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Research article

The defence elicitor AsES causes a rapid and transient membrane depolarization, a triphasic oxidative burst and the accumulation of nitric oxide

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ABSTRACT

The newly characterized elicitor AsES obtained from *Acremonium strictum* induces a strong defence response in strawberry plants and confers plants resistance against the fungal pathogen *Colletotricum acutatum* the casual agent of anthracnose disease. Previous studies showed that AsES causes the accumulation of reactive oxygen species (ROS) that peaked 4 h post treatment (hpt), but due to the experimental approach used it was not clear whether the accumulation of ROS observed was intracellular or extracellular or took place as a single peak. By using a different experimental setup, a more complex early events associated to the activation of the innate immunity were observed. In this paper we report that strawberry plant cells treated with AsES exhibits a triphasic production of H_2O_2 and a rapid intracellular accumulation of NO. The first phase consists in a progressive extracellular accumulation of H_2O_2 that starts immediately after the treatment with AsES and is preceded by a rapid and transient cell membrane depolarization. During this phase takes place also a rapid intracellular accumulation of NO. Microscopic observations of mesophyll cells treated with AsES reveals that NO accumulates at the chloroplast. After the first extracellular H_2O_2 production phase, two intracellular H_2O_2 accumulation events occur, the first 2 hpt, and the second 7 hpt. Cells treated with AsES also show a transient increase of ion leakage, and a progressive alkalinization of the extracellular medium.

1. Introduction

In nature, plants are embedded in ecosystems where growth and survival depend on numerous factors. Microorganisms are one of the most important biotic factors interacting with plants, many of which are pathogens that can cause different diseases. Despite of this, plants can still survive due to the activation of complex defence machinery which helps them to repel the attackers. However, the activation of the defence artillery depends on events that should take places early during the plant–microbe interaction. At this stage of the interaction compounds produced by the pathogens, the plant or both, called elicitors or effectors, change the normal plant metabolism to another one that prepares the plant to repel the invader.

It is well documented that pathogenic fungi are able to deliver elicitors in the apoplastic or intracellular medium of host plants, that may bind to specific host proteins called “elicitor targets” (Dodds and Rathjen, 2010) to activate the plant immune system. Elicitors called “pathogen associated molecular patterns” (PAMPs) need to be recognized by pattern recognition receptors (PRRs) located at the extracellular surface of the host plant cell in order to activate a defence mechanism called PTI (as for PRR-trigger immunity) (Stergiopoulos and de Wit, 2009). The PTI involves the occurrence of series of early biochemical events that take place in plant cells such as H^+ and Ca^{2+} influx, and the generation of reactive oxygen species (ROS), mainly hydrogen peroxide (H_2O_2) and oxygen superoxide ($O_2^{\bullet -}$), that constitute a typical metabolic defence activation fingerprint (Apostol et al., 1989; Bolwell and Daudi, 2009).

We have previously reported that strawberry (*Fragaria ananassa*) plants of the cultivar Pájaro can be protected from a virulent

Abbreviation: AsES, *Acremonium strictum* strawberry elicitor; AU, arbitrary units; H2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; DAF-FM-DA, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate; diSC3-5, 3,3′-dipropylthiacarbocyanine iodide; MES, 2-(N-morpholino) ethanesulfonic acid; NO, Nitric oxide; Pyranine, 8-hydroxypyrene-1,3,6-trisulfonic acid trisodium salt.

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isolate (M11) of *Colletotrichum acutatum* responsible of the anthracnose disease (Freeman and Katan, 1997), when they are pretreated with the avirulent isolates F7 (Salazar et al., 2007) or M23 (Chalfoun et al., 2011) of *Colletotrichum fragariae*. These authors have shown that the protection effect observed is due to the activation of a strong defence response induced by the avirulent isolates F7 and M23, and that are preceded by a noticeable accumulation of H_2O_2 and $O_2^{\bullet -}$; in contrast, plants infected only with the virulent isolate (M11) do not exhibit ROS accumulation (Salazar et al., 2007; Chalfoun et al., 2011).

Similar protection effect against the virulent isolate M11 was also observed when strawberry plants are treated with a protein called AsES obtained from a local isolate (SS71) of the fungus *Acremonium strictum* (Chalfoun et al., 2013). Further studies revealed that AsES is a 34 kDa protein (283 amino acids residues, pI 8.8) that belongs to the subtilisin protease family and exhibits plant defence response and protease activity (Chalfoun et al., 2013; Pat.N°AR/10.03.11/ARA P110100854).

It was reported that Arabidopsis and strawberry plants treated with AsES induce a noticeable peak accumulation of H_2O_2 and $O_2^{\bullet -}$ 4 h post treatment (hpt). However, since the detection of ROS was carried out *in planta* that requires many steps in tissue manipulation, faster and subtler metabolic processes involving ROS are undetectable or overlooked. Accordingly, in this work we investigated the kinetic of ROS accumulation by using an experimental setup that allowed us a better visualization of faster and subtler processes that take place when strawberry plants were treated with the protein AsES. The experimental setup consists in using a cell suspension obtained from mesophyllic cells of strawberry leaves that proved to be highly reliable and reproducible. Using mesophyll cell suspensions we evaluated the time course of H_2O_2 and NO accumulation after AsES treatment. This system also permitted us to detect the effect of AsES on cell membrane potential, ion leakage, and changes of extracellular pH.

2. Materials and methods

2.1. Plants and growth conditions

Strawberry plants of the cv. Pájaro were obtained from the Active Germplasm Bank at National University of Tucumán, Argentina. Plants were *in vitro* propagated according to Vellicce et al. (2003), transferred to sterile substrate (humus:perlome, 2:1) and maintained in growth chambers at 28 °C, 70% relative humidity (RH) with a light cycle of 16 h day^{-1} (white fluorescent, 250 μmol m^{-2} s^{-1}).

2.2. Preparation of the elicitor AsES

The elicitor protein AsES was purified from the supernatant of *A. strictum* fungus according to Chalfoun et al. (2013). Briefly, 5 L culture of the isolate SS71 of *A. strictum* grown up the stationary phase (21 days, 25 °C under fluorescent light) was centrifuged, filtered, vacuum concentrated 30 times and passed, firstly through an ion exchange Q-Sepharose fast flow column (Q-FF 1 mL, Amersham Biosciences), and the top fraction passed through an hydrophobic phenyl-Sepharose high performance column (PS-HP 5 mL, GE Healthcare). The fraction (peak) showing oxidative burst inducing activity was collected and vacuum dried. Water dilution of this fraction containing the purified protein was used in experiments.

2.3. Plant cell suspension

Suspension of mesophyll cells were obtained from young recently expanded leaves of 8 week old strawberry plants. Leaf were homogenized in a potter with 10 mL of W5 modified buffer (154 mM NaCl, 5 mM KCl, 125 mM $CaCl_2$, and 0.5 M sucrose, 2 mM MES, pH 5.6) (Fujikawa et al., 2014; Ramulu et al., 1995), filtered through gauze, and them separated by centrifugation (500× g for 5 min at room temperature). Cells were then washed twice with a sucrose solution (0.5 M) and resuspended in W5 modified buffer at 1×10^6 cells mL^{-1} (Newbauer chamber). The cell suspension was kept in the dark at room temperature 3 h until use. Cell viability was evaluated in cell suspension samples (20 μL, 1×10^6 cells mL^{-1}) obtained at different times after the extraction and treated with Evans Blue dye 0.02% for 10 min (Zhou et al., 2005). The percentage of stained cells was counted in a Newbauer chamber using a light microscope (Leica DM, U.S.A.).

2.4. Oxygen consumption

The oxygen consumption was measured in 1×10^6 cells suspended in a mineral buffer (370 mg $mL^{-1}MgSO_4.7H_2O$, 0.025 mg mL^{-1} $CuSO_4.5H_2O$ 27.8 mg mL^{-1} $FeSO_4.7H_2O$, 22.3 mg mL^{-1} $MnSO_4.4H_2O$, MES 10 mM, pH 7.0), using an Oxigraph-2 K (Oroboros Instruments, Austria) at 25 °C temperature and 100 rpm agitation. Respiration was interrupted with 50 μL of KCN 5 mM.

2.5. Extracellular H_2O_2 determination

The extracellular accumulation of H_2O_2 was evaluated using the fluorescent probe H2DCF (Sigma–Aldrich) according Myhre et al. (2003). H2DCF is a fluorescent probe that reacts with ROS (mainly with H_2O_2) and is not permeable to the cell membrane; therefore was used to detect extracellular H_2O_2. Aliquots of cell suspension (1×10^6 cells mL^{-1}) were mixed with H2DCF (10 μM) and treated with AsES (10 nM) or distilled water as a control. The change of fluorescence was measured continuously with an ISS-APC1 Photon Counting spectrofluorometer (Owingen, Germany) at λex = 485 nm and λem = 522 nm.

2.6. Intracellular H_2O_2 determination

The accumulation of H_2O_2 was evaluated using the fluorescent probe H2DCF-DA (Sigma–Aldrich) (Li et al., 2012; Ye et al., 2013). H2DCF-DA is a fluorescent probe that reacts mainly with intracellular H_2O_2 but only after being transported and deacetylated by cell membrane diacetylases.

Since we were interested to evaluate the change of ROS during 8 h, and the fluorescence produced by the radical 2′,7′-dichlorodihydrofluorescein (H2DCF-) after the reaction with H_2O_2 is unstable (Bonini et al., 2006) and irreversible (Kristiansen et al., 2009), we used a pulse and chase approach to evaluate the H_2O_2 production at different times after AsES treatment. Cell suspension aliquots of 100 μL (1×10^6 cells mL^{-1}) were treated with AsES (10 nM) and at different times 1.5 μL of H2DCF-DA (10 μM) was added, mixed and incubated at 25 °C in the dark for 15 min before measuring. Fluorescence was measured at λex = 485 nm and λem = 525 nm with Photon Counting spectrofluorometer (ISS-APC1, Owingen, Germany). Control experiments consisted in cell suspension treated with water.

2.7. Intracellular Nitrogen oxide determination

The intracellular NO accumulation was observed by fluorescence microscopy according to Zeidler et al. (2004). Cell suspension (200 μL) containing 1×10^6 cells mL^{-1} in W5 buffer was left in the dark during 3 h. After that time DAF-FM-DA (1 μM), and AsES

(10 nM) were added, mixed and immediately observed under fluorescent microscopy using an Olympus microscope mod.BH-FRC (U.S.A.) with an IB filter (λex = 490 nm). Control consisted in water treated cells. The intracellular NO was also analyzed in cell suspension by fluorometry according to Planchet and Kaiser (2006), modified in our laboratory. Aliquots of 1500 μL of cell suspensions (1×10^6 cells mL^{-1}) in W5 buffer were left in the dark during 3 h. After that time the suspensions were incubated with the fluorescent probe DAF-FM-DA (1 μM) during 30 min in the dark. Then the cells were washed once with W5 buffer and treated with AsES (10 nM). The control consisted in cells treated with distilled water. Fluorescence was measured 10 min post treatment at λex = 495 nm and λem = 515 nm with a Photon Counting spectrofluorometer (ISS-APC1, Owingen, Germany).

2.8. Membrane depolarization

The change of cell membrane polarization was evaluated using the fluorescent probe diSC3-5 according to Filippone et al. (2001) modified in our laboratory. 1.5 mL of cell suspension (1×10^6 cells mL^{-1}) was treated with diSC3-5 (4 μM) and left 10 min of stabilization. After that time AsES (10 nM) was added, and fluorescence immediately recorded at 25 °C. Measurements were performed with a low agitation (100 rpm). The ionophore nigericin (50 μM) was added 50 min later as a control of membrane depolarization. A cell suspension without AsES treatment was used as control. Fluorescence was measured continuously using a ISS-APC1 Photon Counting, (Owingen, Germany) spectrofluorometer at λex = 622 nm and λem = 670 nm.

2.9. Ion leakage determination

The ion leakage was evaluated according to Köhle et al. (1985) modified in our laboratory. 20 mL aliquots of 1×10^5 cells mL^{-1} suspended in a recently prepared solution (10 mM KCl, 0.1 mM $CaCl_2$, 40 mM Sucrose, pH 6.5) were treated with AsES (10 nM) or distilled water as a control. The conductivity of cell suspensions was determined continuously at 25 °C, and 100 rpm using an ADWA digital conductimeter (mod. AD31, Rumania). The conductivity is expressed as μSiemens cm^{-1}.

2.10. pH change determination

The pH shift was evaluated according to Kašík et al. (2013), and Wong and Fradin (2013) modified in our laboratory. Briefly, 1.5 mL of 1.3×10^5 cells mL^{-1} suspended in a recently prepared not-buffered solution (10 mM KCl, 0.1 mM $CaCl_2$, 0.4 M Sucrose, pH 6.5) was mixed with Pyranine (1.5 mM), and after 5 min of stabilization AsES was added (10 nM) mixed and the fluorescence measured at different times using a Perkin Elmer LS55 UK spectrofluorometer (U.S.A.) at λex = 370 nm and λem = 510 nm.

2.11. Statistical analyses

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

3. Results

3.1. Evaluation of mesophyll cell suspension

Since strawberry mesophyll cell suspension used in experiments had not been previously tested, before analyzing the effect of AsES on cell suspension, the viability and reactivity of mesophyll cells were evaluated to rule out that the accumulation of ROS is due to previous manipulation (Kristiansen et al., 2009). Preliminary experiments revealed that the mechanical stress provoked to leaf cells during the suspension preparation causes an immediate increase of the intracellular ROS content, but rapidly decreases after 3 h from the cell extraction and remains stable for at least 24 h (Fig. 1A). The loss of viability of cells suspended in W5 buffer is less than 10% during the first 24 h, and 30% at 48 h after the extraction as compared to the initial value (Fig. 1B). Microscopic observations 48 h after extraction show that cells are not damaged and maintained the typical shape expected of mesophyll parenchyma cells (see Fig. 6Ah). To further confirm that the cell suspension maintains an active metabolism the respiratory capacity was evaluated. Results obtained show that 5 h after the extraction cells maintain a constant oxygen consumption rate during 2 h and is immediately halted with KCN (Fig. 1C). These results prove that the cells can be used for experiments within the first 12 h after the extraction.

3.2. Extracellular generation of H_2O_2

Experiments conducted to detect the H_2O_2 generated extracellularly show that cell suspension exhibits an immediate and progressive increase upon AsES addition as compared with not treated cell suspension (Fig. 2). Control experiments carried out with H2DCF without cells but with externally added H_2O_2 confirmed that the deacetylated probe (H2DCF) detects exclusively the extracellular but not the intracellular H_2O_2 (see supplementary Fig. 1). However, since the fluorescence produced by H2DCF after the reaction with H_2O_2 is unstable with time (Bonini et al., 2006) and irreversible (Kristiansen et al., 2009), as mentioned above, it was not possible with this experimental approach to conclude whether the increase of H_2O_2 observed is transient or stable in time.

3.3. Intracellular ROS accumulation

To evaluate whether AsES causes also the intracellular accumulation of H_2O_2 the acetylated fluorescent probe H2DCF-DA was used. Since the accumulation of extracellular H_2O_2 takes place rapidly after AsES treatment (see above) and previous information indicated that the intracellular accumulation of ROS takes place later (Chalfoun et al., 2013), a long term evaluation of H_2O_2 accumulation kinetics was performed. Results showed that cells exhibit two clear accumulation peaks; a first sharp one 2 hpt, and another broader 7 hpt, both preceded by transient decreases 1 hpt and 5 hpt, respectively (Fig. 3). In order to rule out that the fluorescent probe H2DCF-DA detects only the intracellular H_2O_2 preliminary experiments were carried out with exogenously added H_2O_2. Results confirmed that H2DCF-DA can detect exclusively intracellular H_2O_2 whereas H2DCF cannot (supplementary Fig. 1).

3.4. Cell membrane depolarization and ion leakage

When cell suspension that had been previously treated with the fluorescent probe diSC3-5 was treated with AsES exhibited a fast and transient in fluorescence increase (Fig. 4), indicating that AsES causes a rapid and brief cell membrane depolarization. This experiment also shows that the depolarization observed is

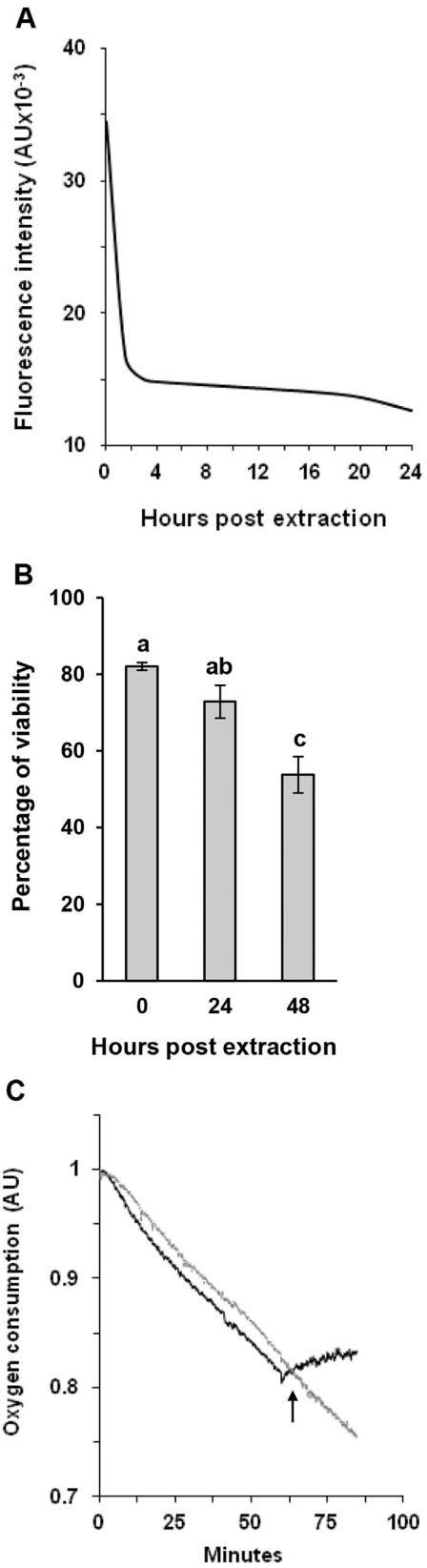

Fig. 1. Viability and respiratory capacity of strawberry mesohpyll cells after the extraction. A) H2DCF-DA fluorescence (λex = 485 nm; λem = 525 nm) produced by the intracellular H_2O_2 after cell extraction. B) Percentage of viable cells at different times. C) Oxygen consumption of strawberry cell suspension 5 h after the extraction expressed relative to the initial value. The arrow indicates the addition of KCN (1 mM); the grey line corresponds to a control experiment without the addition of KCN. (A) and (C) correspond to one of three identical experiments. Bars in (B) represent standard errors (mean value ± SE, n = 5). AU, arbitrary unit.

completely recovered after 8 min, suggesting that the endogenous mechanisms of cell homeostasis is fully active and rapidly compensates the effect induced by AsES which would otherwise bring about the collapse of cellular energy supply and transport systems. Control using nigericin as a membrane decoupler confirms the latter and indicates that the system is still reactive 40 min after the treatment with AsES.

Evaluation of ion leakage revealed that AsES brings about a transient and fast perturbation of the extracellular medium conductivity that decays along the experimental time in an oscillatory fashion. The first peak takes place 15 min after the treatment, lasts approximately 20 min, and is damped around the control value (1547 μS cm^{-1}, Fig. 5). Control experiments consisting in not treated or boiled cell suspension yielded, 1547 and 1830 μS cm^{-1}, respectively, and did not display changes during 120 min.

3.5. Effect of AsES on NO production

Nitric oxide was evaluated in cell suspension by fluorescence microscopy and fluorometry (as mentioned above). Results show that AsES causes on cells a rapid accumulation of NO (Fig. 6A). In Fig. 6Ae, 6Af we can see that the green fluorescence produced by DAF-FM-DA inside the cells increases within the first 3 min, whereas in water treated cells no green fluorescence is observed (Fig. 6Ab, Ac). Microscopic observations allow us also to observe that the NO production is located mainly at the chloroplasts (Fig. 6Ai). Evaluations of the NO accumulated during 10 min by fluorometry corroborate the microscopic observations (Fig. 6B). It is worthwhile mentioning that cells treated with AsES did not show any noticeable symptom of stress or change of the cell structure, at least during the experimental times used. Experiments carried out with leaf discs also confirm results obtained with cell suspensions (not shown).

3.6. Extracellular alkalinization

Since the fluorescence emitted by Pyranine is pH dependent, the effect of AsES on the extracellular pH was measured directly on the mesophyllic cell suspended in a not-buffered solution.

Results obtained indicate that cells treated with AsES (10 nM) exhibit a progressive alkalinization of the extracellular medium during the period analyzed (Fig. 7). Attempts to evaluate the evolution of pH for longer periods were unsuccessful because cells became seriously damage in a not-buffered solution. The increase of fluorescence with pH was previously confirmed by evaluating the fluorescence shift of Pyranine at different pHs at λex = 370 nm and λem = 510 nm (Supplementary Fig. 2).

4. Discussion

It is well known that the activation of the innate immunity is preceded by early biochemical events triggered by the interaction between an elicitor and plant components that are the elicitor targets, and are required to activate a defence signaling cascade (Boller and Felix, 2009; Garcia-Brugger et al., 2006; Thakur and Sohal, 2013; Zhaoa et al., 2005). Among those early events the oxidative burst (e.g ROS accumulation), the proton and calcium influx, and the ion efflux can be mentioned (Schwessinger and Ronald, 2012). Although the occurrence of any of those

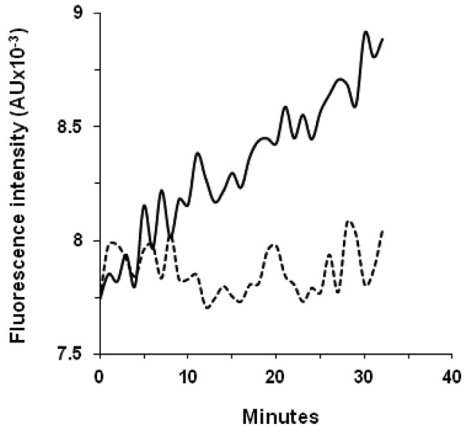

Fig. 2. Effect of AsSE on the extracellular production of H_2O_2. Strawberry mesophyll cell suspensions (1×10^6 cells mL^{-1}) in W5 buffer (pH 6.5) containing H2DCF (10 μM) were treated with 10 nM of AsES. Fluorescence was measured continuously at λex = 485 nm and λem = 522 nm. Solid line corresponds to cell treated with AsES and dashed line corresponds to untreated cells. Results correspond to one of three identical experiments. AU, arbitrary unit.

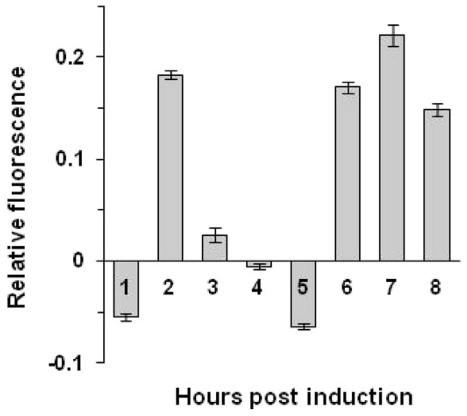

Fig. 3. Time course of the intracellular content of H_2O_2 after AsES (10 nM) treatment. Fluorescence of H2DCF-DA was evaluated at λex = 485 nm and λem = 525 nm and is reported as the change with respect to untreated cells. Values are means of five independent experiments. Bars represent standard errors (mean values ± SE, n = 5).

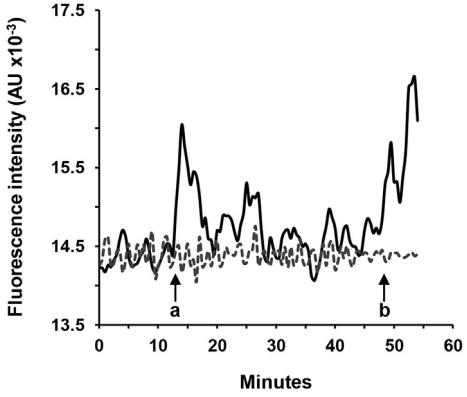

Fig. 4. Effect of AsSE on the membrane potential of strawberry cells. Fluorescence of the cell suspension (1×10^6 cells mL^{-1}) previously treated with diSC3-5 (4 μM) was followed during 60 min. The arrows indicate the moment at which (a) AsES (10 nM) or (b) nigericin (30 μM) was added. The dotted line corresponds to a control experiment without treatments. Fluorescence of diSC3-5 was evaluated at λex = 622 nm and λem = 670 nm, and is expressed in arbitrary units (AU). Results correspond to one of three identical experiments.

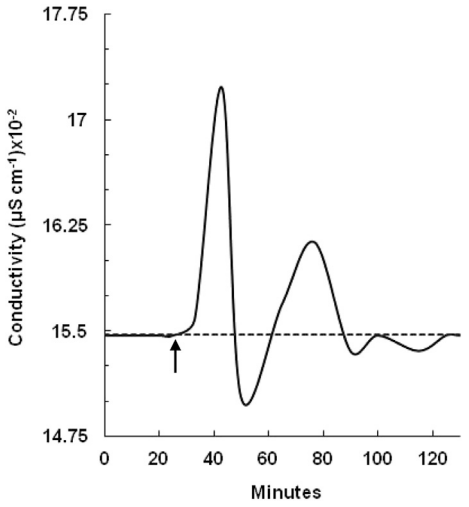

Fig. 5. Effect of AsES on ion leakage of mesophyll strawberry cells treated with AsES. Conductivity was measured directly on the cell suspension (1×10^5 cells mL^{-1}). Solid line corresponds to cells treated with AsES (10 nM), and dotted line corresponds to a control experiment without treatments (1547 μS cm^{-1}). Maximal conductivity of boiled cells was 1855 μS cm^{-1} (not shown). The arrow indicates the moment at which AsES was added. Results correspond to one of three identical experiments.

biochemical markers may not necessarily imply that the innate immunity was fully activated, it is also well documented that the suppression of any of them would seriously impair the expression of genes associated to the defence response such as: *npr1*, *pr1*, *pr3*, *pvnbs-lrr*, *pgip*, *eli7*, *tyrdc* (Jabs et al., 1997; Finkel, 2001; Pignocchi et al., 2006; Knoth et al., 2009; Oliveira et al., 2015). We can assume therefore, that ROS accumulation and proton influx are physiological fingerprints of the activation of the innate immunity.

It was reported that plants of strawberry (*Fragaria ananassa*) and *Arabidopsis thaliana* ectopically treated with AsES induce a strong oxidative burst (Chalfoun et al., 2013). A transient accumulation of both H_2O_2 and $O_2^{\bullet -}$ was observed with a peak 4 hpt (Chalfoun et al., 2013). However, since those experiments were performed *in planta* (e.g. on leaves) it was not possible to evaluate the temporal evolution of ROS with higher precision, or to discriminate whether the ROS accumulation was intra- or extracellular.

For this reason, we used mesophyll cell suspensions to evaluate some of the early events that take place outside and inside the plant cell treated with AsES. But, with the aim to obtain reliable and reproducible information, we had to test some biological parameters to rule out possible experimental artifacts that could mask results or incorporate spurious signals to the measurements. Also, to avoid any experimental artifact that may complicate the evaluation of outcomes cells from mesophyllic tissue were used instead of protoplasts or cell culture (He et al., 2007; Yoo et al., 2007). Results presented in this work show that the strawberry cell suspension is not only suitable for the programmed experiments, but also sensitive to AsES. By using this cell suspension setup, we were able to observe that the oxidative burst takes places in three stages, namely: a first fast extracellular progressive accumulation of H_2O_2 that takes place within 60 min immediately after AsES treatment, followed by two intracellular bursts 2 and 7 hpt, respectively. This pattern of ROS evolution was not observed in experiments carried out with plant leaves (Chalfoun et al., 2013).

However, it is noteworthy that although the physiological state of suspended strawberry mesophyll cells is quite different than cells embedded and packed in leaf tissues, and consequently different reactive properties might arise, they exhibited similar reactivity toward AsES. The latter led us to conclude that, although

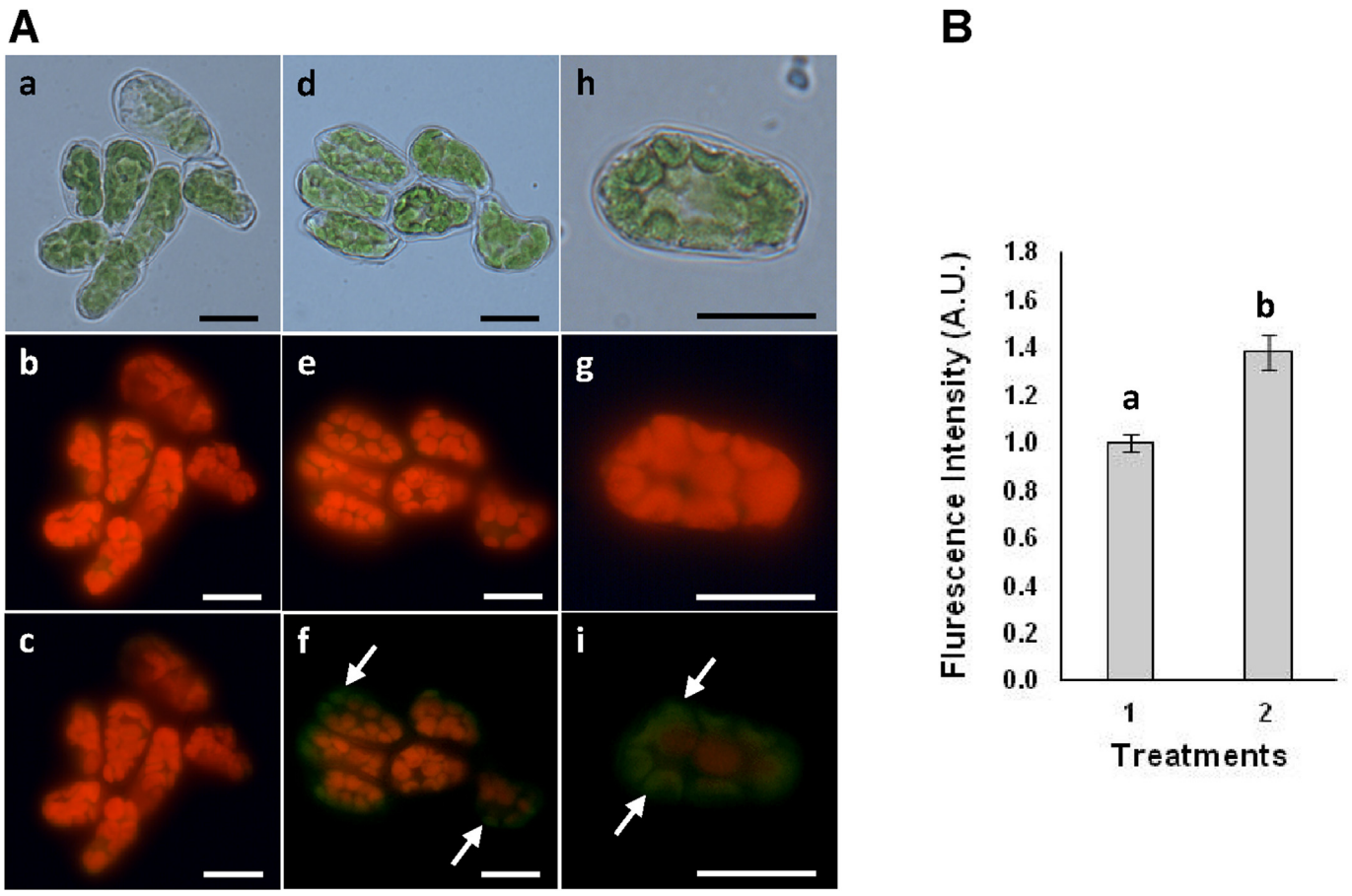

Fig. 6. Effect of AsES on the accumulation of NO in strawberry leaf cells. A) Micrographies of mesophyll cells pre-incubated with the DAF-FM-DA and treated with water as a control (a–c) or treated with AsES (d–i). (a), (d), and (h): bright field of cells treated with distilled water (a) or AsES (d, h), respectively. (b), (e), and (g): fluorescent field (blue light) of cells immediately after treatments with water (b) or AsES (e, g). (c), (f) and (i): fluorescent field of cells 3 min after treatments water (c) or AsES (f, i). (h–i) correspond to a magnified view of a cell treated with AsES. Green fluorescence indicates nitric oxide presence. Arrows indicate the green fluorescence localized in chloroplasts. B) Intracellular fluorescence intensity of DAF-FM-DA (λex = 495 nm; λem = 515 nm) in strawberry cells 10 min after treating with distilled water (1) or AsES (10 nM) (2). The values represent five repetitions. Bars represent relative errors. The comparison between two groups was made using Student's t test, $p < 0.05$. Different letters denote significant difference. Bars represent 3 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

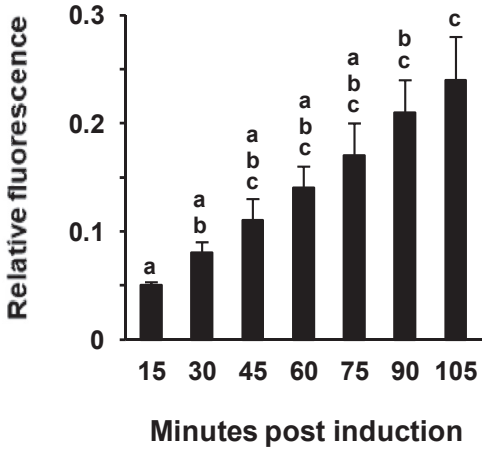

Fig. 7. Increase of Pyranine fluorescence intensity (λex = 370 nm; λem = 510 nm) of the cell suspension after AsES (10 nM) treatment (A). Relative fluorescence was evaluated as the fluorescence change with respect to the control untreated cells at different times. Values are means of three independent experiments. Bars represent standard errors (mean values ± SE, n = 3). Different letters indicate statistical significance (Student's test, $p < 0.05$).

the manipulation exposed to the tissue during the extraction, the cells retain the competence to activate the oxidative burst when treated with AsES, and brings further support to the experimental approach used.

Furthermore, it was observed that the first oxidative burst consists not only in the extracellular accumulation of H_2O_2 but also in a fast intracellular accumulation of NO within the first 10 min after AsES treatment. Microscopic observation of isolated cells let us to observe that most of the fluorescence produced by NO is concentrated in the chloroplasts. The latter suggests that AsES is able to activate a still unknown messenger that translocates rapidly to the chloroplasts where NO is synthesized. We also noticed that the chloroplastic fluorescence decays after 15 min (not shown), presumably due to NO diffusion or probe stability.

Although a biphasic response of oxidative burst was reported earlier (Wi et al., 2012, 2014), a single peak of H_2O_2 accumulation was observed by Grellet-Bournonville et al. (2012) and Chalfoun et al. (2013) when treating plants with an avirulent fungus (M23) or with AsES, respectively. These authors reported a single peak 4 hpt instead of two peaks 2 or 7 hpt. We may speculate that the failure to observe more ROS accumulation peaks in experiments carried out with strawberry plants can be attributed to the use of leaves, which turns out to be less sensitive. Consequently, what they actually observed is a fusion of both peaks. For the same

reason, it is understandable that the fast and less acute extracellular increase of H_2O_2 reported here was completely overlooked.

Attempts to evaluate the effect of AsES on the extracellular production of H_2O_2 in long term experiments were not successful due to the decrease to the fluorescent signal 60 min after the treatment. The latter suggests that the concentration of H_2O_2 becomes lower than the detection limit to the fluorescent probe, or the cells produce changes in the extracellular medium that affect the probe fluorescence emission after AsES treatment. The facts that cells treated with AsES bring about a change of the conductivity and the alkalinization the medium strongly support the latter hypothesis. Anyhow, since the increase of extracellular H_2O_2 was clear and reproducibly observed during the first 40 min we considered being a phase. The extracelluar accumulation of H_2O_2 let us further conclude that AsES does induce the activation of the NADPH oxidase as suggested by many authors (Elmayan and Simon-Plas, 2007; Zhang et al., 2009; Noirot et al., 2014; Kadota et al., 2015).

It is noteworthy that the increase of extracellular H_2O_2 observed during the first 40 min after the treatment with AsES takes place almost simultaneously than the first decrease of the intracellular H_2O_2. We may speculate therefore, that since H_2O_2 can rapidly translocate through the cell membrane in both directions (Jang et al., 2012) the extracellular H_2O_2 detected during the first minutes after AsES treatment could be a contribution not only of the NADPH oxidase activity but also of the transient efflux of intracellular H_2O_2. More experiments are required to confirm this hypothesis.

Experiments conducted to detect whether AsES could also alter the cell membrane potential show that cells treated with AsES cause a rapid and transient depolarization of the cells. The latter suggests that AsES interacts directly with the cell membrane, or there is a receptor that senses the presence of AsES, triggers the defence signaling cascade, and is immediately blocked or internalized allowing the membrane potential to recover. Currently experiments are in course in our laboratory to elucidate this issue. Since AsES triggers a transient cell membrane depolarization we were interested to evaluate whether it was associated with an ion leakage as reported by Marrè et al. (1998). Analysis of the conductivity of the extracellular medium of cell suspensions revealed that a fast and transient ion leakage takes place when treated with AsES; the generated signal is damped over the time, approaching the conductivity values displayed by not treated (control) cells. The latter suggests that although AsES causes the membrane depolarization and ion leakage these processes are transitory, and are rapidly controlled, preventing the cellular collapse, at least during the experimental time and the protein concentration used. The fact that cells can control the perturbation exerted by AsES indicates that AsES does not disrupt the membrane, causing a massive and irreversible ion efflux.

If we analyze the time in which the studied early events occur, namely: membrane depolarization, ion leakage, extracellular ROS and intracellular NO accumulation, and the decrease of intracellular ROS, we may find an interesting correlation, suggesting there is a concerted mechanism orchestrating all the cellular signals involved. Early reports have clearly shown that the ROS accumulation caused by inhibitors of NADPH oxidase was associated to a membrane depolarization and electrolyte leakage (Marrè et al., 1998). The latter would provide further support to the results presented in this paper.

It was reported that when a pathogen attacks a plant, causes the alteration of the cell redox status (Misas-Villamil and van der Hoorn, 2008), the apoplast alkalinization (Felle et al., 2005; Pignocchi et al., 2006; Wilkinson, 1999), an ion efflux (Marrè et al., 1998), and the activation of a defensive response (Felle et al., 2004). By using mesophill cell suspensions we were also able to detect most of the early events above mentioned when cells are treated with AsES. All the information presented here let as envision a metabolic scenario in which we can assume that AsES interacts with a still unknown cell surface component that activates sequentially a cascade of extracellular and intracellular signals that are rapidly propagated to other cellular compartments (i.e. chloroplasts). This signaling program would yield the induction of the plant innate immunity and an effective mechanism of plant protection against virulent pathogens as shown earlier by Chalfoun et al. (2013).

Some pathogens secrete effectors into the apoplast leading to the activation of the innate immunity (Lotze et. at., 2007; Schwessinger and Ronald, 2012). Since *Acremonium strictum* is an endophytic fungus (Morgan-Jones and Gams, 1982) we assumed initially that the protein AsES was excreted to the extracellular space where it acts like an apoplastic elicitor that activates plant defence mechanisms. The latter hypothesis was initially reinforced by the fact that the defence response observed was induced when AsES was applied ectopically on plants without wounds (Chalfoun et al., 2013), suggesting that this protein (elicitor) would interact with targets found at the apoplast (Dodds and Rathjen, 2010). However, results presented in this paper clearly show that AsES does not require the structure, or any other soluble factor that may be present in the apoplast to exert the activation effect. Although these results contribute to reject the initial hypothesis, provide a strong support to the hypothesis that AsES may be a PAMP or some other type of elicitor that does not require an apoplastic target. Results of membrane depolarization discussed above may contribute to confirm this hypothesis. However, since we did not use protoplasts in experiments, we cannot completely rule out that the AsES may still interact with some component of the wall remains left on the surface of the strawberry cells. Currently we are carrying out experiments with protoplasts of different species to answer this question.

Authors' contributions

Experimental design, and data analysis were carried out by GGM and MMT.

The experimental design, data analysis and paper writing was carried out by JCDR.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2015.10.029.

References

Apostol, I., Heinstein, P.F., Low, P.S., 1989. Rapid stimulation of an oxidative burst during elicidation of cultured plant cells. Role in defence and signal transduction. Plant Physiol. 90, 106–116.

Bonini, M.G., Rota, C., Tomasi, A., Mason, R.P., 2006. The oxidation of 2′,7′-dichlorofluorescin to reactive oxygen species: a self-fulfilling prophesy? Free Radic. Biol. Med. 40, 968–975.

Boller, T., Felix, G., 2009. A renaissance of elicitors: perception of microbe-associated

molecular patterns and danger signals by pattern-recognition receptors. Annu. Rev. Plant Biol. 60, 379–406.
Bolwell, G.P., Daudi, A., 2009. Reactive oxygen species in plant-pathogen interactions. In: del Rio, L.A., Puppo, A. (Eds.), Reactive Oxygen Species in Plant Signaling. Springer, Berlin Heidelberg, pp. 113–133.
Chalfoun, N.R., Castagnaro, A.P., Díaz Ricci, J.C., 2011. Induced resistance activated by a culture filtrate derived from an avirulent pathogen as a mechanism of biological control of anthracnose in strawberry. Biol. Control 58, 319–329.
Chalfoun, N.R., Grellet-Bournonville, C.F., Martínez Zamora, M.G., Díaz-Perales, A., Castagnaro, A.P., Díaz Ricci, J.C., 2013. Purification and characterization of AsES: a subtilisin secreted by *Acremonium strictum* is a novel plant defence elicitor. J. Biol. Chem. 288, 14098–14113.
Dodds, P.N., Rathjen, J.P., 2010. Plant immunity: towards an integrated view of plant-pathogen interactions. Nat. Rev. Genet. 11, 539–548.
Elmayan, T., Simon-Plas, F., 2007. Regulation of plant NADPH oxidase. Plant Signal Behav. 2, 505–507.
Felle, H.H., Herrmann, A., Hanstein, S., Hückelhoven, R., Kogel, K.H., 2004. Apoplastic pH signaling in barley leaves attacked by the powdery mildew fungus *Blumeria graminis* f. sp. hordei. Mol. Plant Microbe Interact. 17, 118–123.
Felle, H.H., Herrmann, A., Hückelhoven, R., Kogel, K.H., 2005. Root-to-shoot signaling: apoplastic alkalinisation, a general stress signal and defence factor in barley (*Hordeum vulgare*). Protoplasma 227, 17–24.
Filippone, M.P., Diaz-Ricci, J.C., Castagnaro, A.P., Farías, R.N., 2001. Effect of fragarin on the cytoplasmic membrane of the phytopathogen *Clavibacter michiganensis*. Mol. Plant Microbe Interact. 14, 925–928.
Finkel, T., 2001. Reactive oxygen species and signal transduction. IUBMB Life 52, 3–6.
Freeman, S., Katan, T., 1997. Identification of *Colletotrichum* species responsible for anthracnose and root necrosis of strawberry in Israel. Phytopathology 87, 516–521.
Fujikawa, Y., Nakanishi, T., Kawakami, H., Yamasaki, K., Sato, M.H., Tsuji, H., Matsuoka, M., Kato, N., 2014. Split luciferase complementation assay to detect regulated protein-protein interactions in rice protoplasts in a large-scale format. Rice 7, 11.
Garcia-Brugger, A., Lamotte, O., Vandelle, E., Bourque, S., Lecourieux, D., Poinssot, B., Wendehenne, D., Pugin, A., 2006. Early signaling events induced by elicitors of plant defences. Mol. Plant Microbe Interact. 19, 711–724.
Grellet-Bournonville, C.F., Martínez-Zamora, M.G., Castagnaro, A.P., Díaz Ricci, J.C., 2012. Temporal accumulation of salicylic acid activates the defence response against *Colletotrichum* in strawberry. Plant Physiol. Biochem. 54, 10–16.
He, P., Shan, L., Sheen, J., 2007. The use of protoplasts to study innate immune responses. Methods Mol. Biol. 354, 1–9.
Jabs, T., Tschöpe, M., Colling, C., Hahlbrock, K., Scheel, D., 1997. Elicitor-stimulated ion fluxes and $O_2^{\bullet-}$ from the oxidative burst are essential components in triggering defence gene activation and phytoalexin synthesis in parsley. Proc. Natl. Acad. Sci. 94, 4800–4805.
Jang, J.Y., Rhee, J.Y., Chung, G.C., Kang, H., 2012. Aquaporin as a membrane transporter of hydrogen peroxide in plant response to stresses. Plant Signal Behav. 7, 1180–1181.
Kadota, Y., Shirasu, K., Zipfel, C., 2015. Regulation of the NADPH oxidase RBOHD during plant immunity. Plant Cell Physiol. 56, 1472–1480. http://dx.doi.org/10.1093/pcp/pcv063.
Kašík, I., Podrazký, O., Mrázek, J., Martan, T., Matějec, V., Hoyerová, K., Kamínek, M., 2013. *In vivo* optical detection of pH in microscopic tissue samples of *Arabidopsis thaliana*. Mater Sci. Eng. C Mater. Biol. Appl. 33, 4809–4815.
Knoth, C., Salus, M.S., Girke, T., Eulgem, T., 2009. The synthetic elicitor 3,5-dichloroanthranilic acid induces npr1-dependent and npr1-independent mechanisms of disease resistance in arabidopsis. Plant Physiol. 150, 333–347.
Köhle, H., Jeblick, W., Poten, F., Blaschek, W., Kauss, H., 1985. Chitosan-elicited callose synthesis in soybean cells as a Ca-dependent process. Plant Physiol. 77, 544–551.
Kristiansen, K.A., Jensen, P.E., Møller, I.M., Schulza, A., 2009. Monitoring reactive oxygen species formation and localisation in living cells by use of the fluorescent probe CM-H2DCFDA and confocal laser microscopy. Physiol. Plant 136, 369–383.
Li, X., Ma, H., Jia, P., Wang, J., Jia, L., Zhang, T., Yang, Y., Chen, H., Wei, X., 2012. Responses of seedling growth and antioxidant activity to excess iron and copper in *Triticuma estivum* L. Ecotoxicol. Environ. Saf. 86, 47–53.
Lotze, M.T., Zeh, H.J., Rubartelli, A., Sparvero, L.J., Amoscato, A.A., Washburn, N.R., Devera, M.E., Liang, X., Tör, M., Billiar, T., 2007. The grateful dead: damage associated molecular pattern molecules and reduction/oxidation regulate immunity. Immunol. Rev. 220, 60–81.
Marrè, M.T., Amicucci, E., Zingarelli, L., Albergoni, F., Marrè, E., 1998. The respiratory burst and electrolyte leakage induced by sulfhydryl blockers in *Egeria densa* leaves are associated with H_2O_2 production and are dependent on Ca^{2+} influx. Plant Physiol. 118, 1379–1387.
Misas-Villamil, J.C., van der Hoorn, R.A., 2008. Enzyme-inhibitor interactions at the plant-pathogen interface. Curr. Opin. Plant Biol. 11, 380–388.
Morgan-Jones, G., Gams, W., 1982. Notes on Hyphomycetes. XLI. An endophyte of *Festuca arundinacea* and the anamorph of *Epichloe tvphina*, new taxa in one of two new sections of *Acremonium*. Mycotaxon 15, 311–318.
Myhre, O., Andersena, J.M., Aarnesc, H., Fonnum, F., 2003. Evaluation of the probes 2′,7′- dichlorodihydrofluorescein diacetate, luminal and lucigenin as indicators of reactive species formation. Biochem. Pharmacol. 65, 1575–1582.
Noirot, E., Der, C., Lherminier, J., Robert, F., Moricova, P., Kiêu, K., Leborgne-Castel, N., Simon-Plas, F., Bouhidel, K., 2014. Dynamic changes in the subcellular distribution of the tobacco ROS-producing enzyme RBOHD in response to the oomycete elicitor cryptogein. J. Exp. Bot. 65, 5011–5022.
Oliveira, M.B., Junior, M.L., Grossi-de-Sá, M.F., Petrofeza, S., 2015. Exogenous application of methyl jasmonate induces a defence response and resistance against *Sclerotinia sclerotiorum* in dry bean plants. J. Plant Physiol. 182, 13–22.
Planchet, E., Kaiser, W.M., 2006. Nitric oxide (NO) detection by DAF fluorescence and chemiluminescence: a comparison using abiotic and biotic NO sources. J. Exp. Bot. 57, 3043–3055.
Pignocchi, C., Kiddle, G., Hernández, I., Foster, S.J., Asensi, A., Taybi, T., Barnes, J., Foyer, C.H., 2006. Ascorbate oxidase-dependent changes in the redox state of the apoplast modulate gene transcript accumulation leading to modified hormone signaling and orchestration of defence processes in tobacco. Plant Physiol. 141, 423–435.
Ramulu, K.F., Dijkhuis, P., Rutgers, E., Blaas, J., Verbeek, V.H.J., Verhoeven, H.A., Colinj-Hooymans, C.M., 1995. Microprotoplast fusion technique a new tool for gene transfer between sexually incongruent plant species. Euphytica 85, 255–268.
Stergiopoulos, I., de Wit, P.J., 2009. Fungal effector proteins. Annu. Rev. Phytopathol. 47, 233–263.
Schwessinger, B., Ronald, P.C., 2012. Plant innate immunity: perception of conserved microbial signatures. Annu. Rev. Plant Biol. 63, 451–482.
Salazar, S.M., Castagnaro, A.P., Arias, M.E., Chalfoun, N., Tonello, U., Díaz Ricci, J.C., 2007. Induction of a defence response in strawberry mediated by an avirulent strain of *Colletotrichum*. Eur. J. Plant Pathol. 117, 109–122.
Thakur, M., Sohal, B.S., 2013. Role of elicitors in inducing resistance in plants against pathogen infection. ISRN Biochem. http://dx.doi.org/10.1155/2013/762412. Article ID 762412.
Vellicce, G.R., Coll, Y., Castagnaro, A.P., Díaz Ricci, J.C., 2003. Transformation of a strawberry cultivar using a modified regeneration medium. Hortic. Sci. 38, 277–280.
Wi, S.J., Ji, N.R., Park, K.Y., 2012. Synergistic biosynthesis of biphasic ethylene and reactive oxygen species in response to hemibiotrophic phytophthora parasitica in tobacco plants. Plant Physiol. 159, 251–265.
Wi, S.J., Seo, S.-Y., Cho, K., Nam, H.M., Park, K.Y., 2014. Lysophosphatidylcholine enhances susceptibility in signaling pathway against pathogen infection through biphasic production of reactive oxygen species and ethylene in tobacco plants. Phytochemistry 104, 48–59.
Wilkinson, S., 1999. pH as a stress signal. Plant Growth Reg. 29, 87–99.
Wong, F.H., Fradin, C., 2013. Simultaneous pH and temperature measurements using pyranine as a molecular probe. J. Fluoresc. 21, 299–312.
Ye, W., Hossain, M.A., Munemasa, S., Nakamura, Y., Mori, I.C., Murata, Y., 2013. Endogenous abscisic acid is involved in methyl jasmonate-induced reactive oxygen species and nitric oxide production but not in cytosolic alkalization in *Arabidopsis* guard cells. J. Plant Physiol. 170, 1212–1215.
Yoo, S.D., Cho, Y.H., Sheen, J., 2007. *Arabidopsis* mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat. Protoc. 2, 1565–1572.
Zeidler, D., Zähringer, U., Gerber, I., Dubery, I., Hartung, T., Bors, W., Hutzler, P., Durner, J., 2004. Innate immunity in *Arabidopsis thaliana*: Lipopolysaccharides activate nitric oxide synthase (NOS) and induce defence genes. PNAS 101, 15811–15816.
Zhang, H., Fang, Q., Zhang, Z., Wang, Y., Zheng, X., 2009. The role of respiratory burst oxidase homologues in elicitor-induced stomatal closure and hypersensitive response in *Nicotiana benthamiana*. J. Exp. Bot. 60, 3109–3122.
Zhaoa, J.T., Davisb, L.C., Verpoorte, R., 2005. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Adv. 23, 283–333.
Zhou, J., Wang, B., Zhu, L., 2005. Conditioned culture for protoplasts isolated from chrysanthemum: efficient approach. Colloids Surf. B Biointerfaces 45, 113–119.