H_2O_2 is involved in the dormancy-breaking effect of hydrogen cyanamide in grapevine buds

Francisco J. Pérez · Ricardo Vergara · Sebastián Rubio

Abstract Hydrogen cyanamide (HC) is widely used to induce the breakage of endodormancy (ED) in grape and other deciduous fruit crop, though its mechanism of action is poorly understood. Applications of HC to grapevine buds produce oxidative stress and transient respiratory disturbances which are related to the breakage of ED. Moreover, since the expression and activity of catalase (Cat) is inhibited by HC, enhancements in the levels of H_2O_2 have also been associated to the breakage of ED in grapevine buds. Here, we reported that increases in H_2O_2 level in HC-treated grapevine buds are due to the inhibition of Cat activity and enhancement of the respiratory activity of buds. In addition, exogenous applications of H_2O_2 partially reproduced the inducing effect of HC in the breakage of ED, thus providing further support for the hypothesis that H_2O_2 mediates the effects of HC. On the other hand, Mit isolated from both control and HC-treated buds respired equally well when NADH was used as a respiratory substrate, but when succinate was used as an electron donor Mit respiration was non-detected, suggesting that the stimulatory effect of HC on bud respiration is related to metabolic alterations leading to increase of the concentration of NADH rather than to changes in Mit functionality.

F. J. Pérez (✉) · R. Vergara · S. Rubio
Lab. de Bioquímica Vegetal, Facultad de Ciencias, Universidad de Chile, Casilla 653, Santiago, Chile
e-mail: frperez@uchile.cl

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Introduction

Although hydrogen cyanamide (HC) is widely used to induce the breakage of endodormancy (ED) in grape and in other deciduous fruit crops (Shulman et al. 1983; Or et al. 1999), its mechanism of action is poorly understood (Or et al. 2000, 2002; Pérez and Burgos 2004). Transient respiratory disturbances and oxidative stress produced by applications of HC to grapevine buds have been considered as factors that promotes the breakage of ED. Furthermore, because HC upregulates transcripts encoding key enzymes of the fermentative respiratory pathway, alcohol dehydrogenase (ADH) pyruvate decarboxylase (PDC) and grape dormancy-breaking related protein kinase (GDBRPK), a transcript for sucrose nonfermenting SNF-like protein kinase (Or et al. 2000, 2002), it has been hypothesised that respiratory disturbances triggered by HC are crucial for the breakage of ED in grapevine. Both ADH and PDC transcripts are usually found at low levels in plant tissue and are induced only after the development of respiratory disturbance (Perata and Alpi 1993), whereas SNF-like protein kinase are known as sensors of stress signal (Hardie et al. 1994; Sanz 2003). Since HC inhibits the activity of Cat, the main detoxifying enzyme of H_2O_2 in grapevine buds (Nir et al. 1986;

Pérez and Lira 2005), H_2O_2 has been suggested as the possible stress signal sensed by GDBRPK and associated to the breakage of ED. In plants, H_2O_2 plays a dual role as a toxic byproduct of normal cell metabolism and as a regulatory molecule in stress perception and signal transduction (Neil et al. 2002; Foyer and Noctor 2005). Hence in plants tight regulation of the steady-state levels of H_2O_2 is necessary to avoid cellular injury and to maintain a base level of H_2O_2 on which different environmental and developmental signals can be registered.

To obtain further insight into the hypothesis that H_2O_2 can function as a signal molecule and mediate the effect of HC in inducing the breakage of ED in grapevine, the relationship between Cat activity H_2O_2 levels and respiratory activity were studied in HC-treated and control grapevine buds.

Material and methods

Plant material

Grapevine buds (*Vitis vinifera* L) cv. Thompson Seedless were collected from 8-year-old vines grown at the experimental station from the Chilean National Institute of Agriculture Research (INIA) located in Santiago (33°34′S). The 3.0 × 3.5 m planted vines were drip irrigated and trained in an overhead training system. Canes were collected during the winter recess and cut off at both ends, leaving the central section, 10–12 buds, that were used in further experiments.

Chemical treatments

Single-node cuttings collected on either June 11 or 18 were painted with 2.5% (w/v) hydrogen cyanamide (HC), (Dormex, SKW, Trotsberg, Germany), 2% (w/v) sodium azide (NaN_3) (Sigma, USA), and 2% (w/v) 3-amino-1,2,4-triazole (Sigma, USA) solutions. Cuttings (10× treatments) were mounted on a polypropylene sheet and floated in tap water in a plastic container. The plastic tray was transferred to the growth chamber set at 23 ± 2°C under 14 h light 150 µmol m^{-2} s^{-1} forcing conditions (FC); samples were taken off at the desired times. Experiments inducing the breakage of ED were carried out with buds collected on July 18 and treated with 2.5% (w/v) HC, 1% hydrogen peroxide (H_2O_2) (v/v), and water as control. After treatments, cuttings (15× treatments) were mounted as describe above, settled in the growth chamber under FC and the breakage of buds was assessed every 2 days.

H_2O_2 measurements

H_2O_2 concentration was measured by chemiluminescence (CL) in a cobalt-catalysed oxidation of luminol (5-amino-2, 3-dihydro-1, 4-phtalazinedione) (Pérez and Rubio 2006). Buds (0.1 g fresh weight) were ground to fine powder in liquid N_2, the powder was homogenised with a tenfold volume of 5% trichloroacetic acid (TCA). The homogenate was centrifuged in a bench centrifuge at 13,000 *g* for 5 min. Extracts were diluted with carbonate buffer (1:500) and an aliquot of 20 µl of the diluted extract was incubated for 15 min at 30°C with 5 µl (50 U) Cat (bovine liver, Sigma, USA) or with 5 µl of distilled water. After the incubation luminescence was measured in the Cat-treated and nontreated samples by adding 2 µl of the sample to 1 ml of the diluted mixed reagent solution (Pérez and Rubio 2006). The emitted photons were counted over 5 s with a HY-LITE® 2 luminometer (Merck, Germany). The difference between Cat treated and nontreated sample was considered the H_2O_2-specific CL.

Catalase assays

Cat activity was assayed in extracts of grapevine buds by monitoring the disappearance of H_2O_2 at A_{240} ($\varepsilon = 36$ M^{-1} cm^{-1}) as described by Dorey et al. (1998). Buds (0.1 g fresh weight) were ground in liquid N_2 and the resulting powder was extracted with buffer containing (0.5 M Tris–HCl, 1 mM $MgCl_2$, 10 µM PMSF, 5 mM EDTA, 2% Triton X-100, 2% insoluble PVP and 12.5% glycerol pH 7.5). The homogenate was centrifuged in a bench centrifuge at 13,000 *g* for 5 min, and 5 µl of the supernatant (approximately 10 µg protein) was assayed for Cat activity in buffer phosphate (0.1 M pH = 7) in the presence of 200 µl of 0.1 M H_2O_2.

Bud respiration

The respiration of buds was followed through the release of CO_2 with an infrared gas analyser (IRGA

EGM-4 PP Systems, USA). Single buds were placed in a glass tube purged previously with N_2 to reduce CO_2 contamination; the tube was sealed with a rubber stopper and put on a water bath (25 ± 2°C) for 10 min. Afterwards the tube was punctured with a 1 ml syringe and the collected gas injected in the IRGA. The same operation was repeated after 15 min of incubation and the respiratory rate was calculated as the difference between the two measurements. Values of control and treated samples correspond to the average of four replicates.

Mitochondria purification and respiratory measurements

Approximately 5 g of bud tissue (20-30 buds) was homogenised using mortar and pestle in 10 ml of extraction medium containing 10 mM phosphate buffer (pH 7.5), 0.3 M mannitol, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% bovine serum albumin (BSA), 0.5% cysteine and 0.5% insoluble PVP. The homogenate was filtered through three sheets of gauze and centrifuged for 10 min at 500 g, the pellet was discarded and the supernatant centrifuged again at 12,000 *g* for 10 min. Crude Mit fractions (CMF) were washed twice in washing buffer (10 mM phosphate buffer pH 7.2, 0.3 M mannitol, 0.1% BSA) before being purified by Percoll gradient. A gradient of Percoll (60% v/v) self-generated in a refrigerated centrifuge (2,500 *g* × 1 h) was used to purify the CM. The ring formed in the interface corresponding to the purified Mit fraction (PMF) was rescued and washed three times with washing buffer before protein determinations. Oxygen consumption was monitored polarographically with a Clark-type oxygen electrode (Hansatech, UK) at 30°C in 1 ml reaction mixture. The reaction mixture contained 0.1 mg protein × ml^{-1} of PMF; 1 mM NADH as respiratory substrate and 0.1 mM ADP in respiratory buffer (10 mM phosphate buffer pH 7.2; 0.3 M mannitol, 10 mM KCL, 5 mM $MgCl_2$).

Extraction and purification of RNA

Total RNA was isolated from grapevine buds (0.5–0.7 g fresh weight, 15 buds approx.) using a modification of the method described by Chang et al. (1993). Samples ground in liquid N_2 were homogenised in CTAB buffer (CTAB 2%, Tris–HCL 25 mM, EDTA 2 mM, NaCL 2 M and PVP 2%) and 2% β-mercaptoethanol added to the homogenate immediately after buffer addition. The homogenate was shaken vigorously, heated at 65°C for 30 min and filtered through glass wool. The filtrate was centrifuged for 10 min at 400 *g* and extracted twice with 1 volume of chloroform:isoamylic alcohol (24:1). The aqueous phase was precipitated overnight with 2.5 volumes of ethanol. RNA was recovered by centrifugation at 3,000 *g* for 20 min and washed with 70% (v/v) ethanol. After solvent elimination, RNA was dried at ambient temperature and dissolved in diethylpyrocarbonate (DEPC) water. The RNA solution was incubated with DNAse (70 U/ml) at 36°C for 30 min to eliminate DNA. Binding to silica (1 volume of 6 M NaI and 0.5 volume of silica) further purified RNA. After repeated shaking the supernatant was eliminated by centrifugation, the silica was washed twice with 500 µl washing buffer (Tris–HCL 10 mM; NaCL 50 mM, EDTA 2.5 mM and EtOH 50% v/v, pH 7.5), eliminating the supernatant by centrifugation each time. The pellet was dried at ambient temperature, resuspended in DEPC water and the aqueous solution containing the RNA was separated by centrifugation.

Primers designed and RT-PCR

Specific primers for *V. vinifera* GDBRPK, an SNF-like protein kinase, was designed based on the NCBI database (accession number: AF178575) using the program primer 3 (Skaletsky, 2000). *Fw:* 5′GTTGGG GCGTATCCCTTT3′ *Rv*: 5′TGCCACTGACCATGA ACG3′.

RT-PCR reactions were carried out using the SuperScript™ One-Step RT-PCR System kit with Platinum® Taq DNA Polymerase (Invitrogen, USA) in a Thermocycler MJ Research (PTC-150). The reaction mixture contained 1 µg of total RNA as template, 0.2 µM of each primer, 1 µl RT Platinum Taq mix (Superscrip II RT and Platinum® Taq DNA Polymerase) and 25 µl of 2× Mix, a buffer containing (0.4 mM of each dNTP and 2.4 mM $MgSO_4$. Conditions for c-DNA synthesis were 30 min at 50°C and the reaction was stopped by 2 min at 94°C. Conditions for the 35 PCR cycles were 30 s at 94°C for denaturation, 30 s at 55°C for annealing and 45 s

at 72°C for extension. Final extension was performed at 72°C for 10 min. Primers designed against *V. vinifera* actin gene were used as internal controls, PCR products were separated in 1.5% agarose gel run on TAE buffer (0.038 M Tris, 1 mM EDTA, 1.1% (v/v) glacial acetic acid) at 50 V and visualised by EtBr staining.

Results

Effect of HC and NaN_3 on the level of H_2O_2 in endodormant grapevine buds

The effects of hydrogen cyanamide (HC) and of sodium azide (NaN_3) on the level of H_2O_2 in endodormant grapevine buds are shown in Fig. 1. Grapevine buds were treated chemically with HC and NaN_3, and placed thereafter in the growth chamber under forcing conditions to assess the levels of H_2O_2, 2, 8 and 14 days after treatment. After 2 days of treatment the level of H_2O_2 rose significantly in HC-treated buds, while in NaN_3-treated buds, H_2O_2 dropped to nearly half of the level of the control samples. After 8 days of treatment the level of H_2O_2 decreased in HC-treated and control buds, but still it was higher in HC-treated than in control buds, whereas in NaN_3-treated buds it remained unchanged. Fourteen days post-treatment, control, HC- and NaN_3-treated buds levelled off their concentrations in H_2O_2.

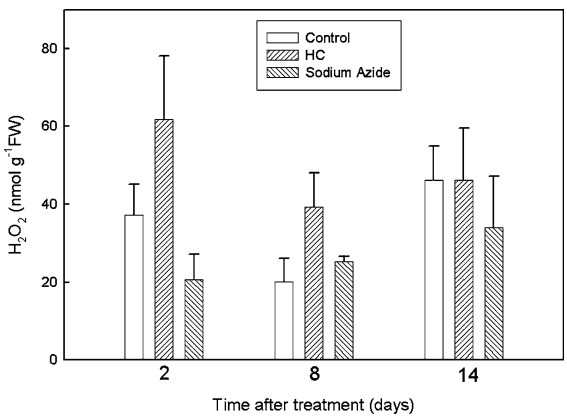

Fig. 1 Changes in the level of H_2O_2 in endodormant grapevine buds after hydrogen cyanamide (HC) and sodium azide (NaN_3) applications. After the chemical treatment grapevine buds were settled in the growth chamber under forcing conditions and H_2O_2 was assessed 2, 8 and 14 days after treatment. Values represent the average of three replicates and bars correspond to standard deviations

Interplay between Cat activity and H_2O_2 levels in HC- and AMT-treated grapevine buds

To verify whether the increase in H_2O_2 levels in HC-treated buds was due exclusively to the inhibition of Cat activity, grapevine buds were treated in parallel with HC and aminotriazole (AMT), a specific Cat inhibitor, and simultaneously H_2O_2 and Cat activity were assessed 6, 24 and 48 h after treatment (Fig. 2a, b). Shortly after treatment the activity of Cat was slightly reduced in HC- and AMT-treated buds, while the level of H_2O_2 increased significantly, increasing more in HC- than in AMT-treated buds. After 1 day of treatment the activity of Cat was reduced significantly by both chemicals, but the difference in H_2O_2 levels between control and treated buds was less pronounced than earlier. After 2 days of treatment the activity of Cat still remained inhibited, but H_2O_2 levels were enhanced only in AMT-treated buds.

HC stimulates bud respiration without modifying mitochondria respiratory capacity

An increase in the release of CO_2 was detected in grapevine buds treated with HC and, after 24 h of treatment, the respiratory activity of HC-treated buds was double that of control buds (Table 1). Nevertheless, Mit purified from HC-treated and control buds respired equally well when NADH was used as a respiratory substrate. Moreover, tightly coupled Mit with a respiratory control ratio greater than 3 were obtained from either control or HC-treated buds (Fig. 3). Mit also respired in the presence of NADPH, but the O_2 uptake and the coupling were lower than with NADH (Fig. 3). Succinate-dependent oxygen consumption was barely detected in Mit purified from both control and HC-treated buds (results not shown).

GDBRPK transcript was induced in grapevine buds by HC but not by NaN_3 treatments

The expression of GDBRPK, an SNF-like protein-kinase transcript, was analysed by RT-PCR in RNA extracted from HC- and NaN_3-treated grapevine buds. The expression of the GDBRPK transcript

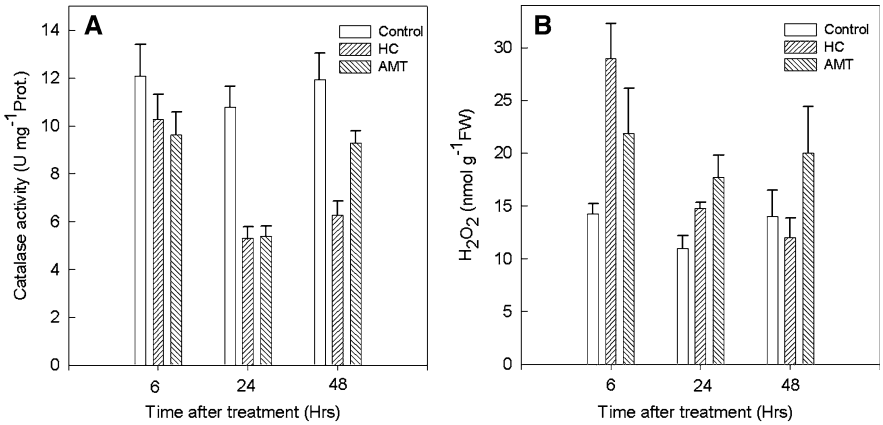

Fig. 2 Decreases in Cat activity (**a**) and increases in H_2O_2 levels (**b**) in endodormant grapevine buds after hydrogen cyanamide (HC) and aminotriazole (AMT) applications. Grapevine buds after chemical treatment were settled in the greenhouse under forcing conditions and the activity of Cat and the levels of H_2O_2 were assessed simultaneously 6, 24 and 48 h post-treatment. Values represent the average of three replicates and bars correspond to standard deviations

Table 1 Effect of hydrogen cyanamide (HC) on CO_2 evolution in grapevine bud cv. Thompson Seedless

Time (h)	Control (nmol CO_2/min/gFW)	HC
6	17.5 ± 0.5	20.6 ± 3.7
12	35.3 ± 1.8	35.8 ± 1.5
24	34.7 ± 2.5	64.4 ± 4.3

Values are average of 4 independent measurements with the corresponding SD

was detected in RNA extracted from HC-treated buds, while in RNA extracted from control and NaN_3-treated buds it was undetectable (Fig. 4). Moreover, in HC-treated buds, GDBRPK expressed 8 days post-treatment and the expression remained until the onset of bud break (14 days after treatment).

Exogenous application of H_2O_2 mimics partially bud-breaking effect of HC

The bud-break response of grapevines measured as percentage of bud-break or as the time required to reach 50% of bud-break under forcing conditions (BR_{50}) was improved by HC and H_2O_2 applications to the buds (Fig. 5). Furthermore, the applications of HC gave the best results, reducing the BR_{50} from 21 days (control buds) to 16 days (HC-treated buds), whereas H_2O_2 applications reduced the BR_{50} of control buds by only 2 days. Advances in the bud-break response

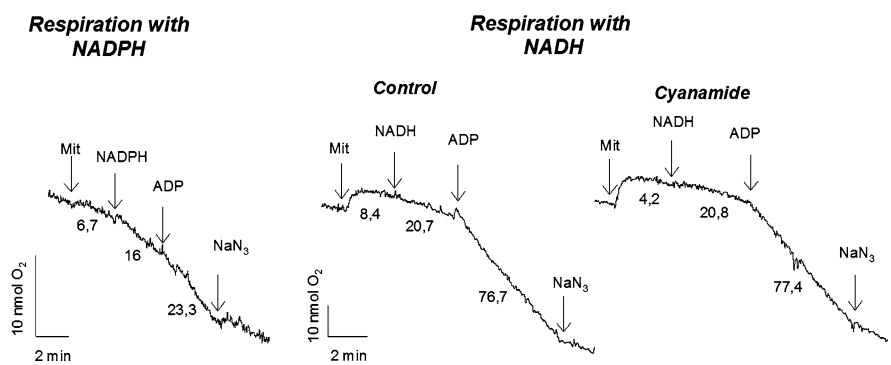

Fig. 3 Rates of oxygen uptake in Mit isolated from grapevine buds. The respiratory activity and the coupling of Mit were higher when NADH was used instead of NADPH as respiratory substrate. Similar rates of oxygen uptake and coupling were found in Mit isolated from both control and HC-treated buds when NADH was used as respiratory substrate. All experiments were carried out with Percoll purified Mit and oxygen uptake is expressed as nmol O_2 min^{-1} mg^{-1} Prot

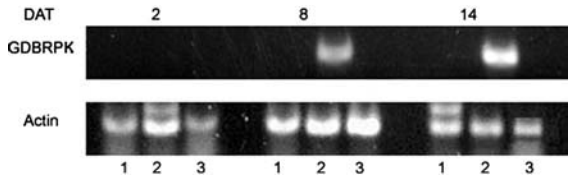

Fig. 4 Effect of hydrogen cyanamide (HC) and sodium azide (NaN_3) on the expression of GDBRPK transcript in grapevine buds. The expression of GDBRPK was analysed by RT-PCR in total RNA extracted from control and chemically treated buds 2, 8 and 14 days after treatment. Lane 1 corresponds to control, lane 2 corresponds to HC-treated buds, and lane 3 corresponds to NaN_3-treated buds. The *V. vinifera* actin gene was used as internal control in all PCR reactions

indicates that buds release earlier from ED, hence the efficiency of H_2O_2 as a dormancy-breaking agent was approximately half that of HC in grapevines.

Discussion

In addition to the well-known oxidative damages caused by increases in H_2O_2 levels in plant tissues, there are compelling evidences that H_2O_2 can function as a signalling molecule in plants (Foyer et al. 1997; Neil et al. 2002; Foyer and Noctor 2005). In grapevine buds, it has been suggested that the breakage of ED triggered by HC could be mediated by H_2O_2 (Or et al. 2000, 2002). Here, we report that the increase in H_2O_2 levels in HC-treated grapevine

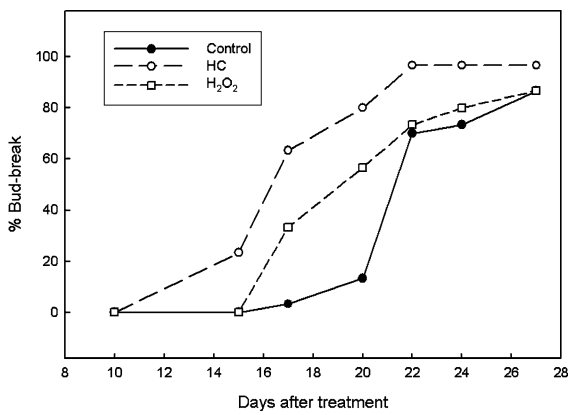

Fig. 5 Effect of hydrogen cyanamide (HC) and hydrogen peroxide (H_2O_2) on the bud-break response in grapevines. HC was applied at the commercial dose of 2.5% (w/v) and H_2O_2 was applied at 1% (w/v) to endodormant grapevine buds collected on July 18. After the treatments, buds were settled in the growth chamber under forcing conditions and breaking of buds was assessed every 2 days

buds was due to an inhibition in the activity of Cat and enhancement in the respiratory activity of buds. The drop in the levels of H_2O_2 in grapevine buds treated with the respiratory inhibitor NaN_3 indicated a dependence of H_2O_2 concentration on the respiratory activity of buds. Furthermore, since the activity of Cat was reduced at the same level by AMT and HC, while the subsequent enhancements in the levels of H_2O_2 was higher in HC- than in AMT-treated buds, we suggest that HC, in addition to inhibiting the activity of Cat, stimulates the respiratory activity of buds. Direct measurements of CO_2 release in grapevine buds confirmed the feature that HC stimulates the respiratory activity of buds, agreeing with previous results (Shulman et al. 1983). However, since Mit purified from control and HC-treated buds respired equally well with NADH, we conclude that the stimulatory effect of HC on bud respiration is related to metabolic alterations rather than to changes in Mit functionality. Mit also respired in the presence of NADPH, signalling the presence of NAD(P)H dehydrogenases (Moller 2001; Rasmusson et al. 2004). NADPH dehydrogenases are non-proton-pumping enzymes, hence they do not contribute to ATP synthesis and their activities is not submitted to respiratory control, thus explaining the low coupling observed when NADPH was used as respiratory substrate. Moreover, because succinate-dependent oxygen consumption was barely detected in Mit purified from control and HC-treated buds (results not shown), the stimulatory effect of HC on bud respiration could be due to alterations in the metabolism, leading to enhancement of the concentration of NADH, which is the main respiratory substrate in Mit isolated from grapevine buds.

Since AMP is known as a stress signal sensed by SNF-like protein kinase, it has been suggested that HC through a transient respiratory disturbance leading to an increase in the ratio AMP/ATP could upregulate the expression of GDBRPK transcript (Or et al. 2000). Our results confirmed that HC upregulates the expression of GDBRPK, but did not support the hypothesis that the mechanism is through a transient respiratory disturbance, because NaN_3, which inhibits the Mit respiratory electron transport chain (ETC), did not induce the expression of GDBRPK. Nevertheless, our results suggest that an increase in the levels of H_2O_2 could induce the expression of GDBRPK transcript.

Exogenous applications of H_2O_2 to endodormant grapevine buds partially reproduced the effect of HC in inducing the breakage of ED, confirming that increases in the level of H_2O_2 are at least partially related with the breakage of ED. The effect of H_2O_2 within the tissue will depend on its steady-state concentration, and therefore, on the rate at which it is synthesised and degraded. When H_2O_2 was applied exogenously to grapevine buds, presumably it was degraded rapidly by the action of Cat. However, under conditions in which Cat activity is inhibited (HC-treated buds) the excess of H_2O_2 can be detoxified alternatively by the ascorbate-glutathione cycle (AGC) (Foyer and Halliwell 1976). During the scavenging of H_2O_2 by AGC, the pentose phosphate pathway (PPP) is activated, thus increasing the concentration of NADPH (Foyer et al. 1997; Salvemini et al. 1999). Therefore, H_2O_2 detoxification by the AGC and the subsequent activation of the PPP could be crucial steps in the breakage of ED triggered by HC, which could not be fully reproduced by the exogenous applications of H_2O_2. However, other metabolic disturbance not signalled by H_2O_2 can not be discarded from the mechanism by which HC exerts its dormancy-breaking effect.

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