Plant Physiology and Biochemistry xxx (2016) xxx-xxx

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com

UNCORRECTED PROOF

Research article

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

Asier Largo-Gosens, María de Castro, Ana Alonso-Simón, Penélope García-Angulo, José L. Acebes, Antonio Encina,* Jesús M. Álvarez

Área de Fisiología Vegetal, Facultad de Ciencias Biológicas y Ambientales, Universidad de León, E-24071 León, Spain

ARTICLE INFO

Article history:
Received 8 May 2016
Received in revised form 7 June 2016
Accepted 7 June 2016
Available online xxx

Keywords:
Antioxidant activities
Cell culture habituation
Herbicide
Oxidative damage
Phaseolus vulgaris
Quinclorac

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.

© 2016 Published by Elsevier Ltd.

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. 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-per-

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

* Corresponding author.
Email address: a.encina@unileon.es (A. Encina)

http://dx.doi.org/10.1016/j.plaphy.2016.06.011
0981-9428/© 2016 Published by Elsevier Ltd.

oxidase (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 15,000*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 ($\varepsilon_{334} = 6.2\ mM^{-1}\ cm^{-1}$). The reaction was performed at 25 °C with 0.1 mL of super-

UNCORRECTED PROOF

natant, 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 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 15,000*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).

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). A Short-term treatment of NH cells with 10 µM quinclorac (NH+10) induced a significant increase in TBARS levels by more than threefold. 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).

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).

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.

UNCORRECTED PROOF

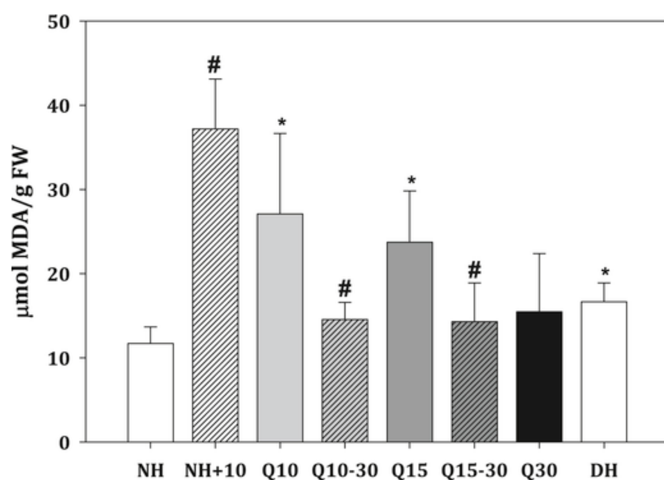

Fig. 1. Lipid peroxidation levels of: non-habituated cells (NH); non-habituated cells treated with 10 µM quinclorac (NH+10); cells habituated to 10 µM (Q10), 15 µM (Q15) and 30 µM quinclorac (Q30); Q10 and Q15 cells transferred to 30 µM quinclorac during 30 days (Q10+30 and Q15+30) and dehabituated cells (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.

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 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. 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.

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 respon-

UNCORRECTED PROOF

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.

sive 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.

Acknowledgements

This work was supported by grants from Junta de Castilla y León (LE 48A07), the Spanish Ministries of “Ciencia e Innovación” (CGL2008-02470/BOS) and “Economía y Competitividad” (AGL2011-30545-C02-2) and a predoctoral grant from the University of León to Asier Largo Gosens. We are grateful to Carlos Romo for providing technical support and to Denise Phelps for correcting the English version of the manuscript.

References

Abdallah, I., Fischer, A., Elmore, C., Saltveit, M., Zaki, M., 2006. Mechanism of resistance to quinclorac in smooth crabgrass (Digitaria ischaemum). Pestic. Biochem. Physiol. 84 (1), 38–48.
Adam, A., Bestwick, C., Barna, B., Mansfield, J., 1995. Enzymes regulating the accumulation of active oxygen species during the hypersensitive reaction of bean to Pseudomonas-syringae pv phaseolicola. Planta 197 (2), 240–249.
Alonso-Simón, A., García-Angulo, P., Encina, A., Acebes, J.L., Álvarez, J.M., 2008. Habituation of bean (Phaseolus vulgaris) cell cultures to quinclorac and analysis of the subsequent cell wall modifications. Ann. Bot. 101 (9), 1329–1339.
Apel, K., Hirt, H., 2004. Reactive oxygen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373–399.
Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. Anal. Biochem. 72 (1–2), 248–254.
Buege, J.A., Aust, S.D., 1978. Microsomal lipid peroxidation. In: Methods in Enzymology. Academic Press, pp. 302–310. pp.
Dat, J., Vandenabeele, S., Vranova, E., Van Montagu, M., Inzé, D., Van Breusegem, F., 2000. Dual action of the active oxygen species during plant stress responses. Cell. Mol. Life Sci. 57 (5), 779–795.
Díaz-Cacho, P., Moral, R., Encina, A., Acebes, J.L., Álvarez, J.M., 1999. Cell wall modifications in bean (Phaseolus vulgaris) callus cultures tolerant to isoxaben. Physiol. Plant. 107 (1), 54–59.
Droillard, M., Paulin, A., Massot, J., 1987. Free radical production, catalase and superoxide dismutase activities and membrane integrity during senescence of petals of cut carnations (Dianthus caryophyllus). Physiol. Plant. 71 (2), 197–202.
Encina, A., Moral, R., Acebes, J.L., Álvarez, J.M., 2001. Characterization of cell walls in bean (Phaseolus vulgaris L.) callus cultures tolerant to dichlobenil. Plant Sci. 160 (2), 331–339.
Encina, A., Sevillano, J.M., Acebes, J.L., Álvarez, J.M., 2002. Cell wall modifications of bean (Phaseolus vulgaris) cell suspensions during habituation and dehabituation to dichlobenil. Physiol. Plant. 114 (2), 182–191.
García-Angulo, P., Alonso-Simón, A., Mélida, H., Encina, A., Acebes, J.L., Álvarez, J.M., 2009. High peroxidase activity and stable changes in the cell wall are related to dichlobenil tolerance. J. Plant Physiol. 166 (12), 1229–1240.
García-Angulo, P., Alonso-Simon, A., Encina, A., Álvarez, J.M., Acebes, J.L., 2012. Cellulose biosynthesis inhibitors: comparative effect on bean cell cultures. Int. J. Mol. Sci. 13 (3), 3685–3702.
Geoffroy, L., Frankart, C., Eullaffroy, P., 2004. Comparison of different physiological parameter responses in Lemna minor and Scenedesmus obliquus exposed to herbicide flumioxazin. Environ. Pollut. 131 (2), 233–241.
Gill, S.S., Tuteja, N., 2010. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol. Biochem. 48 (12), 909–930.

UNCORRECTED PROOF

Grossmann, K., 2000. Mode of action of auxin herbicides: a new ending to a long, drawn out story. Trends Plant Sci. 5 (12), 506–508.
Grossmann, K., 2010. Auxin herbicides: current status of mechanism and mode of action. Pest. Manag. Sci. 66 (2), 113–120.
Grossmann, K., Kwiatkowski, J., 1993. Selective induction of ethylene and cyanide biosynthesis appears to be involved in the selectivity of the herbicide quinclorac between rice and barnyardgrass. J. Plant Physiol. 142 (4), 457–466.
Grossmann, K., Kwiatkowski, J., 2000. The mechanism of quinclorac selectivity in grasses. Pestic. Biochem. Physiol. 66 (2), 73–91.
Grossmann, K., Scheltrup, F., 1997. Selective induction of 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity is involved in the selectivity of the auxin herbicide quinclorac between barnyard grass and rice. Pestic. Biochem. Physiol. 58 (2), 145–153.
Kärkönen, A., Kuchitsu, K., 2015. Reactive oxygen species in cell wall metabolism and developmentin plants. Phytochemistry 112, 22–32.
Kim, Y.H., Kim, Y., Cho, E., Kwak, S., Kwon, S., Bae, J., Lee, B., Meen, B., Huh, G.H., 2004. Alterations in intracellular and extracellular activities of antioxidant enzymes during suspension culture of sweetpotato. Phytochemistry 65 (17), 2471–2476.
Klapheck, S., Zimmer, I., Cosse, H., 1990. Scavenging of hydrogen-peroxide in the endosperm of Ricinus communis by ascorbate peroxidase. Plant Cell Physiol. 31 (7), 1005–1013.
Koo, S.J., Kwon, Y.W., Cho, K.Y., 1991. Differences in herbicidal activity, phytotoxic symptoms and auxin activity of quinclorac among plant species compared with 2,4-D. J. Weed Sci. Technol. 36 (4), 311–317.
Koo, S.J., Neal, J.C., DiTomaso, J.M., 1996. 3,7-dichloroquinolinecarboxylic acid inhibits cell-wall biosynthesis in maize roots. Plant Physiol. 112 (3), 1383–1389.
Koo, S., Neal, J., DiTomaso, J.M., 1997. Mechanism of action and selectivity of quinclorac in grass roots. Pestic. Biochem. Physiol. 57 (1), 44–53.
Largo-Gosens, A., Encina, A., de Castro, M., Mélida, H., Acebes, J.L., García-Angulo, P., Álvarez, J.M., 2016. Early habituation of maize (Zea mays) suspension-cultured cells to 2,6-dichlorobenzonitrile is associated with the enhancement of antioxidant status. Physiol. Plant. 157, 193–204.
Li, G., Xu, M.F., Chen, L.P., Cai, L.M., Bai, L.Y., Wu, C.X., 2016. A novel EcGH3 gene with a different expression pattern in quinclorac-resistant and susceptible barnyardgrass (Echinochloa crus-galli). Plant Gene 5, 65–70.
Mélida, H., Encina, A., Álvarez, J.M., Acebes, J.L., Caparrós-Ruiz, D., 2010. Unraveling the biochemical and molecular networks involved in maize cell habituation to the cellulose biosynthesis inhibitor dichlobenil. Mol. Plant 3 (5), 842–853.
Michalowicz, J., Duda, W., 2009. The effects of 2,4,5-trichlorophenol on some antioxidative parameters and the activity of glutathione S-transferase in reed canary grass leaves (Phalaris arudinacea). Pol. J. Environ. Stud. 18 (5), 845–852.
Michalowicz, J., Posmyk, M., Duda, W., 2009. Chlorophenols induce lipid peroxidation and change antioxidant parameters in the leaves of wheat (Triticum aestivum L.). J. Plant Physiol. 166 (6), 559–568.
Mika, A., Buck, F., Lüthje, S., 2008. Membrane-bound class III peroxidases: identification, biochemical properties and sequence analysis of isoenzymes purified from maize (Zea mays L.) roots. J. Proteom. 71 (4), 412–424.
Miller, G., Schlauch, K., Tam, R., Cortes, D., Torres, M.A., Shulaev, V., Dangl, J.L., Mittler, R., 2009. The plant NADPH oxidase RBOHD mediates rapid systemic signaling in response to diverse stimuli. Sci. Signal. 2 (84). ra45.
Miller, G., Suzuki, N., Ciftci-Yilmaz, S., Mittler, R., 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. Plant Cell Environ. 33 (4), 453–467.
Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. 15 (3), 473–497.
Navrot, N., Rouhier, N., Gelhaye, E., Jacquot, J.P., 2007. Reactive oxygen species generation and antioxidant systems in plant mitochondria. Physiol. Plant. 129 (1), 185–195.
Paradiso, A., Berardino, R., de Pinto, M.C., di Topi, L.S., Storelli, M.M., Tommasi, F., De Gara, L., 2008. Increase in ascorbate-glutathione metabolism as local and precocious systemic responses induced by cadmium in durum wheat plants. Plant Cell Physiol. 49 (3), 362–374.
Passardi, F., Cosio, C., Penel, C., Dunand, C., 2005. Peroxidases have more functions than a Swiss army knife. Plant Cell Rep. 24 (5), 255–265.
San Miguel, A., Faure, M., Ravanel, P., Raveton, M., 2012. Biological responses of maize (Zea mays) plants exposed to chlorobenzenes. Case study of monochloro-, 1,4-dichloro and 1,2,4-trichloro-benzenes. Ecotoxicology 21 (2), 315–324.
Sunohara, Y., Matsumoto, H., 2004. Oxidative injury induced by the herbicide quinclorac on Echinochloa oryzicola Vasing and the involvement of antioxidative ability in its highly selective action in grass species. Plant Sci. 167 (3), 597–606.
Sunohara, Y., Matsumoto, H., 2008. Quinclorac-induced cell death is accompanied by generation of reactive oxygen species in maize root tissue. Phytochemistry 69 (12), 2312–2319.
Sunohara, Y., Shirai, S., Wongkantrakorn, N., Matsumoto, H., 2010. Sensitivity and physiological responses of Eleusine indica and Digitaria adscendens to herbicide quinclorac and 2,4-D. Environ. Exp. Bot. 68 (2), 157–164.
Sunohara, Y., Shirai, S., Yamazaki, H., Matsumoto, H., 2011. Involvement of antioxidant capacity in quinclorac tolerance in Eleusine indica. Environ. Exp. Bot. 74, 74–81.
Tresch, S., Grossmann, K., 2003. Quinclorac does not inhibit cellulose (cell wall) biosynthesis in sensitive barnyard grass and maize roots. Pestic. Biochem. Physiol. 75 (3), 73–78.
Van Eerd, L.L., Stephenson, G.R., Kwiatkowski, J., Grossmann, K., Hall, J.C., 2005. Physiological and biochemical characterization of quinclorac resistance in a false cleavers (Galium spurium L.) biotype. J. Agric. Food Chem. 53 (4), 1144–1151.
Vaughn, K.C., 2002. Cellulose biosynthesis inhibitor herbicides. In: Böger, P., Wakabayashi, K., Hirai, K. (Eds.), Herbicide Classes in Development. Springer, Berlin, pp. 139–150.
Wu, G.L., Cui, J., Tao, L., Yang, H., 2010. Fluroxypyr triggers oxidative damage by producing superoxide and hydrogen peroxide in rice (Oryza sativa). Ecotoxicology 19 (1), 124–132.
Xu, W., Di, C., Zhou, S., Liu, J., Li, L., Liu, F., Yang, X., Ling, Y., Su, Z., 2015. Rice transcriptome analysis to identify possible herbicide quinclorac detoxification genes. Front. Genet. http://dx.doi.org/10.3389/fgene.2015.00306.
Yasuor, H., Milan, M., Eckert, J.W., Fischer, A.J., 2012. Quinclorac resistance: a concerted hormonal and enzymatic effort in Echinochloa phyllopogon. Pest. Manag. Sci. 68 (1), 108–115.

UNCORRECTED PROOF