RESEARCH PAPER

Early changes in membrane permeability, production of oxidative burst and modification of PAL activity induced by ergosterol in cotyledons of *Mimosa pudica*

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Abstract

Ergosterol (a fungal membrane component) was shown to induce transient influx of protons and membrane hyperpolarization in cotyledonary cells of *Mimosa pudica* L. By contrast, chitosan (a fungal wall component with known elicitor properties) triggered membrane depolarization. In the processes induced by ergosterol, a specific desensitization was observed, since cells did not react to a second ergosterol application but did respond to a chitosan treatment. This comparative study correspondingly shows that ergosterol and chitosan were perceived in a distinct manner by plant cells. Generation of O$_2^-$, visualized by infiltration with nitroblue tetrazolium, was displayed in organs treated with ergosterol and chitosan. This AOS production was preceded by an increase in activity of NADPH oxidase measured in protein extracts of treated cotyledons. In all the previously described processes, cholesterol had no effect, thereby indicating that ergosterol specifically induced these physiological changes known to participate in the reaction chain activated by characteristic elicitors. Contrary to chitosan, ergosterol did not greatly activate secondary metabolism as shown by the small change in content of free phenolics and by the low modification in activity of PAL, the key enzyme of this metabolic pathway. Therefore, future studies have to clarify the signalling cascade triggered by ergosterol recognition.

Key words: Elicitor, ergosterol, oxidative burst, PAL, phenolics, plant defence.

Introduction

The interaction of plant cells with pathogenic microorganisms involves rather complex physiological and biochemical reactions. In particular, plant cells possess sensitive perception systems for compounds, named elicitors, released from pathogenic fungi and plant cell walls. Oligosaccharide fragments, glycopeptides, peptides, fatty acids, and ergosterol have been identified as fungal compounds displaying elicitor activity in various plant systems (Dixon and Lamb, 1990). Following binding to receptors, these elicitors induce a signal transduction cascade leading to defence responses (Dixon and Lamb, 1990; Ryan and Farmer, 1991). Within this cascade, three characteristic physiological points need to be considered in the response of plants to fungal attacks. The earliest response to elicitors is indicated by changes in membrane permeability and activation of specific ion channels leading to the influx of Ca$^{2+}$ and H$^+$ and the efflux of Cl$^-$ and K$^+$ (Dixon et al., 1994; Cervone et al., 1997). This step is also characterized by rapid changes in transmembrane potential. In numerous cases, depolarization of the membrane was noted (Mathieu et al., 1966, 1991; Katou et al., 1982; Pelissier et al., 1986; Mayer and Ziegler, 1988; Thain et al., 1995; Kuchitsu et al., 1993). These membrane-located effects were followed by the oxidative burst, the synthesis of phytoalexins, and the activation of defence genes (Templeton and Lamb, 1988; Pugin and Guern, 1996).

It had previously been shown that a factor released from *Cladosporium fulvum* spores and identified as ergosterol was perceived by means of a suspension of tomato cells in culture (Granado et al., 1995). In particular, this compound induced a H$^+$ influx evident from the alkanization

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Abbreviations: AOS, active oxygen species; FC, fusicoccin; FW, fresh weight; JA, Jasmonic acid; NBT, nitroblue tetrazolium; PAL, phenylalanine ammonia lyase; SA, salicylic acid.

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of the growth medium. This process shows a refractory period. In a previous study conducted on pulvinar motor cells of *Mimosa pudica*, similar results were found concerning H⁺ influx in response to ergosterol. However, contrary to the electrophysiological data obtained with other elicitors, it was found that ergosterol hyperpolarized the motor cell membrane (Amborabé et al., 2003). Among different hypotheses, it has been suggested that this result may be due to a characteristic response of the highly specialized status of the pulvinal contractile cell. It is shown here that this was unlikely to be the case, since similar responses were obtained on the common cotyledonary parenchyma cell of *Mimosa pudica*. Based on the measurement of pH variations in a bathing medium and on transmembrane electrophysiological recordings, the characteristics of the ergosterol perception system in this type of cell are described. In addition, this paper shows that ergosterol triggered AOS production derived from a NADPH oxidase activation, but did not induce the secondary metabolism through PAL activation. This last result was validated by the observations made after treatment with chitosan, a major component of fungal cell walls known to produce characteristic reactions of elicitation, in particular, phenolic synthesis and PAL activation (Hadwiger and Beckman, 1980; Köhle et al., 1984).

Materials and methods

Plant materials

Sensitive plants (*Mimosa pudica L.*) were grown in an organic compost and watered daily. They were kept in climate-controlled chambers at 27.5±0.5 °C and 65±5% relative humidity. Illumination was regulated so as to give 16 h of light (photophase 06.00–22.00 h) provided by fluorescent tubes (mixing Osram fluora and Osram daylight types) with a photon flux density (400–700 nm) of 36 μmol m⁻² s⁻¹ at the plant apex. Plantlets were used when the stem began to grow over the cotyledons.

Measurement of pH variations

Cotyledons (about 400 mg FW) were excised from *Mimosa pudica L.* plantlets and the lower epidermis was peeled off. The organs were preincubated for 1 h in 6 ml of medium M: 0.5 mM CaCl₂, 0.25 mM MgCl₂. The organs were then transferred to 6 ml fresh medium M. Variations of the pH of the incubation medium were read on a pH-meter (Expandomatic SS2; Beckman; Roissy, France) and linked to a potentiometric recorder. The incubation medium was aerated by a fine forceps. The cotyledon thus prepared was fixed to the bottom of a 3 ml Plexiglas chamber filled with a buffered medium (10 mM MES, pH 5.5) containing 1 mM NaCl, 0.1 mM KCl, and 0.1 mM CaCl₂ (Abe, 1981). The glass microelectrode was impaled into a cell of the organ. Under these conditions, the resting transmembrane potential was in the range of –105 to –140 mV, the calculated value from 40 assays being –124±16 mV (SD).

**O₂⁻ generation and observation in cotyledons of Mimosa pudica L.**

Six cotyledons were immersed in 5 ml of a reaction mixture (containing 20 mM MES at pH 5.5, 0.5 mM CaCl₂, 0.25 mM MgCl₂, 0.1% Tween 20) supplemented with the studied elicitor. The cotyledons were vacuum-infiltrated with the required solution for 2 min, and then slowly returned to atmospheric pressure. The infiltrated cotyledons were incubated in the presence of elicitor for different durations at 25 °C in the light (36 μmol m⁻² s⁻¹). Histochemical staining for O₂⁻ generation and observation in cotyledons of *Mimosa pudica* L. was excised from the hypocotyl at the base of the pulvinus. The upper epidermis was peeled off with fine forceps. The cotyledon thus prepared was fixed to the bottom of a 3 ml Plexiglas chamber filled with a buffered medium (10 mM MES, pH 5.5) containing 1 mM NaCl, 0.1 mM KCl, and 0.1 mM CaCl₂ (Abe, 1981). The glass microelectrode was impaled into a cell of the organ. Under these conditions, the resting transmembrane potential was in the range of –105 to –140 mV, the calculated value from 40 assays being –124±16 mV (SD).

**Determination of NADPH oxidase activity**

Cotyledons were infiltrated with a solution containing 20 mM MES (pH 5.5), 300 mM sorbitol, 0.5 mM CaCl₂, 0.25 mM MgCl₂, 0.1% Tween 20, and the tested compound (chitosan at 250 μg ml⁻¹, 20 μM ergosterol, or 20 μM cholesterol) for various times. Proteins were extracted as previously described (Fleurat-Lessard et al., 1993). Briefly, cotyledons were washed in the appropriate buffer and ground using a pestle and mortar with 1 g quartz and 4 ml of an extraction solution (pH 7.4) (50 mM TRIS–HCl, 10 mM dithiothreitol, 4 mM thiourea, 200 mg ml⁻¹ polyvinylpyrrolidone, 1 mM phenylmethylsulphonyl fluoride dissolved in 10% isopropanol (w/v), and 100 μg ml⁻¹ polyethylene glycol 20 000) for grinding. The cell homogenate was centrifuged for 20 min at 15 000 g. The supernatant was purified on a PD 10 column (Amersham). The protein content of the extracts was measured according to the method of Bearden (1978).

NADPH-dependent O₂⁻ generation was measured by using the tetrazolium dye NBT as an electron acceptor (Bielski et al., 1980). NBT is rapidly converted to monoformazan by two molecules of O₂⁻. This reduction was detected spectrophotometrically (Beckman DU-65 spectrophotometer) at 530 nm. Monoformazan concentrations (and therefore O₂⁻ concentrations) were calculated using an extinction coefficient of 12.8 mM⁻¹ cm⁻¹. The NBT reduction rates were strictly linear with time up to 10–15 min and were linearly dependent on the protein concentration (25–150 μg) in the sample. The relative reduction of NBT reduction rate was determined in the presence and absence of superoxide dismutase (SOD) (60 units ml⁻¹). The reaction mixture consisted of 50 mM TRIS–HCl buffer (pH 7.4) and 250 mM sucrose. NADPH (0.1 mM) and SOD (60 units ml⁻¹), respectively, were added at 2 min and 8 min to a reaction mixture containing 800 μl of TRIS buffer and 200 μl of the protein extract (100 μg protein).
Determination of phenolic content and PAL activity

Cotyledons were infiltrated with the compounds for various durations (from 3 h to 48 h) as previously described. Extraction of phenolic compounds was made according to Daayf et al. (1997) with some modifications. At the end of treatment, 20 cotyledons were weighted (about 300 mg FW), homogenized in 10 ml absolute methanol in the presence of 1 g quartz. The plant material was extracted for 30 min at 60 °C and then centrifuged for 15 min at 12 000 g. The supernatant was recovered and the pellet resuspended and washed (about 300 mg FW), homogenized in 10 ml absolute methanol in modifications. At the end of treatment, 20 cotyledons were weighted on H+ efflux. Under such conditions, 10

Thus, the pH value fell in 6 h from 6.0 to a steady-state lasting about 1 h, the medium became constantly acidic. The same phenomenon of desensitization was also found described in detail in a previous work on Mimosa pulvinar cells (Amborabé et al., 2003): an initial pH increase induced by 10 μM ergosterol was not followed by a second response subsequent to the application of 20 μM ergosterol 115 min later (Fig. 2A). In contrasting manner, the cells thereby treated remained fully responsive to a chitosan application (Fig. 2B). This effect was specific, since the initial addition of cholesterol did not hinder the response triggered by a subsequent application of ergosterol (Fig. 2C).

These results clearly indicate that chitosan and cholesterol did not interfere with ergosterol perception.

Electrophysiological modifications induced by ergosterol

Figure 3 shows typical electrophysiological recordings noted after the application of ergosterol on peeled cotyledon. Ergosterol induced a hyperpolarization of the cells, whereas cholesterol was without marked effect and chitosan induced a depolarization. Table 1 provides quantification of these effects. The weak response obtained with cholesterol may be attributed to the ethanol effect needed to solubilize the different sterols, as observed in the control experiments (Fig. 3C).

In this model, the process of desensitization previously noted on induced H+ efflux was also observed: initial application of 10 μM ergosterol hindered the expected
hyperpolarization upon a second application of the product (even at a 20 μM concentration), but did not modify the depolarization induced by chitosan (Fig. 3A). This response is also specific to ergosterol since cholesterol did not modify the response induced by a subsequent application of 10 μM ergosterol (Fig. 3B). Fusicoccin (10 μM) added at the end of each experiment triggered a strong hyperpolarization of the cell, the membrane potential reaching an equilibrium around –180 mV in 30 min. This effect of fusicoccin ascertained the bioelectrical viability of the impaled cell and validated the results obtained with treatment showing no induced effect.

Induction of oxidative burst by ergosterol

NBT staining visualizes elevated levels of AOS (in particular O$_2^-$) which turn the dye into an insoluble blue formazan product (Doke and Ohashi, 1988). Figure 4 shows the development of O$_2^-$ formation which was visualized by means of this histochemical method in the treated cotyledons. Ergosterol induced the formation of a blue darkening of the tissues. This positive NBT staining was obvious within 2 h, increased in intensity and extent during the following 12 h, and declined thereafter. Similar observations were made when the cotyledons were treated with chitosan, but the time-course was slightly different since the decline in staining began earlier (10 h). A limited and variable amount of positive NBT staining was sometimes evident near the pulvinar area indicating a probable reaction at the wounding site following sampling of the organs. It may be noted that a transient staining may appear in cholesterol-treated organs, but this occurred later after application of this sterol (nearly 12 h).

Modification of NADPH oxidase activity

Extracts from cotyledons treated with ergosterol for various durations exhibited NBT-reducing activity in the presence of NADPH. The activity was inhibited by the addition of SOD in the reaction mixture, suggesting the involvement of a O$_2^-$ generating reaction in the reduction of NBT. This
result may be linked to increased activity of the NADPH oxidase. Figure 5 shows the time-course of the enzyme activity in tissues treated for various times with the different effectors. Chitosan, ergosterol, and FC to a lower extent, triggered an increase of the activity (respectively 92, 75, and 32%) during the first 30 min. After that, the NADPH oxidase activity decreased. By contrast, treatment with cholesterol did not modify the enzyme activity.

Synthesis of phenolics and modification of PAL activity

Figure 6 shows that the level of phenolics increased rapidly in chitosan-treated sets for up to 24 h. This increase was 70% compared with the initial content. The effect was lower after treatment with ergosterol, the increase totalling only 16%. The preceding data can be paralleled with the variation in PAL activity (Fig. 7). The enzyme activity was greatly increased after treatment with chitosan. This effect was visible after only 6 h treatment and an activation of 207% was achieved after 15 h. By contrast, ergosterol induced a much lower, but significant, increase in activity (46% after 15 h). In comparison with the controls, cholesterol did not modify the activity over the duration of the experimental period. PAL activity increased slightly in all experimental sets after 6 h treatment, an effect that can be related to tissue ageing.

Taken together, these data indicate that, unlike chitosan, ergosterol did not trigger an extensive activation of the phenylpropanoid pathway.

![Fig. 4. Visualization of O2⁻ by localized accumulation of blue formazan in peeled cotyledons of Mimosa pudica. Organs were treated for increasing durations with chitosan at 250 µg ml⁻¹ (K), 20 µM ergosterol (E), and 20 µM cholesterol (CL). Control cotyledons were treated with 0.5% ethanol (C). The experiment were carried out four times on six cotyledons by set.](image)

![Fig. 5. Representative time-course of NADPH oxidase activity in peeled cotyledons of Mimosa pudica treated for various durations with 20 µM ergosterol (E), 20 µM cholesterol (CL), chitosan at 250 µg ml⁻¹ (K), 10 µM fusicoccin (FC), and 0.5% ethanol (control, C). The experiments were conducted at least three times with the same general result.](image)

![Fig. 6. Changes in phenolic content of peeled cotyledons of Mimosa pudica treated for various durations with 20 µM ergosterol (E), 20 µM cholesterol (CL), chitosan at 250 µg ml⁻¹ (K), and 0.5% ethanol (control, C). Means of four experiments with triplicate samples ±SD; n=12. Data in insert represent the increase in phenolic content obtained from difference t²₄–t₀.](image)

![Fig. 7. Changes in PAL activity of peeled cotyledons of Mimosa pudica treated for various durations with 20 µM ergosterol, 20 µM cholesterol, chitosan at 250 µg ml⁻¹, and 0.5% ethanol (control). Means of three experiments ±SD; n=3.](image)
Discussion

Many fungal components secreted or released from wall and membrane surfaces may potentially play a role in the induction of plant defence responses. These molecules with different structures (oligosaccharides, peptides, lipids) are initially recognized by the plant cell at the membrane site. Following this recognition, a cascade of metabolic changes leads to the formation of physical and chemical barriers against spreading of the pathogen. Changes in plasma membrane properties, as shown by modifications of ion fluxes (namely \(H^+\), \(Ca^{2+}\), \(K^+\), and \(Cl^-\)) are the earliest responses of the plant cell to microbial elicitors (Dixon et al., 1994). In many cases, the perception of the fungal elicitor is characterized by a modification of proton flux shown by a transient alkalinization of the incubation medium (Dixon et al., 1994; Mathieu et al., 1996; Pugin et al., 1997). A rise in pH was correspondingly observed following the application of ergosterol on cotyledons of \(M.\) \(pudica\), in the same way as the modifications previously noted on pulvinar motor cells of \(M.\) \(pudica\) (Amborabé et al., 2003) and on tomato cells (Granado et al., 1995). However, the cotyledon model appears much less sensitive than the above-mentioned materials, in particular, tomato cells, if the effective concentration, the lag phase, and the extent of \(H^+\) uptake are taken into consideration. This low sensitivity may be linked to the presence of thick cell walls able to trap exogenous compounds and, therefore, needing higher product concentration in order to be active. A second possibility is that, compared with the other models, cotyledonary cells possess a low receptor density on their membrane surface. A corroborating argument may be derived from the fact that chitosan induced pronouncedly straight responses, in particular a great and rapid \(H^+\) uptake.

By contrast, the response induced by ergosterol on membrane potential completely differs from the manifestations induced by chitosan. Indeed, ergosterol hyperpolarized the cell membrane, whereas chitosan depolarized it. This result corroborated previous ones obtained on pulvinar cells that also showed the dose-dependence of this process (Amborabé et al., 2003). This finding indicated that the underlying mechanism differs basically according to the elicitor and, that, in this particular case, the \(H^+\) influx cannot be explained in the same way. With ergosterol, the causal link between the two events is not direct, since a \(H^+\) entry in cells might correspond with a membrane depolarization. Therefore, it is postulated that ergosterol triggers another ion disturbance, either a release of a cation or an uptake of an anion. Further work will investigate this question.

The results presented here generalize the previous ones on pulvinar motor cells concerning the phenomenon of desensitization that has taken place both for \(H^+\) flux and for electrophysiological events. These results argue for the existence of specific receptors for ergosterol. It is now necessary to determine whether some of the high-affinity binding sites for sterol demonstrated in plasma membrane from sugar beet (Rossard et al., 2003) may correspond to specific bindings of ergosterol. These results also argue in favour of the existence of a particular receptor for chitosan which has been a controversial point (Kauss et al., 1989).

In most cases, such proton influx resulted from the inhibition of \(H^+\)-ATPase (Young et al., 1982; Hagendoorn et al., 1991; Schaller and Oecking, 1999) playing the role of a switch between the elicitor recognition and the pathogen defence-signalling pathways. An alternative explanation comes from data obtained in the case of cryptogein action (Pugin et al., 1997). Cytoplasmic acidification of tobacco cells induced by this elicitor has been correlated with the activation of the plasma membrane redox system through the activation of NADPH oxidase.

More recent data, however, have shown that inhibition of NADPH oxidase resulted in the inhibition of the oxidative burst without modification of the proton flux (Simon-Plas et al., 2002). Whatever the step involved in the action of NADPH oxidase, it is assumed that this enzyme plays a key role in the respiratory burst which is a characteristic process exhibited by plant cells in response to pathogen challenge (Low and Merida, 1996; Wojtaszek, 1997). Treatment of cotyledons with ergosterol and chitosan led to significant increases in the activity of the enzyme. By contrast, the fact that cholesterol did not induce such changes in its activity indicates that, among sterols, ergosterol might act specifically. This increased enzyme activity has been associated with AOS production following the recognition of numerous fungal elicitors (Doke et al., 1996; Pugin et al., 1997; Park et al., 1998). AOS synthesis had previously been noted after application of ergosterol in tobacco suspension cells (Kasparovsky et al., 2003) and in hypocotyls from etiolated cucumber (Kauss and Jeblick, 1996). Development of AOS production was visualized by histochemical staining in cotyledons treated with ergosterol as well as with chitosan. It is stressed that \(\text{in vivo}\) staining appears stronger and more lasting than expected by considering the kinetics of NADPH oxidase activity. This effect might be explained by the accumulation of the dye and its possible compartmentation in the plant cell. Thus, a marked \(O_2^-\) activity was achieved 6 h after the start of treatment. The subsequent fading in staining suggests that SOD and catalase might then be activated. This point requires further verification. Moreover, data obtained with FC may be informative since it has been shown that the toxin treatment induced several components of the plant-pathogen resistance response (Singh and Roberts, 2004). In particular, FC induced a transient induction in gene expression for a homologue of NADPH oxidase (Frick and Schaller, 2002). In this study, FC activated the NADPH oxidase step, but this activation was, however, lower than those induced by ergosterol and chitosan. Furthermore, the respective effects of the three effectors on membrane potential and on
pH variations question the scope of the model proposed by Beffagna et al. (2005) to explain the NADPH oxidase activation. Indeed, since ergosterol and FC hyperpolarized the cell membrane and chitosan depolarized it, it is suggested that the enzyme activation should not be substantially linked to the induced membrane potential change. On the other hand, the pH variations noted with FC (H\(^+\) efflux), compared with those observed with ergosterol and chitosan (H\(^+\) influx), strongly support the hypothesis of a relevant role for the pH\(_{cyt}\) changes in influencing the development of the oxidative wave.

A main result of this work shows that ergosterol does not greatly activate the phenylpropanoid pathway involved following recognition of many elicitors by plant cells (Graham and Graham, 1996). This finding is shown by a smaller change in content of free phenolic compounds and by a low modification in PAL activity, the key enzyme of this metabolic pathway (Maina et al., 1984; Farmer, 1985; Campbell and Ellis, 1992). It is likewise validated by the comparative effects observed after elicitation by chitosan: a strong increase in phenolic synthesis and a strong activation of PAL, shown previously on other materials (Hadwiger and Beckman, 1980; Walker-Simmons et al., 1983; Köhle et al., 1984). A major point concerning the signalling remains to be investigated. The observation that PAL was not activated did not completely exclude the possibility that SA might intervene, since an alternate SA-synthesis pathway involving chorismate has been found (Shah, 2003). Work on nsLTP, metabolites possibly intervening in plant defence mechanisms (Molina et al., 2000) argue for the involvement of JA. Thus, treatment with ergosterol and jasmonic acid on grape cells induced the accumulation of nsLTP mRNAs, whereas salicylic acid did not induce a significant increase of transcripts (Gomès et al., 2003). This hypothesis is also reinforced by the results of Kasparovsky et al. (2004) on tobacco suspension cells showing that ergosterol specifically activated phospholipase A2, an enzyme linked to jasmonic acid synthesis (Chapman, 1998). In order to understand the ergosterol signalling in plant defence, the expression of defence genes in whole plants is likely to constitute a promising line of future research.

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