uzal et al. 2009) using a Thermo Finnigan (MA, Waltham, USA) Trace GC gas chromatograph, a Thermo Finnigan Polaris Q mass spectrometer, and a DB-XLB, J&W (60 m × 0.25 mm I.d.) column.

Histochemical staining of cinnamyl-aldehydes

Intact filtered maize cells were incubated with 1% (w/v) phloroglucinol in 70% (v/v) ethanol for 5 min. Then, the phloroglucinol solution was removed and the cells were further incubated with an 18% (w/v) HCl solution. Stained cells were observed under Nikon SMZ1500 magnifier and photographed using a Nikon Digital Camera DXM1200F.

Relative gene expression analysis

DCB-habituated cells (SH1 and SH6) were collected during their respective exponential growth phases and a set of SNH cells were collected at the same time for comparison. In the case of short-term DCB treatments, SNH+DCB cells and a set of SNH cells were collected on the third day of culture. Total RNA was extracted from homogenized cells of all lines following the procedures established for Trizol reagent (Invitrogen, California, USA). The purity and integrity of the extracted RNA was evaluated spectrophotometrically using a Nanodrop 1000 and running the RNA in 1% agarose gels. RNA (2 μg) was reverse-transcribed with Super Script III First strand retro-transcriptase (Invitrogen) using oligo$(dT)_{20}$ as primer. The synthesized cDNA was used to perform the gene expression analyses by standard and quantitative PCR methods.

Semiquantitative expression analysis by RT-PCR was performed for jasmonic (JA) and salicylic acid (SA) signaling pathway genes: *12-oxophytodienoatereductase* (*ZmOPR1*, AY921638; *ZmOPR2*, AY921639 and *ZmOPR7*, AY921644), *NADPH oxidase* (*ZmNADPHOX*, CK849936), *maize protease inhibitor* (*ZmMPI*, X78988), *pathogenesis related protein 1* (*ZmPR1*, UB2200) and *non-expressor of PR1* (*ZmNPR1*, EU95584). Primers can be found in Table S1. The *ubiquitin* gene was used as a reference gene for this experiment (*ZmUBI*, U29159) (Fornalé et al. 2006).

Reverse transcription-PCR agarose gels were stained with SYBR Safe DNA gel stain (Invitrogen) and gel images acquired with an Alphaimager HP system (Protein Simple 3001, San Jose, California, USA). The quantification of the bands was performed by using the Alpha view v3.4.0.0. software (ProteinSimple). Band intensity was expressed as relative intensity units. For each individual gene, the band intensity was normalized in relation to ubiquitin and then, the normalized intensity ratios for SNH+DCB/SNH; SH1/SNH and SH6/SNH were calculated.

Relative gene expression was determined by qRT-PCR using specific primers for the following genes: *ferulate 5-hydroxylase* (ZmF5H1 (AC210173.4) and ZmF5H2(GRMZM2G100158)), *cinnamoyl-CoA reductase* (*ZmCCR1* (GRMZM2G131205) and *ZmCCR2* (GRMZM2G131836)) and *cinnamyl alcohol dehydrogenase* (*ZmCAD1* (Y13733; GRMZM5G844562), *ZmCAD2* (GRMZM2G118610), *ZmCAD3* (GRMZM2G046070), *ZmCAD4* (GRMZM2G700188), *ZmCAD5* (GRMZM2G443445), *ZmCAD6* (GRMZM2G090980) and *ZmCAD7* (GRMZM167613)) as described by Guillaumie et al. (2007). *Folylpolyglutamate synthase* (*ZmFPGS*; GRMZM2G393334) and the *Ubiquitin carrier protein* (*ZmUBCP*; GRMZM2G102471) genes were used as reference genes (Manoli et al. 2012). Primers can be found in Supplemental Table S1.

The qPCR was carried out in a StepOnePlus platform (Applied Biosystems, California, USA) using Power SYBR green PCR master mix (Applied Biosystems), 2 μL of each cDNA concentration (50 and 100 ng μL^{-1}) and a mix of both primers at 10 μM. All samples were run in triplicate with the following temperature profile: initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 1 min (annealing and elongation). The relative gene expression was calculated by 'δ–δ' method (Livak and Schmittgen 2001) implemented in the StepOne™ Software v2.2.2. A no-template negative control and a melting curve were

performed in each sample set to control the primer dimers and contaminants in the reactions.

CAD enzyme activity assay

CAD enzyme activity was measured by following the method described by Chabannes et al. (2001) modified by Fornalé et al. (2012). Fresh cells were homogenized under liquid nitrogen with a mortar and pestle until a fine powder was obtained, and 5 mL of extraction buffer (100 mM Tris-HCl pH 7.5, 2% (w/v) PEG 6000, 5 mM DTT and 2% (w/v) PPVP) were added. The suspension was centrifuged at 10,000 *g* for 10 min at 4 °C and the supernatant was collected. The centrifugation process was repeated until the supernatant was clear.

CAD activity assays were carried out by measuring the absorbance increment at 400 nm when coniferyl alcohol was oxidized to coniferyl aldehyde. The reactions were performed in 96-well plates containing 140 μL of 140 mM Tris-HCl pH 8.8, 20 μL of 1 mM coniferyl alcohol, 20 μL of 200 μM $NADP^+$ and 20 μL of sample. The mixtures were mixed and incubated at 30 °C for 10 min, and the reactions were measured over the following 10 min in a plate reader Synergy HT (Bio-Tech, Winooski, VT, USA) at 30 °C. Reaction and sample blanks were routinely used.

Apoplastic H_2O_2 content determination

Apoplastic H_2O_2 content was determined with the xylenol orange method as described by Bindschedler et al. (2001). For the reactions, 150 μL of culture media was mixed with 1 mL of reaction mixture (125 μM xylenol orange, 100 mM D-sorbitol, 25 μM $FeSO_4$, 25 μM $(NH_4)_2SO_4$ and 25 μM H_2SO_4), and absorbance (560 nm) was measured after 40 min of incubation.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

Figure S1. Cell wall fractionation

Total sugars in (**A**) CDTA, (**B**) KI, (**C**) KII and (**D**) TFA cell wall fraction obtained from SNH, SNH + DCB, SH1 and SH6 cell lines. For maize cell line annotation see Figure 1 legend. Data represents the means values ± s.d. of 3 technical replicates. Asterisks indicate values that are significantly different from SNH after a Student's t-test ($P < 0.05$).

Table S1. Primers used in RT-PCR and qRT-PCR experiments

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Early habituation of maize (*Zea mays*) suspension-cultured cells to 2,6-dichlorobenzonitrile is associated with the enhancement of antioxidant status

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The cellulose biosynthesis inhibitor 2,6-dichlorobenzonitrile (DCB) has been widely used to gain insights into cell wall composition and architecture. Studies of changes during early habituation to DCB can provide information on mechanisms that allow tolerance/habituation to DCB. In this context, maize-cultured cells with a reduced amount of cellulose (~20%) were obtained by stepwise habituation to low DCB concentrations. The results reported here attempt to elucidate the putative role of an antioxidant strategy during incipient habituation. The short-term exposure to DCB of non-habituated maize-cultured cells induced a substantial increase in oxidative damage. Concomitantly, short-term treated cells presented an increase in class III peroxidase and glutathione *S*-transferase activities and total glutathione content. Maize cells habituated to 0.3–1 μ*M* DCB (incipient habituation) were characterized by a reduction in the relative cell growth rate, an enhancement of ascorbate peroxidase and class III peroxidase activities, and a net increment in total glutathione content. Moreover, these cell lines showed increased levels of glutathione *S*-transferase activity. Changes in antioxidant/conjugation status enabled 0.3 and 0.5 μ*M* DCB-habituated cells to control lipid peroxidation levels, but this was not the case of maize cells habituated to 1 μ*M* DCB, which despite showing an increased antioxidant capacity were not capable of reducing the oxidative damage to control levels. The results reported here confirm that exposure and incipient habituation of maize cells to DCB are associated with an enhancement in antioxidant/conjugation activities which could play a role in incipient DCB habituation of maize-cultured cells.

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Abbreviations – AA, ascorbate; APOX, ascorbate peroxidase; CAT, catalase; CBI, cellulose biosynthesis inhibitor; CIII-POX, class III peroxidase; DCB, 2,6-dichlorobenzonitrile; DHA, dehydroascorbate; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; FW, fresh weight; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; MDA, malondialdehyde; POX, peroxidase; RGR, relative growth rate; ROS, reactive oxygen species; SHx, suspension-cultured cells habituated to 'x' μ*M* DCB; SNH, non-habituated suspension-cultured cells; SNH+DCB(x), non-habituated suspension-cultured cells treated with 'x' μ*M* DCB; TA, total ascorbate; TBARS, thiobarbituric acid-reactive substances; TG, total glutathione.

Introduction

The compound 2,6-dichlorobenzonitrile (DCB) is a broad-spectrum herbicide used to control a wide range of weed species. Although the mechanism of action of DCB still remains unclear, it has been extensively reported to specifically inhibit the incorporation of [^{14}C]Glc into cellulose in a wide range of systems (Hogetsu et al. 1974, Montezinos and Delmer 1980, Hoson and Masuda 1991, Edelmann and Fry 1992, Shedletzky et al. 1992, García-Angulo et al. 2009) supporting its classification as a cellulose biosynthesis inhibitor (CBI) (Acebes et al. 2010).

One possible strategy to study the effect of CBIs on the composition and/or architecture of plant cell walls is to habituate cell cultures to grow in the presence of high concentrations of these herbicides (long-term habituation to DCB). Several studies have been reported in recent years in which plant-cultured cells with both type I and type II primary walls (typical of dicots and commelinoid monocots, respectively) have been habituated to DCB in this way (Acebes et al. 2010 and references therein, de Castro et al. 2015, Mélida et al. 2015). The cell wall modification depended on the type of primary cell wall (types I or II), the concentration of DCB in the culture medium and the number of subcultures in a given concentration of DCB. As a result of the habituation process, cells develop the capacity to grow and divide with a modified cell wall in which the cellulosic scaffold is replaced by a network of highly cross-linked matrix polysaccharides that differ depending on the cell wall type: pectins in the case of type I cell walls (Shedletzky et al. 1992, Encina et al. 2001, 2002, Alonso-Simón et al. 2004, 2010, García-Angulo et al. 2006, 2009, 2009) and feruloylated-arabinoxylans in the case of type II cell walls (Mélida et al. 2009, 2010a, 2010b, 2011, 2015, De Castro et al. 2014, 2015).

However, there is still little information about the cell wall modification associated with a low level of habituation to DCB (incipient or short-term habituation to DCB). To date, only four studies have analyzed the initial stages of DCB habituation in bean (*Phaseolus vulgaris*) cells (type I cell wall) (Alonso-Simón et al. 2004, García-Angulo et al. 2006) and in maize cells (type II cell wall) (de Castro et al. 2014, 2015). Compared with long-term DCB-habituated cells, the reduction in cellulose content was lower (~20% reduction with respect to non-habituated cells), returning to control levels as the time of culture was increased (de Castro et al. 2014). Interestingly, the addition of 0.3–0.5 μ*M* DCB to non-habituated maize cells during one culture cycle (short-term treated cells) induced a ~25% reduction in cellulose content that returned to control levels as the number of subcultures in the presence of the same concentration of herbicide was increased. This reactive behavior was not observed when higher DCB (i.e. 1 μ*M*) concentrations were used (de Castro et al. 2014). These results demonstrate the tight regulatory process governing cell wall metabolism and architecture, but these initial steps have usually been overlooked.

Environmental stresses such as salinity (Hu et al. 2012), temperature (Badiani et al. 1997), nutritional deficiencies (Kováčik et al. 2013), heavy metals (Paradiso et al. 2008, Vuletić et al. 2014), organochlorines (Michalowicz et al. 2009, San Miguel et al. 2012) and herbicides (Geoffroy et al. 2004, Peixoto et al. 2008, García-Angulo et al. 2009, Wu et al. 2010, Karuppanapandian et al. 2011) lead to disruption of the balance between reactive oxygen species (ROS) production and scavenging, which in turn leads to oxidative stress (Apel and Hirt 2004, Gill and Tuteja 2010). Consequently, resistance to oxidative stress is often achieved by developing an antioxidant capacity that comprises enzymatic activities such as class III peroxidase (CIII-POX), ascorbate peroxidase (APOX), catalase (CAT) and glutathione reductase (GR), and antioxidant molecules such as reduced glutathione (GSH), ascorbate (AA) and polyphenols, which scavenge ROS and therefore reduce oxidative damage (Passardi et al. 2005, Ahmad et al. 2008, Gill and Tuteja 2010). In the case of stresses induced by exposure to herbicides, one of the defense responses is catabolism of the molecule in order to reduce the toxic effect. Among other enzymes, glutathione *S*-transferases (GSTs) detoxify herbicides such as DCB by conjugating the drug with the tripeptide glutathione (GSH), and are considered plant stress molecular markers (Edwards et al. 2000, Gill and Tuteja 2010, Cummins et al. 2011).

The treatment of plant cells with CBIs such as isoxaben or DCB has been related to an increase in antioxidant activities in plant cells (García-Angulo et al. 2009, Mélida et al. 2010a, Denness et al. 2011). In addition, DCB habituation of bean suspension-cultured cells is associated with a stable increment of CIII-POX, which has been hypothesized to increase the antioxidant capacity of cells and possibly contribute to cell wall restructuring (García-Angulo et al. 2009). Interestingly, in the case of maize callus-cultured cells, long-term DCB habituation was not associated with an enhancement of antioxidant activities. In the same study, a severe decrease in GST-conjugation levels was reported for such cell lines, both by activity measurements and by proteomics (Mélida et al. 2010a). Taking these results together, an antioxidant/conjugation strategy may seem unlikely in long-term DCB habituation (Mélida et al. 2010a). However, a clue for the involvement of antioxidants in the

early stages of DCB habituation was provided by results indicating that following a short-term treatment with a high concentration of DCB, maize callus-cultured cells presented an increase in CIII-POX, CAT, GR and GST antioxidant activities (Mélida et al. 2010a).

Given the fact that the exposure of maize cells to DCB induces an enhancement of antioxidant and conjugation activities, and that the DCB-dependent decrease in cellulose reverts during the initial stages of habituation, the aim of this study was to gain an insight into the putative role of the antioxidant/conjugation machinery during the initial stages of DCB habituation in maize-cultured cells. To this end, we measured lipid peroxidation and H_2O_2 levels as an indication of oxidative status, followed by the assay of antioxidant (CIII-POX, APOX, GR, CAT) and conjugation (GST) activities as well as GSH and AA contents in maize suspension-cultured cells habituated to 0.3, 0.5 and 1 μ*M* DCB after 11 culture cycles. In addition, in order to investigate differences in antioxidant/conjugation strategies between DCB habituation and acute DCB effects, the same parameters were assayed in maize suspension-cultured cells after a short-term exposure to 0.5 or 1 μ*M* DCB.

Materials and methods

Plant cell cultures

Maize suspension-cultured cells (*Zea mays*, Black Mexican sweetcorn) were obtained from rotary shaken (120 rpm) maize callus cultures obtained as described by Lorences and Fry (1991).

Maize suspension-cultured cells (*Z. mays*, Black Mexican sweetcorn) were routinely grown in Murashige and Skoog (MS) media (Murashige and Skoog 1962) supplemented with 20 g l^{-1} sucrose and 9 μ*M* 2,4-dichlorophenoxiacetic acid at 25°C under photoperiodic conditions (16:8; 3000 lux ≈ 41 μmol m^{-2} s^{-1}), and were rotary shaken (120 rpm) and subcultured fortnightly (Mélida et al. 2011).

Short-term exposure and habituation of maize cells to DCB

In order to determine the effect of short-term DCB treatment, non-habituated suspension-cultured cells (SNH) were transferred to media supplemented with 0.5 or 1 μ*M* DCB for 6 days. These cells were denoted as SNH + DCB (x) where 'x' indicates the DCB concentration (μ*M*) added to the culture media.

Cell cultures habituated to 0.3, 0.5 and 1 μ*M* DCB were obtained from SNH cells after stepwise transfers with gradual increments of DCB in the culture media (de Castro et al. 2014). DCB was dissolved in dimethylsulphoxide, which does not affect maize cell growth at this range of concentrations (0.003–0.01% v:v). For this purpose SNH were treated with 0.3, 0.5 (I_{50}, de Castro et al. 2014) and 1 μ*M* DCB and subcultured in the presence of the herbicide for 10 subcultures (de Castro et al. 2014). Habituated cells were denoted as SHx, where 'x' indicates the DCB concentration (μ*M*) added to the culture media.

Cell growth and viability measurements

Growth curves of all cell lines were obtained at the sixth subculture by measuring the dry weight (DW) gain at different time points in the culture cycle. Relative growth rates (RGRs) were calculated from the slopes of the straight part of the curves after plotting ln DW against time. The doubling time, which is the time that the cell culture takes to double the DW, was calculated as: doubling time = ln 2/RGR.

Viability was measured using the fluorescein diacetate method as described by Duncan and Widholm (1990). Fifty microliter of 0.2% (w/v) fluorescein diacetate (Sigma-Aldrich Quimica SL, Madrid, Spain) stock solution in acetone was diluted with 5 ml of culture medium, and the resulting working solution mixed 1:1 (v/v) with cell suspension on a microscope slide. A Nikon epifluorescence microscope equipped with a Nikon UV-2A filter (330–380 nm excitation, 400 nm dichroic mirror and 435 nm barrier filter) was used for observation of bright green fluorescence emission by viable cells.

Lipid peroxidation levels and enzyme activity assays

Cells from all lines were collected during the exponential growth phase and stored at −80°C until use. One to five grams fresh weight (FW) of these cells was homogenized under liquid nitrogen with a mortar and a pestle until obtaining a powder.

To quantify CAT (EC. 1.11.1.6), GR (EC 1.8.1.7) and GST (EC 2.5.1.18) enzyme activity and lipid peroxidation, the powdered cells were extracted (1 g FW in 5 ml extraction buffer) using extraction buffer 1:0.05 *M* Tris–HCl pH 7.5, 0.1 m*M* ethylenediaminetetraacetic acid (EDTA), 0.1% (v/v) Triton X-100, 10% (v/v) glycerol and 2 m*M* dithiothreitol, and centrifuged at 15 000 *g* for 2 min at 4°C before quantifying the supernatants.

Lipid peroxidation levels were determined by quantification of thiobarbituric acid-reactive substances (TBARS) using malondialdehyde (MDA) as the reference molecule (Buege and Aust 1978). One milliliter of reaction buffer, 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid and 0.01% (v/v) butylated hydroxytoluene in

0.25 *M* HCl) was mixed with 20 µl of sample and incubated at 100°C for 15 min. The samples were cooled, centrifuged at 2500 *g* for 15 min and A_{535} was measured in the supernatants. CAT activity was measured as the reduction in A_{240} induced by the catalysis of H_2O_2 for 2 min (Droillard et al. 1987). The activity assay was performed by mixing 3 ml of 50 m*M* phosphate buffer pH 7.0 with 37.5 m*M* H_2O_2 and 0.1 ml of sample supernatant. CAT activity was calculated using the molar extinction coefficient for H_2O_2 at 240 nm: $\varepsilon = 39.58\ M^{-1}\ cm^{-1}$. Quantification of GR activity was performed in accordance with the method described by Edwards et al. (1990), which is based on the reduction in A_{340} due to the oxidation of NADPH for the conversion of glutathione disulfide (GSSG) to its reduced form (GSH). Activity was measured by mixing 0.1 ml of sample supernatant with 1.35 ml of reaction buffer (100 m*M* Tris–HCl pH 8.0, 0.1 m*M* EDTA, 1 m*M* GSSG and 3 m*M* $MgCl_2$) and 0.05 ml of 10 m*M* NADPH, and calculated using the molar extinction coefficient for NADPH at 340 nm: $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$. GST enzyme activity was determined following the method described by Habig et al. (1974), which is based on an increase in A_{340} due to the formation of a complex between a reduced GSH and the compound chloro-2,4-dinitrobenzene. The reaction was performed by mixing 0.93 ml of 0.1 *M* potassium phosphate buffer pH 7.5 with 0.02 ml of 0.001 *M* chloro-2,4-nitrobenzene and 0.05 ml of sample supernatant, and was measured for 2 min at 30°C. GST activity was calculated using the molar extinction coefficient of the GSH-chloro-2,4-dinitrobenzene complex ($\varepsilon = 9.6\ mM^{-1}\ cm^{-1}$).

For peroxidases (CIII-POX; EC 1.11.1.7 and APOX; EC 1.11.1.11), the powdered cells were resuspended in extraction buffer 2 (1 g FW in 5 ml extraction buffer): 0.04 *M* Tris–HCl pH 7.2, 1 m*M* EDTA-2Na-2H and 5% (v/v) glycerol, then centrifuged at 15 000 *g* for 2 min at 4°C before measuring activities in the supernatants. Quantification of CIII-POX activity was performed as described by Adam et al. (1995), based on the increase in A_{470} due to guaiacol oxidation. The reaction was performed with 3 ml of reaction buffer (100 m*M* sodium acetate pH 5.5 and 1 m*M* guaiacol), 0.3 ml of 1.3 m*M* H_2O_2 and 0.05 ml of sample supernatant at 25°C for 2 min. Activity was calculated using the molar extinction coefficient for guaiacol at 470 nm: $\varepsilon = 26.6\ mM^{-1}\ cm^{-1}$. APOX activity was measured following the method described by Hossain and Asada (1984), in which a reduction in A_{290} due to AA oxidation occurs. Sample supernatants (0.01 ml) were mixed with 0.98 ml of 50 m*M* HEPES-NaOH buffer pH 7.6 and 20 m*M* ascorbic acid. The reaction was started by the addition of 0.01 ml of 1.3 m*M* H_2O_2. Activity was calculated using the molar extinction coefficient for AA at 290 nm: $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$. Protein content was determined by the Bradford method (Bradford 1976).

Total GSH and GSSG measurement

For GSH and GSSG extraction, cells were homogenized under liquid nitrogen with a mortar and pestle. Then, powdered cells (1 g FW) were extracted with 5 ml of 5% (w/v) metaphosphoric acid. Assays were conducted rapidly to avoid oxidation of GSH to GSSG.

Total glutathione content (TG = GSH + GSSG) was measured using the DTNB recycling method described by Griffith (1980). GSSG determination was performed using the same method but with a previous treatment with acrylonitrile, a thiol-blocking reagent, following the indications of Matsumoto et al. (1996). GSH content was calculated as the difference between TG and GSSG values.

Total ascorbate and dehydroascorbate measurement

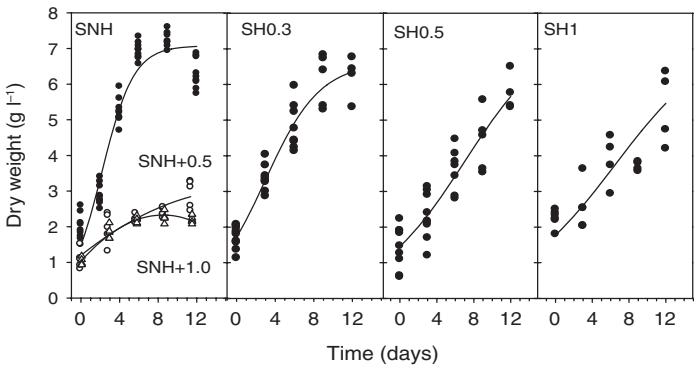
For total AA (TA = AA + DHA) and dehydroascorbate (DHA) extraction, cells in the exponential growth phase were homogenized under liquid nitrogen with a mortar and pestle. Powdered cells (1 g FW) were extracted with 5 ml of 5% (w/v) metaphosphoric acid and kept on ice for 20 min. Extracts were clarified by centrifugation at 19 000 *g* for 5 min at 4°C and samples from the supernatant were collected for measurements.

AA and DHA were measured spectrophotometrically following the method described by Takahama and Oniki (1992) and modified by Kärkönen and Fry (2006). Extracts (30 µl) were mixed with 1 ml of reaction mix (38 m*M* Na^+-succinate in 90 m*M* NaH_2PO_4, pH 6.8) and A_{265} was measured. Furthermore, 2 U of AA oxidase from *Cucurbita* sp. (Sigma) was added to oxidize AA to DHA and A_{265} was re-measured after 1 min. AA was measured by calculating the reduction in A_{265} upon addition of AA oxidase. In an independent sample, extracts (30 µl) were added to 1 ml of reaction mix (38 m*M* Na^+-succinate in 90 m*M* NaH_2PO_4, pH 6.8) and A_{265} was measured. Then, dithiothreitol (freshly prepared, to 14.8 m*M*) was added to reduce DHA again and A_{265} was re-measured. DHA was determined by measuring the increase in A_{265} upon DHA reduction.

H_2O_2 determination

The H_2O_2 content of spent medium in all cell lines was determined by using the ferrous ammonium sulfate/xylenol orange method as described by Cheeseman

A



B

Cell line	Doubling time	Relative growth rate	% viability at stationary phase
SNH	2.3	0.30	91.0
SNH+DCB(0.5)	5.3	0.12	47.1
SNH+DCB(1.0)	6.0	0.13	39.6
SH0.3	2.9	0.23	68.6
SH0.5	3.9	0.17	75.9
SH1	3.8	0.17	64.4

Fig. 1. (A) Growth curves of non-habituated —SNH—, DCB short-term treated —SNH + DCB(x)—, and DCB-habituated —SHx— maize suspension-cultured cells. (B) Growth parameters of maize suspension-cultured cell lines. Data represent growth curves for at least four replicates. 'x' indicate the DCB concentration (μ*M*) added to the culture medium.

(2006). Aliquots (150 μl) of the spent medium (cell free) were collected during the culture cycle and mixed with 1 ml of reaction buffer (100 μ*M* xylenol orange, 100 μ*M* D-sorbitol, 250 μ*M* $FeSO_4$, 250 μ*M* $(NH_4)_2SO_4$ and 1% ethanol in 25 m*M* H_2SO_4). A blank reaction was prepared by adding 150 μl of distilled water to 1 ml of reaction buffer. Samples were incubated at room temperature for 40 min with shaking, and A_{550} was measured. Absorbance values obtained for spent medium were corrected by measuring the A_{550} of 150 μl of fresh culture media mixed with 1 ml of reaction buffer and incubated for 40 min as described above.

To obtain the H_2O_2 concentration, a standard curve with different concentrations of H_2O_2 (from 0.5 to 40 μ*M*) was performed, following the same procedure. Standards were prepared by dilution of reagent grade, 30% H_2O_2 (Sigma). The concentration of H_2O_2 in the reagent was calculated by using absorbance at 240 nm and an extinction coefficient: $\varepsilon = 43.6\ M^{-1}\ cm^{-1}$

Statistical analyses

All results are expressed as the means ± SD of at least four replicates. When indicated, differences between means were statistically analyzed by using a Student's *t*-test.

Results

Growth measurements in DCB-habituated cells

SNH cells showed a threefold increase in DW after 7 days of culture in fresh medium (Fig. 1A). An RGR of 0.3 was calculated for these cell lines. In the stationary phase, SNH-cultured cells maintained high cell viability (91%; Fig. 1B). The addition of 0.5 or 1.0 μ*M* DCB to the culture medium of SNH cells markedly reduced the accumulation of biomass and cell viability. Consequently, the RGR of DCB short-term treated SNH cells (SNH + DCB(0.5) and SNH + DCB(1)) was reduced by more than 50%. Growth curves of maize cells habituated to low DCB concentrations were obtained throughout the culture cycle (Fig. 1A). As the level of DCB habituation increased, cultured cells accumulated less biomass during the stationary phase. DCB-habituated cell lines had higher doubling times, and the RGR decreased as the habituation level rose, in such a way that the RGR in SH0.5 and SH1 cells was almost half the one estimated for SNH cells (Fig. 1B).

Oxidative status

In order to determine the oxidative status of SH and SNH cells, lipid peroxidation levels were measured as

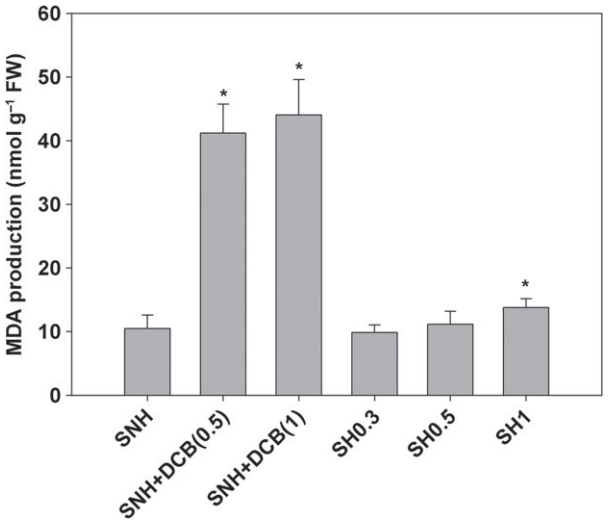


Fig. 2. Lipid peroxidation levels measured as MDA production in non-habituated —SNH—, DCB short-term treated —SNH + DCB(x)—, and DCB-habituated —SHx— maize suspension-cultured cells. Data represents means ± SD of at least four replicates. Asterisks indicate significant differences with respect to SNH cells by Student's *t*-test ($P < 0.05$). 'x' indicates the DCB concentration (μ*M*) added to the culture medium.

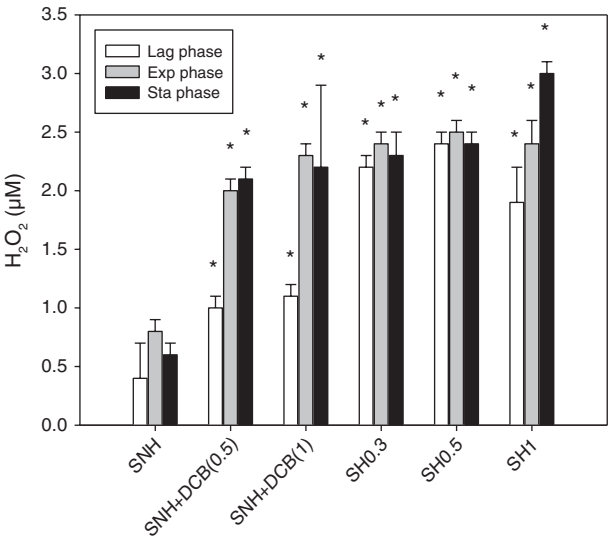


Fig. 3. Changes in H_2O_2 concentration measured in the spent medium of non-habituated —SNH—, DCB short-term treated —SNH + DCB(x)— and DCB-habituated —SHx— maize suspension-cultured cells at the lag (white), exponential (gray) and stationary (black) phase of the cell culture cycle. Data represent means ± SD of three replicates. For each cell culture phase, asterisks indicate significant differences with respect to SNH cells by Student's *t*-test ($P < 0.05$). 'x' indicates the DCB concentration (μ*M*) added to the culture medium.

a function of TBARS formation using MDA as reference molecule (Fig. 2). Short-term treatment of SNH cells with DCB (0.5 or 1 μ*M*) caused more than a fourfold increase in lipid peroxidation levels when compared with cells cultured in media lacking DCB (SNH). Among DCB-habituated cultured cells, only SH1 cells showed a significant increment in lipid peroxidation levels when compared with SNH cells.

The H_2O_2 accumulated in the spent medium during the cell culture cycle was measured in all cases (Fig. 3). SNH cells accumulated H_2O_2 in the cell culture medium in a concentration ranging from 0.4 to 0.8 μ*M*. In these control cells, H_2O_2 accumulation peaked in the exponential phase.

Short-term treatments of SNH cells with DCB significantly increased H_2O_2 accumulation during the culture cycle (Fig. 3). In these cell lines, H_2O_2 began accumulating in the lag phase, reaching a plateau in the exponential growth phase. In the exponential phase (the growth phase selected for lipid peroxidation assays) the H_2O_2 concentration measured in SNH + DCB(0.5) and SNH + DCB(1) spent medium was on average 2.5 to 3-fold higher with respect to SNH cells.

In the same way, DCB-habituated cells accumulated a significantly higher concentration of H_2O_2 with respect to SNH cells (Fig. 3). H_2O_2 accumulation in the exponential phase of SH cells did not markedly differ from that obtained for DCB short-term treated cells. However, differences were found in the kinetics of H_2O_2 accumulation, since SH cells maintained a high H_2O_2 level from the lag phase throughout the cell culture cycle.

Antioxidant and conjugation enzyme activities

In order to gain an insight into the ROS scavenging capacity, the activity of the antioxidant enzymes APOX, CIII-POX, CAT and GR (Fig. 4) were measured in all the cell lines in their exponential phase.

APOX and CIII-POX activities increased during the DCB habituation process (Fig. 4A, B). When SNH and SH1 cells were compared, a significant increase (~2.5-fold) in both peroxidase activities was found. In contrast, the habituation process was associated with a gradual decrease in CAT activity (Fig. 4C). DCB-habituated cells showed similar levels of GR activity to that of control cells (Fig. 4D).

The short-term treatment of SNH cells with 0.5 μ*M* or 1 μ*M* DCB resulted in a significant enhancement of CIII-POX activity and a slight reduction in APOX activity (Fig. 4A, B). In the case of CAT activity (Fig. 4C), no clear trends were found as this activity significantly increased in SNH + DCB(0.5) but did not vary with respect to SNH cells or SNH + DCB(1). As in the case of DCB habituation, GR activity did not vary with respect to control in DCB short-term treated cells (Fig. 4D).

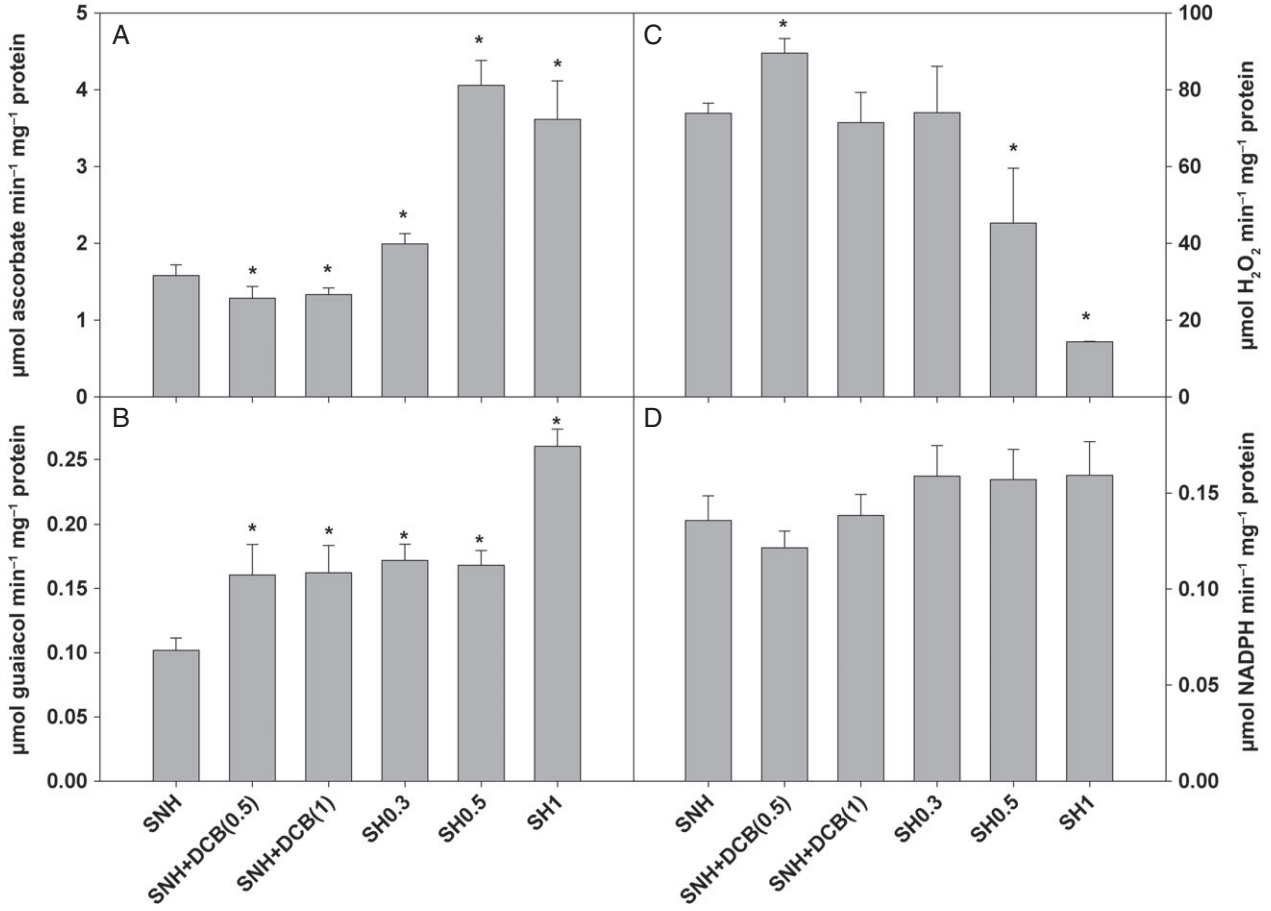


Fig. 4. Activity of APOX (A), CIII-POX (B), CAT (C) and GR (D) measured in non-habituated —SNH—, DCB short-term treated —SNH + DCB(x)— and DCB-habituated —SHx— maize suspension-cultured cells. Data represent means ± SD of at least four replicates. Asterisks indicate significant differences with respect to SNH cells by Student's *t*-test ($P < 0.05$). 'x' indicates the DCB concentration (µ*M*) added to the culture medium.

Besides the enzymatic activities regularly involved in the antioxidant defense mechanism, the involvement of GST activity as a putative DCB detoxifying system was tested (Fig. 5). The results obtained showed that GST activity significantly increased with respect to control both after a DCB short-term treatment and in DCB-habituated cells.

Glutathione and ascorbate measurements

To study the levels of non-enzymatic antioxidant molecules, TG (GSH + GSSG) and the GSH/TG ratio were quantified in all cell lines in the exponential growth phase (Fig. 6). The short-term DCB treatment of SNH cells induced a significant increment in TG content (more than 28-fold in both treatments) and an increase in the GSH/TG ratio, indicating that 97–98% of glutathione was in its reduced form. DCB habituation was also characterized by a significant increment in TG content in comparison with SNH cells. The GSH/TG ratio of DCB-habituated cells was only slightly higher than that estimated for SNH cells.

TA measured in SNH cells during the exponential phase was on average 1 µmol g^{-1} FW (Fig. 7). In SNH cells, ~ 50% of TA was in its reduced form as the average AA/TA ratio was 0.52 (Fig. 7). Both the short-term DCB treatment and DCB habituation resulted in a reduction in the TA cell content. Moreover, the redox status of AA changed as an increase in DHA (oxidized AA) was detected, with average ratios ranging from 0.29 to 0.17 (Fig. 7).

Discussion

The habituation of cells to DCB leads to a modified cell wall. This modification depends on the type of cell wall, the concentration of DCB to which cells are habituated and the number of subcultures in a given concentration of DCB. In contrast to cells with a high-level

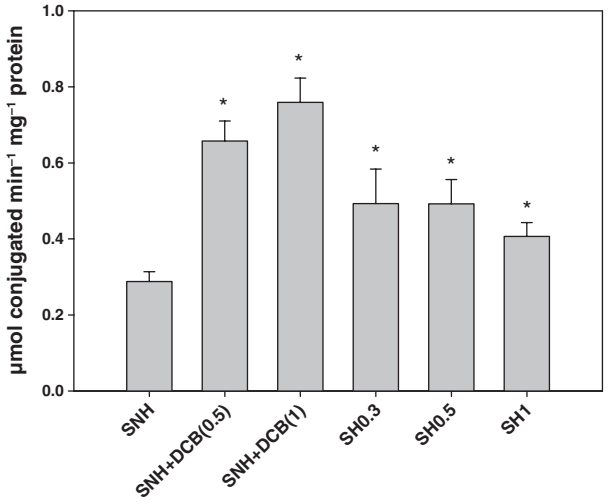


Fig. 5. GST activity measured in non-habituated —SNH—, DCB short-term treated —SNH + DCB(x)— and DCB-habituated —SHx— maize suspension-cultured cells. Data represent means ± SD of at least four replicates. Asterisks indicate significant differences with respect to SNH cells by Student's *t*-test ($P < 0.05$). 'x' indicates the DCB concentration (µ*M*) added to the culture medium.

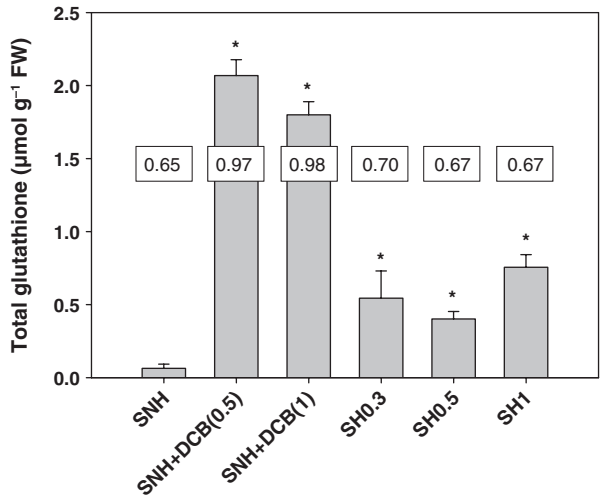


Fig. 6. Total glutathione (TG) measured in non-habituated —SNH—, DCB short-term treated —SNH + DCB(x)— and DCB-habituated —SHx— maize suspension-cultured cells. Squared values represent the GSH/TG ratio of each cell line. Data represent means ± SD of at least six replicates. Asterisks indicate significant differences with respect to SNH cells by Student's *t*-test ($P < 0.05$). 'x' indicates the DCB concentration (µ*M*) added to the culture medium.

of habituation, FTIR and biochemical analysis revealed that the wall modification of cells with a low level of habituation reverts to that of non-habituated cells if the concentration is maintained under a certain threshold (Alonso-Simón et al. 2004, de Castro et al. 2014). Hence, cell wall changes are not stable during these

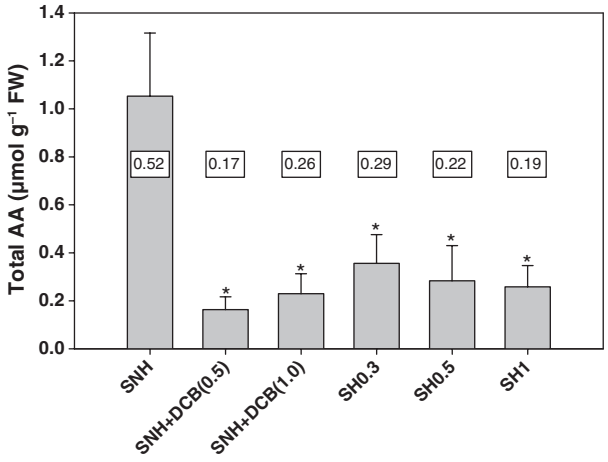


Fig. 7. Total ascorbate (TA) content measured in non-habituated —SNH—, DCB short-term treated —SNH + DCB(x)— and DCB-habituated —SHx— maize suspension-cultured cells at the exponential phase of the cell culture cycle. Squared values represent the AA/TA ratio of each cell line. Asterisks indicate significant differences with respect to SNH cells by Student's *t*-test ($P < 0.05$). 'x' indicates the DCB concentration (µ*M*) added to the culture medium.

initial stages of habituation, and therefore the notion of a non-cell wall-related mechanism that allows maize cells to grow under DCB presence is plausible.

Early habituation to DCB, as was the case of SH0.3, SH0.5 and SH1 cells reduced cell wall cellulose content by 20% in comparison to SNH cells (de Castro et al. 2014). At this stage, DCB habituation was associated with a dose-dependent enhancement of antioxidant activities, mainly CIII-POX and APOX (Fig. 4). In light of the results reported here, we suggest that CIII-POX and APOX activities play an active role in the early DCB habituation process of maize-cultured cells by maintaining ROS at a low level. The role of APOXs in the detoxification of H_2O_2 by oxidizing AA as part of the ascorbate–glutathione and water-water cycle has been observed previously (Gill and Tuteja 2010). Physiological and gene expression analysis have widely shown that a common pattern in the response to abiotic stresses is the overexpression of cytosolic APOX isoenzymes and the increase in APOX activity (Mittler 2002, Shigeoka et al. 2002, Gill and Tuteja 2010). Recently it has been demonstrated that nitric oxide positively regulates cytosolic APOX activity by *S*-nitrosylation, enhancing the resistance of plants to oxidative stress (Yang et al. 2015). This result opens up the possibility of nitric oxide being a factor contributing to regulate the oxidative stress response of DCB-habituated cells.

The reported increase in the level of CIII-POX activity associated with DCB habituation of maize-cultured cells (Fig. 4B) is consistent with previous results obtained

by our group (García-Angulo et al. 2009). As previously indicated, DCB-habituated bean cells have been shown to feature stable and constitutively high levels of CIII-POX (García-Angulo et al. 2009). In the regular peroxidative cycle, CIII-POX reduces H_2O_2 by oxidation of a variety of co-substrates (Passardi et al. 2005). Furthermore, a CAT-like activity has been reported for type-III POXs, which efficiently detoxifies H_2O_2 (Mika et al. 2004 and references therein). Peroxidase-mediated hydroxylation could also play a role in DCB detoxification as it has been shown that this process occurs by glutathione conjugation of hydroxylated DCB derivatives (Brittebo et al. 1992).

In addition, a role for CIII-POXs in maize cell wall remodeling may be proposed as CIII-POXs oxidatively cross-link cell wall hemicelluloses by di-ferulate bonding of arabinoxylans (Fry 2004). In accordance with this, an increased level of di-ferulates has been recorded in both low (de Castro, personal communication) and high levels (Mélida et al. 2009, 2010b, 2011) of DCB habituation. However, in maize cells habituated to high DCB levels, no relationship was found between high ferulate dimerization and increased peroxidase activity (Mélida et al. 2010a), indicating that cell wall CIII-POX activity is not a limiting factor for ferulate dimerization. In accordance with this, it is likely that changes in CIII-POX activity associated with low levels of DCB habituation do not account for cell wall remodeling. Besides oxidative reinforcement of cell wall, it is possible that CIII-POXs contribute to cell wall loosening by producing hydroxyl radicals (Schopfer 2001).

The incipient habituation of maize cells to DCB induced a significant increment in TG content and a slightly higher proportion of its reduced form (GSH) (Fig. 6), both factors being related to protection against a build-up of stress-induced ROS (Szalai et al. 2009). Several studies have related high GSH/TG ratios to efficient protection of the plant cell against abiotic stresses and the control of cell redox homeostasis through a reduction in ROS content (Szalai et al. 2009, Gill and Tuteja 2010). The increase in GSH observed in abiotic-stressed cells is frequently explained by a higher rate of *de novo* GSH synthesis (Szalai et al. 2009). This finding would explain why our short-term treated cells showed a high TG content with no significant changes in GR activity (Fig. 4D). Early DCB habituation of maize cells was also associated with an increase in GST activity (Fig. 5), pointing to high TG contents and further GSH conjugation of DCB as mechanisms implicated in the habituation of maize cells to low DCB concentrations (<1 μ*M*). High GSH availability and GST activity would be consistent with high rates of DCB-conjugation and would partially explain the recovery in cellulose content reported as the number of subcultures in presence of low concentrations of DCB increases (de Castro et al. 2014). Intriguingly, maize cells habituated to high DCB levels (>6 μ*M*) did not show increased levels of GST activity (Mélida et al. 2010a), prompting us to speculate that GST activity is solely implicated in early habituation to DCB.

SH0.5 and SH1 cells showed a reduction in CAT activity (Fig. 4C). Although in a context of increased antioxidant protection a reduction in CAT activity might appear contradictory, it seems a consistent result as this same effect has already been reported for maize cells habituated to high DCB levels (Mélida et al. 2010a). Interestingly, it has been widely reported the association between reduced CAT activity, H_2O_2 accumulation and GSH biosynthesis as it may occur in our experiment (Smith 1985, Queval et al. 2009, Noctor et al. 2012). In the same way, DCB-habituation is associated with a reduction in TA content and a relative increase in its reduced form (Fig. 7), contrary to what might be expected of an antioxidant strategy. However, in some systems it has been shown that abiotic stress reduces AA content (Gill and Tuteja 2010).

The enhancement of antioxidant machinery in SH0.3 and SH0.5 cells was apparently sufficient to cope with the oxidative stress provoked by the herbicide because their lipid peroxidation levels did not significantly differ from that of SNH cells (Fig. 2), although SH cells accumulated a significantly higher level of H_2O_2 in the culture medium (Fig. 3). In the case of SH1 cells, DCB habituation induced activation of the antioxidant system, but this defense response was apparently insufficient to control the putative formation of ROS, leading to a slight but significant increase in oxidative damage when compared with control cells (Fig. 2). The oxidative damage that SH1 cells putatively sustained could explain their lower RGR, which was reduced by almost half compared with SNH cells (Fig. 1B). An alternative explanation for the reduced RGR of SH1 cells could be a more effective inhibition of cell wall expansion through DCB inhibition of cellulose biosynthesis (de Castro et al 2014). It should be borne in mind that unlike SH0.3 and SH0.5 cells, cellulose content of SH1 cells did not revert to control levels as the number of subcultures in the presence of DCB increased (de Castro et al. 2014).

The response of SNH cells to a short-term treatment with DCB was characterized by cell growth impairment expressed both as DW gain and cell viability and a significant rise in oxidative stress measured as lipoperoxidation (increased by more than fourfold in comparison with SNH cells), indicating that DCB induces ROS formation as indicated by the build-up of H_2O_2 measured in the spent medium. It can be speculated that short-term treated maize cells, even when not controlling oxidative

damage, putatively display an antioxidant/conjugation strategy as has previously been found (Mélida et al. 2010a). The antioxidant response is supported by an enhancement in CIII-POX and GST activities, high TG levels and GSH/TG ratios. In summary, our results indicate that in an attempt to cope with oxidative stress, short-term DCB-treated cells responded with substantial *de novo* GSH biosynthesis and an enhancement of CIII-POX and GST activities. GSH content and GST activity were reduced during the subsequent subcultures, although it nevertheless remained at high levels in comparison with SNH cell levels, and CIII-POX and APOX activities increased. In contrast to a long-term DCB habituation, where DCB cannot be efficiently detoxified and cells cope with the herbicide by a cell wall remodeling strategy, during incipient habituation the antioxidant–conjugation machinery seems to be good enough to revert the initial stress situation and successfully cope with the herbicide. Results reported here show that depending on the level of DCB-induced stress maize cells develop alternative coping strategies.

Author contributions

A. L. G. designed research, performed research, analyzed data, wrote manuscript. A. E. provided research opportunity, designed research, performed research, analyzed data, wrote manuscript. M, d. C. performed research, revised manuscript. H. M. designed research, assisted in performing the research, revised manuscript. J. L. A. provided research opportunity, designed research, assisted in performing the research, revised manuscript. P. G. A. designed research, assisted in performing the research, revised manuscript. J. M. A. provided research opportunity, supervised project, designed research; wrote manuscript.

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Research article

Quinclorac-habituation of bean (*Phaseolus vulgaris*) cultured cells is related to an increase in their antioxidant capacity

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ABSTRACT

The habituation of bean cells to quinclorac did not rely on cell wall modifications, contrary to what it was previously observed for the well-known cellulose biosynthesis inhibitors dichlobenil or isoxaben. The aim of the present study was to investigate whether or not the bean cells habituation to quinclorac is related to an enhancement of antioxidant activities involved in the scavenging capacity of reactive oxygen species. Treating non-habituated bean calluses with 10 μM quinclorac reduced the relative growth rate and induced a two-fold increase in lipid peroxidation. However, the exposition of quinclorac-habituated cells to a concentration of quinclorac up to 30 μM neither affected their growth rate nor increased their lipid peroxidation levels. Quinclorac-habituated calluses had significantly higher constitutive levels of three antioxidant activities (class-III peroxidase, glutathione reductase, and superoxide dismutase) than those observed in non-habituated calluses, and the treatment of habituated calluses with 30 μM quinclorac significantly increased the level of class III-peroxidase and superoxide dismutase. The results reported here indicate that the process of habituation to quinclorac in bean callus-cultured cells is related, at least partially, to the development of a stable antioxidant capacity that enables them to cope with the oxidative stress caused by quinclorac. Class-III peroxidase and superoxide dismutase activities could play a major role in the quinclorac-habituation. Changes in the antioxidant status of bean cells were stable, since the increase in the antioxidant activities were maintained in quinclorac-dehabituated cells.

1. Introduction

Quinclorac (3,7-dichloro-8-quinolinecarboxylic acid) is a highly selective auxin-type herbicide mainly used to control broad-leaved weeds and harmful grass weeds in rice crops and lawns (Grossmann, 2000, 2010).

It was previously reported that quinclorac inhibits the incorporation of glucose into cellulose in a dose and time-dependent manner (Koo et al., 1996, 1997), being regarded as a cellulose biosynthesis inhibitor (CBI) (Vaughn, 2002). However, other works challenged the correlation of cellulose inhibition effect and quinclorac mechanism of action (Tresch and Grossmann, 2003). In an attempt to elucidate whether quinclorac directly inhibited cellulose biosynthesis, our group proceeded to habituate bean callus-cultured cells to grow in otherwise lethal concentrations of the herbicide. In addition, modifications in cell wall composition due to the habituation process were analysed (Alonso-Simón et al., 2008). The results obtained showed that the mechanism of bean cells habituation to quinclorac differed from that reported for well-known CBIs such as dichlobenil (Encina et al., 2001, 2002) or isoxaben (Díaz-Cacho et al., 1999). In the dichlobenil and isoxaben-habituation processes, bean cells developed the capacity to divide and expand, with a modified cell wall in which the xyloglucan-cellulose network had been partially replaced by pectins.

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; CIII-POX, class III peroxidase; CAT, catalase; CBI, cellulose biosynthesis inhibitor; DTT, dithiothreitol; DH, quinclorac-dehabituated cells; DMSO, dimethylsulphoxide; DW, dry weight; EDTA, ethylenediaminetetraacetic acid; FW, fresh weight; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; isoPOX, peroxidase isoforms; MDA, malondialdehyde; NADPH, nicotinamide adenine dinucleotide phosphate; NH, non-habituated cells; PAGE, polyacrylamide gel electrophoresis; POX, peroxidase; Qn, quinclorac-habituated cells to "n" μM quinclorac; RGR, relative growth rate; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; SOD, superoxide dismutase; TBARS, thiobarbituric acid reacting substances.

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0981-9428/

Quinclorac habituated cells did not show a decrease in the cellulose content, and the minor changes observed in the distribution and post-depositional modifications of homogalacturonan and rhamnogalacturonan I during the habituation process seemed to be due to a side-effect of quinclorac presence (Alonso-Simón et al., 2008). Moreover, short-term treatment of bean suspension-cultured cells with quinclorac concentrations that significantly reduced their dry weight gain (10 μM) did not decrease the incorporation of [^{14}C] glucose to cell wall polysaccharides; in fact, the glucose incorporation increased (García-Angulo et al., 2012). Therefore, the mechanism of quinclorac-habituation did not seem to rely on a modification of cell wall structure and/or composition.

In some species, habituation of cell cultures to CBIs leads to an increase in antioxidant capacity. This is the case of bean cell cultures where habituation to dichlobenil is associated with high class III-peroxidase (CIII-POX) activity (García-Angulo et al., 2009). In the case of maize cells, an increased antioxidant capacity seems to take part in changes associated to the incipient dichlobenil-habituation process (Largo-Gosens et al., 2016), however, antioxidant activities are not implicated in the long-term habituation to high dichlobenil concentrations (Mélida et al., 2010).

In sensitive species, quinclorac induces the activity of the enzyme 1-aminocyclopropane-1-carboxylic acid (ACC) synthase, which increases the level of ACC (Grossmann and Scheltrup, 1997). The subsequent oxidation of this compound to ethylene leads to cyanide accumulation which can interrupt electron flow in chloroplast and mitochondria leading to reactive oxygen species (ROS) overproduction (Navrot et al., 2007) and is considered to be responsible for the phytotoxic effects of quinclorac (Grossmann and Kwiatkowski, 2000; Abdallah et al., 2006). In susceptible dicots, the response to quinclorac is related to increased abscisic acid biosynthesis, which also leads to overproduction of ROS (Van Eerd et al., 2005; Grossmann, 2010). By transcriptomic analysis, it has been recently demonstrated that quinclorac treatment of rice plants (*Orzya sativa*), provoked the enhancement of several groups of genes related with drug detoxification (Xu et al., 2015). Additionally, the induction of the expression of the gene *EcGH3.1*, that belongs to Gretchen Hagen 3 gene family and regulates the auxin homeostasis, has been demonstrated to play a key role in *Echinochloa crus-galli* resistance to quinclorac (Li et al., 2016).

Quinclorac has been reported to induce the overproduction of ROS causing oxidative injury in several sensitive species. Sunohara and Matsumoto (2004) demonstrated the relationship between antioxidant capacity and quinclorac tolerance in several monocots. Later, same authors suggested that the cell death of a quinclorac-sensitive variety of maize may be caused by the overproduction of ROS, but not by ethylene or cyanide action (Sunohara and Matsumoto, 2008). However, tolerant species (*Oryza sativa* and *Eleusine indica*) and resistant biotypes of susceptible species (*Echinochloa* spp., *Digitaria* spp. and *Galium* spp.) neither increase ethylene and cyanide production, nor overproduce ROS in response to quinclorac treatment (Grossmann, 2000; Grossmann and Kwiatkowski, 1993, 2000; Van Eerd et al., 2005; Abdallah et al., 2006; Sunohara et al., 2010, 2011; Yasuor et al., 2012).

Given that i) bean cells habituation to quinclorac does not seem to rely on cell wall modifications; ii) bean cells habituation to other herbicide such as dichlobenil is associated with high CIII-POX activity and iii) quinclorac treatment in sensitive species provokes an overproduction of ROS, the aim of the present study was to investigate whether or not the habituation of bean cells to quinclorac is related to an enhancement of antioxidant activities involved in the scavenging capacity of reactive oxygen species. Moreover, the stability of a putative antioxidant capacity was further investigated by using quinclorac-habituated cells transferred for several subcultures in a medium lacking quinclorac (dehabituated cells).

To our knowledge, this is the first time that a quinclorac-habituated cell line has been used to investigate the role of the antioxidant machinery connected to the tolerance to quinclorac. For this purpose, CIII-POX, glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) activities, as well as lipid peroxidation as an indicator of oxidative damage, were measured in a set of cell lines grown on solid medium: non-habituated, habituated to different quinclorac concentrations (ranging from 10 to 30 μM), and dehabituated, as well as non-habituated cells cultured in the presence of 10 μM quinclorac and quinclorac-habituated cells treated with 30 μM quinclorac. Lastly, polyacrylamide gel electrophoresis (PAGE) to separate the peroxidase isoforms (isoPOX) of all cell lines was performed.

2. Materials and methods

2.1. Plant material and quinclorac habituation

Bean (*Phaseolus vulgaris* L.) cell lines were obtained and subcultured as described by Encina et al. (2001) on Murashige and Skoog (1962) solid basal medium supplemented with sucrose (30 g L^{-1}), 10 μM 2,4-D (2,4-dichlorophenoxyacetic acid) and agar (8 g L^{-1}).

Quinclorac was dissolved in dimethylsulphoxide (DMSO). Non-habituated bean cell lines (NH) were habituated by adding stepwise increments in the concentration of quinclorac to the culture medium, beginning at the I_{50} value for quinclorac (10 μM) and continuing until obtaining bean calluses that were capable of growing under otherwise lethal concentrations of the herbicide (Alonso-Simón et al., 2008). In order to account for DMSO effects, during the habituation process NH cells were supplemented with DMSO ranging from 0.1% to 0.3% (v/v). The highest DMSO concentration used in this experiment, 0.3% (v/v), did not affect the parameters determined in this study (data not shown). Habituated cells were denoted as Qn, where n indicates the quinclorac concentration in μM. In summary, NH, Q10, Q15 and Q30 cell lines were used in this study. Q30 cells were transferred to a medium lacking quinclorac for five subcultures, obtaining dehabituated (DH) cells. All different cell lines were regularly subcultured every 30 days.

A set of NH calluses was subcultured in the presence of 10 μM quinclorac for 30 days and denominated as NH+10, while sets of Q10 and Q15 calluses were subcultured in the presence of 30 μM quinclorac for 30 days, and were denominated Q10+30 and Q15+30 respectively.

2.2. Effect of quinclorac on bean callus growth

To evaluate the effect of quinclorac on callus cell growth, fresh weight (FW) gain was measured in NH, NH+10 and Q10. The relative growth rate (RGR) was determined as follows:

$$RGR = [(FWf - FWi)/FWi]$$

where FWi and FWf indicate the fresh weight of calluses at 0 and 30 days respectively. To determine the dry weight (DW), calluses were dried at 60 °C for 72 h and were weighed. Data for RGR and DW/FW ratio of Q15 and Q30 were taken from Alonso-Simón et al. (2008) for comparison.

2.3. Activity assays of antioxidant enzymes and lipid peroxidation

In order to measure GR (EC 1.8.1.7), SOD (EC 1.15.1.1) and CAT (EC 1.11.1.6) activities, as well as lipid peroxidation levels, cells of all lines were collected at their exponential growth phase and stored at −80 °C until use. Calluses (1 g FW) were homogenized in liquid

nitrogen using a mortar and pestle and 5 mL 0.05 M Tris- HCl pH 7.5, 0.1 mM EDTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol and 2 mM DTT were added. These extracts were centrifuged at 15,000g for 2 min and the supernatants were used for the measurements.

GR activity was determined as described by Klapheck et al. (1990) measuring the decrease in A_{334} caused by NADPH oxidation during 3 min for the conversion of GSSG to GSH ($\varepsilon_{334} = 6.2\ mM^{-1}\ cm^{-1}$). The reaction was performed at 25 °C with 0.1 mL of supernatant, 1.35 mL reaction buffer (100 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM GSSG and 3 mM $MgCl_2$) and 0.05 mL 10 mM NADPH.

SOD activity was evaluated using a SOD assay kit (Sigma, Alcobendas, Spain). Briefly, SOD activity was measured as the inhibition of the formation of a colored compound which can be detected at 440 nm and activity was determined from the inhibitory curve made with different concentrations of standards of a commercial SOD (from 0.001 U/mL to 2000 U/mL). For the determination of SOD activity, 0.02 mL of sample supernatant or standard was used.

CAT activity was measured by Droillard's method (Droillard et al., 1987), based on absorbance reduction at 240 nm due to H_2O_2 reduction to water ($\varepsilon_{240} = 39.58\ M^{-1}\ cm^{-1}$). Measurements were performed during 2 min at 25 °C by adding 0.1 mL of supernatant extract to 3 mL reaction buffer (50 mM phosphate buffer pH 7.0 and 37.5 mM H_2O_2).

Lipid peroxidation levels were determined by measuring the concentration of thiobarbituric acid reacting substances (TBARS), using malondialdehyde (MDA), an end product of lipid peroxidation, as the reference molecule (Buege and Aust, 1978). Aliquots of supernatant (20 μL) were mixed with 1 mL 15% (w/v) trichloroacetic acid, 0.375% thiobarbituric acid and 0.01% butylated hydroxytoluene in 0.25 M HCl. The mixture was incubated at 100 °C for 15 min, then cooled to room temperature and centrifuged at 2500 g for 15 min. Absorbance at 535 nm was measured in the supernatants. Blank reactions (sample blank) were used. Lipid peroxidation levels were expressed as μM MDA per g FW calculated on the basis of a calibration curve obtained with MDA dimethyl acetal (0–10 μM, Merck, Hohenbrunn, Germany).

For CIII-POX (EC 1.11.1.7) assay, 1 g FW of bean cells from all cell lines was homogenized in liquid nitrogen using a mortar and pestle and 5 mL 0.04 M Tris-HCl pH 7.2, 1 mM EDTA-2Na-2H and 5% (v/v) glycerol were added. The homogenate was centrifuged at 15,000g for 2 min and the supernatant was used to perform the assay. CIII-POX was measured following the method described by Adam et al. (1995), based on the increase in absorbance at 470 nm due to guaiacol oxidation ($\varepsilon_{470} = 26.6\ mM^{-1}\ cm^{-1}$). The reaction was performed with 3 mL reaction buffer (100 mM sodium acetate pH 5.5 and 1 mM guaiacol), 0.3 mL 1.3 mM H_2O_2 and 0.05 mL supernatant.

Protein content was determined following the method described by Bradford (1976).

2.4. Polyacrylamide gel electrophoresis (PAGE)

To detect peroxidase isoforms (iso-POX), supernatant of extracts obtained as indicated for the measurement of CIII-POX activities (see paragraph above) were used. Samples were concentrated using Vivaspin 500 (cutoff 5 KDa; GE Healthcare) and a final amount of 6 μg of proteins were loaded into polyacrylamide gel. Proteins were separated using a non-denaturing gel (12% acrylamide) and running denaturing conditions with Tris-glycine SDS buffer (25 mM Tris-glycine, pH 8.8, and 0.1% SDS (w/v)) at 120 V/h. Peroxidase protein spots were detected by the guaiacol-H_2O_2 procedure as described by Mika et al. (2008); gels were washed with water during 5 min (×3) to remove the SDS, and were incubated with 50 mL of H_2O_2-guaiacol solution (1.0% guaiacol (v/v) and 0.03% H_2O_2 (v/v) in 250 mM sodium acetate buffer pH 5.0) for 30 min until brown spots were visible. For molecular weight determination, EZ-RUN pre-stained protein marker 100 was used as external marker (Fisher Scientific, Spain).

2.5. Statistical analyses

All results are represented as the means ± s.d. of at least 3 replicates. Differences between means were statistically analysed using a Student's *t*-test ($p < 0.05$). When indicated, a one-way ANOVA analysis followed by the Tukey-test was used to compare treatments ($p < 0.05$). Statistics were performed with Statistica software after the data had been tested for normality.

3. Results

3.1. Effect of quinclorac on bean callus growth

A RGR of 1.65 was calculated for NH bean callus-cultured cells. The presence of 10 μM quinclorac (I_{50} concentration, Alonso-Simón et al., 2008) in the culture medium for 30 days was found to reduce the growth of NH cells by half (Table 1). Quinclorac-habituated (Q) cells did also show an impaired growth capacity. In average, a 64%–72% reduction of RGR was measured in Q cells when compared with NH controls (Table 1). The culture of Q30 cells in a medium lacking quinclorac (DH cells) for five subcultures did not rendered an increment in the RGR. No clear trends were observed when DW/FW ratios were compared between cell lines (Table 1).

3.2. Effect of quinclorac on oxidative status and antioxidant activities

As an indicator of oxidative status, the membrane lipid peroxidation level was quantified in all cell lines by MDA formation (Fig. 1). Short-term treatment of NH cells with 10 μM quinclorac (NH+10) induced a significant increase in TBARS levels by more than three-fold. Quinclorac habituation was associated to a steep reduction in the oxidative damage to such an extent that the level of lipid peroxidation measured in Q30 cells did not significantly differ from that in NH cell lines. A short-term treatment of quinclorac habituated cells with 30 μM quinclorac (Q10+30 and Q15+30) did not increase their lipid peroxidation level, but reduced it significantly. In the case of DH cells the lipoperoxidation level measured was slightly higher than that found in NH cells.

To determine the antioxidant capacity of quinclorac-habituated cells, GR, SOD, CAT and CIII-POX activities were determined in crude cell extracts. A short-term treatment of NH cells with 10 μM quinclorac (NH+10) induced an enhancement of CIII-POX activity. No changes in GR and SOD activities were detected (Fig. 2).

Table 1
Relative growth rate (RGR) and final DW/FW ratio of non-habituated calluses (NH), non-habituated calluses treated with 10 μM quinclorac (NH+10), calluses habituated to 10, 15 or 30 μM quinclorac (Q10, Q15 and Q30), and dehabituated calluses (DH), Data represent average values of at least 6 replicates. Different letters indicate significant differences between cell lines by one-way ANOVA ($p < 0.05$).

Cell line	RGR	DW/FW ratio
NH	1.65 ± 0.84^{a}	$0.054 \pm 0.015^{a,b,c}$
NH+10	0.81 ± 0.34^{b}	0.064 ± 0.003^{b}
Q10	0.52 ± 0.20^{b}	$0.055 \pm 0.018^{a,b,c}$
Q15[§]	0.59 ± 0.10^{b}	0.044 ± 0.001^{a}
Q30[§]	0.47 ± 0.15^{b}	0.050 ± 0.003^{c}
DH	0.58 ± 0.49^{b}	$0.055 \pm 0.021^{a,b,c}$

[§]Data from Alonso-Simón et al. (2008), included for comparison.

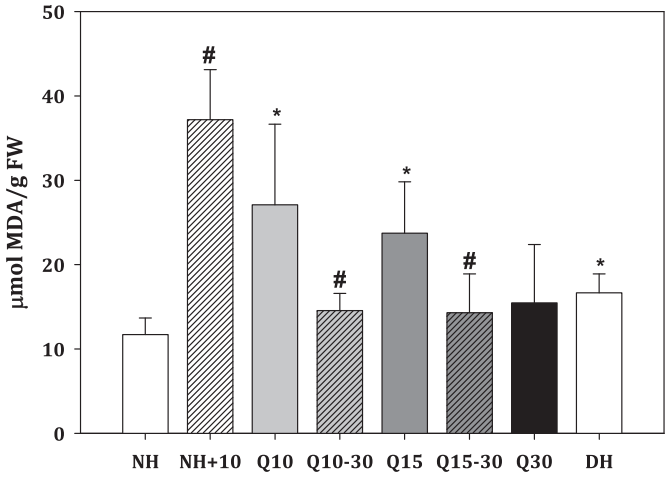

Fig. 1. Lipid peroxidation levels of: non-habituated cells (NH); non-habituated cells treated with 10 µM quinclorac (NH+10); cells habituated to 10 µM (Q10), 15 µM (Q15) and 30 µM quinclorac (Q30); Q10 and Q15 cells transferred to 30 µM quinclorac during 30 days (Q10+30 and Q15+30) and dehabituated cells (DH). Data represent the average values ± s.d. of at least 3 replicates. Asterisks indicate significant differences with respect to NH cells by using Student's t-test. Hashtag indicate the significant differences using Student's *t*-test between treated and the corresponding untreated cell line.

All quinclorac-habituated cells showed a significantly increased GR activity in comparison with NH cells. Moreover, GR activity steeply increased over the course of the habituation process (Fig. 2A). The short-term exposure of Q10 and Q15 to 30 µM quinclorac did not induce changes in GR activity when compared with their respective untreated cell lines (Fig. 2A).

The pattern of changes in SOD and CIII-POX activities over the course of quinclorac-habituation differed from those reported for GR activity. SOD and CIII-POX antioxidant activities only differed from control cells when measured at the highest habituation level (Q30). However, Q10 and Q15 cells significantly increased both antioxidant activities (Fig. 2B and C) upon 30 µM quinclorac treatment (Q10+30 and Q15+30). CAT activity was not detected in any cell line tested.

Quinclorac-dehabituated cells showed no significant differences in lipid peroxidation levels (Fig. 1), and exhibited increased levels of GR, SOD and CIII-POX activities when compared with those of NH cells (Fig. 2).

3.3. *Peroxidase isoforms*

To obtain further information about isoPOX, a semi-native PAGE of cell extracts was performed, followed by H_2O_2-guaiacol staining (Fig. 3).

Two isoPOX (I-II) were detected in NH calluses (Fig. 3). IsoPOX II was present in all cell lines analysed. Contrary, isoPOX I was not detected in Q10 and Q15 cells and it was again detected in Q30. Interestingly this same isoPOX was detected when Q10 and Q15 cells were treated with 30 µM quinclorac (Q10+30 and Q15+30).

The staining intensity of the isoPOX I and II markedly increased in Q30, Q10+30 and Q15+30 when compared with NH cells.

Quinclorac-dehabituation is associated with the maintenance of a high staining intensity for isoPOX I and II.

4. Discussion

The exposure of plant cells to a variety of abiotic stresses such as heavy metals (Paradiso et al., 2008), organochlorines (Michalowicz and Duda, 2009; Michalowicz et al., 2009; San Miguel et al., 2012) and herbicides (Geoffroy et al., 2004; García-Angulo et al., 2009) often unbalances ROS production and scavenging, leading to oxidative stress (Apel and Hirt, 2004; Gill and Tuteja, 2010). Excessive ROS production can damage cellular components such as proteins, lipids, DNA and polysaccharides, which interrupts normal metabolism (Dat et al., 2000). The triggering of these oxygen intermediates leads to the activation of defence mechanisms in the plant cells, which consist of antioxidants, such as ascorbate and glutathione, and enzymatic antioxidant activities such as CIII-POX, CAT, SOD and the ascorbate–glutathione cycle enzymes, ascorbate peroxidase (APOX) and GR (Apel and Hirt, 2004; Passardi et al., 2005; Gill and Tuteja, 2010). Additionally, stress-induced ROS can act as signaling molecules specifically inducing several cell and molecular responses (Miller et al., 2009, 2010; Kärkönen and Kuchitsu, 2015).

It has been postulated that the mode of action of quinclorac differs between grasses and broad-leaved weeds (Koo et al., 1991). However, quinclorac toxicity assays on FW gain in bean callus-cultured cells (Alonso-Simón et al., 2008; García-Angulo et al., 2012) indicated that quinclorac is active in the range of concentrations previously reported for maize seedlings, regarded as a model for quinclorac-susceptible grasses (Sunohara and Matsumoto, 2008). Our study suggests that in the same way as a series of other sensitive species (Sunohara and Matsumoto, 2004, 2008), bean calluses are subjected to oxidative harm, since treatment of NH cells with quinclorac (NH+10) increased lipid peroxidation above the level observed in the other cell lines analysed (Fig. 1).

The habituation of bean calluses to quinclorac is associated with an increased constitutive level of antioxidant activities (Fig. 2). The level of the activities increased as the level of quinclorac in the culture medium rose. Thus Q30, the cell line habituated to the highest concentration of quinclorac, also had the highest level of GR, SOD and CIII-POX activities (Fig. 2). These results were also confirmed by a higher staining intensity in both of the isoPOX separated by PAGE (Fig. 3).

This enhancement of antioxidant capacity is associated with a steep reduction in lipid peroxidation levels to the extent that Q30 and NH cells had similar levels of oxidative damage. Results reported here point to bean cells habituated to quinclorac having developed an antioxidant strategy that allows them to cope with quinclorac-induced oxidative stress.

Despite of enhanced controlling the quinclorac-induced oxidative stress, Q cells maintain a lower growth capacity in comparison to NH cells as previously reported by our group (Alonso-Simón et al., 2008). These results point to quinclorac affecting growth of habituated cell lines by mechanisms related neither to cell wall modification nor oxidative damage (Grossmann, 2010).

During the habituation process, bean cultured cells have to cope with stepwise increments of quinclorac concentration added to the culture medium. In a way that mirrored the habituation procedure, Q10 and Q15 calluses where subcultured in a medium with 30 µM quinclorac for a short period of time (Q10+30 and Q15+30 cells respectively). The results showed that Q10 and Q15 cells responded to this treatment by significantly increasing CIII-POX and SOD activities, but not GR activity. In addition, upon 30 µM quinclorac short-time treatment, Q10 and Q15 cells showed a higher staining intensity for isoPOX II, and the recovery of isoPOX I.

These findings suggest that CIII-POX and SOD antioxidant activities play a primary role in quinclorac habituation process. Interestingly, an association between increased lipid peroxidation levels and low CIII-POX and SOD activities was found in Q10 and Q15 cells.

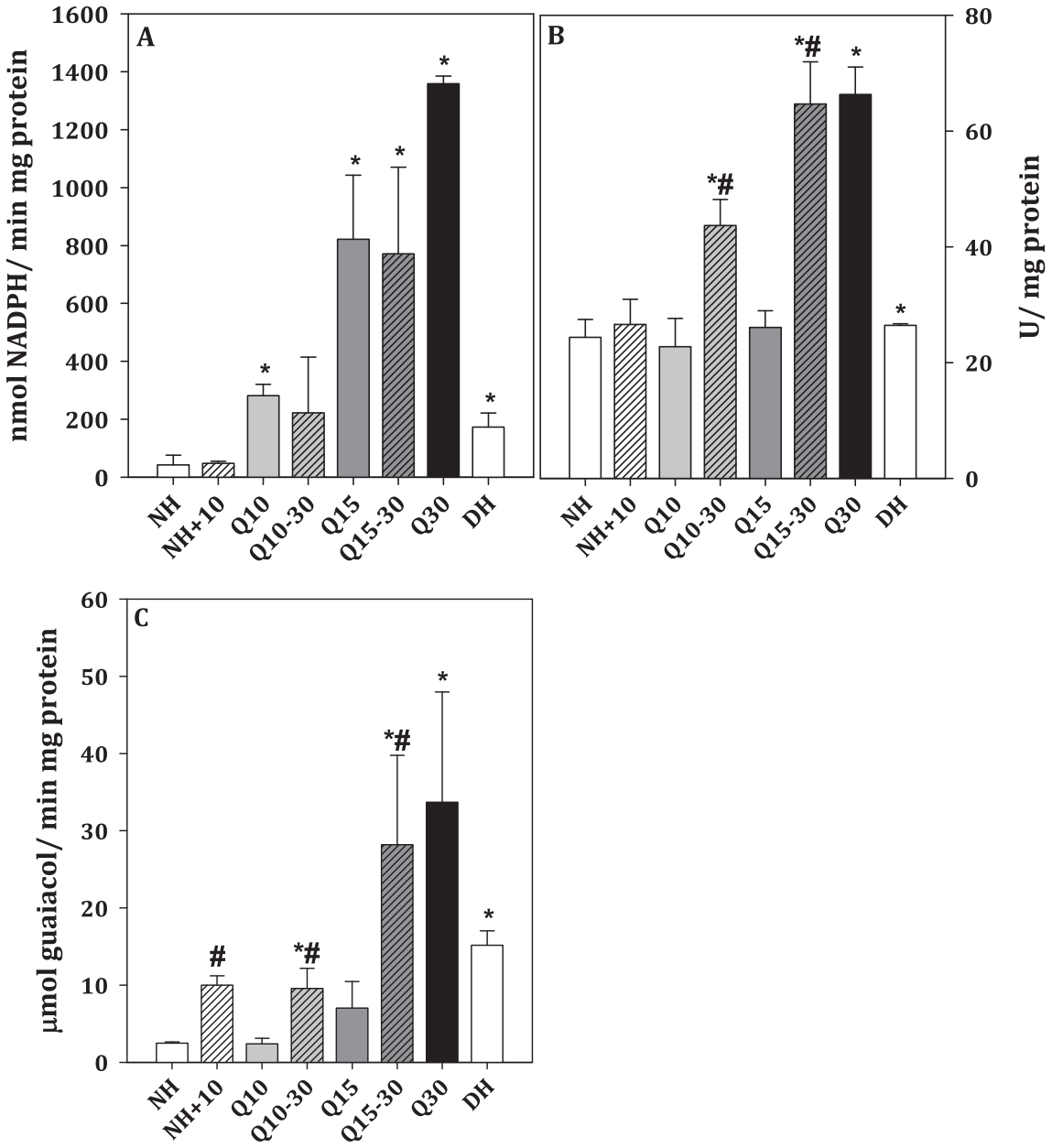

Fig. 2. Activity of GR (A), SOD (B) and CIII-POX (C) in non-habituated, quinclorac-habituated and quinclorac-dehabituated cells. Data represent means ± s.d. of at least 3 replicates. One SOD unit (U) will inhibit the rate of reduction of cytochrome *c* by 50% in a coupled system, using xanthine and xanthine oxidase, at pH 7.8 at 25 °C in a 3 ml reaction mixture (Sigma). Cell lines, asterisks and hashtag as in Fig. 1.

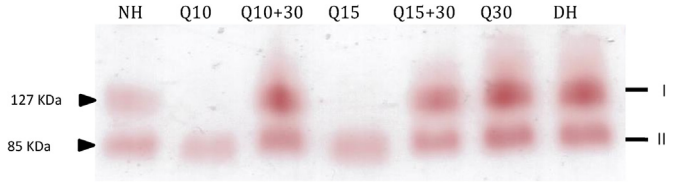

Fig. 3. Semi-native PAGE of peroxidase isoforms in non-habituated, quinclorac-habituated and quinclorac-dehabitutated cells. Cell lines indicated as in Fig. 1. Different isoPOX were indicated with italic numbers. Molecular weights of external markers are indicated at left.

A short treatment of NH cells with 10 µM quinclorac (NH+10) provoked an enhancement of CIII-POX activity, but GR and SOD activities did not experienced any changes (Fig. 2). These results strengthen the hypothesis that CIII-POX activity is especially responsive to quinclorac-induced stress. Interestingly, same results have already been reported for bean cultured cells habituated to diclobenil (García-Angulo et al., 2009). According to our results, the increment in CIII-POX activity measured in NH+10 cells did not seemed to have the capacity to cope with quinclorac-induced oxidative stress, since NH+10 cells had the highest lipid peroxidation levels (Fig. 1). Moreover, NH+10 cells had almost half RGR by comparison with NH cells (Table 1), indicating that quinclorac treatment provokes a reduction in NH cell growth that could be closely related to oxidative stress.

Quinclorac-dehabituated bean cells (DH) were obtained by subculturing Q30 cells in a medium lacking quinclorac for five subcultures. The most remarkable characteristics of DH calluses were: i) a growth rate similar to quinclorac-habituated cells,

therefore lower to that found in NH cells (Table 1), ii) slightly higher lipid peroxidation levels than NH cells (Fig. 1), iii) higher antioxidant activities than NH calluses but much lower than quinclorac-habituated calluses (Fig. 2) and iv) the upkeep of the enhanced staining of the two isoPOX (Fig. 3). These results allow us to suggest that the increase in antioxidant activities that putatively confers habituated cells with the capacity to cope with quinclorac is stable and independent of the presence of quinclorac in the culture medium. Interestingly, an acquired high POX activity has also been found in dichlobenil-dehabituated bean calluses grown in a medium lacking the inhibitor (García-Angulo et al., 2009).

The undetectable levels of CAT activity would indicate that this enzyme does not play a role in quinclorac habituation. This finding is consistent with previous results in which no CAT activity was detected in non-habituated and dichlobenil-habituated bean cells (García-Angulo et al., 2009). One possible explanation could be the plant material used, as heterotrophic calluses, grown under dark conditions have been reported to have a lower CAT activity than plant tissues (Kim et al., 2004).

We have previously observed that bean calluses can be habituated to herbicides following one or more mechanisms. The habituation of bean calluses to dichlobenil was associated with a high scavenging capacity of ROS, mainly by CIII-POX activity (García-Angulo et al., 2009) and also with the capacity of the cells to divide and expand with a modified cell wall in which the xyloglucan-cellulose network had been partially replaced by pectins (Encina et al., 2001, 2002). However, quinclorac-habituated bean cells have a non-modified cell wall (Alonso-Simón et al., 2008) and, as was observed in this study, this habituation seemed to be related to a high antioxidant capacity. Other herbicides have also provoked oxidative stress in plant materials other than bean cultured cells. This is case for fluroxypyr, where increasing concentrations of this herbicide caused accumulation of various ROS and at the same time reduced the shoot growth of *Oryza sativa*. Fluroxypyr-induced oxidative stress significantly changed SOD, CAT, APOX and POX activities. With the exception of POX activity, the rest of the antioxidant activities showed a general increase at low herbicide concentrations and a decrease at high fluroxypyr levels (Wu et al., 2010).

In sum, based on the observations of the present study, it can be concluded that quinclorac exerts oxidative stress on bean calluses. The acquisition of a prominent antioxidant capacity against quinclorac-induced oxidative stress seems to be an important factor in habituation to quinclorac. Lastly, this adaptative antioxidant response to quinclorac remains stable in the absence of the herbicide.

5. Conclusions

The habituation of bean calluses to high quinclorac concentrations (30 μM) was associated with increased constitutive levels of class III-peroxidase, glutathione reductase, and superoxide dismutase activities. These findings correlated with a reduction in the lipid peroxidation level in habituated cell lines, which was always significantly lower than that found in non-habituated cells following short-term treatment with 10 μM quinclorac. Changes in the antioxidant status of bean cells were maintained when quinclorac-habituated cells were cultured in a medium lacking quinclorac.

Author's contribution

ALG: designed research; performed research; analysed data; wrote manuscript; MdC: performed research; revised manuscript; AAS: writing and editing work; PGA: designed research; assisted in performing the research, revised manuscript; JLA: designed research; assisted in performing the research; revised manuscript; AE: provided research opportunity, supervised project, designed research; performed research; analysed data; wrote manuscript; JMA: provided research opportunity, supervised project, designed research, wrote manuscript.

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