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Lithium alters elicitor-induced H_2O_2 production in cultured plant cells

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Abstract

Lithium pollution may seriously influence the metabolic and signalling processes of plants. In the present paper, we investigate the effect of lithium chloride on fungal elicitor-triggered H_2O_2 generation in *Rubia tinctorum* L. cell cultures. Our results show that Li^+ strongly influences elicitor-induced H_2O_2 formation and time-course in the cells and culture medium. Neomycin, a phospholipase C inhibitor, and 2-APB, an inositol-1,4,5-triphosphate (IP_3) receptor-mediated Ca^{2+} release blocker, strongly affected the elicitor-induced H_2O_2 production and had a similar effect on elicitor-triggered H_2O_2 formation as Li^+. We monitored changes in H_2O_2 location at subcellular level and our observations confirmed the changes measured by quantitative methods. The obtained results enabled us to deduce that the IP_3 pathway might be involved in the early signalling events leading to the moderation of elicitor-induced reactive oxygen species generation.

Additional key words: elicitation, inositol-1,3,5-triphosphate, oxidative stress, *Rubia tinctorum*, signal transduction.

Introduction

Lithium ion cells have become one of the most frequently used portable accumulators (Nishi 2001) and nowadays they are widely used in mobile and wireless phones, camcorders, notebooks, *etc*. In air conditioning systems lithium bromide and chloride are used widely in the dehumidification of air. One fifth of the marketed lithium is extensively used in its hypochlorite form for bleaches and sanitizers and in swimming pools as disinfectants (Garrett 2004). This large-scale consumption raises questions about the effects of lithium on living organisms. Inorganic and organic lithium compounds are usually water-soluble, so, an excess Li^+ content in the environment may harm plants more than other less water-soluble chemical substances.

At different concentrations, Li^+ influences various physiological processes in plants such as microtubule depolarisation (Bartolo and Carter 1992), mitotic progression of stamen hair cells (Wolniak 1987), callus induction (Bagga *et al*. 1987), respiration (Laties 1963), cellular transport (Wissocq *et al*. 1991), enzyme activities (Boller 1984, Monreal *et al*. 2007), gene expression (Liang *et al*. 1996), and certain developmental events (Boyer *et al*. 1979, Belyavskaya 2001, Gillaspy and Gruissem 2001).

Phosphoinositides play a central role in the regulation of various processes within eukaryotic cells (Silverman-Gavrila and Lew 2002, Ischebeck *et al*. 2010). With regard to plant signal transduction, Li^+ inhibits inositol monophosphatase isoenzymes (IMPs) (Gillaspy *et al*. 1995, Banerjee 2007) and this reduced activity results in a depletion of the intracellular inositol content leading to a lowered supply of inositol lipids for signalling. Lithium blocked IMPs are unable to dephosphorylate inositol phosphate forms, particularly inositol 1,4,5-triphosphate (IP_3), an important molecule in the phospholipase C (PLC) signalling pathway. Like several other signalling components, PLC activation and IP_3 level modulation are robust parts of elicitor-induced plant signal transduction (Xiong *et al*. 2002, Zhao *et al*. 2005). In plants, the secondary messenger IP_3 mobilizes Ca^{2+} from intracellular calcium stores such as the endoplasmic reticulum, Golgi apparatus, or vacuoles (Berridge 1993, DeWald *et al*. 2001). Accordingly, the modified IP_3 levels may alter further signalling responses (Kovács

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Abbreviations: 2-APB - 2-aminoethyldiphenyl borinate; CDPK - calcium-dependent protein kinase; IP_3 - inositol-1,4,5-thriphosphate; IPMs - inositol monophosphatase isoenzymes; MAPK - mitogen-activated protein kinase; PLC - phospholipase C; ROS - reactive oxygen species.

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et al. 2009, Nyitrai *et al*. 2009, Distefano *et al*. 2010).

In unstressed plants, Li^+ treatment increased the IP_3 content in *Sorghum vulgare* leaves within 15 min, while a longer (1 h) exposure markedly decreased it (Monreal *et al*. 2007). Under anaerobic conditions, Li^+ also increases IP_3 content together with the accumulation of γ-aminobutyric acid and this accumulation was abolished by the PLC inhibitor neomycin (Reggiani and Laoreti 2002). The IP_3 content of soybean cell suspension cultures increased rapidly within 1 - 2 min after treatment with different elicitors; however, this spike only lasted for 5 - 10 min. Shortly after the IP_3 spike, H_2O_2 accumulation was also detectable indicating that PLC activation might constitute a crucial pathway by which elicitors trigger oxidative burst (Legendre *et al*. 1993). Rapid, transient IP_3 increase was detected in salt-stressed *Arabidopsis* suspension cultures which was lowered by the PLC inhibitor U-73122 (DeWald *et al*. 2001). In contrast to this, other works reported decreased IP_3 accumulation as an early answer to *Pseudomonas syringae* pv. *glycinea* infection (Shigaki and Bhattacharyya 2000, Chou *et al*. 2004).

The oxidative burst, *i.e.* enhanced production of reactive oxygen species (ROS), is an important part of the complex defence response of plants to cope with harmful biotic/abiotic factors. ROS compounds are generated by various stimuli and the altered redox state of the cells leads to subsequent signalling events (Lecourieux *et al*. 2002, Zhao *et al*. 2005, Chen *et al*. 2009, Darehshouri and Lütz-Meindl 2010, Tamás *et al*. 2010), such as lipid peroxidation, modification of down-stream signal transduction steps, cell wall reinforcement, hypersensitive cell death, defence gene activation, *etc*. (Kawano 2003, Apel and Hirt 2004, Zhao *et al*. 2005, Iannone 2010). The most investigated ROS compound is H_2O_2 because of its relatively long lifetime and ability to diffuse freely through different membranes. These facts allow it to play a substantial role in the generation and modification of the defence response in plants. H_2O_2 reacts with protein phosphatases (Gupta and Luan 2003) and mitogen activated protein kinases (Jonak *et al*. 2002) as well as histidine kinases (Hwang *et al*. 2002) and can directly modulate developmentally important genes (Desikan *et al*. 1996). Moreover, H_2O_2 is also able to non-enzymatically oxygenate other second messengers which may modify the effects of these signalling elements (Apel and Hirt 2004). After elicitor treatment, production of H_2O_2 is not enhanced continuously, but instead displays the sequential appearance of several transient maxima, where each distinct maximum probably has its particular meaning for further signalling processes (Zhao *et al*. 2005, Bóka *et al*. 2007, Bóka and Orbán 2007).

Rubia tinctorum L. cell suspension cultures are simple and useful model systems for evaluating the elicitor-triggered events of signal transduction (Bóka *et al*. 2002, Perassolo *et al*. 2007, Vasconsuelo and Boland 2007); the location and dynamics of elicitor-induced H_2O_2 production have been studied in this system (Bóka *et al*. 2007). Results show that prolonged H_2O_2 production has more maxima than has been previously described and H_2O_2 formation was obvious in the cytoplasm, at the plasma membrane, or in the cell wall, depending on the sampling time.

Neomycin is extensively used in plants to inhibit PLC (Legendre *et al*. 1993). Neomycin binds to phosphatidylinositol-4,5-biphosphate and prevents its hydrolysis by PLC, thus inhibiting rapid IP_3 accumulation. Compound 2-aminoethyl diphenylborinate (2-APB) is an inhibitor of IP_3 receptor-mediated Ca^{2+} release from inner stores (Vasconsuelo *et al*. 2003), mainly from endoplasmic reticulum and vacuoles (Peiter 2011). Li^+, neomycin, and 2-APB all act on the PLC signalling pathway, but they inhibit it at different steps.

The aim of this study was to investigate the effects of lithium treatment on the elicitor-triggered H_2O_2 production of *R. tinctorum* suspension cultures. We examined the early alterations of H_2O_2 production in the cells and in the supernatant of the cultures using two distinct quantitative methods and we also investigated the subcellular location of the produced H_2O_2 in the cells. We have also checked the effect of neomycin and 2-APB on elicitor-triggered H_2O_2 generation.

Materials and methods

Cell suspension cultures were prepared from *Rubia tinctorum* L. roots and grown in 250 cm^3 Erlenmayer flasks with Murashige-Skoog liquid medium (containing 30 g dm^{-3} sucrose, 1 mg dm^{-3} indoleacetic acid, 0.2 mg dm^{-3} naphtaleneacetic acid and 0.2 mg dm^{-3} kinetin) at room temperature and in natural light (approximately 14-h photoperiod). Medium pH was set to 5.8 before sterilization. Suspensions were maintained on a rotary shaker at 125 rpm and were subcultured every 14 d (Orbán *et al*. 2008). Since cell division in the cultures reached a maximum rate between the 7^{th} and 14^{th} days and this period was followed by a stationary phase, elicitations were made at the end of the exponential phase of the cell divisions, *i.e.* on the 14^{th} day (Bóka *et al*. 2002).

The *Botrytis cinerea* Pers *ex*. Pers isolates were obtained from the Department of Cell and Molecular Biology of Drugs, Comenius University, as a gift from Dr. M. Psenak (Bratislava, Slovakia) and were cultured on a 3 % (m/v) malt medium. The isolation and purification of the cell wall material of *B. cinerea* and its hydrolysis with trifluoroacetic acid were performed as described in detail previously (Bóka *et al*. 2002). Hyphae prepared from the *in vitro* cultured mycelium of fungus were cleaned of proteins and lipids and the pure cell walls were hydrolysed. The dialysed and neutralized hydrolysate composed of oligosaccharides (mainly the 1.5 - 67.5 kDa fraction; stock elicitor from one hydrolysis) was used in all experiments.

$LiCl_2$ (*Sigma-Aldrich*, St Louis, USA) was dissolved in

distilled water (DW) and, according to the literature data, applied in 1 and 20 mM concentrations to the cell cultures. In the case of Li^+ altered elicitations, a 15 min Li^+ pre-treatment was performed before elicitor application.

The *Botrytis* elicitor (Bot) was applied at a concentration of 1.25 cm^3 of extract per 100 cm^3 of suspension culture to achieve the optimal effects established previously (Bóka *et al*. 2002). Samples collected at 0 min were taken from the treated cultures just after the elicitor or Li^+ administration.

R. tinctorum suspension cultures were also treated with 250 μM neomycin (250 Neom mock) and subjected to a 15 min 125 or 250 μM neomycin pre-treatment prior to elicitation (125 Neom+Bot and 250 Neom+Bot, respectively). Similarly, in the cultures, 15 μM 2-APB (2-APB mock) was applied or cells were pre-treated for 15 min with 15 μM 2-APB before *Botrytis* elicitor administration (2-APB+Bot). Samples collected at 0 min were taken from the cultures just after the start of measurements, *i.e*. the administration of elicitor and/or inhibitors.

We used three methods for H_2O_2 detection. For characterization of H_2O_2 content changes in the cells and culture medium, two quantitative methods were chosen, whereas for subcellular localization of H_2O_2 generation, the cerium method was used. All three methods are specific for H_2O_2 but all of them have their benefits and drawbacks which determine their utilities and limitations dependent on H_2O_2 concentration, experimental conditions, and manner of detection. The titanium complex method is appropriate for momentary H_2O_2 content determination in cells. Cold acetone stops H_2O_2 generation which ensures the exact measuring of the actual H_2O_2 content of cells. Additionally, the autofluorescence of the cells does not disturb the measurement. The disadvantage of this method is the time consuming sample preparation. The scopoletin fluorescence quenching method is quick and highly sensitive (after optimization) even at lower H_2O_2 concentrations but the autofluorescence of cells can hinder its accuracy. Furthermore, because of the necessary incubation during the measurement, results are delayed after sampling. Fortunately, this is a negligible factor in the case of the culture medium where H_2O_2 level changes are much slower than in cells. Both of the previously mentioned approaches are appropriate for quantitative determination of H_2O_2 production but they do not provide information on its localization. The electron dense precipitate of cerium perhydroxides shows the location of H_2O_2 generation in the cells analysed using transmission electron microscope. The results of these three methods provide a complete picture of the H_2O_2 formation in the treated cell cultures.

The H_2O_2 concentration of cells was determined as a peroxide-titanium complex using a slightly modified version of the method described by Brennan and Frenkel (1977). Briefly, 5 g sample of cells were suspended in 10 cm^3 of cold acetone and homogenized. Then, 5 cm^3 DW was added and the homogenate was filtered. A 2.5 cm^3 aliquot was treated with 0.25 cm^3 of titanium reagent (20 %, m/v, titanic tetrachloride in concentrated HCl) and 0.75 cm^3 of concentrated ammonia solution. The precipitated peroxide-titanium complex was separated out by centrifugation (8000 *g*, 10 min) and dissolved in 15 cm^3 of 2 M H_2SO_4. The absorbance of this solution was measured at 415 nm with a *Ultrospec Plus* spectrophotometer (*Pharmacia,* Cambridge, UK). H_2O_2-free samples were also created as reference samples from the cell suspension cultures (Bóka *et al*. 2007).

The H_2O_2 accumulation was quantified in the suspension culture medium by oxidative quenching of scopoletin fluorescence (Cazalé *et al*. 1998) measured with a *Fluoromax-3* spectrofluorimeter (*Horiba Jobin Yvon*, Longjumeau, France).

In our preliminary experiments, we checked the effect of lithium on cell free H_2O_2 solution and we did not detect any change in the H_2O_2 content.

Each experiment was performed in triplicate and all the treatments were repeated three times. Control treatments were carried out by adding appropriate amounts of lithium solution (Li mock) or DW (DW mock) to the cell suspension cultures.

For the subcellular localization of H_2O_2, Bestwick's cytochemical method was used which is based on the generation of cerium perhydroxides (Bestwick *et al*. 1997). Briefly, small clumps of cells from control cultures, elicited cultures, and Li^+ pre-treated elicited cultures were incubated in freshly prepared 5 mM cerium chloride for 30 min before fixation. Samples were collected at 0, 5, 30, 50, 90, and 240 min after treatment. Cells were fixed in two steps (2 %, m/v, glutaraldehyde for 3 h, 1 %, m/v, osmium tetroxide for 2 h, both fixatives were dissolved in 0.1 M K-Na-phosphate buffer at pH 7.2) and embedded in *Durcupan* resin after dehydration in an ethanol series. Sections (60 to 80 nm) were cut with a *Reichert Ultracut E* microtome (*Leica Microsystems*, Vienna, Austria) mounted on uncoated copper grids and stained with 5 % (m/v) uranyl acetate dissolved in methanol for 4 min and lead citrate for 6 min. Samples arose from one fixation-embedding process. Three cell aggregates were sectioned per sample and two grids from each of the aggregates (8 - 10 sections on the grids) were investigated. Sections were observed with a *Hitachi 7100* transmission electron microscope (TEM) (Tokyo, Japan) at an accelerating voltage of 75 kV and a *JEOL JEM 1011* TEM (Tokyo, Japan) at 60 kV.

Results

Compared to DW mock, Li^+ mock treatments (at both concentrations) slightly elevated the H_2O_2 production of cells. However, this weak increase lacked any signs of significant transient maxima/minima. Application of two different Li^+ concentrations (1 and 20 mM) did not show essential differences. The 1 mM Li^+ treatment slightly

increased the H_2O_2 concentration and the measured H_2O_2 content remained around 0.3 - 0.4 mg g^{-1}(f.m.) up to 240 min of examination (Fig. 1).

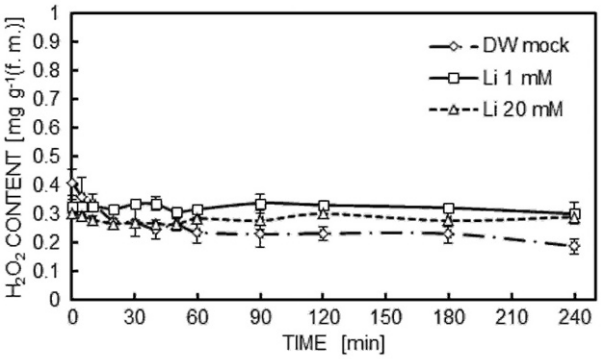

Fig. 1. The effects of LiCl on H_2O_2 production in *R. tinctorum* suspension cultures measured using the titanium method. Comparison of H_2O_2 content after distilled water (DW) and 1 and 20 mM Li^+ mock treatments. Means ± SE, $n = 9$.

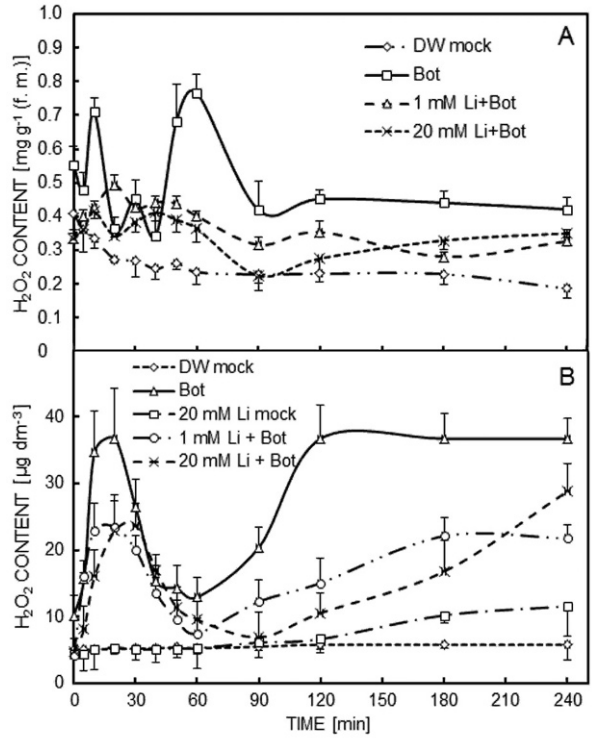

Fig. 2. H_2O_2 content in Li^+ and elicitor treated *R. tinctorum* suspension cultures. *A*: Changes of H_2O_2 content in DW mock, *Botrytis* elicitor treated (Bot), and LiCl pre-treated elicited cells (1 mM Li+Bot and 20 mM Li+Bot, respectively). *B*.: H_2O_2 content in the medium of DW mock, Li^+ mock, Li^+ pre-treated and elicited cultures. Means ± SE, $n = 9$.

In cell suspension, elicitation resulted in three distinct maxima (at 10, 30, and 60 min), and three distinct minima (at 5, 20, and 40 min) of H_2O_2 content (Fig. 2*A*). Moreover, H_2O_2 content remained 0.45 mg g^{-1}(f.m.) after 120 min. 1 mM Li^+ pre-treated and elicited cells showed lower H_2O_2 content and had altered H_2O_2 production dynamics compared to the elicited cells. This treatment resulted in four maxima (at 20, 50, 120, and 240 min; the last two were slight), and three distinct moderate minima (at 30, 90, and 180 min). The H_2O_2 formation kinetics of 20 mM Li^+ pre-treated elicited cells also changed: two moderate and wide maxima (at 10 and 40 min) and two pointed minima (at 20 and 90 min) were detectable and following this H_2O_2 content increased slightly up to the end of the experiment.

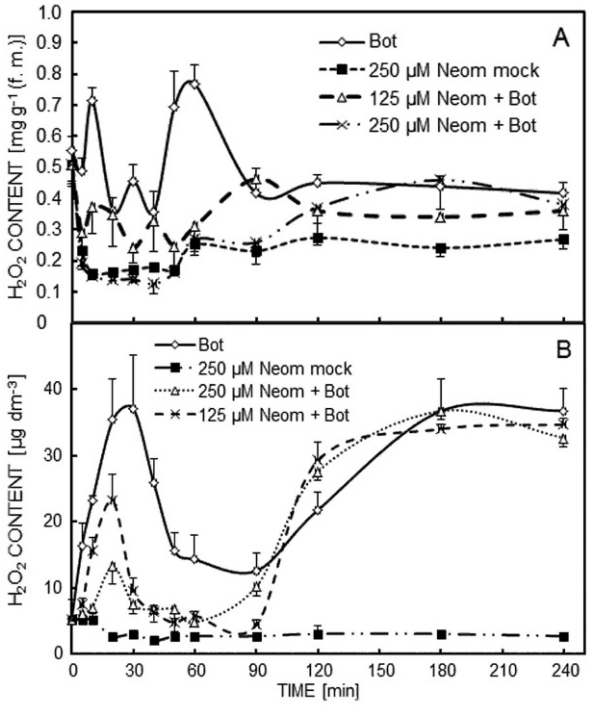

Fig. 3. H_2O_2 content in neomycin and elicitor treated *R. tinctorum* suspension cultures. *A*: Changes of H_2O_2 content in *Botrytis* elicitor treated (Bot), 250 µM neomycin mock, and neomycin pre-treated elicited cells (125 µM Neom+Bot and 250 µM Neom+Bot, respectively). *B*.: H_2O_2 content in the medium of the same cultures. Means ± SE, $n = 9$.

The H_2O_2 content of the medium was also detected after DW mock, lithium mock, elicitor, and lithium pre-treated elicitor treatments (Fig 2*B*). To simplify the graph, we only indicated the 20 mM Li mock treatment because there was no significant difference between the 1 and 20 mM Li^+ mock treatment. There was no significant difference between DW mock and 20 mM Li^+ mock up to 120 min. However, after that point, a moderate H_2O_2 accumulation began and it lasted up to the 4th h in the case of the 20 mM Li^+ mock. Elicitation generated maximum H_2O_2 accumulation (around 35 - 40 µg dm^{-3}) at 10 - 20 min and 120 - 240 min. Li^+ pre-treatment altered these maxima; 1 mM Li^+ pre-treatment reduced the first maximum to around 20 - 25 µg dm^{-3} and also reduced the later accumulation. 20 mM Li^+ pre-treatment not only decreased the H_2O_2 content but also delayed its formation: the first maximum appeared at 30 min and the later accumulation started at 120 min (the

value detected at 240 min was higher than that measured in the case of 1 mM pre-treatment).

The effect of the PLC inhibitor neomycin is shown on Fig 3. To simplify graphs, results of the 125 μM neomycin mock treatment are not presented because they were not significantly different from the 250 μM neomycin mock treatment. The DW mock graphs obtained in the cells and medium are visible in Figs. 1 and 4, respectively. In cells pre-treated with 125 μM neomycin, elicitor treatment (125 Neom+Bot) significantly decreased H_2O_2 content at the maxima and slightly shifted them. The H_2O_2 production dynamism was completely different in the 250 Neom+Bot treated cells. The first H_2O_2 formation maximum almost totally ceased and the H_2O_2 content of the cells was lowered later on but from 90 min it increased (Fig. 3*A*). Elicitation of the pre-treated cultures (125 Neom+Bot and 250 Neom+Bot) resulted in similar H_2O_2 content dynamics in the culture media as Bot did, but the H_2O_2 content of the first maximum dropped in a neomycin concentration-dependent manner. Interestingly, the H_2O_2 content in both 125 Neom+Bot and 250 Neom+Bot treatments increased after 90 min to values similar to those in the Bot treatment (Fig. 3*B*).

2-APB decreased H_2O_2 content in the cells continuously up to 0.2 mg g^{-1}(f.m.) at 60 min and it remained

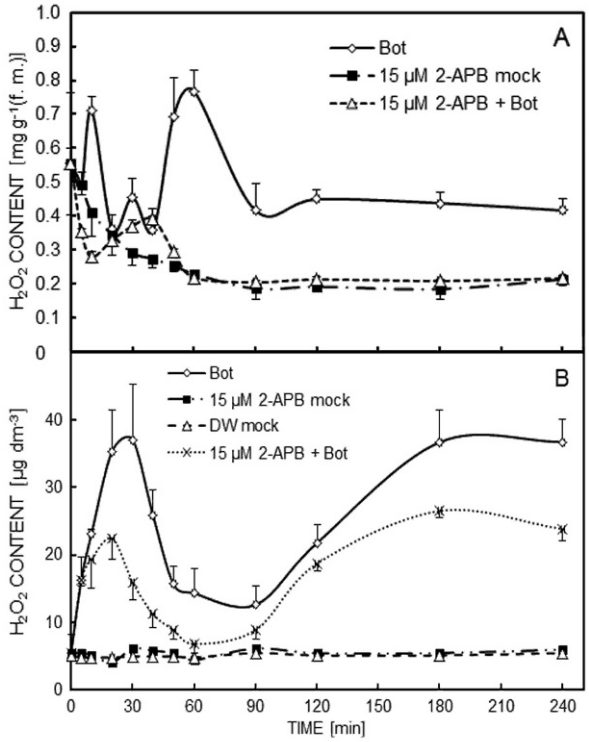

Fig. 4. H_2O_2 content in 2-APB and elicitor treated *R. tinctorum* suspension cultures. *A*: Changes of H_2O_2 content in *Botrytis* elicitor treated (Bot), 15 μM 2-APB mock, and 2-APB pre-treated elicited cells (15 μM 2-APB+Bot). *B*.: H_2O_2 content in the medium of DW mock, 2-APB mock, elicited and 2-APB pre-treated elicited cultures. Means ± SE, $n = 9$.

at that level (2-APB mock). Elicitation after 2-APB pre-treatment (15 μM 2-APB+Bot) reduced H_2O_2 generation in the cells with a local minimum at 10 min and a maximum of 0.4 mg g^{-1}(f.m.) at 40 min. Subsequently, the H_2O_2 content returned to 0.2 mg g^{-1}(f.m.) at 60 min (Fig 4*A*). In the medium of the cultures, 2-APB mock treatment was similar in its characteristics to the DW mock displaying a base-line like H_2O_2 formation. In contrast to this, 15 min pre-treated and elicited cells (15 μM 2-APB+Bot) displayed a similar H_2O_2 generation dynamism to the elicitor-treated cells, but the measured H_2O_2 concentrations at all time-points were significantly lower (Fig. 4*B*).

The DW mock samples did not contain cerium perhydroxide precipitates at any point during the whole experiment time (Fig 5*A* represents the characteristic cytochemical staining of these samples). In the 20 mM Li mock treated cells, a weak reaction was observed mainly after 50 min of Li^+ treatment (Fig. 5*C*). However, most of the region in the cell walls was free of precipitates in the observed cells. Only some granulated staining was seen in the cytoplasm near the plasma membrane at 240 min (Fig. 5*B*,*D*). Exposure of cells to the elicitor resulted in significant H_2O_2 production. It appeared mainly in the cell walls or just inside the plasma membranes (Fig. 5*E*). Moreover, the intensity of the reaction seen in the electron microscope was in accordance with the peaks obtained using quantitative methods (*e.g.* in 90 min samples almost no precipitates were detectable, Fig. 5*F*). A significant and unexpected cytochemical reaction was detected in the 1 mM Li^+ pre-treated elicited sample after 5 min when heavy precipitate deposition was regularly observed at the tonoplast in numerous cells. On the other hand, in some cases we could even see differences in this feature in the neighbouring cells or in the different vacuolar compartments of the same cell (Fig. 5*G*). After this time (from 30 to 240 min), no reaction was observed close to the tonoplasts in these samples. In comparison with the elicitor treated cultures, weaker H_2O_2 appearance was detectable in the cells and the intensity of cytochemical coloration appearing in limited areas was in accordance with the intensity of peaks obtained by quantitative methods (Fig. 5*H*). In the 20 mM Li^+ pre-treated elicited cells, no reaction was at the tonoplast. After 5 min, H_2O_2 formation was found in the cell walls (Fig. 5*I*). However, as in the 1 mM pre-treated elicited samples, H_2O_2 production also accorded well with the quantitative peak data, like in the samples collected at 50 min, where cells scarcely contained any cytochemical precipitates (Fig. 5*J*). In the 240 min samples, a significant but not equally strong reaction was observable in the case of elicitor treated, 1 mM and 20 mM Li^+ pre-treated elicited samples, indicating that H_2O_2 accumulation was efficiently modified by Li^+ pre-treat-ment; however, H_2O_2 formation of cells was not totally blocked by the pre-treatments (Fig. 5*K*,*L*). Mitochondria and plastids did not display staining.

Compared to the Bot treatment, both the neomycin

and 2-APB caused a reduction in the amount of the cerium precipitate depositions although the cell area where they appeared was more extensive. Staining the tonoplast region of vacuole was not detected in any of the pharmacologically treated samples (data not shown).

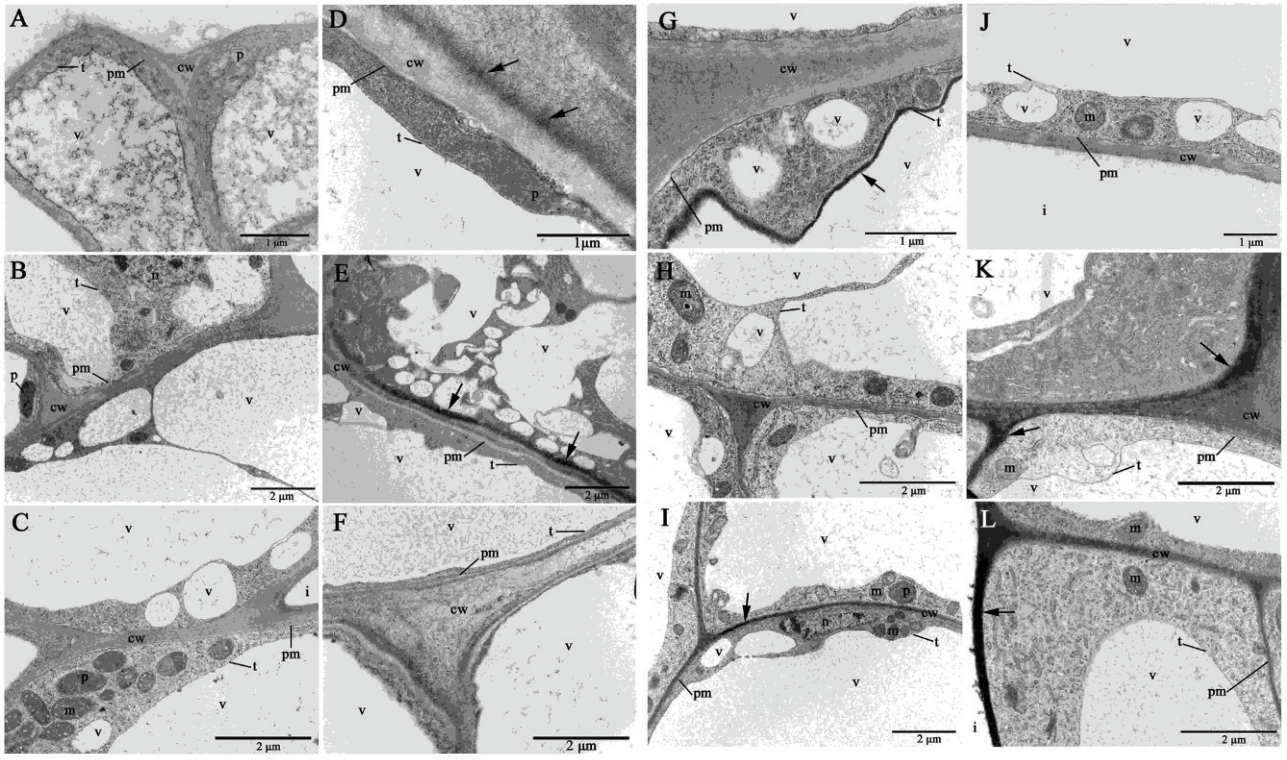

Fig. 5. Cytochemical detection of H_2O_2 production in *R. tinctorum* cells visualised using the electron-dense cerium perhydroxide precipitates (*arrows*) after different treatments and at various time points. The precipitate depositions in the cell wall and/or near the plasma membrane of treated cells display a characteristic pattern. The strong staining of tonoplast was unique for the 1 mM Li^+ pre-treated elicited cells after 5 min. *A*: DW mock control cells. Appearance of this 240 min sample also represents the reaction in other DW mock samples. *B*: 20 mM Li^+ mock, 5 min. *C*: 20 mM Li^+ mock, 90 min. *D*: 20 mM Li^+ mock, 240 min. *E*: *Botrytis*-elicitor-treated, 50 min. *F*: *Botrytis*-elicitor treated, 90 min. *G*: 1 mM Li^+ pre-treated elicited cells, 5 min. *H*: 1 mM Li^+ pre-treated elicited cells, 50 min. *I*: 20 mM Li^+ pre-treated elicited sample, 5 min. *J*: 20 mM Li^+ pre-treated elicited cells, 50 min. *K*: *Botrytis*-elicitor treated, 240 min. *L*: 1 mM Li^+ pre-treated elicited cells, 240 min. Legends: cw - cell wall, i - intercellular space, m - mitochondrium, n - nucleus, p - plastid, pm - plasma membrane, t - tonoplast, v - vacuole.

Discussion

H_2O_2 generation after different stresses appears as a dynamic process, a series of transient minima and maxima, and the time, amplitude, and duration of these transients might refer to their different sites of action in the signal transduction pathways (Zhao *et al*. 2005, Bóka *et al*. 2007, Bóka and Orbán 2007, Chen *et al*. 2009, Cardenas 2009, Mittler *et al.* 2011). Several questions arise about the regulation of the H_2O_2 generation transients because the actual concentration of ROS at any given moment depends on the balance between their generation and scavenging (Mehdy *et al*. 1996, Iannone 2010, Mittler *et al*. 2011). Bearing in mind the multiple roles of H_2O_2 in plants (direct toxicity, second messenger, redox state establishment, and gene expression modification), the H_2O_2 formation transients may be modified by several processes (protein phosphorylation, ion channel activa-tion, Ca^{2+} peak, *etc*.), and the regulatory events could also be influenced by other factors at any time (Hao *et al*. 2006, Elmayan *et al*. 2007). Several authors have investigated the regulation of the elicitor induced oxidative burst; however, they did not concentrate on its dynamics (Olmos *et al*. 2003, Zhao *et al*. 2005).

Biotic and abiotic stresses activates the phospholipase C /inositol-1,4,5-triphosphate - diacylglycerol/protein kinase C (PLC/IP_3 - DAG/PKC) cascade resulting in altered IP_3 and DAG content and polyphosphoinositol turnover (Meier and Munnik 2003, Vasconsuelo *et al*. 2003, Kovács *et al*. 2009, Nyitrai *et al*. 2009, Distefano *et al*. 2010). IP_3 is known to play a substantial role in Ca^{2+} release from internal stores (vacuole, endoplasmic reticulum, and non-vacuolar vesicles) and the increased Ca^{2+} concentration affects its targets (*e.g*. MAPK, CDPK, NADPH oxidase, ion channels, and protein phospha-

tases), leading to the next steps of the signal transduction. The linkage between the PLC pathway and the oxidative burst is not fully understood, however, the role of signalling components (such as direct or CDPK-dependent NADPH oxidase activation by calcium released from internal stores) implies a close relationship between these two processes (DeWald *et al*. 2001, Huang and Huang 2008, Garrido *et al*. 2009, Curran *et al*. 2011). The functioning of the MAPK pathway also seems to be more complex than was previously supposed. From the numerous MPK forms, it is mainly MPK4 that would appear to be activated by biotic stresses but recent data indicate that MPK3 and MPK6 may also perform a similar role (Pitzschke *et al*. 2009, Komis *et al*. 2011, Kong *et al*. 2011). An approach to revealing some components of this relationship is the use of well-defined inhibitors to block characteristic physiological steps. One of these inhibitors is Li^+ which inhibits the IMPs determining IP_3 content (Gillapsy *et al*. 1995, Belyavskaya 2001, Banerjee 2007). Neomycin and 2-APB also influence inositol signalling in the cells but they act at different steps of the inositol pathway. Neomycin prevents conversion of PIP_2 to IP_3 and DAG by PLC inhibition (Silverman-Gavrila and Lew 2002) and blocks both the consecutive IP_3 transient formation and H_2O_2 accumulation; however, the inhibitory effect is incomplete (Vasconsuelo *et al*. 2003). Cross-talk of inositol and ROS pathways is coupled by a Ca^{2+} signalling network and all up-stream and down-stream events are influenced by cytoplasmic and nuclear Ca^{2+} concentration-altering compounds (Xiong *et al*. 2002, Garrido *et al*. 2009, Mazars *et al*. 2010, Li *et al*. 2011). 2-APB is an IP_3 receptor-mediated Ca^{2+} release blocker which has a powerful inhibitory effect on ROS generation after PLC activation arresting both slow and rapid Ca^{2+} channels activated by IP_3 (Silverman-Gavrila and Lew 2002). The stunted cytoplasmic Ca^{2+} level results in supressed ROS production affecting several functions in the cell (Cardenas 2009).

Based on our results, Li^+ exposure in non-elicited cultures (Li mock treatment) resulted in slightly higher H_2O_2 content after 2 h, also detected in the cells by TEM. These weak and prolonged changes in H_2O_2 production may play a role in the previously described altered physiological processes induced by Li^+ (Bartolo and Carter 1992, Wissocq *et al*. 1991, Liang *et al*. 1996, Belyavskaya 2001).

Li^+ pre-treatment markedly affected the elicitor-induced H_2O_2 generation; moreover, there were significant differences in the effect of the two used concentrations. However, both of them reduced the amplitude of peaks both in the cells and in the culture medium. 1 mM Li^+ pre-treatment altered the timing and the duration of peaks of the elicitor-induced H_2O_2 generation and caused an unexpected H_2O_2 localization at the tonoplast after 5 min. The role of this is unknown but a similar staining pattern was also detected in the case of cadmium treatment (Olmos *et al*. 2003) and salt and osmotic stress (Wi *et al*. 2006). In contrast, in other samples (taken after 30, 50, 90 and 240 min) staining close to the tonoplast was not detected. 20 mM Li^+ pre-treatment also decreased the H_2O_2 content and altered its dynamics both in the cells and the culture medium.

Apart from an obvious decrease in H_2O_2 content, the shifted maxima and inhibition of the early events at 250 mM Neom+Bot treatment were observed in the cells. Similarly, changes were almost negligible after 90 min whereas the early H_2O_2 accumulation was strongly affected in the medium. In the 2-APB treated cells, only one maximum appeared whereas the others were demolished and steady H_2O_2 accumulation after 120 min was lowered significantly. H_2O_2 content changes in the medium showed an intermediate dynamics between Li^+ and neomycin inhibition, with mainly amplitude being modified. These divergences in the H_2O_2 production dynamics caused by the used inhibitors point to the differences in their inhibition mechanisms and underline the eventual multiplicity in the early signal transduction steps and/or source of H_2O_2 generation.

The subcellular localization of the perhydroxide precipitates in the pre-treated elicited samples had a similar appearance to that described in pathogen infected lettuce (Bestwick *et al*. 1997) and elicited *R. tinctorum* suspension culture cells (Bóka *et al*. 2007) indicating a possible pre-determined polarity of H_2O_2 formation in the cells. A less marked cytochemical reaction was observed in the Li^+ pre-treated 240 min elicited samples than in the only elicited ones. Neomycin and 2-APB treatment resulted in weaker precipitate depositions even at H_2O_2 accumulation maxima but they did not alter the appearance of deposits apart from the complete lack of tonoplast staining seen in the 5 min 1 mM Li+Bot sample. This fact indicates that Li^+ and the used inhibitors might act at different compartments during the early signalling process.

In summary, according to the observed dynamics of H_2O_2 formation in the Li^+ pre-treated elicited cells, the changed IP_3 content might be the reason for the altered H_2O_2 production in the first 90 min but later the H_2O_2 formation seems to recover to a certain level (Monreal *et al*. 2007). Another hypothetical reason for the decreased early H_2O_2 production is that Li^+ may retard some enzyme activities involved in the early stages of the oxidative burst inasmuch as lithium is a known regulator of several enzyme activities (Boller 1984, Monreal *et al*. 2007). However, in the case of Li mock samples there was a slight difference in the early H_2O_2 content in comparison with DW mock samples (Fig. 1) whereas in elicited *Rubia* cultures, Li^+ strongly stunted the H_2O_2 generation (Fig. 2). According to these facts, Li^+ seems to be a regulator of elicitor-inducible H_2O_2 generation and IP_3 might play a crucial role in the tuning of the early steps of elicitor-induced oxidative response.

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