

RESEARCH PAPER

Inhibition of catalase activity as an early response of *Arabidopsis thaliana* cultured cells to the phytotoxin fusicoccin

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Abstract

In *Arabidopsis thaliana* cells, fusicoccin (FC) treatment induced an early and marked increase in the extracellular H₂O₂ level. It also increased the huge hypo-osmotic stress-induced oxidative wave and, in addition, prevented the H₂O₂ peak drop. These effects were apparently not linked to changes in either cytoplasmic pH or cytoplasmic free calcium concentration, since they occurred independently of the activity state of the plasma membrane (PM) H⁺-ATPase and neither influx nor efflux of ⁴⁵Ca²⁺ was modified by FC. In the presence of diphenylene iodonium (DPI), inhibiting the PM NADPH oxidase presumably responsible for reactive oxygen species (ROS) production, no apoplastic H₂O₂ development was detected either with or without FC. However, no increase in DPI-sensitive ferricyanide reduction, but rather a gradual decrease, occurred with FC. These results suggested that the H₂O₂ increase observed with FC was not due to a overproduction of ROS but, more probably, to a reduced capability of FC-treated cells to degrade the H₂O₂ formed. This view, at first supported by the finding that FC-treated cells failed to break down exogenously supplied H₂O₂, was clearly confirmed by a series of measurements on exogenous catalase activity, tested in cell-free media of FC-treated samples. This assay, in fact, allowed ascertainment and partial characterization of an as yet unidentified factor increasingly accumulating in the incubation medium of FC-treated cells, behaving as a non-competitive catalase inhibitor and able to reduce markedly the cell's capability for H₂O₂ scavenging.

Key words: *Arabidopsis thaliana* cells, catalase activity, cytoplasmic pH, fusicoccin, H₂O₂ scavenging, reactive oxygen species, stress.

Introduction

Several lines of evidence obtained both *in vivo* and *in vitro* (Beffagna *et al.*, 1977; Rasi-Caldogno and Pugliarello, 1985; Rasi-Caldogno *et al.*, 1986) proved that fusicoccin (FC), a fungal phytotoxin produced by *Fusicoccum amygdali*, is a powerful activator of the P-type H⁺-ATPase, the major ion pump in the plasma membrane (PM) of all plant tissues. Most of the plant cell secondary transporters depend on the H⁺-ATPase activity, which is essential for generating the electrochemical gradient across the PM utilized by the plant for a number of physiological functions (reviewed by Palmgren, 2001). Stimulation of cell enlargement, stomatal opening, and breaking of seed dormancy are some of the effects induced by FC, and much effort has been made during the last 30 years to elucidate the mechanism of its action. It is now widely accepted that FC activates the H⁺-ATPase by binding to a regulatory protein belonging to the 14-3-3 family, whose association with a specific binding sequence located at the end of the C-terminal autoinhibitory domain of the PM H⁺-ATPase releases the autoinhibitory action. FC binding would promote and stabilize this association, thus leading to the formation of a complex in which the enzyme is stable in its activated form (Baunsgaard *et al.*, 1998; Olivari *et al.*, 1998; Fuglsang *et al.*, 2003, and references therein).

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Abbreviations: BTP, BIS-TRIS propane; [Ca²⁺]_{cyt}, cytoplasmic free calcium concentration; DMO, 5,5-dimethyl oxazolidine-2,4-dione; DPI, diphenylene iodonium; EY, eosin yellow; FC, fusicoccin; IBA, isobutyric acid; pH_{cyt}, cytoplasmic pH; PM, plasma membrane; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance.

FC has been largely used as a tool to understand both the regulation mechanism of the PM H⁺-ATPase and the role of H⁺-ATPase in modulating various physiological processes (for a review, see Marrè, 1979; Marrè *et al.*, 1992). Moreover, FC was utilized to obtain information on the signal transduction pathway during the hypersensitive response (HR), typical of an incompatible plant-pathogen interaction. A transient production of reactive oxygen species (ROS) accompanied by changes in ion fluxes and transmembrane electric potential difference (E_m) are among the earliest signalling events in plant-pathogen interactions (Blumwald *et al.*, 1998). Pathogen elicitors most commonly cause extracellular alkalinization associated with an E_m decrease, and the large FC-induced increases in E_m and H⁺ extrusion were exploited to counteract the electrochemical gradient changes occurring in many pathogen-elicited plants (Messiaen and van Cutsem, 1994; Simon-Plas *et al.*, 1997; Schaller and Oecking, 1999; Schaller and Frasson, 2001).

More recently, the toxin itself was proved to act as a stress factor, independently of its main role in activating the PM H⁺-ATPase. In tomato plants, for instance, FC was reported to induce an increase in salicylic acid (SA) synthesis and the expression of some pathogenesis-related (PR) genes involved in the defence responses (Schaller *et al.*, 2000). In *Acer pseudoplatanus* cells, prolonged treatments with FC were found to induce several responses which are typical for environmental stress: ethylene production, alternative oxidase activation, cyanide-sensitive H₂O₂ overproduction accompanied by leakage of cytochrome *c* from the mitochondria, and also a massive extracellular H₂O₂ accumulation (Malerba *et al.*, 2003, and references therein). Various systems generating active oxygen intermediates, such as PM NADPH oxidases, apoplastic amine oxidases, and cell wall-bound peroxidases (producing O₂⁻, H₂O₂, or both) have been characterized and recognized as the major sources for extracellular ROS production triggered by stress conditions (Bolwell, 1999; Mittler, 2002). Among these, a main role has been attributed to the PM NADPH oxidase on the basis of several lines of evidence obtained in different plant materials exposed to a variety of biotic and abiotic stresses (Mathieu *et al.*, 1991; Auh and Murphy, 1995; Desikan *et al.*, 1996; Lamb and Dixon, 1997; Pugin *et al.*, 1997). In particular, Cazalè *et al.* (1998) proved that glucosamine treatment suppressed the hypo-osmotic- or mechanical stress-induced oxidative wave by inhibiting the pentose phosphate pathway (PPP), which essentially supplies the demand for NADPH. Most of these studies were substantially based on the capacity of diphenylene iodonium (DPI, an inhibitor of the mammalian NADPH oxidase also effective for plant NADPH oxidases; Levine *et al.*, 1994) to inhibit this enzymatic system specifically. More recently, however, DPI was proved also to inhibit peroxidases and amine oxidases (Frahry and Schopfer,

1998; Cona *et al.*, 2006). With regard to the DPI-sensitive extracellular H₂O₂ accumulation induced by FC, Malerba *et al.* (2003) reasonably interpreted this effect as substantially due to an increased PM NADPH oxidase activity, since in a medium strongly acidified by FC no significant contribution to ROS production should come from amine oxidases and peroxidases (active at neutral to alkaline pH).

ROS production mediated by the PM NADPH oxidase is known to be regulated by several factors. A key role was ascribed, for instance, to the early evolution of a large and transient Ca²⁺ wave (Atkinson *et al.*, 1990; Pugin *et al.*, 1997; Lecourieux *et al.*, 2002; Orozco-Cárdenas *et al.*, 2001; Sanders *et al.*, 2002) and also to Cl⁻ efflux, postulated as a step in hypo-osmotic signal transduction leading to oxidase activation (Cazalè *et al.*, 1998). A decrease in cytoplasmic pH (pH_{cyt}), controlled by protein phosphorylation, was recognized as a common response to elicitors and hypothesized to play a role in the early events of signal transduction, including ROS production (Mathieu *et al.*, 1996; Pugin *et al.*, 1997). In addition, a close correlation between pH_{cyt} changes and NADPH oxidase-mediated ROS production was recently proved in *Arabidopsis thaliana* cells, and a role for the PM H⁺-ATPase in modulating the development of the oxidative wave through the pH_{cyt} changes was proposed (Beffagna *et al.*, 2005). Therefore, an FC-induced increase in NADPH oxidase-mediated ROS production such as that described by Malerba *et al.* (2003) did not actually seem easy to explain. In fact, the strong pH_{cyt} increase induced by FC through the PM H⁺-ATPase activation (Marrè *et al.*, 1987; Beffagna and Romani, 1994), shown to reduce NADPH availability through inhibition of the PPP (Marrè *et al.*, 1992, and references therein), was expected to produce a drop in ROS production (Pugin *et al.*, 1997; Beffagna *et al.*, 2005).

In order to understand why the correlation between pH_{cyt} changes and ROS production levels apparently failed for FC treatments, the early effects of FC on a number of parameters known to be affected by stress situations (Ca²⁺ and H⁺ fluxes across the PM, pH_{cyt} and extracellular ROS generation) were investigated in *A. thaliana* cells. In particular, various experimental approaches were combined to analyse the FC-induced changes in apoplastic H₂O₂ content at both the production and consumption levels.

Materials and methods

Plant material and general conditions

Cell suspension cultures of *A. thaliana* L. (ecotype Landsberg 310-14-2) were grown in 250 ml Erlenmeyer flasks containing 90 ml of Murashige and Skoog salt solution (Sigma catalogue no., M5524), supplemented with a vitamin mixture (Sigma catalogue no. M3900, modified by increasing thiamine content to 30 µM) and with 2 µM biotin, 0.9 µM 2,4-D, 1.1 µM folic acid, 0.23 µM kinetin, 88 mM

sucrose, and 5 mM MES, adjusted to pH 5.5 with BIS-TRIS propane (BTP). Cultures were grown in a rotatory shaker at 150 rpm under constant white light ($7.2 \mu\text{E m}^{-2} \text{s}^{-1}$) at 24 °C and transferred into fresh medium every 7 d (1 ml of packed cells into 90 ml of fresh medium). Seven-day-old subcultures ($\sim 50\text{--}60 \text{ mg FW ml}^{-1}$) were used for all the experiments.

Sample preparation

Cell suspensions ($\sim 10 \text{ g FW}$) were filtered through a 25 μm nylon cloth with suction, transferred into 100 ml of a solution containing 0.2 mM CaSO_4 and 0.5 mM MES/Li (pH 6), and rinsed four times under gentle shaking, renewing the medium every 10 min. Each wash was followed by cell filtration. After the washes, filtered cells were weighed and the samples (100 mg FW) were put in 'Transwell' cell culture chambers with permeable supports (24 mm diameter, 8 μm pore size, from Costar, Cambridge, MA, USA). Before supplying the different treatments, cells were always pre-equilibrated under shaking for 30 min in 3 ml of a basal medium containing 0.2 mM CaSO_4 and 2 mM MES/Li (pH 6). This pre-equilibration period was omitted when samples for the evaluation of cytoplasmic pH were prepared, given the long time required for loading and equilibrating the labelled pH probe before supplying the different treatments (see below). A weight/volume ratio of 100 mg FW/3 ml was also maintained for all the following treatments. Experiments were carried out in a thermoregulated water bath at 24 °C under shaking (100 cycles min^{-1}).

Experimental conditions

The following treatment solutions, known to affect the activity of the PM H^+ -ATPase, and hence the pH_{cyt} values, differently, were chosen: (i) plain basal medium, a condition in which the absence of permeating cations allows only a very low activity of the H^+ -ATPase and therefore the pH_{cyt} value is substantially unvaried during the whole incubation time; (ii) basal medium supplemented with K^+ , a condition of partial stimulation of the H^+ -ATPase activity, typically leading to a gradual pH_{cyt} increase which is further amplified in the case of direct activation of the enzyme by FC (Marrè *et al.*, 1987; Beffagna and Romani, 1991, 1994); and (iii) K^+ -containing hypo-osmotic medium, a condition characterized by a very strong reduction of K^+ influx that, in turn, causes a drop of the H^+ -ATPase activity and, consequently, a marked pH_{cyt} decrease (Beffagna *et al.*, 2005).

Osmotic treatments

To impose the hypo-osmotic jump, at the end of the pre-equilibration samples were drained by gentle blotting of the Transwell supports on filter paper, resuspended in basal medium supplemented with 1 mM K_2SO_4 and 200 mM betaine, and incubated for 60 min. At the end of the pre-treatment with the osmoticum, samples were moved to an equal betaine-free medium containing different effectors, as specified in the individual experiments, and incubated for up to 60 min.

H_2O_2 assay

Apoplasmic H_2O_2 content was measured by the colorimetric method of Jiang *et al.* (1990), based on the peroxide-mediated oxidation of Fe^{2+} (catalysed by sorbitol) under acidic conditions, followed by the reaction of Fe^{3+} with the dye, xylenol orange. The assay was performed on 800 μl aliquots of the incubation medium collected at the desired treatment times. Each aliquot was added to 800 μl of the reaction mixture containing 500 μM ammonium ferrous sulphate, 50 mM sulphuric acid, 200 μM xylenol orange, and 200 mM sorbitol. After 45 min at room temperature, absorbance data were gathered on a Jasco 7800UV/VIS spectrophotometer (A_{560}). The

calibration curve was made with increasing concentrations of H_2O_2 in the range between 0 μM and 2.5 μM by dilution of H_2O_2 in treatment solution. A calibration curve for each treatment solution was made.

Measurements of H^+ net fluxes and of cytoplasmic pH

Net H^+ extrusion was measured by back-titration of the medium, after removal of CO_2 according to Lado *et al.* (1981). Cytoplasmic pH values were calculated by the distribution at equilibrium of the weak acid 5,5-dimethyl oxazolidine-2,4-dione (DMO), a technique previously proved to be suitable for this kind of measurements for both *Elodea densa* leaves, and *A. thaliana* seedlings or cells (Beffagna and Romani, 1989, 1994; Beffagna *et al.*, 2005). In *A. thaliana* cells, DMO equilibration between the intra- and extracellular compartments was completely reached within 90 min. Thus, to evaluate the effects of the different treatments on pH_{cyt} , samples were first pre-incubated for 90 min in a medium containing 0.2 mM CaSO_4 , 10 mM MES (adjusted to pH 6 with BTP), and 5 μM [$2\text{-}^{14}\text{C}$]DMO (4.63 kBq per sample), and then treated for a further 30 min in the same medium supplemented with small aliquots of the different effectors, fed at the final concentrations routinely used. An additional series of samples was collected at the end of the 90 min pre-treatment and taken as time zero (t_0). All samples collected either at t_0 or at the desired treatment times were drained on filter paper and washed for 3 min at 0 °C under agitation with the corresponding unlabelled solutions. The radioactivity incorporated was measured by liquid scintillation counting and determined by a Packard TriCarb counter after addition of 10 ml of scintillation liquid (Hionic Fluor, Packard). The calculations were carried out by the following equation derived from that of Henderson-Hasselbalch:

$$\text{pH}_{\text{cyt}} = \text{pK} + \log \left\{ \frac{1}{V_{\text{cyt}}} [C_{\text{tot}} - V_{\text{vac}} \times (\text{AH} + 10^{\text{pH}_{\text{vac}} - \text{pK} + \log \text{AH}}) - \text{AH}] \right\} - \log \text{AH}$$

For a detailed description of the equation terms, see Marrè *et al.* (1987), Beffagna and Romani (1989), and Beffagna *et al.* (2005).

Ferricyanide reduction

After pre-equilibration in basal medium, samples were transferred into the same fresh medium supplemented with 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$ and incubated for up to 30 min with or without FC and DPI, fed at the final concentrations routinely used. At the desired treatment times, samples were withdrawn and ferricyanide reduction was measured spectrophotometrically by the decrease in A_{420} in the medium.

Catalase activity measurement

The activity of commercially available catalase purified from bovine liver (Sigma-C3155) was determined by following at 560 nm the consumption of exogenous H_2O_2 [measured according to Jiang *et al.* (1990), as described above], supplied at room temperature at different concentrations in 800 μl of K^+ -containing basal medium (taken as a control) and of the cell-free incubation media of samples treated for different times with or without FC, as specified in the individual experiments. The reaction was started by H_2O_2 addition, after a 5 min incubation period of the media with 0.5 U of catalase (taken as t_0) and stopped after 2 min by addition of 800 μl of the xylenol orange reaction mixture. Catalase activity was calculated by subtracting the amount of H_2O_2 remaining in the various media from that initially supplied, measured in a parallel series of samples. For measurements carried out in the cell-free incubation media, to measure accurately the amount of H_2O_2 initially supplied (essential to calculate the activity of catalase properly), it was necessary to

remove the H_2O_2 produced by the cells during the incubation before adding the exogenous H_2O_2 . Therefore, these media were first incubated for 5 min with catalase, then the enzymatic activity was stopped by addition of the reaction mixture, and finally H_2O_2 was supplied. Before carrying out the enzymatic assay, all the tested media were adjusted to pH 6 or 7 according to the experimental requirements.

(K^{*}) medium preparation

After the usual four washes in 0.2 mM $CaSO_4$ and 0.5 mM MES/Li (pH 6), batches of 3 g of cells were weighed, placed into 250 ml Erlenmeyer flasks containing 90 ml of basal solution, pre-equilibrated for 30 min, and then transferred for a further 30 min into the same volume of fresh basal medium supplemented with 1 mM K_2SO_4 and 10 μ M FC. At the end of this pre-treatment, cells were moved to a new medium of the same composition but free of FC. After 60 min of treatment, cells were finally withdrawn and the incubation medium collected to give the (K^{*}) medium, namely a K⁺-containing basal medium integrated with a blend of compounds released by the cells during the treatment.

Chemicals

FC was dissolved in methanol and added drop by drop with continuous stirring to an appropriate volume of hot (70 °C) distilled water [final methanol concentration (v/v)=0.6%] to give a 1 mM stock solution, stored in the dark at 4 °C. FC was always fed at a concentration of 10 μ M, proved to give an increase in net H⁺ efflux, representative of PM H⁺-ATPase activity, equivalent to ~85% of the maximum stimulation (not shown). Eosin Y (EY) was dissolved in distilled water to yield a 10 mM stock solution, and stored in the dark at 4 °C. EY was supplied at a final concentration of 0.2 μ M (proved to be optimal to inhibit selectively the PM Ca²⁺-ATPase in *A. thaliana* cells; not shown) starting from the last 10 min of pre-equilibration in basal solution. DPI was first dissolved in dimethylsulphoxide (DMSO) at 50 mM and then diluted with distilled water to yield a 2.5 mM stock solution that was stored at room temperature. DPI was supplied at the final 25 μ M concentration, starting from the last 15 min preceding the different treatments.

Results and discussion

Changes of apoplastic H_2O_2 content, pH_{cyt} and H⁺ fluxes induced by FC

The effect of FC on H_2O_2 accumulation was tested in a series of different experimental conditions, known to differently affect the activity of the PM H⁺-ATPase, and hence the pH_{cyt} values, whose changes strongly influence the extent of ROS production in *A. thaliana* cells (Beffagna *et al.*, 2005). Data reported in Fig. 1A show that treatment of cells in plain basal conditions (C) resulted in a moderate rise in apoplastic H_2O_2 content, gradually increasing with time. The same pattern was observed for the K⁺-treated controls where, however, the amount of the accumulated H_2O_2 was about half that detected in the absence of K⁺. This finding was in agreement with a reduced ROS production, as expected for a drop in PM NADPH oxidase activity due to the K⁺-induced pH_{cyt} increase (Beffagna *et al.*, 2005 and Fig. 1B).

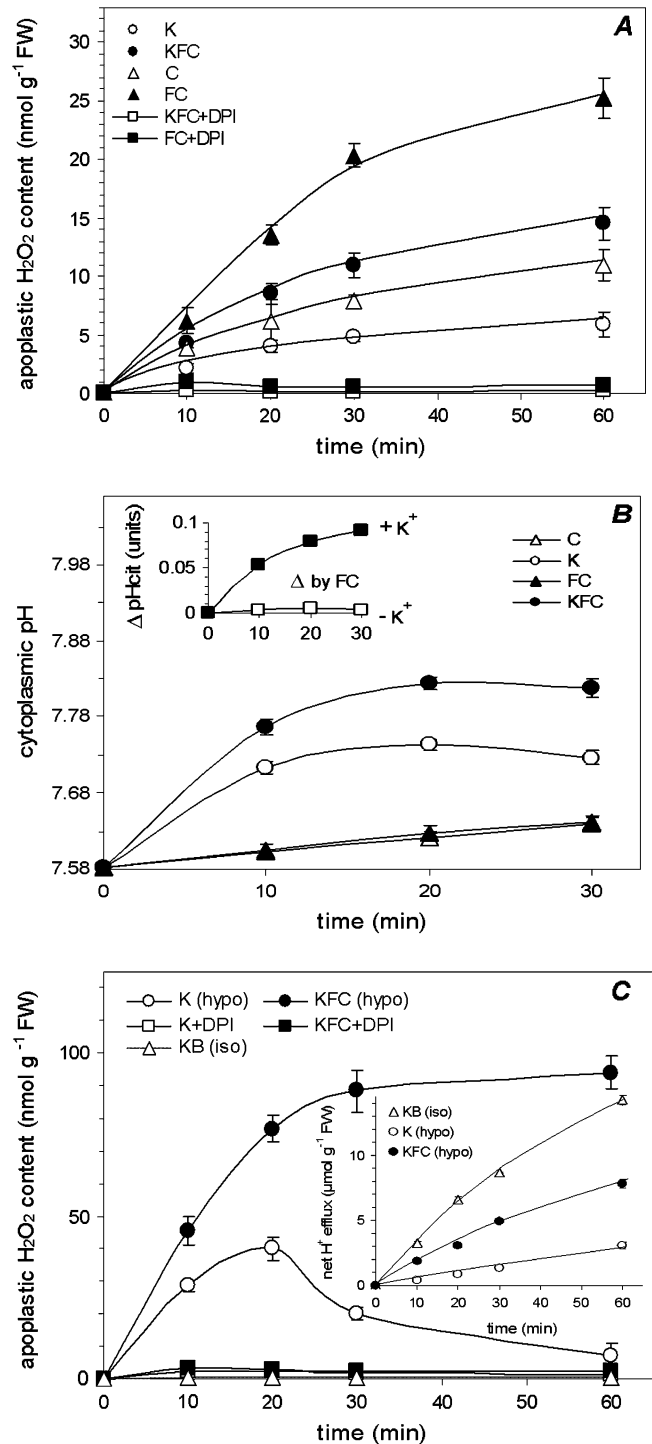


Fig. 1. Time course of FC effects on apoplastic H_2O_2 content, pH_{cyt} , and H⁺ fluxes. (A) Apoplastic H_2O_2 content measured in basal medium (C), supplemented or not with K⁺ and/or FC. The pH_{cyt} changes occurring in the same experimental conditions are reported in B. The insert shows the pH_{cyt} differences induced by FC, calculated from the data reported in the figure. (C) Effects of FC on the H_2O_2 accumulation induced by hypo-osmotic stress. The time course of the concurrent net H⁺ efflux, measured on the external media collected at the indicated times, is reported in the insert. Values are means of at least three experiments run in triplicate \pm SE.

The addition of FC to samples incubated in plain basal medium was ineffective on the pH_{cyt} time course (Fig. 1B), but led to a marked increase in the extracellular H_2O_2 content, which from the first 10 min of treatment was more than double that found in the controls. Surprisingly, also when FC was added to a K^+ -containing medium, the extracellular H_2O_2 level was >2-fold higher than that measured in the media of samples treated with K^+ alone, in spite of the strong further pH_{cyt} rise occurring in this condition (see the insert of Fig. 1B), which should lower ROS production. Also, the huge increase in extracellular H_2O_2 content induced by hypo-osmotic treatment (Cazalè *et al.*, 1998; Beffagna *et al.*, 2005) was further enhanced by FC (Fig. 1C). In addition, with FC, the H_2O_2 level did not drop after the imposition of the osmotic jump, but remained high throughout the whole course of the experiment. Here again, the FC-induced rise in H_2O_2 accumulation occurred in a condition in which ROS development should be reduced by the pH_{cyt} increase following the PM H^+ -ATPase activation (proved by the marked rise in net H^+ efflux shown in the insert). On the other hand, the strong inhibiting effect of DPI (used as an NADPH oxidase inhibitor) seemed to rule out the possible involvement of some other DPI-insensitive systems in ROS production (Fig. 1A, C).

Time course of FC effects on Ca^{2+} fluxes and ferricyanide reduction

In order to investigate the discrepancies between pH_{cyt} changes and ROS production levels resulting from FC treatment, different experimental approaches were employed. First of all, the possibility was considered that FC might modify Ca^{2+} movements across the PM, thus altering the usual pattern of ROS production. In fact, a potential FC-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase might provoke a greater ROS production, in spite of the unfavourable pH_{cyt} changes. However, the results of a series of experiments in which FC effects on the time course of the unidirectional $^{45}\text{Ca}^{2+}$ fluxes were measured proved that both influx and efflux were substantially unchanged (data not shown), thus ruling out this hypothesis.

Changes in ferricyanide reduction were then investigated to verify whether a larger efflux of reducing agents might actually be the starting point for the observed FC-induced increase in apoplastic H_2O_2 content. Measurements were performed both with and without DPI, in order possibly to discriminate between whole efflux of reducing agents and NADPH oxidase-mediated transfer of electrons across the PM. Data of Fig. 2 show that, in all conditions tested, FC gradually increased ferricyanide reduction, in agreement with previous observations with other plant materials (Lass *et al.*, 1986; Marrè *et al.*, 1988), and the increase became greater for longer treatment times. However, the comparison between FC

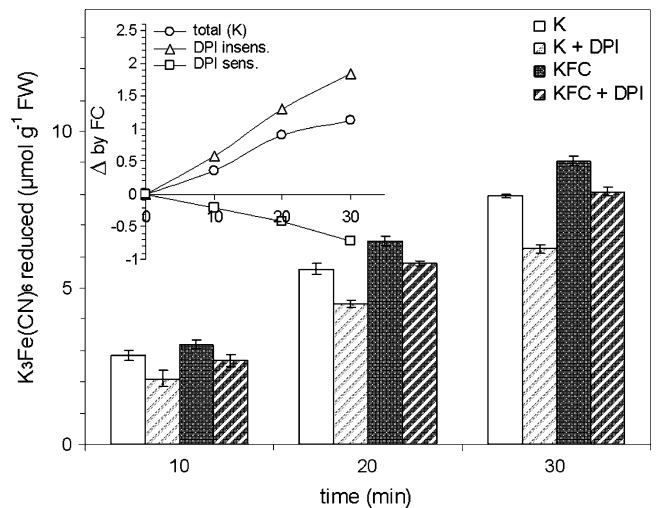


Fig. 2. Time course of FC effects on ferricyanide reduction. After pre-equilibration in basal medium, samples were transferred into the same fresh medium supplemented with 1 mM $\text{K}_3\text{Fe}(\text{CN})_6$, in the presence or absence of FC and DPI. The insert shows the differences induced by FC in total, DPI-insensitive, and DPI-sensitive ferricyanide reduction, calculated from the data reported in the figure. Units on the y-axis are the same as in the main figure. Values are means of at least four experiments run in triplicate \pm SE.

effects in the absence of DPI (total) and in its presence (DPI-insensitive) indicated that the increase in DPI-insensitive reduction was always larger than that measured in the samples without DPI. It follows that the effect of FC on the DPI-sensitive reduction (calculated by the difference between the individual FC effects on the reduction values measured with and without DPI) would be progressively less, as highlighted by the data reported in the insert. This gradual decrease in ferricyanide reduction (taken as indicative of a decreased PM NADPH oxidase activity) occurred in a condition characterized by an increasing pH_{cyt} rise (see insert of Fig. 1B). Therefore, the data indicated that the correlation between pH_{cyt} changes and PM NADPH oxidase-mediated ROS production was also effective for FC treatments, confirming the role of pH_{cyt} in modulating the level of DPI-sensitive ROS production mediated by this PM enzymatic complex. These results, however, did not yet explain the origin of the higher DPI-sensitive increase in apoplastic H_2O_2 content occurring under FC treatment.

Synergism between FC and EY effects on the apoplastic H_2O_2 content

To obtain further information about the mechanism leading to the FC-induced increase in extracellular H_2O_2 content, FC effects were compared with those induced by EY, a PM Ca^{2+} -ATPase inhibitor known to keep ROS production at a sufficiently high level (presumably by hindering the return of $[\text{Ca}^{2+}]_{\text{cyt}}$ to basal levels) to suppress even the transient feature of the oxidative burst

induced by either oligogalacturonides or hypo-osmotic stress (Romani *et al.*, 2004; Beffagna *et al.*, 2005). Data of Fig. 3 indicate that at all considered times the extracellular H_2O_2 content measured when cells were treated with EY was $\sim 40\%$ higher than that found in the presence of FC that, in turn, was about double that of the controls, as previously seen (see Fig. 1A). However, when samples were simultaneously treated with FC and EY, the apoplastic H_2O_2 content was ~ 2 -fold higher than that observed with EY alone. This synergism between FC and EY in affecting H_2O_2 accumulation (indicative of distinct modes of action of these two compounds) is better highlighted in the insert, in which the H_2O_2 accumulation rise separately induced by FC and by EY is reported, and a comparison is made between the sum of these individual effects and the H_2O_2 accumulation induced by the simultaneous presence of the two effectors.

H_2O_2 degradation by FC-treated cells

Taken as a whole, the findings that: (i) the FC-induced changes in extracellular H_2O_2 content were not apparently linked to $[Ca^{2+}]_{\text{cyt}}$ or pH_{cyt} changes; (ii) no increase in DPI-sensitive ferricyanide reduction resulted from FC treatments; and (iii) FC and EY acted synergistically in increasing the H_2O_2 level in the external medium, indicated that events other than those controlling ROS production were responsible for the H_2O_2 increase induced by FC. Moreover, the evident correlation between the amount of ROS generated by the controls and the changes in extracellular H_2O_2 level induced by FC (a

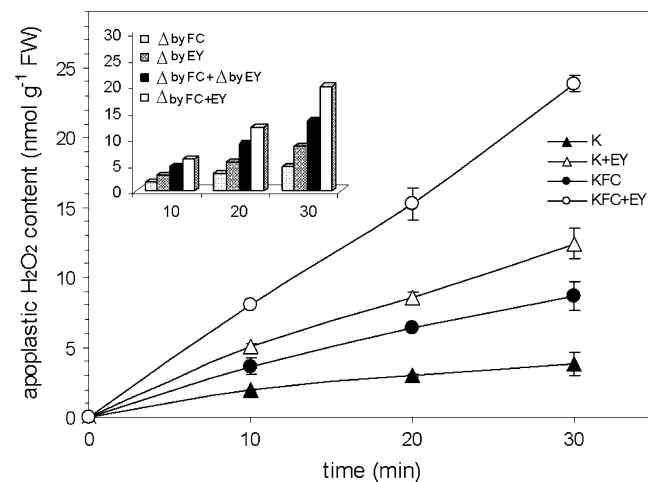


Fig. 3. Synergism between FC and EY effects on apoplastic H_2O_2 content. After pre-equilibration, samples were transferred into a fresh K^+ -containing basal medium supplemented with FC and/or EY and incubated for up to 30 min. The insert shows the increase in apoplastic H_2O_2 content (calculated from the data reported in the figure) induced by FC and by EY, respectively, and the comparison between the sum of these individual effects and the increase in H_2O_2 content induced by the simultaneous administration of the two compounds. Units on the y-axis are the same as in the main figure. Values are means of three experiments run with three replicates \pm SE.

higher basal ROS production being accompanied by a larger FC-induced H_2O_2 increase), and the finding that in hypo-osmotically treated cells FC prevented the drop of the H_2O_2 peak, strongly suggested a reduced capability of the FC-treated cells to decompose the H_2O_2 derived from basal ROS production. Indeed, the possibility that ROS are controlled at both the production and consumption level and that during the HR of plant-pathogen interaction a decrease in the activity of antioxidant enzymes occurs has been extensively discussed by Van Breusegem *et al.* (2001), who proposed that this event could probably contribute to the achievement of the threshold H_2O_2 level required for triggering the plant defence mechanisms against invading pathogens (for an overview of the literature on the signalling role of ROS in plant defence responses, see Vranovà *et al.* 2002).

This suggestion would also explain the apparent incongruity between the results obtained by measuring the effects of FC on the DPI-sensitive ferricyanide reduction (Fig. 2) and the DPI-sensitive H_2O_2 accumulation (Fig. 1A, C). In fact, both parameters should reflect the same ROS-producing machinery which, in the case of FC treatment (strongly acidifying the external medium), should mainly correspond to the PM NADPH oxidase. However, the ferricyanide reduction data clearly indicated an FC-induced decrease in DPI-sensitive activity (as expected for a pH_{cyt} rise), whereas the H_2O_2 measurements showed an unpredicted increase in DPI-sensitive H_2O_2 accumulation. However, if FC would somehow reduce the activity of some cell system involved in H_2O_2 scavenging, its addition to cells treated with DPI (which prevents ROS formation) would be substantially ineffective. Thus, the absence of extracellular H_2O_2 found with FC in the presence of DPI would simply reflect the DPI-induced inhibition of the basal NADPH oxidase-mediated ROS generation and not the suppression of an FC-induced increase in ROS production.

To check this hypothesis, the cell's capacity to decompose exogenously added H_2O_2 was first tested. The experiments were carried out with cells exposed to a hypo-osmotic jump (a condition characterized by a very evident FC effect on H_2O_2 accumulation, in particular for the longer treatment times; Fig. 1C). Figure 4 shows a comparison between the actual amounts of H_2O_2 found in the media supplemented or not with exogenous H_2O_2 (expressed as OD values at 560 nm) and those theoretically calculated by summing the OD contribution of the added H_2O_2 and the OD values measured in the absence of exogenous H_2O_2 . Data show that, in the absence of FC, the measured OD values in the presence of exogenous H_2O_2 were 65-70% lower than those theoretically calculated, consistent with a marked aptitude of cells to decompose the supplied H_2O_2 . Conversely, in the presence of FC, there was only a negligible difference between measured and calculated values, in agreement

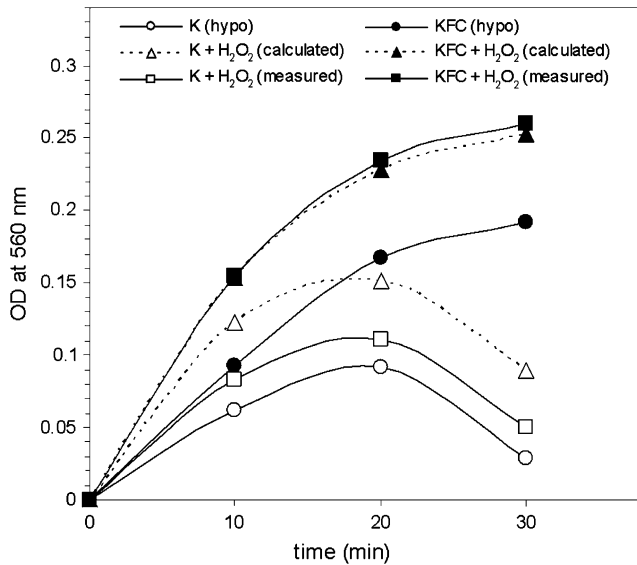


Fig. 4. FC effect on the cell's capability to decompose exogenously supplied H_2O_2 . Data refer to samples exposed to hypo-osmotic stress, imposed by transferring cells from a basal medium supplemented with K^+ plus betaine either to equal betaine-free media, with or without FC (KFC hypo and K hypo), or to equivalent hypo-osmotic media supplemented with $0.4 \mu\text{M}$ H_2O_2 . The induced changes in H_2O_2 concentration in the external medium are expressed as OD changes at 560 nm. The dotted lines indicate the theoretical OD values resulting from the sum of 0.06 (accounting for the OD contribution by the added H_2O_2) and the OD values measured in the incubation media, in the absence of added H_2O_2 . Values are means of three experiments run with three replicates \pm SE.

with the view that FC could trigger the formation of some factor able to inhibit H_2O_2 scavenging.

Catalase activity inhibition by FC-treated cells

Catalase and ascorbate peroxidase (APX) can be considered the major H_2O_2 -scavenging enzymes of plants. Given the high affinity of APX for H_2O_2 (μmol range), a key role for this ubiquitous enzyme has been proposed in the fine modulation of the ROS level during signalling. Conversely, catalase, characterized by a low affinity for H_2O_2 (mM range) but a very high reaction rate, might be responsible for the removal of the excess of ROS during stress. Though catalase is mainly localized in peroxisomes, its involvement in the degradation of H_2O_2 from other compartments is possible, and it was proved to be essential for detoxification when high ROS levels are produced (Mittler, 2002, and references therein). Moreover, a role as a cellular sink for H_2O_2 was proposed for this enzyme on the basis of some evidences proving that catalase-deficient transgenic tobacco leaf discs were strongly impaired in their ability to remove extracellular H_2O_2 (Willekens *et al.*, 1997). Therefore, the finding that the FC-induced increase in extracellular H_2O_2 level was particularly evident for cells exposed to hypo-osmotic stress (a condition leading to a huge extracellular H_2O_2

accumulation) seemed to indicate catalase as the likely target for the putative inhibitor of H_2O_2 scavenging. Actually, the involvement of catalase inhibition in the induction of disease resistance by signalling of H_2O_2 and activation of the defence genes was proposed by Mehdy (1994), and the induction of the expression of pathogenesis-related proteins by FC was proved in tomato leaves (Schaller *et al.*, 2000; Schaller and Frasson, 2001). In addition, transgenic tobacco plants expressing antisense RNA for cytosolic catalase were found to be hyper-responsive to pathogen attack (Mittler *et al.*, 1999), and a fall in catalase activity has been described as an event occurring in many plant species under different stress conditions (Shim *et al.*, 2003).

To verify this hypothesis, some experiments were carried out in which the activity of a commercially available catalase was assayed by measuring its capacity to decompose exogenous H_2O_2 , supplied in a K^+ -containing basal buffer or in equal cell-free solutions collected after different incubation periods of the samples with or without FC (Fig. 5). The rationale for this kind of experiment was that, if the hypothetical inhibitor produced during FC treatment would pass through the PM and accumulate in the external medium at a sufficiently high concentration, this approach would allow detection of its presence in the incubation media and, possibly, its characterization in a relatively easy way. Figure 5A shows the results of catalase activity measurements performed at pH 6 (routinely used for all the above-described experiments) either in a K^+ -containing basal solution (taken as a control) with or without FC, or in the incubation media of samples exposed for 30 min to a hypo-osmotic jump in the presence or absence of FC. When the assay was run in the incubation medium of cells treated with K^+ alone (ex K hypo), the measured activity was equal to that detected in the control solution, signifying that none of the compounds released by cells in these conditions affected catalase activity. Conversely, a marked activity decrease ($\sim 37\%$) was observed when the test was carried out in the medium deriving from FC-treated samples (ex KFC hypo), suggesting the presence of some inhibitor released by the cells during FC treatment. The data also show that the addition of FC to the control medium did not alter catalase activity, thus ruling out the possibility that FC, by itself, might be responsible for the detected activity changes.

To substantiate these data and exclude that they were due to a possible overlap between FC and osmotic stress effects, the analyses were repeated with the incubation media of cells treated for 30 min with FC alone (ex KFC). Measurements were carried out not only at pH 6, as before, but also at the pH optimum for catalase activity (pH 7), in order to improve the assay conditions (Fig. 5B). When the assay was run at pH 6, catalase activity was decreased by $\sim 30\%$ (an inhibition somewhat lower than

that observed in the incubation media of samples treated with both FC and osmotic stress). For measurements carried out at pH 7 (at which catalase activity in basal buffer was markedly higher than that detected at pH 6) the activity measured in the external medium of FC-treated cells was nearly halved. These data confirmed that, independently of the occurrence of other stress conditions, FC treatment could specifically promote the production of some factor inhibiting catalase, the presence of which could be detected even in the incubation media of FC-treated cells.

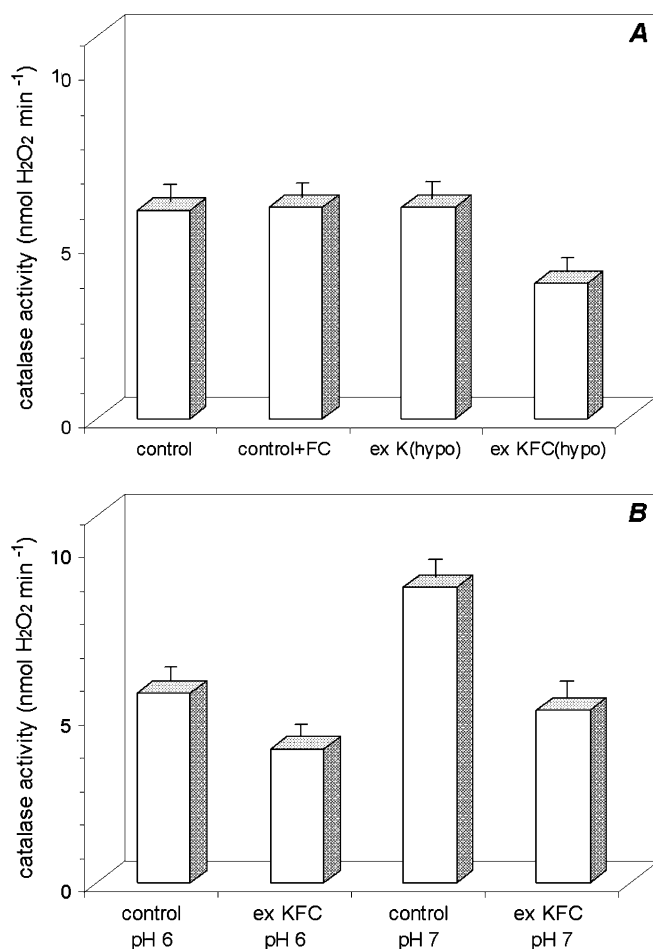


Fig. 5. FC effects on exogenous catalase activity. Catalase activity was tested by following the consumption of H₂O₂ (200 μM) added either to aliquots of K⁺-containing basal medium (taken as a control) supplemented or not with FC, or to aliquots of cell-free incubation media deriving from samples treated in different conditions, as follows. (A) After a 30 min hypo-osmotic treatment, imposed in the absence or presence of FC, cells were withdrawn and the incubation media (indicated as ex K hypo and ex KFC hypo) were collected. Before carrying out the enzymatic assay all media were adjusted to pH 6. (B) After pre-equilibration, all samples were incubated for 30 min in basal medium supplemented with K⁺ and FC. Before running the enzymatic assay, the incubation media collected at the end of treatment (ex KFC), as well as the K⁺-containing basal medium (control), were divided into two parts and adjusted to pH 6 or pH 7, respectively. Values are means of at least four experiments run in triplicate ±SE.

The following step was a study of catalase activity as a function of H₂O₂ concentration, aimed to define which type of inhibition was induced by the factor produced during FC treatment. The data reported in Fig. 6 show that at all concentrations tested the activity of catalase measured in a K⁺-containing basal medium (control) was ~2-fold higher than that found when the assay was carried out in the medium deriving from FC-treated cells (ex KFC 30). Kinetic analysis of the data (shown in the insert) indicates that in the medium from FC-treated cells the apparent K_m value of catalase for H₂O₂ was substantially unchanged (from 39.4 mM in the basal medium to 36.6 mM), whereas V_{max} dropped from 2.17 μmol min⁻¹ to 1.07 μmol min⁻¹, respectively, indicating that the compound released in the incubation medium upon FC treatment behaved as a non-competitive inhibitor. To characterize this inhibitor further, some experiments were also carried out in which the enzymatic assay was run in a medium derived from cells treated for up to 60 min with FC in the same experimental conditions described for Figs 5B and 6. The results obtained showed that in this case catalase activity was more than halved (from ~9 nmol H₂O₂ min⁻¹ in basal medium to ~4 nmol H₂O₂ min⁻¹), indicating a progressive release of the inhibitor from the FC-treated cells.

Effect of the putative FC-induced inhibitor on the IBA-induced H₂O₂ accumulation

The results so far presented clearly indicated that FC promotes the formation of a compound that is gradually

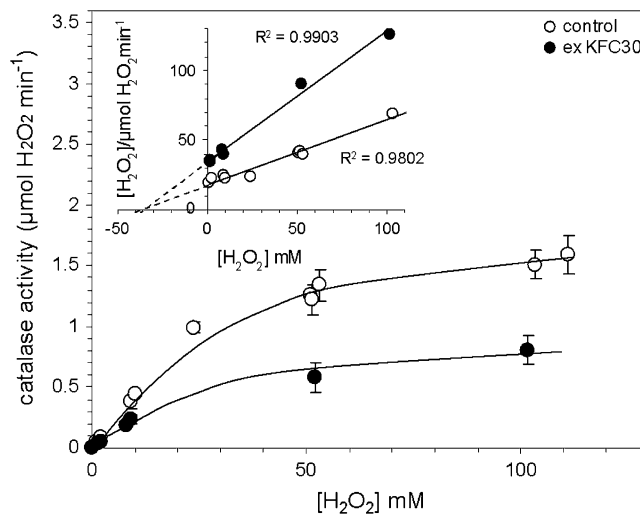


Fig. 6. Catalase activity as a function of H₂O₂ concentration. Catalase activity was tested at pH 7 in a [H₂O₂] range between 1 mM and 120 mM. Measurements were carried out in basal medium supplemented with K⁺ (control) and in a cell-free medium deriving from samples incubated for 30 min in a K⁺-containing basal medium supplemented with FC (ex KFC 30). Kinetic analysis of the data is reported in the insert. Values are means of three experiments run with three replicates ±SE.

released in the external medium and markedly inhibits catalase activity. The effects of this inhibitor, however, were proved only on exogenous catalase, assuming that it behaved as the *A. thaliana* enzyme. Some information about its efficacy on endogenous catalase might come from an analysis of the extracellular H_2O_2 accumulation induced by various treatments, with or without the inhibitor. Unfortunately, the medium deriving from FC-treated samples, in which the inhibitor was proved to accumulate, could not be used for these measurements, since FC, by itself, would largely increase the H_2O_2 level, as shown before (see Fig. 1). However, given that FC is known to bind irreversibly to its receptor on the PM (Dohrman *et al.*, 1977), the inhibitor accumulation could probably also be obtained in a FC-free medium, after switching on inhibitor formation by prior treatment of cells with FC. Therefore, some tentative experiments were carried out in which the external medium of cells treated with a K^+ -containing basal solution, after a previous FC treatment, was utilized as incubation medium (referred to as K^*) for the subsequent treatments, carried out on freshly prepared samples. Before applying the new treatments, cells were pre-incubated in the K^* medium for 30 min, a period assumed to be long enough to allow the inhibitor to enter the cells at least partially.

Figure 7 shows the time course of H_2O_2 accumulation induced by isobutyric acid (IBA) in two parallel series of samples, incubated for up to 30 min in a K^+ -containing basal solution (taken as the control) or in the (K^*)

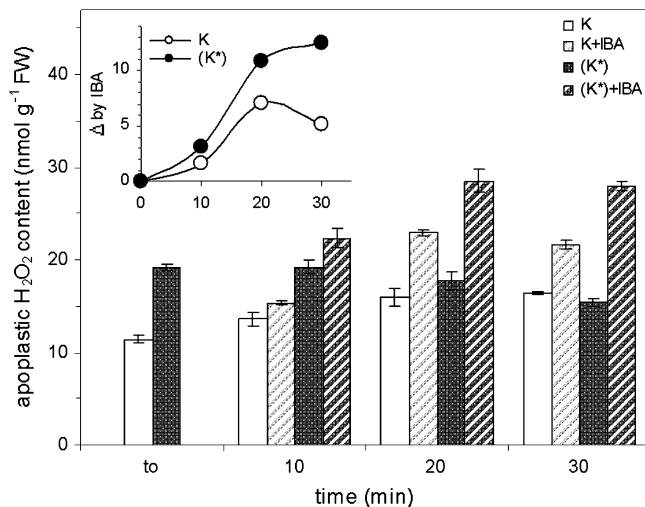


Fig. 7. Effect of the putative FC-induced inhibitor on the time course of IBA-induced H_2O_2 accumulation. After pre-equilibration, cells were transferred either to a K^+ -containing basal solution (K) or to a (K^*) medium (prepared as described in Materials and methods). IBA was fed at the final 2 mM concentration after a 30 min pre-equilibration period (taken as time 0) and treatments were carried out for up to 30 min. The insert shows the H_2O_2 accumulation rise induced by IBA, calculated from the data reported in the figure. Units in the y-axis are the same as in the main figure. Values are means of three experiments run with three replicates \pm SE.

medium, respectively. These experimental conditions were chosen since the large ROS production resulting from the IBA-induced pH_{cyt} decrease (Beffagna *et al.*, 2005) would better highlight the hypothesized inhibitory effects. The data reported show that the H_2O_2 level measured in the (K^*) medium at the beginning of the treatment (t_0) was higher than that found in the control buffer, as expected for a medium also containing some H_2O_2 accumulated during the previous pre-incubation period. Upon IBA addition, H_2O_2 accumulation increased in both control solution and (K^*) medium. However, in this last case the effect was always larger and became more significant for longer treatment times, as better highlighted by the data reported in the insert of Fig. 7. In addition, in the (K^*) medium, the transient feature of the stress-induced rise in apoplastic H_2O_2 content was also suppressed, as previously observed for the FC-treated cells (Fig. 1C). These data clearly indicated the presence of the inhibitor in the (K^*) medium and confirmed that it was actually possible to induce its accumulation also in an FC-free medium by simply pre-treating cells with the toxin. Above all, however, these results proved the efficacy of such a putative inhibitor on cell catalase activity.

Comparison between the effects of FC and salicylic acid on exogenous catalase activity

At this point there was some speculation as to the possible identity of this FC-induced inhibitor. A series of literature data, obtained with different plant materials, suggested that an increase in the level of salicylic acid (SA) might be responsible for the inhibition of catalase activity observed in the present experimental conditions. In fact, FC was proved specifically to stimulate an increase in SA synthesis (Schaller *et al.*, 2000; Singh and Roberts, 2004) and SA, proposed to be the putative endogenous signal for systemic acquired resistance (SAR) (Malamy *et al.*, 1990; Métraux *et al.*, 1990), was suggested to mediate SAR by binding to and inhibiting a catalase, thus leading to an increase in H_2O_2 which may act as a second messenger in the induction of defence mechanisms (Chen *et al.*, 1993; Dempsey and Klessig, 1994). In addition, a soluble SA-binding protein identified as a catalase, whose enzymatic activity is inhibited by SA binding, was purified from tobacco (Conrath *et al.*, 1995).

To check this hypothesis, the effects of SA on exogenous catalase activity were tested (Fig. 8) and the obtained results were compared with those previously found using the media derived from FC-treated cells. The data of Fig. 8A show that the activity of catalase, assayed as a function of [SA] in the K^+ -containing basal solution routinely used, was gradually reduced with the increase in [SA]. However, as indicated in the insert, at the lowest concentration considered the inhibition was only somewhat higher than 20%. A degree of inhibition comparable

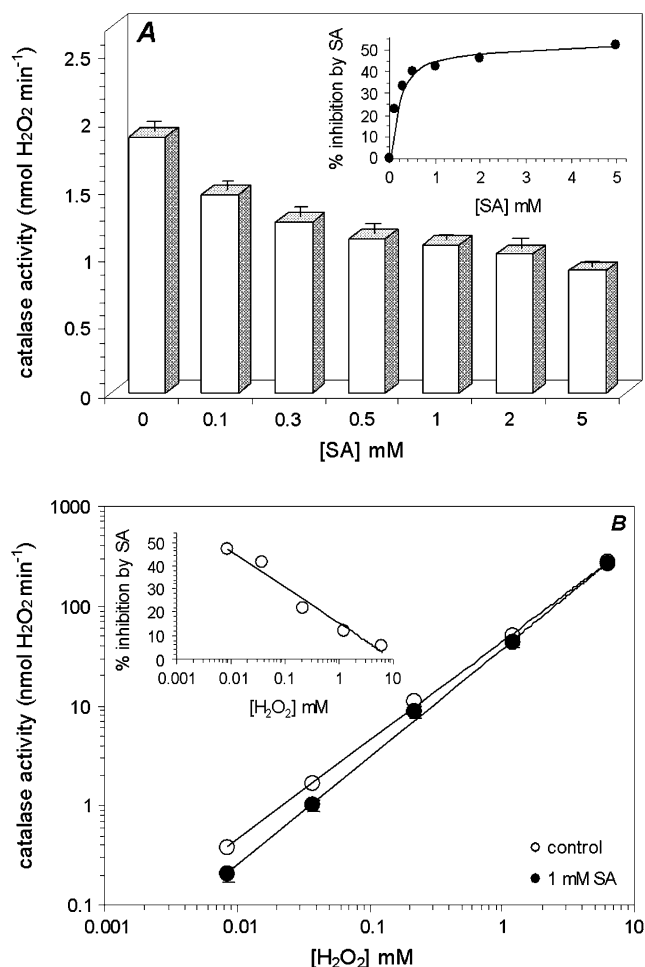


Fig. 8. Salicylic acid (SA) effects on exogenous catalase activity. (A) Catalase activity as a function of SA concentration. The enzymatic activity was assayed at pH 7 in a K⁺-containing basal medium supplemented with SA at the indicated concentrations. The reaction was started by the addition of 40 μ M H₂O₂. (B) SA effects on catalase activity as a function of H₂O₂ concentration. The enzymatic activity was assayed as described in (A) in the indicated [H₂O₂] range. SA was added at the 1 mM concentration. The inhibiting effects of SA, calculated on a percentage basis from the data reported in the figures, are reported in the inserts of A and B. Values are means of at least four experiments run with three replicates \pm SE.

with that observed when measurements were performed in the media of cells treated with FC for 30 min (Fig. 5B) was reached only for an [SA] \geq 0.5 mM. To achieve an inhibition of \sim 50%, similar to that produced by the medium derived from cells treated with FC for 60 min, a 5 mM [SA] was required.

These results did not favour the view that SA could be the inhibitor contained in the medium of FC-treated cells. In fact, taking into account the strong dilution of the inhibitor in the external medium, [SA] inside the cell should be incredibly high to account for the release needed to give the observed inhibition. Some further experiments were carried out, in which the effects of SA on catalase activity were studied as a function of [H₂O₂]

to define the type of inhibition exerted by this compound in the present experimental conditions. The enzymatic assay was run in a K⁺-containing basal medium supplemented or not with 1 mM SA. As shown in Fig. 8B, the inhibitory effect of SA gradually decreased with the increase in [H₂O₂] and became substantially negligible at the higher concentrations tested (as better highlighted by the data reported in the insert showing the percentage inhibition by SA calculated from the data of the figure). These results clearly indicated that, differently from what was observed for the inhibitor contained in the incubation medium of FC-treated cells, SA behaved as a competitive inhibitor, thus definitely ruling out the possibility that SA is the inhibitor released in the incubation medium by FC-treated cells.

Concluding remarks

The results presented in this study show that in *A. thaliana* cells FC induces an early and marked increase in the level of apoplastic H₂O₂, and suggest that this effect is, at least partly, due to a decreased capability of the cell to degrade the H₂O₂ formed. Strongly supporting this view are, in particular, a series of data proving that FC promotes the production of an as yet unidentified factor, which increasingly accumulates in the external medium and behaves as a non-competitive catalase inhibitor. Evidence is also presented that the observed catalase inhibition is not apparently mediated by SA, supposed to be the most likely candidate for this role. To identify and further characterize this putative catalase inhibitor, methods more adequate than those adopted in the present study are required, and further work in this direction is in progress.

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