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The ellagitannin HeT induces electrolyte leakage, calcium influx and the accumulation of nitric oxide and hydrogen peroxide in strawberry

Gustavo Gabriel Martos[a], Alicia Mamani[a], María Paula Filippone[b], Atilio Pedro Castagnaro[b] and Juan Carlos Díaz Ricci[a*]

[a] Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT, and Instituto de Química Biológica "Dr. Bernabé Bloj", Facultad de Bioquímica, Química y Farmacia, UNT. Chacabuco 461, T4000ILI, San Miguel de Tucumán, Argentina.
[b]Sección Biotecnología de la Estación Experimental Agroindustrial Obispo Colombres (EEAOC)-Unidad Asociada al INSIBIO, Av. William Cross 3150, Las Talitas, 4101, Tucumán, Argentina.

* Corresponding author. juan@fbqf.unt.edu.ar

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Abbreviation: HeT, ellagitanin (1-*0*-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose); H2DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; DAF-FM-DA, 4-amino-5-methylamino-20,70-difluorofluorescein diacetate; diSC3-5, 3,30-dipropylthiacarbocyanine iodide; MES, 2-(N-morpholino) ethanesulfonic acid; EC, electric conductivity; NO, Nitric oxide; s, seconds; min, minutes; h, hour; hpt, hours post treatment; cPTIO, 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; AOX, alternative oxidase capacity; NOXs, NADPH oxidases; ROS, reactive oxygen.species.

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Highlights

- HeT induces a rapid electrolyte leakage and hyperpolarization on strawberry cells
- HeT produces the Ca^{2+} influx which inhibits the nitric oxide accumulation
- HeT causes a biphasic accumulation of H_2O_2 and the AOX activation

Authors' contributions

The experimental design, data analysis and paper writing was carried out by GGM and JCDR. MPF and MA obtained HeT. JCDR and APC critically reviewed the paper.

Abstract

HeT (1-*0*-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-*β*-D-glucopyranose) is a penta-esterified ellagitannin obtained from strawberry leaves. Previous studies have shown that foliar application of HeT prior to inoculation with a virulent pathogen increases the resistance toward *Colletotrichum acutatum* in strawberry plants and to *Xanthomonas citri* subsp. *citri* in lemon plants. In this work we report that HeT induces an immediate leak of electrolytes, the hyperpolarization of the cellular membrane, a rapid Ca^{2+} influx to the cytoplasm during the first few seconds, which in turn modulates the accumulation of nitric oxide 5 min after treatment. At longer times, a biphasic accumulation of H_2O_2 with peaks at 2 and 5 hours post treatment could be observed. In addition, HeT elicited the increase of alternative oxidase capacity during the first 12 hours post treatment.

1. Introduction

Plants are constantly exposed to attack by pathogens, and to defend themselves against them they have evolved an effective immune system that can be activated by elicitors. It is known that some elicitors that are capable to cause ion leakage and changes in membrane polarity as part of the activation signaling of the defense response (Baker et al., 1993; Pike et al., 2005). It has also been reported that the hyperpolarization of cell membrane can induce the influx of Ca^{2+} (Ebel and Cosio, 1994; Pei et al., 2000; Qu et al., 2007; Shang et al., 2005). Calcium is a key molecule in the plant immune system acting as a second messenger that through specialized sensors and proteins would transmit and decode the signals (Hetherington and Brownlee, 2004; Sanders et al., 2002). The mode in which Ca^{2+} levels modulates many different specific responses in plant cells is only partially known. It has been well documented that the induction of innate immunity in plant requires the calcium influx for the activation of membrane NADPH oxidases (NOXs) causing an oxidative burst characterized by the accumulation of the superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) (Kadota et al., 2014; Wang et al., 2016). At the same time, plants have a variety of detoxification systems, which include enzymes such as catalase, peroxidase, superoxide dismutase and alternative oxidases that together work to reduce the levels of reactive oxygen species in cells (Mittler 2002). Another important mediator in the activation of the immune response is nitric oxide (NO), which can be generated endogenously by enzymes such as nitric oxide synthase (NOS) or nitrate reductase (NR), or by chemical reactions of plant metabolites containing nitrogen (Cooney et al., 1994; Wendehenne et al., 2001). The calcium influx is also involved in nitric oxide signaling in various physiological processes (Chen and Kao, 2012; Li et al.,

2009; Wany et al., 2017) and defense in plants (Delledonne et al., 1998; Downie, 2014), although the details in the cross talk among the major defense signaling pathways have not yet been completely unveiled. Previous studies have shown that foliar application of an ellagitanin (1-*0*-galloyl-2,3;4,6-bis-hexahydroxydiphenoyl-β-D-glucopyranose) called HeT, obtained from strawberry leaves, prior to inoculation with a virulent pathogen increased the resistance toward *Colletotrichum acutatum* in strawberry plants and to *Xanthomonas citri* subsp. *citri* in lemon plants (Mamani et al., 2012). The induced resistance in strawberry was associated with a transient increase of salicylic acid in phloem and the upregulation of gene responsive to salicylic acid (*Fa-PR1*). In addition, the accumulation of ROS (e.g. $O_2^{\cdot -}$, H_2O_2) and callose deposition was observed (Mamaní et al., 2012). Since HeT may be used as the active agent of a plant diseases biocontrol commercial product (INPI, Pat. N° P-080103098), a more detailed biochemical characterization of HeT induced defense response, and how the signals of calcium, nitric oxide and hydrogen peroxide are involved, are required to understand its mechanism of action.

2. Materials and methods

2.1. Plants and growth conditions

Strawberry plants (*Fragaria ananassa*) of the cv. Pájaro were obtained from the Active Germplasm Bank at National University of Tucumán, Argentina. Plants were *in vitro* propagated according to Vellicce et al. (2003), transferred to sterile substrate (humus: perlome, 2:1) and maintained in growth chambers at 28 °C, 70% relative humidity (RH) with a light cycle of 16 h day^{-1} (white fluorescent, 250 mmol m^{-2} s^{-1}). For leaf disc experiments, 5-mm diameter discs were obtained by punching leaflets of 8-week-old plants. Cell extraction was performed according to Martos et al. (2015) and cells were suspended in W5 modified solution (154 mM NaCl, 5 mM KCl, 125 mM $CaCl_2$, and 0.5 M sucrose, 2 mM, MES, pH 5.6) at 10^6 cells mL^{-1}.

2.2 HeT Extraction and Purification

Leaves of *Fragaria ananassa* were ground by a mortar and pestle in liquid nitrogen. The dried powder of plant tissues was extracted three times with 80% methanol for 24 h with continuous agitation at 25 °C. The homogenate was filtered through a Whatman filter (N° 1) (Springfield Hill, UK), and the filtrate concentrated in a rotary evaporator under reduced pressure (Speed Vac Plus SC110A; SAVANT, NY, U.S.A.). The dry

extract was resuspended in distilled water and purified according to Mamaní et al, (2012).

2.3 Electrolyte leakage determination

The electrolyte leakage was evaluated according to Köhle et al. (1985) modified in our laboratory. Briefly, 20 mL aliquots of 10^5 cells mL^{-1} suspended in distilled sterile water were treated with HeT (10 µM) or water as a control. The conductivity was determined continuously at 25 ℃, and 100 rpm using an Metrohm E 527 conductimeter, constant of the conductimetric cell = 0.77 cm^{-1}. The conductivity was expressed as µSiemens. cm^{-1}.

2.4 Membrane depolarization

The change of cell membrane polarization was evaluated using the fluorescent probe diSC3-5 according to Filippone et al. (2001) modified in our laboratory. Briefly, 1.5 mL of cell suspensions (10^6 cells mL^{-1}) was treated with diSC3-5 (4 µM) and left 10 min of stabilization. After that time HeT (10 µM) was added. Measurements were performed with low agitation (100 rpm). The ionophore nigericin (50 µM) was added 20 min later as a control of membrane depolarization. An untreated cell suspension treated was used as control (mock). Fluorescence was measured continuously using the ISS-APC1 Photon Counting, (Owingen, Germany) spectrofluorometer at λ_{ex}= 622 nm and λ_{em}= 670 nm.

2.5 Calcium Determination

Accumulation of intracellular calcium was measured in leaf discs using the fluorescent probe Fura2-AM (Invitrogen) according to Nakamura et al. (1996) and Kong and Lee (1995) adapted in our laboratory. The 5 mm diameter discs were obtained from recently expanded young strawberry leaves. Discs were placed in test tubes with 1000 µl of W5 buffer and Fura2-AM (15 µM), 5 vacuum pulses were applied, and allowed to stand in the dark for 3 hours. Then discs were rinsed twice with distilled water and placed on a multiwell plate with 100 µl of the modified W5 solution per well. Immediately before each measurement, HeT (10 µM) or Mock as control was added to the discs suspension. Measurements were also performed with disks pretreated with calcium inhibitors: $LaCl_3$ (75 µM), Verapamil (50 µM), EDTA (5 mM), or the calcium ionophore a23187 (100 µM). Discs with inhibitors were incubated for 60 minutes prior to treatment with HeT or Mock. 10 leaf discs were used per treatment. The fluorescence excitation spectra from 350 nm to 420 nm were obtained at λ_{em} = 532 nm using a Photon Counting spectrofluorometer (ISSAPC1, Owingen, Germany). The

calcium level was expressed as the ratio between the relative intensities of fluorescence measured at 362 nm and 402 nm. Spectra were obtained every 30 seconds during 10 minutes. The spectra did not change in the range of the time analyzed. Values showed correspond to the first measurement at 30 seconds.

2.6 Intracellular nitric oxide determination

The intracellular NO accumulation was analyzed by fluorometry according to Planchet and Kaiser (2006), modified in our laboratory. Briefly, leaf discs were loaded with DAF-FM-DA fluorescent probe (10 µM) for 30 minutes at 25 °C in the dark. Five vacuum pulses were applied to let the probe enter into the cells. The discs were washed three times with distilled water, treated during 30 minutes with: cPTIO (15 µM), a23187 (100 µM), EDTA (5 mM), Verapamil (50 µM) or $LaCl_3$ (75 µM), and then treated with HeT (10 µM). The disks were then placed on a multiwell plate with 100 µL of modified W5 solution per well. Then discs were treated with HeT (10 µM) or mock as a control. 10 leaf discs were used per treatment. The fluorescence intensity was measured in a Perkin Elmer LS55 (USA) spectrofluorometer equipped with a 96-well microplate reader at λ_{ex} = 495 nm and λ_{em} = 515 nm. Measurements were taken every 2.5 minutes up to 30 minutes. The values showed correspond to the measurement at 5 minutes. Intracellular NO production was also visualized using an Olympus microscope mod.BH-FRC (U.S.A.) with an IB filter (λex = 490 nm) (He et al., 2013) on guard cells of *Vicia faba*. Epidermal fragments isolated from 8 week old plants were preloaded with 10 mM DAF-FM DA for 30 min before washing in W5 solution three times for 5 min. Then, fragments were treated with HeT (10 µM) or Mock. For each treatment 10 guard cells in five independent epidermal fragments were observed.

2.7 Intracellular H_2O_2 determination

The accumulation of H_2O_2 was evaluated using the fluorescent probe H2DCF-DA (SigmaeAldrich) (Li et al., 2012; Ye et al., 2013) according to Martos et al. (2015). We used a pulse and chase approach to evaluate the H_2O_2 production at different times after HeT treatment. Five cell aliquots of 100 mL (10^6 cells mL^{-1}) were treated with HeT (10 µM) and at different times 1.5 µL of H2DCF-DA (10 mM) was added, mixed and incubated at 25 ℃ in the dark for 15 min before measuring. Measurements were performed every hour until 8 hours post-treatment with HeT. To evaluate the effect of NO-scavenger cPTIO on H_2O_2 accumulation, aliquots of cell suspensions were pretreated with cPTIO (15 µM) for 30 minutes and subsequently treated for two hours with HeT (10 µM) or not treated (mock). Fluorescence was measured at λ_{ex}= 485 nm

and λ_{em}= 525 nm with a Photon Counting spectrofluorometer (ISSAPC1, Owingen, Germany).

2.8 Alternative oxidase capacity

Alternative oxidase capacity (AOX) was evaluated according to Hilal et al. (1998). Breafly, 0.5 g of pieces of three months old of strawberry leaves were immersed in 2 mL of Hoagland nutrient solution (Hoagland and Arnon, 1950). 1 mM KCN was used as a cytochrome pathway inhibitor and 3 mM salicylhydroxamic acid (SHAM) as an inhibitor of the alternative respiratory chain pathway. Two inhibition treatments were performed: i) KCN + SHAM and ii) KCN. 10 µM HeT or Mock was added and oxygen consumption was measured every 2 h during 12 h. The AOX capacity was determined as the difference between the oxygen consumption of the KCN treated tissue and the oxygen consumption of the KCN + SHAM treated tissues. The measurements were made with a Wilson electrograph with Clark's electrode.

2.9 Enzymatic activity

The catalase activity was determined spectrophotometrically at 20 ℃ (Aebi, 1986; Monti et al., 2003). Aliquots of 1 mL H_2O_2 (10 mM) in 0.1 M buffer phosphate (pH 7.4) were treated with: Mock, HeT 1 µM, 10 µM and 20 µM. 5 min after the measurement, 30 $U.mL^{-1}$ of bovine liver catalase enzyme (sigma-EC 1.11.1.6) was added. The H_2O_2 consumption was monitored at 240 nm for 15 min. Peroxidase activity was measured according to Pine et al. (1984). The absorbance change at 460 nm of 1 mL aliquots of: 8 mM O-dianisidine, 1.7 mM H_2O_2, 0.2 $U.mL^{-1}$ of horseradish peroxidase (Sigma-EC 1.11.1.7) was measured at 37 ℃ for 15 min in 0.1 M buffer phosphate (pH 7.4) treated with: Mock, HeT 0.1 µM, 10 µM and 20 µM. All measurements were made on a Beckman DU 7500 spectrophotometer. Five replicates of each test were made.

2.10 Statistical analyses

The statistical analyses of the data were carried out using the INFOSTAT software (professional version 1.1). All data were obtained from at least three independent experiments, and expressed as mean ± standard error, where corresponded. The data were also analyzed by one-way variance analysis test (ANOVA), and the means were evaluated by Tukey's test for $p < 0.05$.

3. Results and Discussion

3.1 Electrolyte leakage and membrane hyperpolarization

Electrolyte leakage and polarity changes in the plasmatic membrane are among the earliest events defense activation in plants (Dixon et al., 1994). The effect of HeT on the leakage of ions in cells extracted from the mesophyll of strawberry leaves was studied (Fig. 1A). The application of HeT on the cell suspension produced the sustained increase of the EC (Electric Conductivity) indicating the leakage of ions to the extracellular medium. This effect is similar to that produced by the harpin elicitor from *Erwinia amylovora* when applied on tobacco (*Nicotiana tabacum*) suspension cells (Baker et al., 1993). The initial increase of EC after the addition of HeT in both the suspension cells and in the solution may be due to the intrinsic electrolytic property of ellagitannins as observed by Buso et al. (2000) and Rodríguez Martins et al. (2015). The mock experiment did not produce a change in conductivity. The effect of HeT on the polarity of cell membranes was also tested (Fig. 1B). The application of HeT on a suspension of cells preloaded with the probe diSC3-5 produced the rapid decrease of the fluorescence due to the fluorophore uptake, concentration and fluorescence quenching at the membrane (Filippone et al., 2001). The latter indicates that HeT produces hyperpolarization of cells. Ion leakage and changes in cell membrane polarity produced by HeT are early effects closely associated with the activation of immune responses in plants (Baker et al., 1993; Elmore and Coaker, 2011; Gelli and Blumwald, 1997).

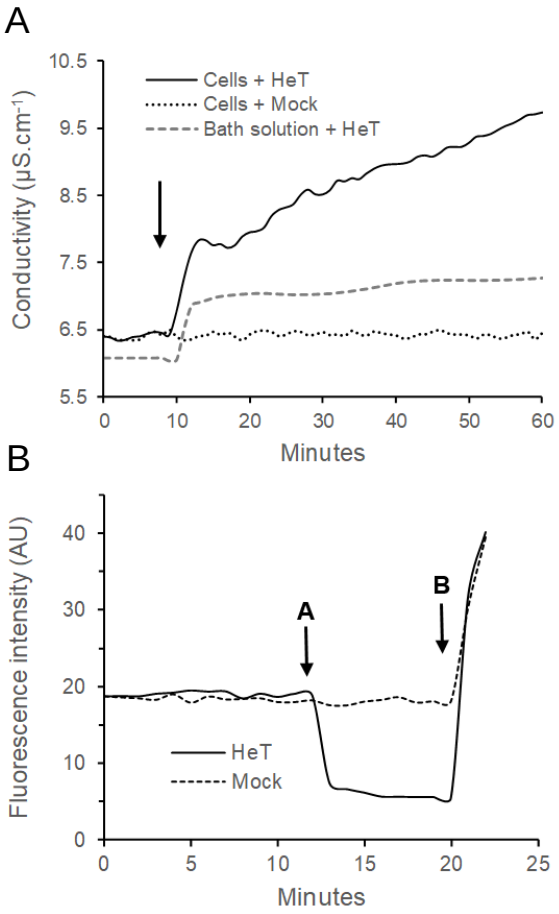

Fig. 1. A) Effect of HeT on electrolyte leakage of mesophyll strawberry cells. Conductivity was measured directly on the cell suspension (10^5 cells mL^{-1}) and the bath solution. The arrow indicates the moment at which HeT (10 µM) was added to cells suspension. Results correspond to one of five identical experiments. **B)** Effect of HeT on the membrane potential of strawberry cells. Fluorescence of the cell suspension (10^6 cells mL^{-1}) previously treated with diSC3-5 (4 µM) was followed in the time. The arrows indicate the moment at which (A) HeT (10 µM), and (B) nigericin (30 µM) was added. The dashed line corresponds to the mock experiment without HeT treatment but nigericin. Fluorescence of diSC3-5 was evaluated at λ_{ex}= 622 nm and λ_{em}= 670 nm, and is expressed in arbitrary units (AU). Results correspond to one of five identical experiments.

3.2 Calcium influx

It is known that hyperpolarization can induce the influx of Ca^{2+} into the cell producing elevation of free cytosolic Ca^{2+} concentration which in turn modulates the induction of several biochemical pathways many of which are associated to the plant defense response (Ebel and Cosio 1994; Pei et al., 2000; Qu et al., 2007; Shang et al., 2005). The ectopic application of HeT on leaf discs of strawberry (Fig. 2) caused a rapid

increase in Ca^{2+} content in the cytoplasm. The spectrum change of the fluorophore Fura2 observed 30 seconds after the application of HeT indicates that the ellagitannin causes a rapid increase of the intracellular concentration of calcium. The effect was similar to that produced by Ca^{2+} ionophore a23187. Leaf discs pretreated with the Ca^{2+} chelator EDTA, and Ca^{2+} channel blockers Verapamil and $LaCl_3$ did not increase the cytosolic Ca^{2+} level after treatment with HeT.

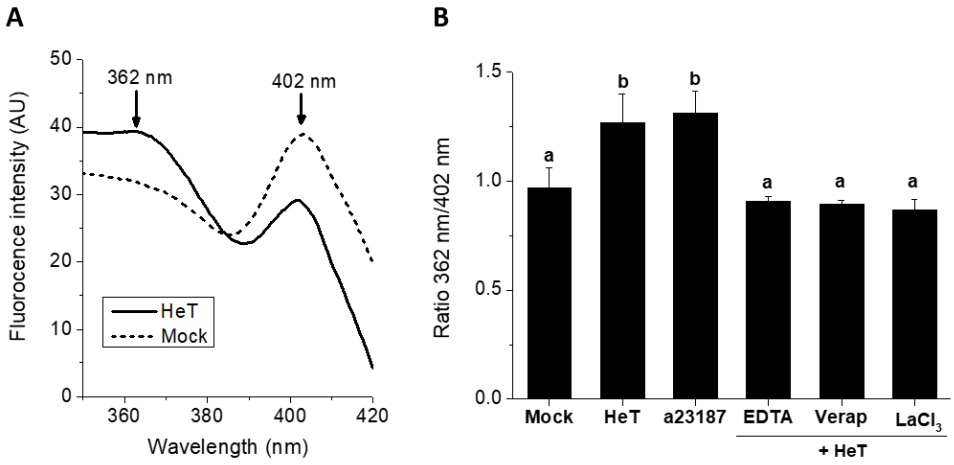

Fig. 2. Calcium influx in the leaf disc of strawberry induced by HeT. **A)** Excitation spectrum change of the Fura2-AM probe at λ_{em} = 532 nm in leaf discs 30 seconds after HeT (10 µM) treatment or Mock. The increase in intensity in the band at 362 nm and the decrease in the intensity of the band at 402 nm are directly related to the intracellular content of free calcium. Fluorescence is expressed in arbitrary units (AU). Spectra correspond to one of five identical experiments. **B)** Intracellular calcium content in leaf discs preincubated with Fura2-AM and subsequently treated with: Mock, HeT (10 µM) and the Ca^{2+} ionophore a23187 (100 µM). Intracellular Ca^{2+} increased in leaf discs pretreated with the calcium inhibitors: EDTA (5 mM), Verapamil (50 µM) or LaCl3 (75 µM) and later treated with HeT (10 µM) are also shown. All measurements were taken 30 second after the treatment with HeT. The calcium concentration is expressed as the ratio of the band intensities 362 nm / 402 nm. Bars represent the standard deviation (mean values ± SE). The comparison between two groups was carried out by Tukey's test ($p < 0.05$). Different letters denote significant difference.

3.3 Accumulation of nitric oxide

It is known that both hyperpolarization signals and Ca^{2+} influx are events that are associated with accumulation of nitric oxide in cells (Garcia-Mata et al., 2003; Garcia-Brugger et al., 2006). Strawberry leaf discs produced a peak of NO accumulation 5 minutes after treating with HeT, and was completely suppressed on discs pretreated with the NO-scavenger cPTIO (Fig. 3). The effect of HeT on the intracellular NO was confirmed by fluorescence microscopy on *Vicia faba* guard cells, where NO

accumulation occurs mainly in chloroplasts (Supplementary Fig. A1). The accumulation of NO was further measured in leaf discs pretreated with the ionophore of Ca^{2+} a23187 and with different Ca^{2+} inhibitors. It was observed that HeT induces a higher NO accumulation in the leaf discs where the Ca^{2+} flux was inhibited. Experiments with calcium inhibitors carried out with in grape cells (Vandelle et al., 2006) showed a strong dependence between calcium influx and NO production triggered by the elicitor endopolygalacturonase1 (BcPG1) from *Botrytis cinerea*. This relationship has also been observed in the process of stomatal closure induced by different elicitors (Desikan et al., 2004; Srivastava et al., 2009). However, Ca^{2+} chelator and Ca^{2+} channel blockers had no effect on NO accumulation induced by indole-3-butyric acid (IBA) in rice seedlings (Chen and Kao, 2012). Previous studies have related the accumulation of nitric oxide induced by elicitors or other stress factors with a Ca^{2+} dependent regulation of the NO synthase activity (Delledonne et al., 1998; Vandelle et al., 2006; Rodríguez-Serrano et al., 2009). However, conclusions about the effect of HeT on NO accumulation should be further evaluated, since NO can also be produced by non-enzymatic pathway which may be regulated by other variables such as the ion concentration as suggested by Zweier et al. (1999).

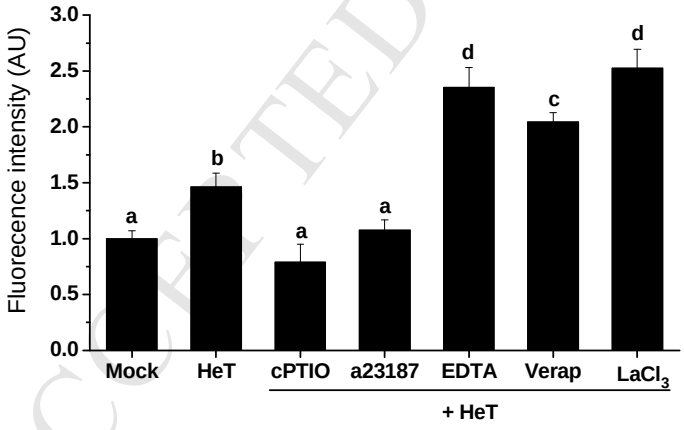

Fig. 3. Effect of HeT on the accumulation of NO in strawberry leaf pre-loaded with the DAF-FM-DA probe and treated with: HeT (10 µM) or Mock as a control. Results of NO accumulation in leaf discs pretreated with cPTIO (15 µM), a23187 (100 µM), EDTA (5mM), Verap (50 µM) and $LaCl_3$ (75 µM) and later treated with HeT (10 µM) is also presented. All measurements were performed 5 minutes after HeT treatment. Bars represent the standard deviation (mean values ± SE). The comparison between two groups was carried out by Tukey's test ($p < 0.05$). Different letters denote significant difference.

3.4. Oxidative stress

Intracellular H_2O_2 accumulation was measured in suspensions of strawberry cells (Fig. 4). It was observed that the treatment with HeT induces a biphasic accumulation of H_2O_2 content, with peaks at 2 and 5 hpt (Fig. 4-A). Biphasic oxidative burst has been described as a fairly common feature in the induction of immune response (Lamb and Dixon, 1997; Baxter et al., 2014; Martos et al., 2015; Wi et al., 2012). The observed H_2O_2 increase is also consistent with the early accumulation of Ca^2 also induced by HeT. Increased intracellular calcium may cause accumulation of reactive oxygen species that would directly modulate the NOXs, as well as to calcium binding proteins (i.e. CDPK, CBL, CaM) that would regulate the activity of NOX (Kadota et al., 2014; Wang et al., 2016). Previous studies have reported that HeT causes the accumulation of reactive oxygen species (ROS) that peaked 4 hpt (Mamaní et al, 2012); however, by using a different and more sensitive experimental approach, a more complex pattern of ROS accumulation was observed. To investigate the relationship between the increase of Ca^{2+} and NO and subsequent accumulation of H_2O_2, we performed experiments with Ca^{2+} inhibitors and NO scavenger in strawberry cell suspensions, but the results were not conclusive (not shown). Perhaps, since the cells were exposed to the inhibitors for an excessive time, artifacts occured that did not allow us to obtain reliable results. It has been demonstrated that the oxidative burst in plants can enhance the activity of the alternative oxidase pathway as part of the mechanism of response to oxidative stress (Dat et al., 2002). The capacity of alternative oxidase was evaluated in strawberry leaves and an increase was observed during the first 12 hpt. Evaluations at later times (24 and 36 hpt) exhibited values close to the control (not shown).

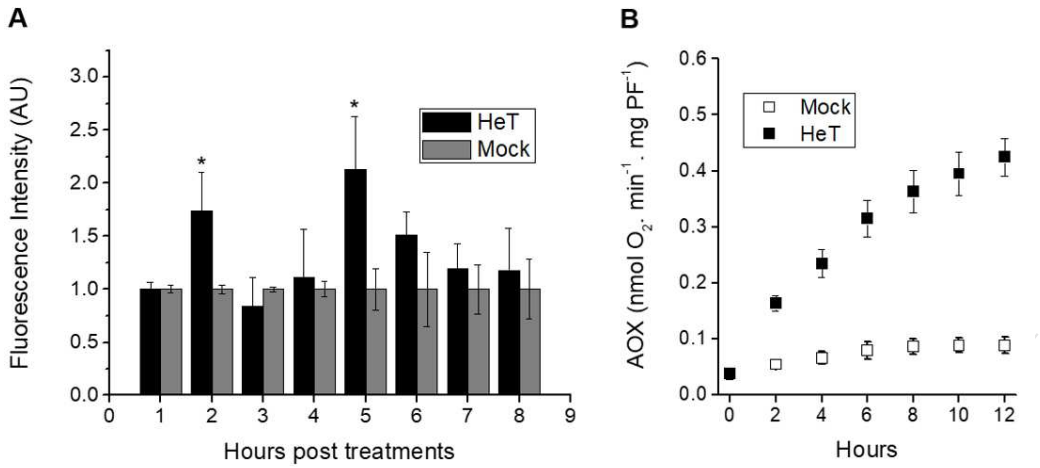

Fig. 4. Intracelullar H_2O_2 acumulation induced by HeT. **A)** Time course post treatments of the intracellular content of H_2O_2 in cell suspensions of strawberry treated with HeT (10 µM) or Mock. Fluorescence of H2DCF-DA was evaluated at λex = 485 nm and λem = 525 nm. **B)** AOX capacity in strawberry leaves. The measurement was performed every two hours after treatment with HeT or Mock as a control. Bars represent standard errors (mean values ± SE), asterisk mean significant difference Tukey's test ($p < 0.05$).

It is known that certain ellagitannins such as geraniin can modify the activity of some key antioxidative enzymes e.g. catalase, peroxidase and superoxide dismutase (Lin et al., 2008, Londhe et al., 2012). Attempts to evaluate catalase and peroxidase activities on strawberry leaves were unsuccessful due to the high contain of chemical compounds that interfere with the measurements. However, with the aim to evaluate the effect of HeT on antioxidant enzymes *in vitro* experiments were carried out using the orthologous enzymes horseradish peroxidase and bovine liver catalase. The results showed that HeT has a dose dependent inhibitory effect on both enzymes (Supplementary Fig. A2). Assuming that HeT affects the strawberry catalase and peroxidase in the same way as observed with these orthologous enzymes, we may speculate that the biphasic accumulation of H_2O_2 observed in Fig 4-A can be partially due to a balance between the inhibitory effect of antioxidant enzymes and the increase of AOX produced by HeT (Fig. 4-B).

4. Conclusion

HeT produces the immediate leakage of electrolytes and hyperpolarization of the plasmatic membrane. These signals are preceded by a rapid influx of calcium (30 s) and subsequent accumulation of NO (5 min). Experiments with Ca^{2+} chelator (EDTA) and inhibitors of Ca^{+2} channels (Verapamil and $LaCl_3$) showed that the early accumulation of NO is regulated by the increase of intracellular Ca^{2+}. It was also

observed that HeT produces the biphasic accumulation of H_2O_2 with peaks at 2 and 5 hpt. Further experiments are being conducted to elucidate the relationship between the early events of Ca^{+2} influx and NO accumulation with the ROS metabolism.

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