Influence of PAMP-induced systemic resistance in potato suspension cells and seedlings on defense signaling markers and infection with *Phytophthora infestans*

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ABSTRACT

Colonization of roots by selected strains of fluorescent Pseudomonas spp. or their elicitors can trigger induced systemic resistance (ISR) against foliar pathogens in a plant species-specific manner. It has been suggested that early responses in cell suspension cultures in reply to rhizobacterial elicitors, such as generation of active oxygen species (AOS) and extracellular medium alkalinization (MA), are linked to the development of ISR in whole plants. In the present study, the pathogen-associated molecular patterns (PAMPs) i.e. lipopolysaccharides (LPS), siderophores, and the flagellin were tested for their effects on the induction of ISR in either potato cell suspension or seedlings. LPS among all PAMPs provoked an almost immediate burst in potato cell suspensions (H_2O_2 24.8 nmoles /g f. w. after 20 min). Filagellin showed a slower and weaker induction of AOS effect compared to LPS, whereas Siderophores did not show such induction. Siderophores were almost as active as LPS in induction of extracellular medium alkalinization, whereas flagellin was approximately half-so. In contrast to the refractoriness of the potato cells to re-stimulation of an oxidative burst with PAMPs, the cells here were able to almost fully re-alkalinize the medium upon repeated stimulation. All studied PAMPs were able to generate cell death in potato cell suspension to different levels. Northern blotting of the defense marker genes PAL, GST and ACO showed a remarkable induction at 3-9 hours post inoculation with the PAMPs and the induction effect subsided at 12-24 h. Potato suspension cells induced by Siderophores completely inhibited sporangia germination, whereas those induced by LPS led to deterioration of the sporangia. Elicitation of potato seedlings with all studied PAMPs resulted in significant reduction of disease index, AUDPC. Obtained significant correlation between ISR induction in potato cell suspension and seedlings suggested the potential to explore the response of potato plant to new elicitors with cell suspension first as it is a time and effort saving technique.

***Key words**: Potato, Phytophthora infestans, ISR, Cell culture, Resistance genes.*

INTRODUCTION

Priming, in plants, is a state of enhanced ability to mobilize pathogen or elicitor-induced defense responses (Conrath *et al.*, 2002 and Heller and Tudzynski, 2011). In order to set up this state, plants or cell suspensions are mostly challenged with inducers of systemic resistance. Priming can also be obtained with elicitors of defense reactions used at concentrations insufficient to induce alone a measurable response (Conrath

et al., 2002). These primers strongly enhance a broad spectrum of cellular responses, such as an early oxidative burst, incorporation of various phenolic compounds, secretion of phytoalexins, and accumulation of defense gene products, upon subsequent challenge of pre-treated cells by avirulent bacteria or products of virulent ones (Val *et al*., 2007). Plant roots support large populations of soil bacteria, notably of the genus *Pseudomonas* (Lugtenberg *et al*., 2001). These rhizosphere bacteria derive nutrients from root cell exudates and lysates and, in return, can promote plant growth and antagonize soilborne plant pathogens through multiple mechanisms (Handelsman and Stabb, 1996; Van Loon, 2007 and Van Loon *et al*., 2008). In addition, specific bacterial strains can induce a systemic protection against both soilborne and foliar pathogens, a phenomenon known as induced systemic resistance (ISR) (Van Loon and Bakker, 2005 and Ralph *et al*., 2014). Rhizobacteria-mediated ISR is independent of salicylic acid (SA) but requires jasmonate (JA) and ethylene signaling in the plant (Pieterse *et al*., 1998). This type of induced resistance confers priming of defense reactions in response to subsequent challenge with, particularly, necrotizing pathogens It should be (Conrath *et al*., 2002). ISR has been extensively studied in *Arabidopsis* but has also been convincingly demonstrated in tobacco and tomato plants that are unable to express pathogen-inducible, SA-dependent systemic acquired resistance (Van Loon *et al*., 2008). In contrast to SAR, ISR is not commonly associated with an accumulation of pathogenesis- related (PR) proteins (Ralph *et. al*., 2014), even though varying changes at the transcriptional level in reaction to several resistance-inducing rhizobacteria have been documented (Van Loon, 2007). For a number of resistance-inducing bacterial strains, the ISR-eliciting compounds have been identified and shown to comprise the cell wall outer membrane lipopolysaccharide (LPS), iron-chelating siderophores, example flagellin (structural subunit of bacterial flagella filaments), antibiotics, quorum- sensing *N*-acylhomoserine lactones, and volatile compounds, such as 2,3-butanediol (Van Loon, 2007). Bacterial LPS, Fe-free siderophores and flagellin have been demonstrated to act as elicitors of innate immunity in animals and to function likewise as PAMPs that are perceived by plants (Nürnberger *et al*., 2004 and Sayyed and Patel, 2011). Recognition of these PAMPs leads to the activation of inducible plant defense responses that may confer non-host resistance to non-host plants and activate basal resistance and reduce disease severity in host plants (Val *et al*., 2007). For example, infiltration of LPS reduced hypersensitive tissue collapse when the same leaf area was subsequently infiltrated with live pathogen (Graham *et al*., 1977). In tobacco cell suspensions, the LPS induced a rapid influx of Ca^{2+} into the cytoplasm, an oxidative burst associated with the production of AOS and alkalinization of the extracellular culture medium (Gerber *et al*., 2004, Heller and Tudzynski, and 2011 and Chen *et al*., 2015). On the other hand, cell cultures of several species, including tobacco, reacted to bacterial siderophores with a rapid alkalinization of the culture medium (Felix et al. 1999, Sayyed and Patel, 2011). Also, flagellins were found to induce an oxidative burst accompanied by cell death in tomato cells (Taguchi *et al*., 2003). Iron is a limiting nutrient for both plant and microbial growth (Robin *et al*., 2006). Disease-suppressing rhizobacteria can directly antagonize soilborne pathogens by competition for iron through the release and specific uptake of iron-chelating siderophores in the rhizosphere (Höfte, 1993). However, bacterial siderophores can also be perceived by plants and induce systemic resistance (Bakker *et al*., 2007 and Sayyed and Patel, 2011); for example, as shown by the

lack of systemic protection of tobacco against black root rot by a mutant of *P. fluorescens* (CHA0) lacking the ability to produce the pseudobactin siderophore (Maurhofer *et al*., 1994). Siderophores has been demonstrated to induce resistance in *Arabidopsis*, bean, carnation, radish, and tomato (Ran *et al*., 2005 and Van Loon, 2007). In *Arabidopsis*, the LPS, flagellin, and the siderophores all triggered ISR whereas, in tomato and bean, the LPS and the siderophores were effective but flagellin was not (Meziane *et al*., 2005). Thus, these three bacterial components were differentially active in these plant species. These differences are likely to be the result of different structures of the elicitors due to gene sequence variations and post-translational modifications (Taguchi *et al*., 2003).

Due to the differential specificities of the rhizobacterial elicitors, this study was initiated to compare early responses thought to represent initial signaling steps in the elicitation of ISR in potato cells by comparing LPS, flagellin, and siderophores of well-characterized plant system (i.e., suspension-cultured cells of potato cv. Lady Rosetta) and to study their early responses to elicitors which include production of AOS, extracellular medium alkalinization and cell death. These responses were examined and related to the effectiveness of the three rhizobacterial elicitors to induce systemic resistance in intact potato plants against late blight disease. The results could provide more insight to plant differential specificities of the rhizobacterial elicitors.

MATERIALS AND METHODS

Rhizobacterial elicitors

Lipopolysaccharides (LPS, L9143) produced by *Pseudomonas aeruginosa* prepared by phenol-extraction purification technique was obtained from Sigma-Aldrich (Germany); Fe-free siderophores (S4019) from Genaxxon Bioscience Co., (Germany); and partially purified flagellin (AIX-522-058-C010) from Alexis Biochemicals, (Axxora Co., USA).

Plant material

Establishment of callus culture and embryogenic cell suspension

The study was carried out according to Desender *et al*. (2006) with modifications. Well-developed leaflets from 20-day *in vitro* cultured Lady Rosetta potato plantlets were used as explants for callus induction. Leaflets were wounded perpendicularly by sterilized scalpel then transferred with the abaxial side-down onto callus induction medium (CIM) consisting of MS basal salts and vitamins (Murashige and Skoog, 1962), 2 % (w/v) sucrose, 0.8% (w/v) Phytoagar supplemented with 1-naphtalene-acetic acid (2 mg /l), and 6-benzylaminopurine (0.5 mg/l) at pH 5.8. Produced calli were maintained at 23 ºC under 8-hr photoperiod (white light, 40 µE m–2 s–1), (Loyola-Vargas and Vázquez-Flota, 2006) and sub-cultured to fresh medium at 4-week intervals for further investigations.

For initiation of cell suspensions, small portions (2.5 g) of light green active callus that produced directly from plant tissue were transferred into 100-ml aliquots of liquid suspension culture medium (SCM) consisting of MS basal salts and vitamins, 3 % (w/v) sucrose and supplemented with 1-naphtalene-acetic (1 mg/l) acid and Kinetin (1 mg/l). A pure single cell line was produced by picking up a single viable cell with Narishige micromanipulator (Japan) attached to an inverted Olympus microscope (IX70, USA). The cell, contained in a permeable 45 µ-sigma membrane (USA), was incubated in dual culture with tobacco cell suspension prepared as mentioned earlier. Produced cells were pelleted by centrifugation at 100 *g* for 5 min and resuspended in fresh medium. If necessary, sub-culturing was carried out by transferring 10 ml of homogenous cell suspension at the end of the exponential

growth phase (*ca*. 7 days) to 50 ml of fresh medium in 250-ml round bottom flasks. Cell suspension cultures were held in an orbital shaker at 125 rpm in the dark at 24 ºC. The pH of all media was adjusted to 5.8 with 1M NaOH. Hormones and NaOH preparations were filter sterilized (0.2 µ) and added to media just before pouring.

Determination of AOS

The production of AOS by the cells was assayed by chemiluminescence, using luminol as reagent (Pugin *et al*., 1997). Briefly, cells were washed by filtration and resuspended to 0.1 g fresh weight /ml in buffer (10 mM HEPES, 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, pH 5.75), and then equilibrated for 2 h at 24°C on a rotary shaker (150 rpm) before treatments. Typically, 3×10^5 cells are present per 0.1 g fresh weight in the suspensions. Cells were counted using the Haemocytometer with Olympus IX-70 inverted microscope (UAS) under a 10X objective. Phase contrast system was used to clearly distinguish the cells. Upon addition of test compounds, samples of 250 µl were withdrawn every 5 min and added to 350 µl of 50 mM HEPES, 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, pH 6.5, and luminol (final concentration 25 µM). Chemiluminescence was measured using a luminometer (Lumat LB9507, Berthold, Bad Wildbad, Germany) for 10 s and expressed in nanomoles of H_2O_2 per gram fresh weight of cells, based on a standard calibration curve obtained by H_2O_2 addition to potato cell suspension aliquots.

Extracellular medium alkalinization

In sub-samples from the AOS experiment, the extracellular pH was measured with a glass combination electrode every 10 min on pre-equilibrated 1-g aliquots of cells in 2 mM HEPES, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 175 mM mannitol, pH 5.5.

Determination of defense-related gene expression

To determine the effect of different elicitors on defensive genes PAL (phenylalanine ammonia-lyase), GST (glutathione S-transferase), and ACO (aminocyclopropane-1-carboxylate oxidase), the elicitors LPS, flagellin or the siderophores were added (10 µg/ml) to the potato cell suspension maintained in the culture medium, then, 2.5-ml aliquots were withdrawn, filtered and immediately frozen at hourly intervals. Total RNA was extracted from leaf tissue by using an RNA isolation kit (TRI reagent, USA), according to the manufacturer's manual. Various amounts of RNA were size-fractionated *via* electrophoresis through a 1% (v/v) agarose gel that contained 5 mM methyl-mercury hydroxide and transferred onto a MagnaGraph nylon membrane (Micron Separations), (GE Osmonics, USA). Equal loading of RNA samples and uniform transfer onto a nylon membrane were confirmed by visualizing cross-linked RNA stained with ethidium bromide under UV light. The cDNA clones used as probes for hybridization were labeled by random priming (Ready-To-Go DNA Labeling Beads-dCTP; Amersham Biosciences, USA). Membrane hybridization was performed at 65°C as described by Church and Gilbert (1984). The membrane was washed with 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7) twice for 5 min at room temperature, with 0.5% sodium dodecyl sulfate (w/v) and 2× SSC twice for 30 min at 65°C, and subsequently with 0.1% SSC twice for 30 min at room temperature. The membrane was then exposed to a Kodak X-Omat AR film (VWR Co., USA).

Quantification of cell death as a reflection of elicitor treatment

Cell death induced by different elicitors was quantified using the conventional viability indicator fluorescein diacetate (FDA)-spectrofluorimetric method according to

Amano *et al*. (2003). Briefly, 4-day-old potato suspension cells were collected and washed by filtration and centrifugation in H10 medium containing 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 10 mM HEPES (Loyola-Vargas and Vázquez-Flota, 2006). Finally, H10 medium was adjusted to pH 5.8 with KOH. One ml of cell suspension was incubated in the presence or absence of the elicitor (1.0 µM). After 24 h, 500 µl of the suspension was diluted in 1.5 ml of H10 medium in a quartz cuvette with final cell density of 1 x 10^5 cells/ml. Cells were gently stirred, then, FDA was added at a final concentration of 12 µM, and the fluorescence increase was monitored over a 2-min time period, using a Hitachi F-2000 spectrofluorimeter (Perkin-Elmer LS 50 B spectrofluorimeter, USA) programmed for FDA excitation at 490 nm and emission at 510 nm. Slope of fluorescence increase, representing cell viability, was calculated for each treatment and directly compared with non-treated cells. Cell death induced by different elicitors was estimated in percent with respect to the standard level, corresponding to non-treated cells and was calculated as follows :

% of cell death = (slope of treated cells/slope of non-treated cells) x100.

Cell culture bioassay for ISR

A sub-feeding layer technique was used in this study as described by Loyola-Vargas and Vázquez-Flota (2006). A homogenous layer of active embryogenic potato cells was established in Petri dishes, 18-mm height, supplemented with SCM mixed with tobacco cell suspension (3 x 10^5) as a sub-feeding layer. A permeable cellophane membrane was laid over the tobacco cell layer and a smooth layer of potato cells previously treated (1.0 µM) or untreated (control) with elicitors was equally spread over and covered with a second cellophane membrane. One ml of *P. infestans* sporangia suspension (0.5 x 10^3) was then equally spread over the top membrane with aid of L-shape glass rod and was carefully left up after 12 h for microscopic examination.

Growth room bioassay for ISR

The study was applied as described by Van Loon *et al*. (2008) with modifications. Two-week-old potato seedlings were uprooted and treated by dipping the root system in a 200-ml suspension of each bacterial elicitor (0.01, 0.1 and 1.0 µg/ml) in 10 mM $MgSO_4$ for 15 s before transplanting into a sand-potting soil mixture that had been autoclaved twice with a 24-h interval. Two days later, plants were challenged with *P. infestans* by spraying their foliar with sporangia suspension (0.5 x 10^3 sporangia / ml) and kept for 5 days at 18C and over 95% relative humidity with aid of a humidifier. Foliage infection was recorded as area under disease progress curve (AUDPC) as described by Yuen and Forbes (2009) and Simko and Piepho (2012). Three replicates served for each treatment each containing five plants. According to the following equation:

$$\mathrm{AUDPC} = \sum_{i=1}^{n}\left[\left(Y_{i+1} + Y_i\right)\right]\left[\left(t_{i+1} - t_i\right)\right]$$

Where *yi* is an assessment of a disease (percentage or score) at the *i*th observation, *ti* is time (in days) at the *i*th observation, and *n* is the total number of observations.

RESULTS

Generation of active oxygen species as a determinant of induction of resistance

Addition of LPS to potato cell suspensions provoked an almost immediate burst of AOS that reached a maximum after approximately 15 min and then returned to control level within 1 h (Fig. 1A). No further increase occurred at longer times as a gradual decrease took place till AOS activity reached the basic level (start point level). Adding flagellin to cell suspension resulted in a milder production of AOS that reached its maximum peak after 40 min then gradually declined till reaching the basic line fifty min later. On the other hand, the siderophores did not lead to the production of AOS in the potato cells, and their effect all along the experiment was negligible.

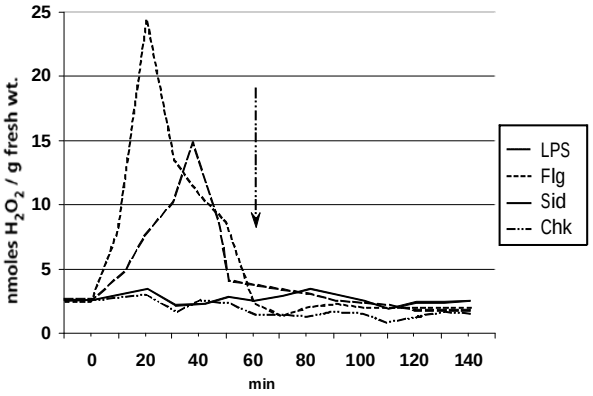

Fig. (1A): Production of active oxygen species by potato cv. Lady Rosetta cell suspensions in response to 1 µM of LPS, flagellin (Flg.) and siderophores (Sid.).

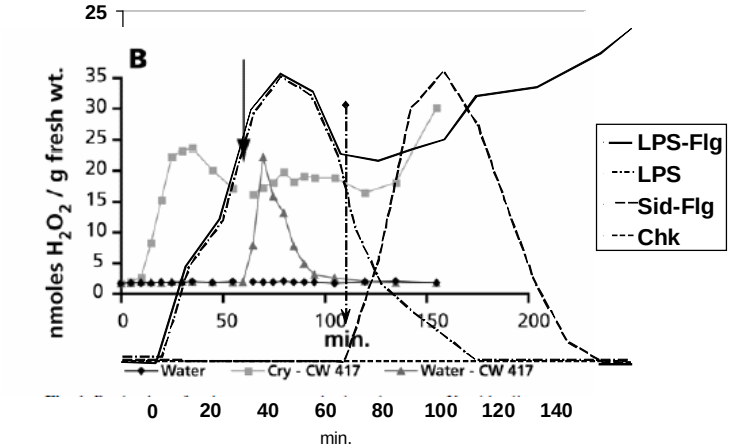

Fig. (1B): Production of active oxygen species by potato cv. Lady Rosetta cell suspensions in response to 5 µM combinations of different elicitors. LPS-Flg, primary elicitation with LPS with 60-min re-induction with flagellin; Sid-Flg, primary elicitation with siderophores with 60-min re-induction with flagellin, LPS, only single primary dose induction with LPS; Chk, cells treated only with buffer.

Data in Fig. 1B. show that when the bacterial LPS was added as a primary elicitor, then the flagellin was introduced at 60 min, a strong production of AOS started after approximately 15 min, reached a peak around 45 min and after second induction it remained elevated for the duration of the experiment. This additional oxidative burst was not comparable in duration or magnitude with the 60-min flagellin-induced burst of AOS produced by potato cells that were previously treated with siderophores at zero time (Fig. 1B). Thus, a synergistic effect of LPS-Flg elicitors mostly took place in the first case as neither the single primary treatment with flagellin (Fig. 1A) nor the 60-min dose of it (Fig. 1B) lasted consistently all along the experiment. This remark was supported by the fact that a secondary induction with flagellin after primary siderophores induction gradually collapsed within 80 min.

Extracellular medium alkalinization

Bacterial elicitors that induced an oxidative burst were also active in generating a rapid alkalinization of the extracellular medium (Fig. 2). However, LPS were almost as active as those of the siderophores while flagellin was approximately half-so. LPS caused a transient increase in extracellular pH of more than one unit within 10 min, with a peak between 10 and 20 min, followed by a gradual subsidence over the next hour, whereas the siderophores induced a response similar to LPS.

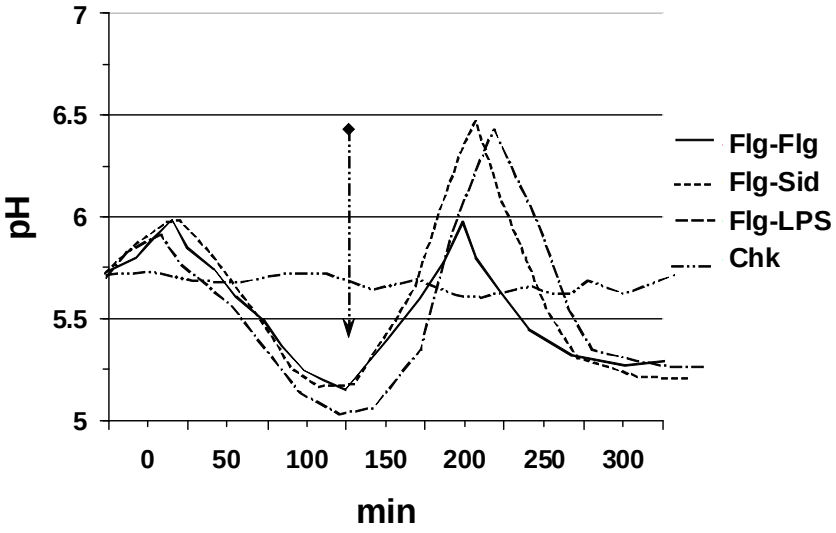

Fig. (2A): Medium primary alkalinization by flagellin (Flg) with re-stimulation at 110 min with Flagellin, siderophores (Sid) or LPS. The arrow indicated the re-stimulation point.

The flagellin started to raise the extracellular pH only after 15 to 20 min, and a plateau was reached after 20 to 40 min (Fig. 2A). In contrast to the refractoriness of the potato cells to restimulation of an oxidative burst, the cells here were able to almost fully re-alkalinize the medium upon repeated stimulation (Fig. 2A-C). It made almost no difference whether the cells were re-stimulated by the same elicitor or by a different one (Fig. 2A-C).

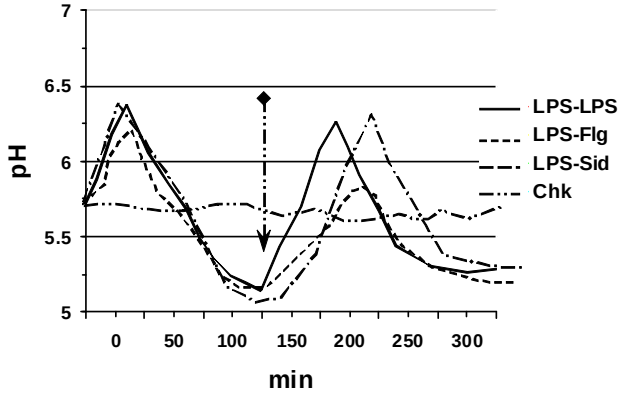

Fig. (2B): Medium primary alkalinization by siderophores (Sid) with restimulation at 110 min with siderophores, Flagellin (Flg) or LPS.

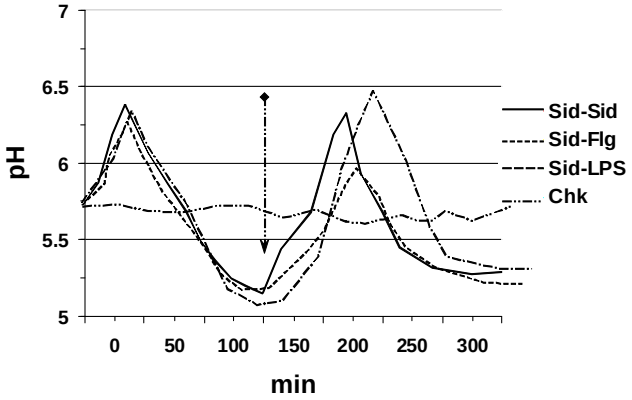

Fig. (2C): Medium primary alkalinization by LPS with restimulation at 110 min with LPS, siderophores (Sid) or Flagellin (Flg).

Even when flagellin was used as a secondary stimulant to potato cells after primary treatment with any of the three elicitors, its activity was demolished to almost half of the other elicitors activity expressed as media pH level.

Expression of defense-related genes

To determine whether LPS, flagellin or siderophores induced expression of defensive genes in potato cell culture, activation in response to cell treatment with different elicitors was studied by Northern blotting (Fig. 3). All tested genes showed a remarkable induction at certain point with different elicitors but in all cases, induction subsided after 12 or 24 h.

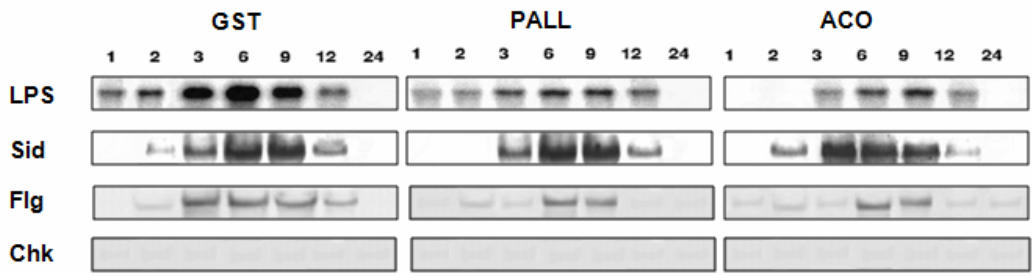

Fig. (3): Expression of defense-related genes PAL (encoding phenylalanine ammonia-lyase), GST (glutathione S-transferase), and ACO (aminocyclopropane-1-carboxylate oxidase) in potato cv. Lady Rosetta suspension cells upon elicitation with LPS, Sid or Flg.

Flagellin induced the weakest expression in all tested genes as no remarkable activity was noticed with PAL and ACO till 6h after the induction with a sho rt peak between 6 and 9h in both, extended to 3-12h with GST. Gene expression was subsided before 12h in PAL and ACO. On the other hand, siderophores induced strong expression in all genes between 3-9h which subsided before 12 to 24h. No immediate expression was noticed at all after 1h of elicitation except with LPS in the case of GST and PAL genes where the induction extended for 12h.

GST was the most responsive to this elicitor with distinguished bands starting from 1 to 12h while ACO was the least one.

Cell death

All bacterial inducers were able to generate cell death in potato cell suspension to certain level. Percentage of dead cells amounted to 28, 14.6 and 8.1% comparable with the water controls for LPS, Sid and Flg respectively after 24h of the elicitation.

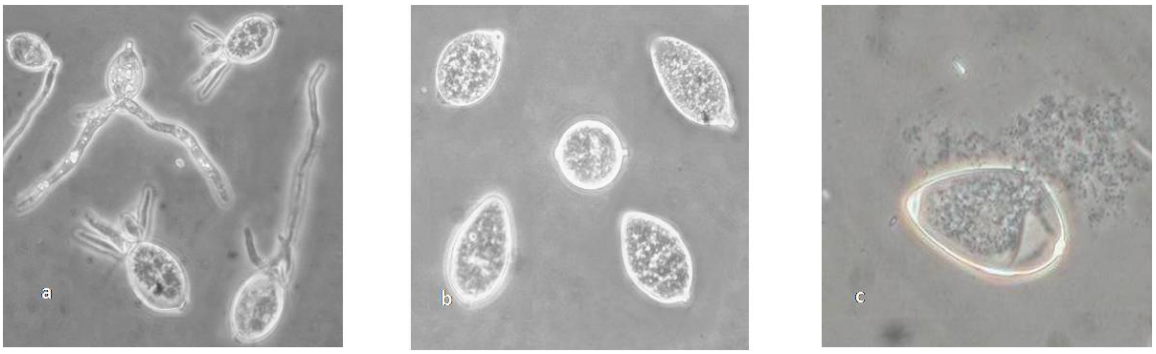

Fig. (4): Effect of elicitation of potato cells with LPS, Siderophores and flagellin on P. infestans sporangia germination and viability via cellophane membrane technique.

Cell culture bioassay for ISR

Potato cells treated with flagellin elicitor did not affect the viability and the germination of *P. infestans* sporangia as shown in Fig. 4a. No remarkable differences were noticed between control cells (only treated with elicitor-free media) and the flagellin-treated cells concerning their effect on pathogen sporangia. Elicitation of cells with siderophores resulted in a complete absence of sporangia germination (Fig. 4b). When ungerminated sporangia were removed from the cellophane membrane, washed and placed on green peas medium, a 92% germination rate was obtained indicating that the effect was basically on inhibition of germination not on the sporangia viability. Sporangia placed over potato cells treated with LPS not only lost their germination capabilities but they also started to deteriorate after about 6h of exposure. A single tiny hole was noticed on the side of each sporangium from which the inner content oozed out. Couple of hours later the whole sporangia collapsed (Fig. 4c). Obviously, testing sporangia viability in this case was not considered.

Growth room bioassay for ISR

To be able to relate the observed early effects on potato suspension cells to the induction of systemic resistance in whole plants, bioassays were conducted in which the roots of potato seedlings were dipped in suspensions of eliciting compounds and plants were challenged 2 days later with sporangia suspension of *P. infestans*.

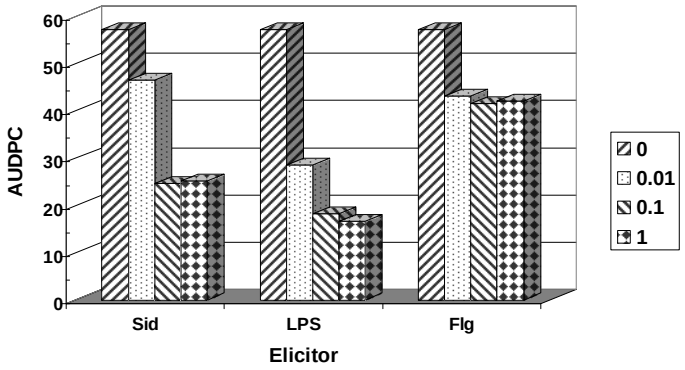

Fig. (5): Effect of elicitation of potato plants with LPS, Siderophores and flagellin at 0.01, 0.1 and 1.0 µg/ml on resistance-inducing activity against late blight expressed as AUDPC. All elicitors significantly reduced disease incidence estimated as AUDPC.

All test concentrations with no significant differences between the doses 0.1 and 1.0 µg/ml (24.7 and 25.2 in the case of siderophores, 18.5 and 16.7 for LPS and 41.6 and 42.1 for flagellin). LPS induced the highest reduction in disease occurrence (only 16.7) while flagellin was the least effective. Increase elicitor dose from 0.01 up to 1.0 µg/l in flagellin didn't result in any significant increase in the resistance-inducing activity.

DISCUSSION

All along the study, cell suspension cultures were used to estimate the direct response of potato cells to different elicitors except in case of ISR estimation where it was a must that potato seedlings had to be the subject of the test. A major advantages of using cell culture is the ability to manipulate the physico-chemical, i.e., temperature, pH, osmotic pressure, O_2 and CO_2 tension and the physiological environment, *i.e.*, hormone and nutrient concentrations in which the cells propagate which assure them a steady behavior affected only by the introduced factor. Moreover, cell culture technique, especially the pure cell line one, is known for its homogeneity and simplicity compared to the difficulties of carrying out studies with whole plant or even plant organs composed of many different cell types. With cell culture, it is possible to observe, in a well-defined environment, any tiny development and to easily uptake samples according to previously determent schedule. Finally, cell cultures don't require huge space to apply as in greenhouse trials (Loyola-Vargas and Vázquez-Flota, 2006).

The limitations of cell culture include the complexity of the techniques and that expertise is needed for initiation and preservation of cultures and the possibility for unexpected infection with viruses or microorganisms as media used to propagate cells are rich in nutrients and, therefore, support growth of a multitude of organisms, or even cross-contamination with other cell types if single cell method was not carried out. Accordingly, most culture methods require restricted sterile conditions. Also, continuous culture may lead to changes occur in cell culture properties

generating a new cell line due to mutation (Hunter-Cevera and Belt, 1996).

Bacterial elicitors have been demonstrated to induce systemic resistance in several plant species, including *Arabidopsis* spp and tobacco. The O-antigenic side chain of the LPS is one of the determinants involved, but the nature of other elicitors has not been investigated (Van Loon *et al.*, 2008 and Heller and Tudzynski, 2011). As for generation of active oxygen species (AOS), LPS among all elicitors provoked an almost immediate burst in potato cell suspensions. Filagellin had milder effect compared to LPS while Siderophores did not lead to any AOS production. An additional oxidative burst occurred when a second elicitor was added one hour after the primary one. For example, when the LPS were added as primary elicitors, then the flagellin was introduced at 60 min as a secondary one, the oxygen burst remained elevated for the duration of the experiment. This phenomenon suggested that a synergistic effect of both elicitors mostly took place.

The burst initiated by LPS and flagellin resembled the one induced by the well-characterized flg22 elicitor peptide (Van Loon *et al.*, 2008) and except that the bursts induced here by the commercial bacterial elicitors were much stronger. However, AOS have been associated with stress adaptation as well as programmed cell death (Gechev *et al.*, 2006 and Heller and Tudzynski, 2011) and are known to act as signals mediating both defense gene activation and the execution of the hypersensitive reaction in response to pathogens (Torres *et al.*, 2006). Similar results were obtained with other elicitors in plant cell suspensions like cryptogein and have been well characterized (Garcia-Brugger *et al.*, 2006), involving not only an oxidative burst, rise in extracellular pH, cytosol acidification, and [Ca^{2+}] influx but also a rapid efflux of nitrate anions, generation of NO, activation of protein kinases, defense related gene expression, and, finally, cell death. In our opinion, the siderophores in our trials proved inactive in initiation of AOS burst because, as indicated by the producing company, the bacterial cell wall preparations were derived from killed bacterial cells. Extraction of siderophores from filtrates of living cell cultures might be an option in future research in order to explore the actual effect of such elicitors. These results suggest that LPS and flagellin can independently provoke the same response in potato cell suspensions. The results suggested a synergistic effect between LPS and flagellin which was not announced or reported in previous work (Van Loon *et al.*, 2008). Though, the incompetence of siderophores in AOS elicitation, all three tested elicitors have been shown to induce systemic resistance in different plants, indicating that mechanisms other than AOS might be involved in such induction. The data were generally in agreement with those reported by Van Loon *et al.* (2008). Bacterial elicitors that induced an oxidative burst were also active in generating a rapid alkalinization of the extracellular medium. LPS were almost as active as those of the siderophores while flagellin was approximately half-so. Even when flagellin was used as a secondary stimulant to potato cells after primary treatment with any of the three elicitors, its activity was demolished to almost half of the other elicitors activity expressed as media pH level. The steadiness of flagellin data confirmed the inferiority of flagellin as an elicitor compared to LPs or siderophores**.**

These reactions appear typical of microbial effectors triggering elicitation that lead to a hypersensitive response in different plants (Van Loon, 2007) and Elicitation by living nonpathogenic rhizobacteria that induce systemic resistance prime plants for enhanced defense reactions upon challenge inoculation with a pathogen but are characteristically not associated with extensive transcriptional

reprogramming or with cell death like chemical elicitors (Van Loon, 2007 and Verhagen *et al.*, 2004). For example, induction of systemic resistance was proved by challenging tobacco plants with *Erwinia carotovora* after been induced by nonpathogenic rhizobacteria. Induced plants were sensitive to SA-independent and ethylene-dependent ISR defenses (Geraats *et al.*, 2003 and Van Loon and Bakker, 2005).

When northern blotting technique was applied to determine whether LPS, flagellin or siderophores induced expression of defensive genes in potato cell culture, it was proved that all elicitors were capable of such induction. Tested genes showed a remarkable induction at certain point of time with various elicitors but in all cases, induction subsided at 12 or 24 h. Flagellin induced the weakest expression in all tested genes while siderophores induced the strongest one. LPS was the intermediate and GST was the most responsive gene to this elicitor. In contrary to the results of Van Loon *et al*. (2008), all bacterial inducers used in our experiments were able to generate cell death in potato cell suspension to certain level. When the effect of elicitors on sporangia viability and germination was investigated using the cellophane membrane technique, no remarkable differences were noticed between control cells (only treated with elicitor-free media) and the flagellin concerning sporangia germination. Siderophores resulted in a complete reversible inhibition of sporangia germination. Sporangia placed over potato cells treated with LPS not only lost their germination capabilities but they also started to deteriorate and to ooze out their cytoplasmic contents. In whole plants, all elicitors significantly reduced disease incidence at all test concentrations. LPS induced the highest reduction in disease occurrence while flagellin was the least effective. Oxidative burst was demonstrated to be associated with increased defense-related gene expression and induced resistance in intact plants against bacterial and fungal pathogens. The data was supported by the findings of Van Loon *et al*. (2008) except that they reported that the expression of ISR against *E. carotovora* in treated tobacco plants did not correlate with the reactions of the tobacco suspension cells to the rhizobacterial compounds. Whereas, in potato and tomato cell suspensions, the LPS and the siderophores all induced the production of AOS, medium alkalinization (Chen *et al*., 2015), cell death and suppression of pathogen spore germination and viability that were all related with disease resistance in whole potato plants. These results clearly demonstrate that the ability of a rhizobacterial elicitors to trigger early signaling events commonly related to defense responses in potato suspension are necessarily corresponding to its resistance-inducing properties in intact potato plants. Although suspension cells differ physiologically from cells in intact plant tissues, as the chlorophyll-containing potato cells do not visibly resemble the chlorophyll-lacking root cells that must be the site of perception of the bacterial compounds *in vivo*. Additionally, the reactions measured in suspension cells are local reactions, whereas ISR is a systemic response that must be mediated by distinct signaling compounds that are generated locally and transported throughout the plant. But even though, the results in both cases were somehow clearly interrelated. The poor correlation between the defense-related reactions in suspension-cultured cells and the elicitation of ISR in whole tobacco plants indicates that the relationship between early reactions in that case was not straightforward like the case with potato. The parameters used in current study are obviously useful in analyzing the perception and early signal transduction of rhizobacterial compounds and their relationship to the generation and establishment of ISR in potato plants.

As a conclusion, different bacterial elicitors tested in our work proved to be quite effective in inducing ISR either in potato cell suspension or in whole plants. A correlation was established between early signaling events commonly related to defense responses in potato cell suspension and the systemic resistance induced in plants with same elicitors. LPS and siderophores were more promising than flagellin and require more future examination on greenhouse or open field levels.

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الملخص العربي

استحثاث المقاومة الجهازية فى معلق خلايا البطاطس والنباتات الكاملة بواسطة المستحثات البكتيرية وتأثيرها علي جينات دفاعية محددة وعلى الاصابة بـ Phytophthora infestans

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ان استعمار الجذور بواسطة سلالات من *Pseudomonas* spp. الفلورسنتية او المستحثات الناتجه عنها يمكن أن يستحث المقاومة الجهازية ضد المسببات المرضية للمجموع الخضري فى تفاعل خاص بين النوع النباتي والكائن المستحث. ولقد اقترح ان الاستجابات المبكرة لمزارع الخلايا ضد المستحثات البيكتيرية مثل انتاج انواع الاكسجين النشطة وقلوية البيئة يمكن ربطها بتطور المقاومة الجهازية المكتسبة فى النبات الكامل. وفى الدراسة الحالية فان المستحثات البكتيرية مثل الليبوبولي سيكريدز ، السيديروفورات ، والفلاجيللين قد تم اختبار تأثيرها فى احداث ظاهرة المقاومة الجهازية المكتسبة فى كل من معلق خلايا البطاطس وبادراتها. ووجد ان الليبوبولى سيكريدز استطاعت احداث ارتفاع فوري فى مستوى الاكسجين النشط فى معلق الخلايا بينما كان تأثير الفلاجيللين منخفضاً فى حين فشلت السيديروفورات فى احداث اى تأثير. اما بالنسبة لقلوية البيئة فقد تساوى تأثير السيديروفورات مع الليبوبولي سكريدز فى هذا الصدد بينما كان تأثير الفلاجيللين فى نصف مستوى تأثيرهما. وعلى العكس من فشل معلق الخلايا فى استعادة الاستحثاث بالنسبة لانواع الاكسجين فانه نجح فى ذلك بالنسبة لقلوية البيئة عند اعادة معاملته بعد فترة بمستحث جديد. جميع المستحثات المستخدمة استطاعت ان تتسبب فى موت خلايا المعلق بدرجة او بأخرى. وعند استخدام طريقة نورثيرن بلوتينج للكشف عن جينات المقاومة فى البطاطس وجد ان جينات PAL , GST , ACO قد اظهرت استجابة واضحة لكل المستحثات المستخدمة في فترة تتراوح بين ٣ -٩ ساعات. ولم يتضاءل تأثير الاستحثاث الا بعد ١٢- ٢٤ ساعة. و قد استطاعت خلايا البطاطس المستحثة بالسيديروفورات ان تحدث تثبيط كامل لانبات جراثيم الكائن الممرض بينما تسببت الخلايا المعاملة بالليبو بولي سكريدز فى انهيار الجراثيم وتحللها. ولم يكن هناك تأثير يذكر للفلاجيللين. وعند معاملة بادرات البطاطس بالمستحثات حدث انخفاض معنوي فى معدل حدوث المرض فى كل المعاملات بمختلف المستحثات. ان الارتباط الملاحظ بين الاستحثاث في النبات الكامل وفي مزارع الخلايا يفتح بابا لدراسة تاثير المستحثات الجديدةعلي الخلايا اولا توفيرا للوقت والجهد.