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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

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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.

* Corresponding author.
E-mail address: a.encina@unileon.es (A. Encina).

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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