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Hydrogen peroxide induced phenylpropanoids pathway eliciting a defensive response in plants micropropagated in Temporary Immersion Bioreactors (TIBs)

Ariel D. Arencibia[a,b,*], Aydiloide Bernal[a], Carlos Zayas[a], Elva Carmona[a], Cecilia Cordero[b], Gloria González[b], Rolando García[b], Ignacio Santana[a]

[a] National Institute for Sugarcane Research, CUJAE Road km 2½, Boyeros 19390, Havana, Cuba
[b] Catholic University of Maule, Ave San Miguel 3605, Talca 617, Chile

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ABSTRACT

The relation between the oxidative burst and phenylpropanoid pathways has been studied using the sugarcane cultivar C86-56, which does not release phenolics in agar-base micropropagation systems. In stationary liquid culture, a significant production of phenolic compounds and plant survival were determined in sugarcane plants treated with 5 mM H_2O_2. The spectrophotometer determinations and the gene expression analysis corroborated that releasing of phenolics and soluble θ-quinones was induced during the first 24 h of treatment. In comparison with the control treatments, sugarcane plants treated with H_2O_2 demonstrated differences in the micropropagation-related variables when multiplied in Temporary Immersion Bioreactors (TIBs) supplemented with polyethyleneglycol (PEG 20%). Expression of selected genes related to photosynthesis, ethylene, auxins, oxidative burst, and defense pathways were confirmed during the entire PEG 20% stress in the plants coming from the 5 mM H_2O_2 treatment; whereas, much more heterogeneous expression patterns were evidenced in plants stressed with PEG but not previously treated with H_2O_2. RT-PCR expression analysis supports the hypothesis that while H_2O_2 induces the oxidative burst, the phenylpropanoids pathways elicit and maintain the defensive response mechanism in micropropagated sugarcane plants.

1. Introduction

Reactive oxygen species (ROS) are highly reactive by-products of aerobic metabolism and can lead to oxidative damage of cells. Plants possess a complex ROS network consisting of antioxidant enzymes, antioxidants, and ROS producing enzymes. This network operates to keep ROS levels under control [1]. Although it is known that high concentrations of ROS can cause irreversible damage and cell death, they can also influence signaling and gene expression controlling several biological pathways [2].

Plants have developed specific and efficient strategies for targeted production of ROS. Reactive oxygen species were found to play a role in programmed cell death (PCD), in development, and in stress response [2–4]. During the course of photosynthesis and respiration, ROS are constantly produced in the cell, and redox homeostasis is tightly controlled by redundant protective mechanisms [5].

Tolerance to oxidative stress is gained through a number of different mechanisms [6]. It can be hypothesized that enhancing the ability of plants to scavenge reactive oxygen will also enhance the ability of plants to tolerate other stresses. Several studies have shown a direct correlation between enhanced tolerance to oxidative stress and enhanced tolerance to different abiotic stresses [4,7,8]. Plant defense is based on a complex response to external inputs. The redox state of the cells and its alteration through the oxidative burst is an important regulatory element of this defense response. H_2O_2 signaling pathways are a special part of signal transduction influencing the entire signal transduction framework [9].

Recently, a review by Ramakrishna and Ravishankar [10] stated that accumulation of plant secondary metabolites often occurs in plants under stresses including various elicitors or signal molecules, altogether playing a crucial role in the adaptation of plants to the environment and in overcoming stress conditions. The authors summarized the influence of abiotic factors on secondary metabolites in plants, as well as the effective role of plant cell culture technologies for both studying and producing plant secondary metabolites under *in vitro* conditions.

As a defense response, plants also synthesize an array of natural products based on the phenylpropane skeleton. These molecules have multiple functions such as lignification and other cell wall modifications [11,12] cell wall expansion, and homeostasis control during plant development [13], protection from UV irradiation [14].

* Corresponding author at: Faculty of Agronomy and Forestry Sciences, Catholic University of Maule, Ave. San Miguel 3605, Talca 617, Chile.
E-mail address: arieldarencibia@gmail.com (A.D. Arencibia).

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These molecules are also antioxidants [15,16] and allelochemicals [17].

In sugarcane plants micropropagated in Temporary Immersion Bioreactors (TIBs), phenolic metabolites were found to induce expression of genes related to cell metabolism/development, plant defenses, phenylpropanoids, methyl jasmonate response, ethylene, oxidative burst, and auxins pathways [18]. As a consequence of this observation, a practical application of plant phenolic metabolites as elicitors of resistance to tomato bacterial wilt in the *Solanum lycopersicum–Ralstonia solanacearum* pathosystem was identified. Phenolic metabolites sprayed onto tomato plants infected with *R. solanacearum*, elicited and/or maintained an early defense signaling mechanism that resulted in the protection of the plant against the tomato bacterial wilt disease [19].

In this paper, the relation between the oxidative burst and phenylpropanoid pathways has been investigated in a plant micropropagation system. The sugarcane cultivar C86-56 was selected because it does not turn brown in standard tissue culture conditions. Experiments were conducted in Temporary Immersion Bioreactors (TIBs) with an enhancement of the plant–air contact in a CO_2 enrichment atmosphere [20]. Results showed that sugarcanes propagating in medium supplemented with H_2O_2 (5 mM) secreted phenolic metabolites; furthermore, when plants propagating in TIBs were under an osmotic stress (PEG), the induction of a defensive response was verified with a significant influence on the micropropagation-related traits.

2. Materials and methods

2.1. Plant materials

In vitro sugarcane plants (*Saccharum* spp., cv. C86-56) were established by sterile meristem culture and micropropagated following a standard protocol [21]. Shoots regenerated by organogenesis were used to originate a stock. Cultures were maintained at 110 $\mu M\, m^{-2}\, s^{-1}$ luminosity, 12 h photoperiod, at 27 °C. Pathogen-free plants of 15 days old (after subcultures) were selected for the experiments. For both conventional (H_2O_2 treatments) and TIBs (PEG treatments) five plant clusters (3–4 plants/cluster) were inoculated by single bottle (treatment). Experimental treatments were replicated three times on three different dates.

2.2. Oxidative burst induction

Five selected sugarcane plants were transferred to sterile glass bottles containing 50 mL of multiplication liquid medium composed of MS salts [22], 100 mg/L myo-inositol, 4 mg/L 6-BAP (benzylaminopurine), 30 g/L sucrose, pH 5.6. For the experimental treatments, cultures were supplemented with H_2O_2 (5, 10, 20 and 50 mM). Control treatments were bottles without H_2O_2. The induction treatments were also replicated three times and, experiments were repeated in three different dates. The cultures were maintained at 27 °C under a combination of both natural light and cool-white fluorescent tubes (light intensity of 110 $\mu M\, m^{-2}\, s^{-1}$). Hydrogen peroxide was determined in the culture medium each 8 h during five days. Absorbance was measured at 560 nm according to Elnemma [23] and following standard procedures (National Diagnostic, CL-204, USA) in a microplate reader spectrophotometer (Rayto, RT-2100C).

2.3. Phenolic compound determination

The concentration of phenolic compounds released in to the sugarcane multiplication medium was measured according to Campos-Vargas et al., [24]. Briefly, 1 mL of culture medium was mixed with 1 mL methanol (HPLC grade). Absorbance was read at 320 nm (potential browning) and 437 nm (soluble θ-quinones) in a microplate reader spectrophotometer (Rayto, RT-2100C). Determinations were conducted during the 25 days of the micropropagation cycle. The time zero (T_0) point was considered just after plantlets were transferred to the culture medium supplemented with H_2O_2.

2.4. Osmotic stress in Temporary Immersion Bioreactors

Sugarcane plants propagated in medium containing H_2O_2 5 mM were transferred to TIBs (glass bottles of 1 L capacity). TIBs contained 250 mL of culture medium composed of MS salts [22], 100 mg/L myo-inositol, 4 mg/L 6-BAP (benzylaminopurine), 0.1 mg/L PBZ (Pacrobutazole), 20 g/L sucrose, pH 5.6. The immersion frequency was 3 min every 3 h. Incoming air was supplemented with 0.4 MPa CO_2 and cultures were maintained at 27 °C under a combination of both natural light and cool-white fluorescent tubes at a light intensity of 110 $\mu M\, m^{-2}\, s^{-1}$. Experimental treatments were TIBs supplemented with Polyethylene-glycol (PEG 20%). TIBs plus 20% PEG containing sugarcane plants previously propagated in medium without H_2O_2 were the control treatments. Shoots number, plantlets diameter and number of brown leaves were evaluated after 30 days of osmotic stress.

2.5. RT-PCR

RNA was extracted from *in vitro* sugarcane leaves (−80 °C) pooled from five separate replicates. Extractions were performed using TRIzol reagents (Invitrogen, Carlsbad, CA). One gram of the pooled, frozen leaf tissue was ground to a fine powder in the presence of liquid nitrogen, and then mixed with 10 mL TRIzol. The RNA sample concentrations were quantified by determining the 260:280 and 260:230 nm ratios by spectrophotometry and further confirmed on a 1.2% agarose/0.4 M formaldehyde gel. Twenty nanograms of high quality RNA were added as template to each reaction using the Enhanced Avian HS RTPCR kit (Sigma, St. Louis, MO). For specific RT-PCR amplification, genes involved in different plant defense pathways were selected and the oligonucleotide primers were designed using sequences from the GenBank public databases (www.ncbi.nlm.nih.gov). Information on primer source, nucleotide sequence, and PCR annealing temperatures are listed in Table 1. The PCR reaction mixture consisted of 3 μL of first strand cDNA, 5 μL of 10× PCR buffer, 5 μL of 25 mM MgCl2, 1 μL of dNTPs (10 mM each), 1 μL each of 10 μM primers (forward and reverse), 1 U of Taq DNA polymerase (Invitrogen), and ultrapure water (Sigma) to 50 μL. PCR reactions were conducted with the following parameters: 94 °C for 3 min, 30 cycles of 94 °C for 30 s, annealing according to specific gene (Table 1) for 30 s, 72 °C for 1 min followed by a final incubation at 72 °C for 10 min. RT-PCR products were electrophoretically separated on a 1% agarose gel and stained with ethidium bromide.

3. Results

3.1. Oxidative burst and phenylpropanoids production

Sugarcane *in vitro* propagated plants (cv. C86-56) were transferred to multiplication liquid medium supplemented with 5, 10, 20, 50 mM H_2O_2. Experiments were conducted under stationary (standard) conditions, and the number of replicates was indicated in Section 2. After only 24 h of culture, the browning color typical for the presence of phenolic metabolites was evidenced in both 5 mM and 10 mM H_2O_2 treatments; instead, in the control ($-H_2O_2$), 20 mM H_2O_2 and 50 mM H_2O_2 treatments, the culture medium remained transparent (data not shown). After 20 days, both color browning of the culture medium and plants survival were clearly