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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Title: "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-diclorobenzonitrile (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 characterised 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.

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Abbreviations

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

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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 (type 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 scarce of 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 analysed 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 to 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 behaviour was not observed when higher DCB (i.e. 1 μM) concentrations were used (de Castro et al. 2014). These results

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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 defence 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). Additionally, DCB habituation of bean suspension-cultured cells is associated with a stable increment of CIII-POX, which has been hypothesised to increase the antioxidant capacity of cells and possibly to 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

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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 eleven culture cycles ~~at these DCB concentrations~~. Additionally, 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.

Materials and methods

Plant cell cultures

Maize suspension-cultured cells (*Zea mays* L., 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 (*Zea mays* L., Black Mexican sweetcorn) were routinely grown in MS media (Murashige and Skoog 1962) supplemented with 20 g l^{-1} sucrose and 9 μM 2,4-dichlorophenoxiacetic acid, at 25ºC 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% to 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 ten subcultures (de Castro et al. 2014). Habituated cells were denoted as SHx, where "x" indicates the DCB concentration (μM) added to the culture media.

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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 by the fluorescein diacetate method as described by Duncan and Widholm (1990). Fifty µl of 0.2% (w/v) fluorescein diacetate (Sigma) 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.

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Lipid peroxidation levels and enzyme activity assays

Cells from all lines were collected during the exponential growth phase and stored at -80ºC until use. One to five g fresh weight (FW) of these cells was homogenised 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 mM 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 4ºC 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 ml 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 100ºC 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 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: $\varepsilon = 39.58\ M^{-1}\ cm^{-1}$. 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

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disulphide (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 mM Tris-HCl pH 8.0, 0.1 mM EDTA, 1 mM GSSG and 3 mM $MgCl_2$) and 0.05 ml of 10 mM 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 30ºC. 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 mM EDTA-2Na-2H and 5% (v/v) glycerol, then centrifuged at 15000 *g* for 2 min at 4ºC 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 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 25ºC 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 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: $\varepsilon = 2.8\ mM^{-1}\ cm^{-1}$.

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

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Total GSH and GSSG measurement

For GSH and GSSG extraction, cells were homogenised 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 by 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 homogenised 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 4ºC 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 1ml 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 oxidise 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 absorbance at 265 nm 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 sulphate/xylenol orange method as described by Cheeseman et al. (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 H_2O_2 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: $\varepsilon = 43.6\ M^{-1}\ cm^{-1}$

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Statistical analyses

All results are expressed as the means ± s.d. of at least 4 replicates. When indicated, differences between means were statistically analysed by using a Student's t-test.

Results

Growth measurements in DCB-habituated cells

SNH cells showed a 3-fold increase in DW after 7 days of culture in fresh medium (Fig. 1A). A 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 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. 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 a function of TBARS formation using MDA as reference molecule (Fig. 2). Short-term treatment of SNH cells with DCB (0.5 or 1 μM) caused more than a 4-fold 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 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. 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.

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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) was 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 μM or 1μM DCB resulted in a significant enhancement of CIII-POX activity and a slightly 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) ones. As in the case of DCB habituation, GR activity did not vary with respect to control in DCB short-term treated cells (Fig. 4D).

Besides the enzymatic activities regularly involved in the antioxidant defence 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 characterised 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.

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3 TA measured in SNH cells during the exponential phase was on average 1µmol/g 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 (oxidised 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 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.

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 oxidising 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,

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a catalase-like activity has been reported for type-III POXs, which efficiently detoxifies H_2O_2 (Mika et al. 2004 and refs. 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).

Additionally, a role for CIII-POXs in maize cell wall remodelling 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, pers. comm.) and high (Mélida et al. 2009, 2010b, 2011) levels of DCB habituation. However, in maize cells habituated to high DCB levels, no relationship was found between high ferulate dimerisation and increased peroxidase activity (Mélida et al. 2010a), indicating that cell wall CIII-POX activity is not a limiting factor for ferulate dimerisation. 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 remodelling. 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 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 SH1cells 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

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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 since 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 defence 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 characterised by cell growth impairment expressed both as dry weight gain and cell viability and a significant rise in oxidative stress measured as lipoperoxidation (increased by more than 4-fold 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 remodelling strategy, during incipient habituation the antioxidant-

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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 contribution

ALG: designed research; performed research; analysed data; wrote manuscript

AE: designed research; performed research; analysed data; wrote manuscript

MdC: performed research; revised manuscript

HM: designed research; revised manuscript

JLA: designed research; revised manuscript

PGA: designed research; revised manuscript

JMA: supervised project; designed research; wrote manuscript

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Figure legends

Figure 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 4 replicates. "X" indicate the DCB concentration (μM) added to the culture medium.

Figure 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 ± s.d. of at least 4 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.

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Figure 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 (grey) and stationary (black) phase of the cell culture cycle. Data represent means ± s.d. of 3 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.

Figure 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 ± s.d. of at least 4 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.

Figure 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 ± s.d. of at least 4 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.

Figure 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 ± s.d. of at least 6 replicates. Asterisks

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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.

Figure 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 (µM) added to the culture medium.

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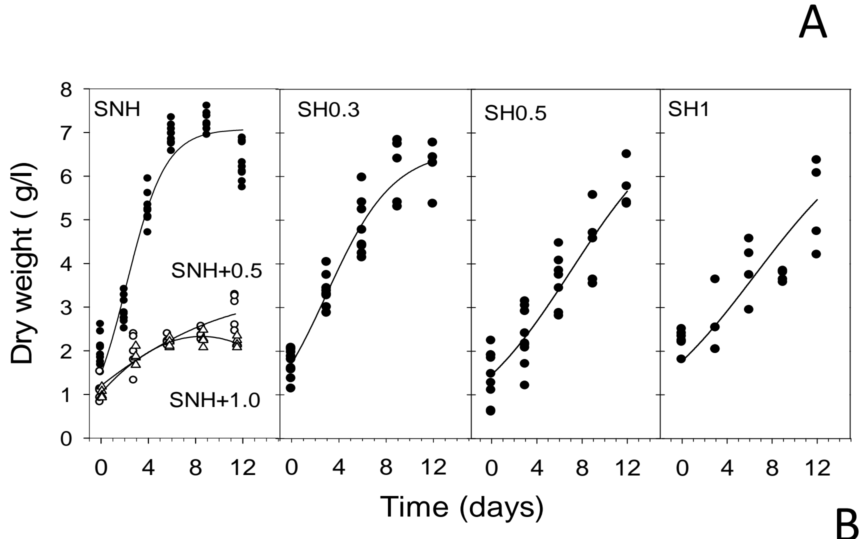

B

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

Figure 1

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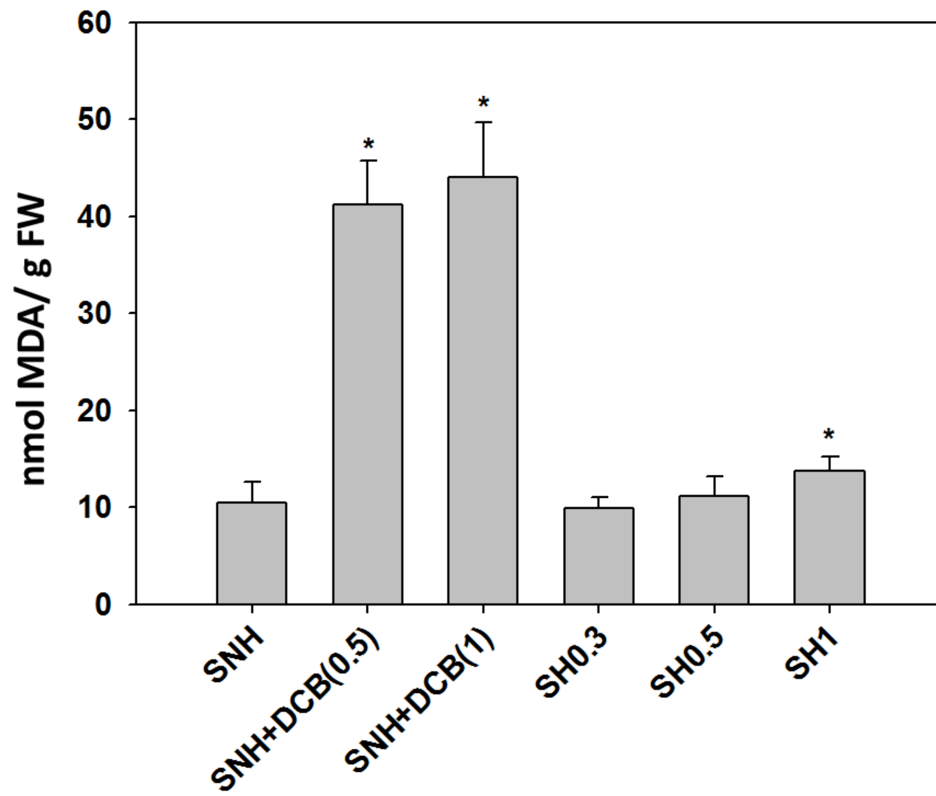

Figure 2

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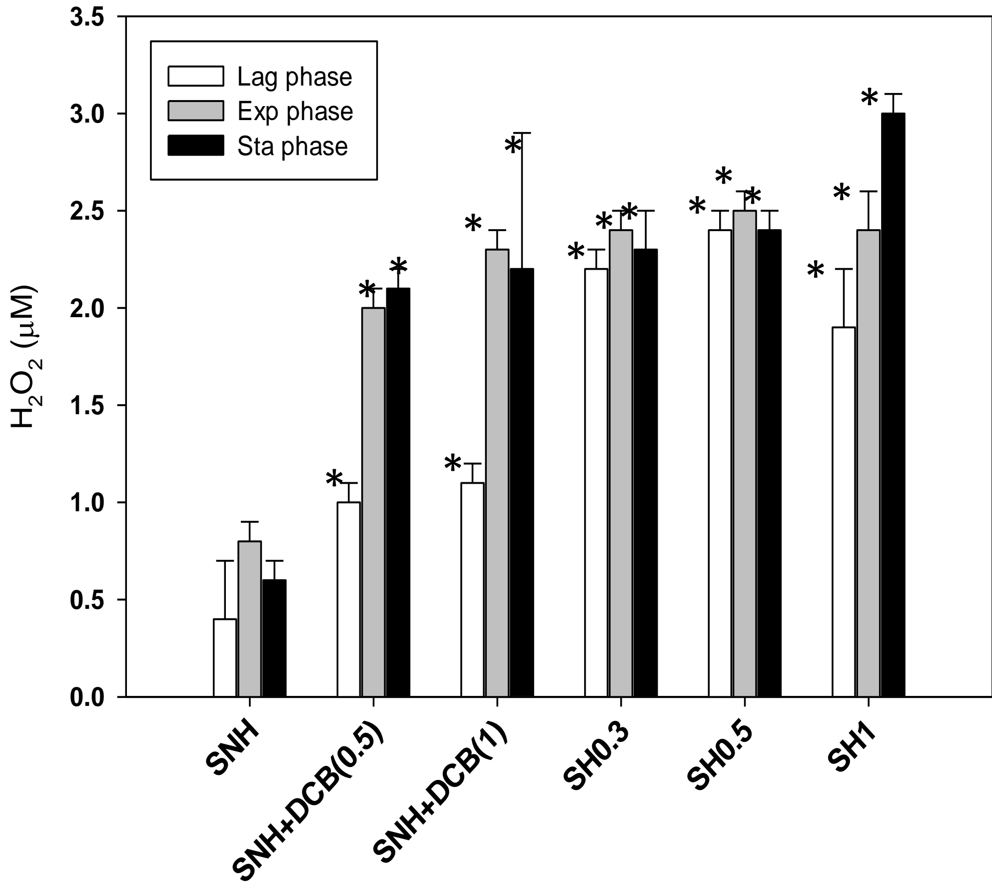

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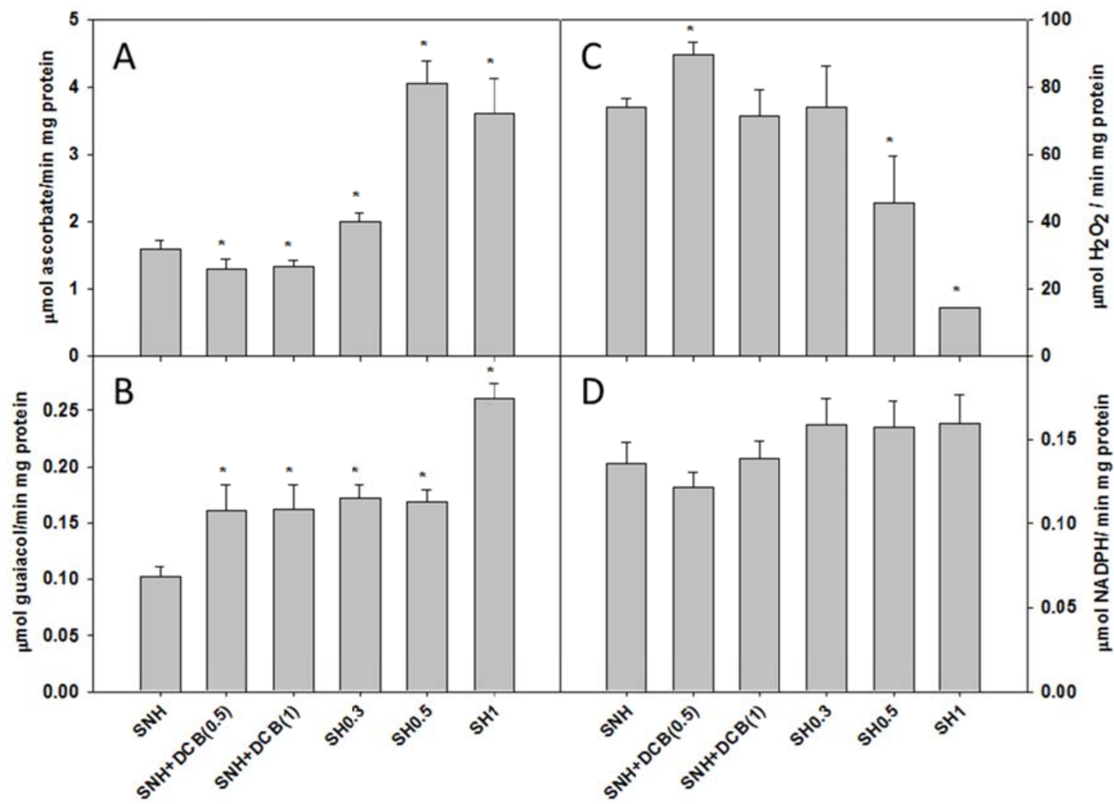

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Figure 4

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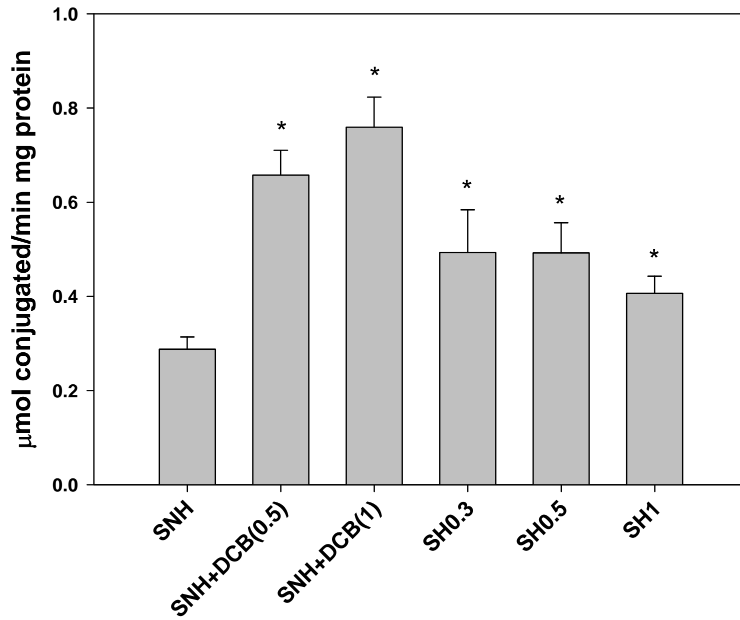

Figure 5

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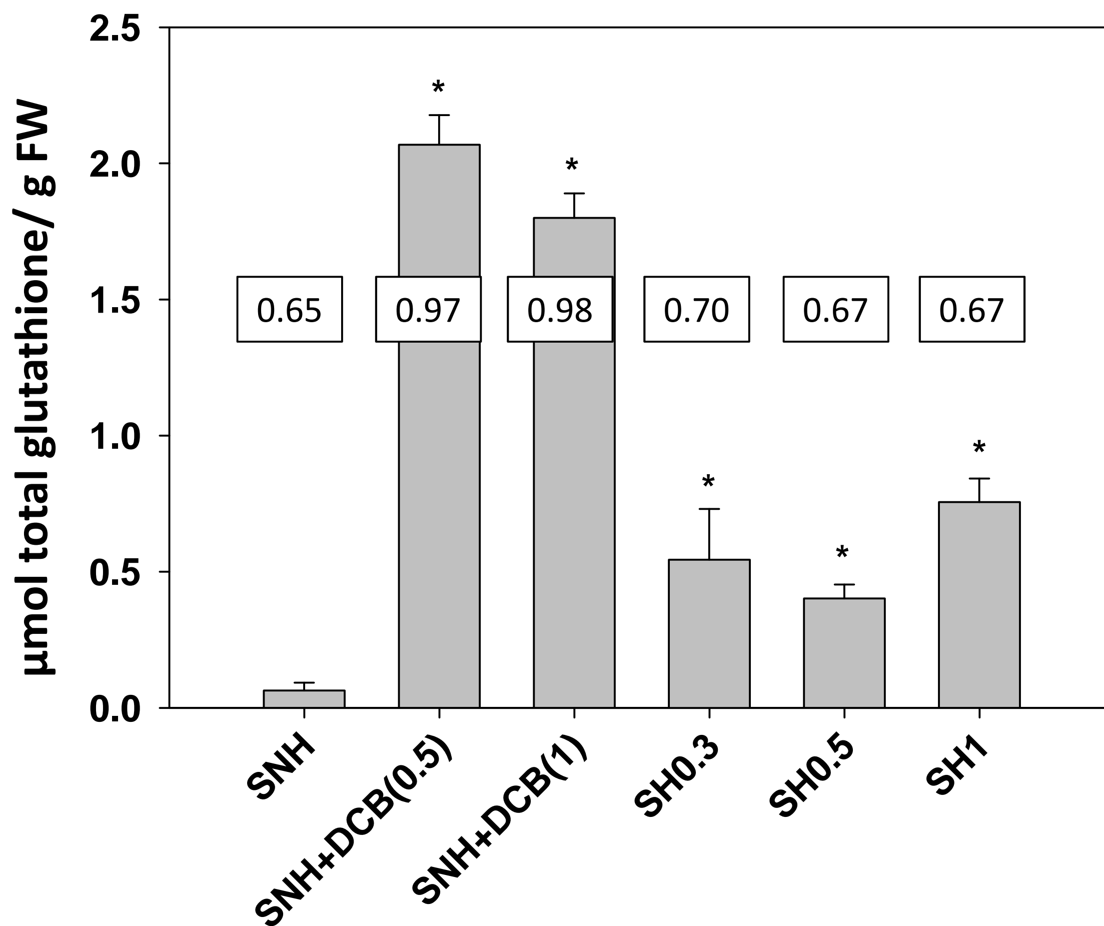

Figure 6

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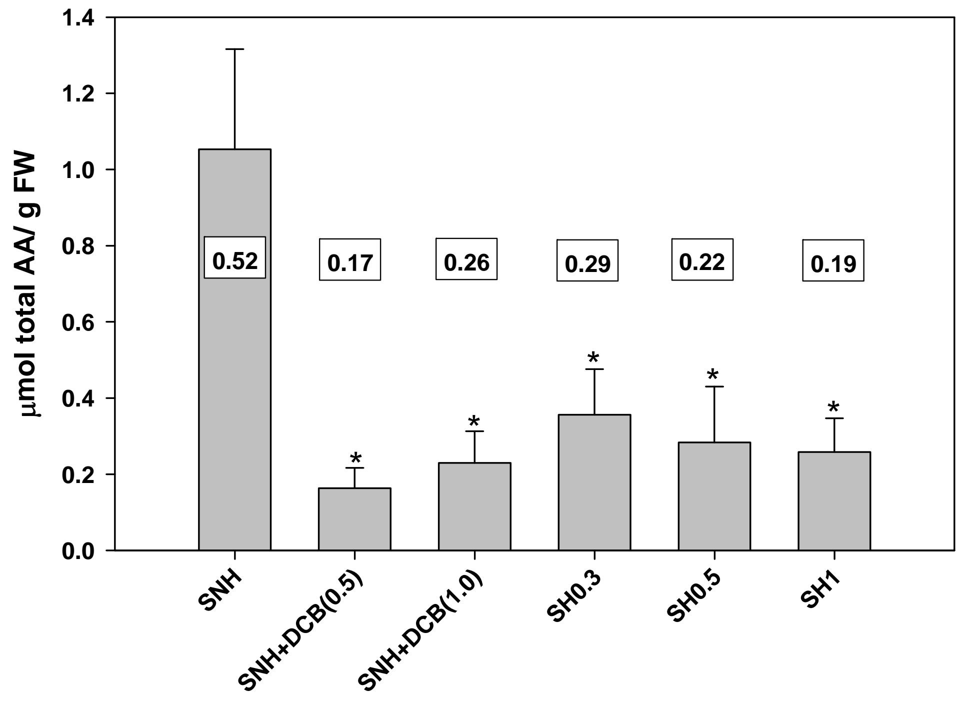

Figure 7.

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