

Phospholipid signaling pathway in *Capsicum chinense* suspension cells as a key response to a consortium infection

Eugenia Sánchez-Sandoval

Centro de Investigacion Cientifica de Yucatan

Graciela E Racagni Di-Palma

Universidad Nacional de Rio Cuarto

Victor M González-Mendoza

Centro de Investigacion y Desarrollo en Criotecnologia de Alimentos

Yahaira A Cab-Guillén

Centro de Investigacion Cientifica de Yucatan

Armando Muñoz-Sanchez

Centro de Investigacion Cientifica de Yucatan

Ana Luisa Ramos-Diaz

Centro de Investigacion y Asistencia en Tecnologia y Diseno del Estado de Jalisco

Teresa Hernandez-sotomayor (✉ ths@cicy.mx)

Centro de Investigacion Cientifica de Yucatan <https://orcid.org/0000-0001-8261-3318>

Research article

Keywords: *C. chinense*, *Colletotrichum* species, phosphatidic acid, plant-pathogen, biochemical response, phospholipase

Posted Date: May 1st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-24845/v1>

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Version of Record: A version of this preprint was published at BMC Plant Biology on January 25th, 2021.
See the published version at <https://doi.org/10.1186/s12870-021-02830-z>.

Abstract

Mexico is considered to be a diversification center for the chili species, but at the same time, these crops have been susceptible to infection by pathogens, such as *Colletotrichum*, which causes anthracnose diseases and postharvest decay in general. Different studies have been carried out with isolated strains of *Colletotrichum* in *Capsicum* plants, however, under growing conditions, the microorganisms are interacting with others, which can increase or decrease their infective capacity. This study presents the first report between plant-pathogen interactions and their biochemical responses in phospholipid pathways for *C. chinense* to microbial consortium with mainly *Colletotrichum ignotum* pathogen. The results showed morphological changes in the first hours (h) in the presence of the microbial consortium, and high levels of diacylglycerol pyrophosphate (DGPP) and phosphatidic acid (PA) were found after 6 h postinoculation (hai). These metabolic changes were correlated with high transcription levels of diacylglycerol-kinase (CchDGKs) expressed for 3, 6 and 12 hai and related to pathogen gene markers, such as CchPR1 and CchPR5. Finally, this study shows how the phospholipase C/DGK (PLC/DGK) pathway offers insight into the microbial infection responses of chili crops with damping-off diseases in the Yucatan.

Introduction

Chili was domesticated in Mexico, where 4 of the 5 species of *Capsicum* are cultivated: *C. frutescens*, *C. annuum*, *C. pubescens* and *C. chinense*. However, *C. chinense* is the only species of chili thought to have originated in Mexico, given the uses and customs by settlers from the Yucatan region. In field conditions, these crops are susceptible to pathogen infections, and special attention has been paid to *Colletotrichum* sp. In recent years, there have been numerous anthracnose diseases cases that were related to fungi belonging to over a dozen species, of the genere *Colletotrichum*.

Colletotrichum sp. causes anthracnose disease and postharvest decay in a wide range of tropical, subtropical and temperate fruits, crops and ornamental plants (Damm et al. 2012; Nam et al. 2013; Aiello et al. 2015; Guarnaccia et al. 2016). Four *Colletotrichum* species complexes have been described: *C. gloeosporioides*, *C. acutatum*, *C. boninense*, and *C. truncatum* are the primary causal agents of these diseases (Weir et al. 2012; Huang et al. 2013).

Recently, a strain of *C. ignotum* was identified as part of a microbial consortium isolated from a infected plant of *C. chinense* root (unpublished, CIATEJ). In the literature, *C. ignotum* belongs to the *C. gloeosporioides* species complex and has only been associated with generalist endophytes in cacao plants (Rojas et al. 2010).

In plant-pathogen interactions, a precise signaling process is indispensable to the successful adaptation and survival of the plant. There are many studies on host plant defense systems and pathogenic invasion effectors with their phytohormone targets, such as salicylic acid (SA) and/or jasmonic acid (MJ) (Kazan and Lyons 2104). However, only a few studies have been conducted that relate to the phospholipid signal

transduction pathway in response to pathogens and, in particular, the pathway that involves phospholipid-derived molecules as second messengers (Munnik and Testerink 2009). Phosphatidic acid is a very important signaling molecule that can modulate the activities of kinases, phosphatases, phospholipases and other proteins involved in membrane traffic, calcium signaling and biotic and abiotic stress responses (Testerink and Munnik 2011; Hou et al. 2016; Altuzar-Molina et al. 2011).

In a previous study, we evaluated the effect of SA and MJ on phospholipid signaling in *C. chinense* Jacq. suspension cells. Treatments with SA inhibited phospholipase C (PLC) and phospholipase D (PLD) activities, while treatments with MJ produced increases in PLC and PLD activities (van der Luit et al. 2000). Studies on the transient accumulation of PA and DGPP were conducted in tomato suspension cells (van der Luit et al. 2000; Yamaguchi et al. 2005). The cells were treated with a pathogenic elicitor, and high levels of PA were found that were subsequently metabolized to DGPP (van der Luit et al. 2000). When DGPP and PA were added, there was also an induction of the expression of elicitor-responsive genes in the absence of the elicitor (Yamaguchi et al. 2005). We can use a *C. chinense* suspension cell system to study and correlate phytohormone targets related to plant defense with phospholipid signal transduction. We propose to first correlate the generation of important phospholipid-derived molecules and the expression of their genetic pathway in response to the microbial consortium in *C. chinense* plants to understand the phospholipid signal transduction that occurs in the interaction between *C. chinense* suspension cells and a microbial consortium isolated from infected plants that is primarily formed by *C. ignotum*.

Materials And Methods

Materials

Radiolabeled [^{32}P] γ -ATP was obtained from Amersham Pharmacia Biotech (UK). Bicinchoninic acid (BCA) protein assay reagent was purchased from Pierce Chemical Co. Other chemicals were provided by Sigma Aldrich. Murashige and Skoog (MS) media were supplied by Phytotechnologies Inc. The commercial kit ZymoBiomix DNA was purchased from Zymo Research. Chloroform, methanol, pyridine, and formic acid were purchased from J.T. Baker Co. TLC plates were supplied by Merck®.

Biological Material

C. chinense suspension cells were obtained by the disaggregation of callus and cultured in Murashige and Skoog media (MS) (Murashige and Skoog 1962) at pH 5.6. This media was supplemented with 0.5 mM myo-inositol, 0.02 mM thiamine, 0.2 mM cysteine, 4 μM 2,4-dichlorophenoxyacetic acid and 3% sucrose. The cells were subcultured every 14 days, as previously reported (Altuzar-Molina et al. 2011), and grown with shaking (100 rpm) in continuous light at 25 °C.

The consortium was obtained from *C. chinense* plants isolated from infected crops in the Yucatán region. The selection method for growing the microbial consortium was used, and the consortium was

maintained at 20 °C in the dark on modified agar media (10% vegetable juice*, 2% Bacto agar; *The vegetable juice was a Herdez V8 juice that contained 8 vegetables, such as carrot, tomato, beetroot, spinach, kale/leaf cabbage, celery, parsley and lemon juice) (Gou and Ko 1993). The microbial consortium was cultivated in Petri dishes; this material was flooded with water and rubbed with a sterile glass slide. The suspension was filtered through a 50 µm Miracloth as described in (Sharma et al. 2015), incubated at 37 °C for 3 has suggested in (Shipton 1985) and maintained at 4°C, and the suspension was filtered (Kamoun et al. 1993). The filtrate was called "conidial suspension" (cs). We standardize the amount of inoculum by quantifying the amount of *C. ignotum* conidia in the conidial suspension. The cs were released as it was mentioned above and heated at 85 °C for 5 min.

Analysis of the consortium microbial profile by next-generation sequencing (NGS)

Previously, a microbial consortium had been isolated from the rotted roots of *C. chinense* seedlings and routinely maintained as described above. DNA isolation was performed using the commercial kit ZimoBionics DNA (Zimo Research). The quality of the extracted DNA was examined by agarose electrophoresis (1%) with ethyl bromide (0.01%) and visualized under UV light. DNA samples were sent for analysis to LABSERGEN (CINVESTAV) where ITSs or 16S amplicons were made with 300,000 PE readings, and MySeq sequences were generated. Bioinformatics analyses were processed using readpipeline and MG_RAST. The identification of the strains was conducted in the massive BLAST in MG_RAST, and the taxonomy of each strain was determined using the following databases: the Encyclopedia of Life (<http://www.eol.org/>), Global Catalog of Microorganisms (<http://www.gcm.wfcc.info>), Integrated Taxonomic Information System (<https://www.itis.gov/>) and Livemap (<http://lifemap-ncbi.univ-lyon1.fr/>).

Infection assay establishment

One gram of *C. chinense* suspension cells from 14 days of culture was used to establish the infection assay, and the cells were subcultured in 25 mL of MS media and kept overnight at 24 °C on a shaker at 100 rpm for acclimation (Altuzar-Molina et al. 2011). Then, the suspension cells were inoculated using 1×10^4 of cs and harvested at different inoculation times (indicated for each experiment) like hour after infection (hai).

Epifluorescence analysis

Suspension cells of *C. chinense* were infected (or not) with the consortium and washed three times with sodium phosphate buffer (PBS) at 0.1 M and pH 5.7, and the suspension was diluted 1:10 in PBS. The cells were stained with 1 µM of 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma) and 1.76 µM of FM4-64 Dye (*N*-(3-triethylammoniumpropyl)-4-(6-(4-(diethylamino) phenyl) hexatrienyl) pyridinium dibromide) (Invitrogen™), 10 µM CellMask™ Plasma Membrane Stains (molecular probes™)

and 2 μM Calcofluor White Stain (WCF) (Fluka™), and after 30 min of incubation at room temperature, the fluorescence was observed using epifluorescence microscopy (Axioplan, Zeiss, Germany).

Cell fixation and scanning electron microscopy

For scanning electron microscopy (SEM), the MS media was discarded, and the *C. chinense* cells were washed with PBS (to eliminate the MS media) and incubated with FAA solution (40% formaldehyde, 50% ethanol, 5% acetic acid, and 5% distilled water) for 72 h at 25 °C with gentle agitation every 3 h. The samples were washed with PBS to eliminate the FAA solution. The cells were dehydrated in ethanol solutions in a sequential gradient at 30%, 50%, 70%, 96%, and 100% for 12, 12, 3, 2 and 1 h, respectively. After the cells were fixed, the samples were dried to the critical point with liquid CO₂ using a Sandri-795 critical point dryer (Tousimis), metalized with gold (Denton vacuum Desk II) and observed using SEM (JEOL JSM 6360LV).

Cell viability assay

C. chinense suspension cells infected with the consortium were washed 3 times with phosphate buffer, resuspended in 1 mL of PBS and gently mixed. Then, a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) solution was added to a final concentration of 0.5 mg mL⁻¹ and the mixture was incubated at 25 °C for 8 h in the darkness. The formazan salts were then solubilized with 1.5 mL of methanol solution (50% final concentration) and incubated at 60 °C for 30 min. Finally, the cells were centrifuged at 1,500 x g for 5 minutes. The supernatant was recovered, washed 5 or 6 times with methanol and mixed to quantify the absorbance at 570 nm.

Protein extract preparation

Frozen *C. chinense* cells, previously infected or not, were macerated in liquid nitrogen and homogenized with solution A (50 mM HEPES, pH 7.2, 0.25 M sucrose, 5 mM KCl, and 1 mM EDTA) with protease inhibitors (1 $\mu\text{g mL}^{-1}$ leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 $\mu\text{g mL}^{-1}$ aprotinin). The extract was centrifuged at 20,000 x g for 30 min at 4 °C, and the supernatant was centrifuged at 105,000 x g for 1 h at 4 °C. The precipitate obtained (membrane fraction) was resuspended in 200 μL of 50 mM HEPES, pH 7.4. The protein concentration in the extracts was determined with a modified bicinchoninic acid protein assay reagent (Smith et al. 1985), using bovine serum albumin (BSA) as the standard.

Lipid kinase activity

Lipid kinases were determined using [γ -³²P] ATP incorporation into the corresponding endogenous substrate (Racagni-Di Palma et al. 2002). The phosphorylation assay was performed using a reaction mix with 50 mM HEPES at pH 7.4, 1 mM EDTA, 10 mM MgCl₂, 1 mM ATP, 0.2 mM sodium vanadate, 0.5 mM DTT, [γ -P³²] ATP (370 MBq) and 80 μg of protein membrane fraction. The mixture was incubated for 2 min at 30 °C, and the reaction was finally stopped with 1.5 mL chloroform:methanol (1:2, v/v).

Lipid extraction and separation

Lipid extraction was conducted as described previously in Racagni-Di Palma et al. (Racagni-Di Palma et al. 2008). In each sample, 0.5 mL of 2.4 N HCl and 0.5 mL of chloroform were added, and then the bottom phase was carefully extracted and mixed with 2 mL of 1 N methanol:HCl (1:1, v/v). The lipids were dried under vacuum and resuspended in 200 µL chloroform:methanol (9:1, v/v). Finally, the lipids were analyzed using thin layer chromatography (TLC) plates impregnated with solution I [1% potassium oxalate, 2 mM EDTA, and methanol:water (2:3, v/v)] and activated for 40 min at 110 °C as described in Racagni-Di Palma et al. (Racagni-Di Palma et al. 2008). The plates were developed with a solution of chloroform:methanol:acetone:acetic acid:water (40:15:14:12:7, v/v) and chloroform:pyridine:formic acid (35:30:7, v/v) for the first and second dimension in the TLC, respectively (Racagni-Di Palma et al. 2002). Positions of radiolabeled lipids were determined by autoradiography.

Phylogenetic analysis

Phylogenetic testing was conducted on DGK proteins using complete amino acid sequences obtained from the SOL Genomics Network database (<https://www.sgn.cornell.edu/>). The sequences were aligned using ClustalW and displayed with MEGA 6 software, and a maximum likelihood method was employed with a robustness of 1000 bootstrap replicates. The *C. chinense* DGK homologs *CanDGK1*, *CanDGK2*, *CanDGK3*, *CanDGK5*, *CanDGK5L*, *CanDGK6* and *CanDGK7* were tested against the predicted protein from tomato (*L. esculentum* ITAG release 2.4), coffee (*C. canephora* v1.0) and *Arabidopsis* to obtain their phylogenetic relationship.

Expression gene assay and data analysis

Cultures of *C. chinense* previously treated with the consortium according to the infection conditions were used to determine the transcription levels of candidate genes involved in the infection response. For the expression analysis, RNA was isolated using a TRIzol™ RNA Reagent (Invitrogen™), and the cDNA was synthesized using 500 ng of total RNA with Revert Aid Reverse Transcriptase (Thermo Scientific). Treatments at 0.5, 1, 3, 6 and 12 h with or without 1×10^4 cs of the consortium were analyzed; the treatment at 0.5 h was used as the control. For the reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays, amplification was conducted using a Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) and a PikoReal 24 real-time PCR system (Thermo Fisher Scientific, Ratatsie 2, FI-01620 Vantaa, Finland). The conditions of the RT-qPCR were as follows: 1) Initial denaturation step at 95 °C for 10 min; 2) Three-step cycling at 95 °C for 40 s and at T_m °C for 40 s with 40 or 45 cycles, respectively, for each gene; and 3) Final melting curve step from 56 °C to 95 °C. The primers used were designed based on pepper genome sequences (*C. annuum* cv. CM334 genome CDS) and tested in *C. chinense* (Table S1). Finally, for the fold change determination, a $2^{-\Delta\Delta CT}$ method and EF1α as an internal control were used.

Results

The roots of plants establish a relationship with the microorganisms present in the rhizosphere. This interaction can occur with beneficial or pathogenic microorganisms. Both types trigger a complex

response that will decide the success of the proliferation and development of the plant. In recent years, the response of plant cells to pathogenic microorganisms has been studied independently; however, the cellular response is even more complex under field conditions, due to the fact that microorganisms form consortia with compatible microbes (Lamichhane et al. 2017). These microbes may have an inhibitory or synergistic effect on the development of an infection. In the present work, we propose to study the cellular and biochemical responses to the microbial consortium isolated from the interaction of rotting roots and fruits in *C. chinense* plants.

The isolated consortium of the rotten roots of *C. chinense* seedlings was characterized by NGS to identify the microorganisms present in the microbial consortium. The metadata were stored in the database of the NCBI with the registration number ID PRJNA479448. The bioinformatic analysis of the sequences enabled the identification of the microorganisms present in this consortium with *C. ignotum* comprising most of the eukaryotic microorganisms (Fig. S1), and the predominant genus of prokaryotes was that of the bacteroids (Fig. S2). The low proportion of readings obtained from 16S compared to the readings obtained from the ITS amplicon does not limit their relationship with the plant or with the majority strain. In this sense, we consider the set of identified microorganisms as a consortium.

Infection establishment

To understand the relationship between the host and pathogen, *C. chinense* suspension cells were inoculated with the microbial consortium that contains primarily *C. ignotum*. The most important challenge to be resolved in this work was to determine the infection conditions and periods that may provide more information regarding this topic. *C. chinense* suspension cells were challenged with the consortium, and different times and conditions were tested until a contrasting response was established at the morphological level, growth rate and/or exacerbation of death when a known cs concentration was used.

C. chinense cells that were infected with 1×10^8 cs (empty triangles) showed a decrease of approximately 35% at 12 h and decreases up to 50% and 90% at 24 and 48 h after inoculation, respectively (Fig. 1a). With 1×10^4 cs (filled triangles), a decrease of approximately 15%, 30% and 40% at 12, 24 and 48 h after inoculation was shown, and when cells were inoculated with 1×10^1 cs (open circles), a clear trend was not obtained (Fig. 1a). The decrease in viability was also correlated with a decrease in the fresh weight (Fig. 1b). When the cells were exposed to a higher concentration of cs, a larger decrease in viability, as well as in fresh weight, was observed. Finally, 1×10^4 cs allowed us to generate slight (30%) and/or severe cell damage (50%) at 24 and 48 h of treatment, respectively.

Images of *C. chinense* suspension cells in the presence of the microbial consortium showed a blow-up cell phenotype after 24 h and a clearer one at 48 h after inoculation when compared to the control cells (Fig. 2, 3). Interestingly, when a viable consortium was compared with the attenuated version, both had the same response to a blow-up cell phenotype (Fig. 2), which could be related to secreted elicitors, as

described by (van der Luit et al. 2000). Finally, 1×10^8 cs at 48 h after inoculation showed a null viability browning culture (data not shown) culture with dark brown colour.

With respect to pathogens, the consortium showed an appreciable amount of growth after 12 h, and major changes were shown in hyphae abundance to 48 h after inoculation when 1×10^4 cs were used (Fig. 2, 3) and when 1×10^8 cs showed an exacerbated hyphae abundance (data not shown).

Morphological changes during infection

C. chinense suspension cell morphology was evaluated using a barrier microscope after the cells were inoculated at 24 and 48 h with 1×10^4 cs from the microbial consortium; all treatments were conducted in constant light conditions. With 24 h of inoculation, the cells showed changes, such as increased cell turgescence and low-abundance hyphae, with *C. chinense* cells and a microbial consortium, respectively (Fig. 3). Some *C. chinense* cells began to be broken when inoculated with 1×10^8 cs (6 h after treatment, data not shown). The broken cells could be caused by programmed cell death in response to inoculation with the consortium. In contrast at 48 h after inoculation, a complete reversal of the rate for the plant cell vs hyphae populations occurred, and the *C. chinense* cells died when the microbial consortium reached the largest hyphae population (Fig. 3).

On the other hand, the cells were evaluated using fluorophores to observe the cell wall (WCF), cytoplasmic membrane (Cell Mask), endoplasmic membrane (FM4-64) and DNA integrity (DAPI; Fig. 4). During the experiments without the consortium, the structure of the *C. chinense* cells remained unchanged even 24 to 48 h after inoculation with the mock solution (Fig. 4). When the *C. chinense* cells were inoculated with, they showed several damages in the plasma and endoplasmic membrane even after 12 h after inoculation (Fig. 4). However, at 48 h after inoculation, the damage to both membranes (plasma and endoplasmic) and in the cell wall was severe (Fig. 4).

Based on these results, the damage observed could be associated with DNA integrity as evaluated with DAPI. The results showed that prior to 24 h after inoculation, the cells accumulated moderate DNA damage, and the cells had DNA aggregation and fragmentation at 48 h (Fig. 4).

Changes in lipid kinase activity are involved in infection events

The effect of a microbial consortium on the phospholipid-derived molecules in *C. chinense* cells was evaluated in a time-course experiment. Cells were incubated for different times in the presence or absence of the consortium. Lipid kinases activities were assayed, as described in the Material and Methods section. We observed that during the inoculation, the levels of PA, lysophosphatidic acid (LPA) and DGPP increased (Fig. 5 levels showed a rapid increase during the 6 hai) and then decreased until disappeared (at 48 hai, data not showed) (Fig. 5). In general, the resulting PA can be obtained by two different pathways: via PLD, which generates PA directly by hydrolysing structural phospholipids, such as phosphatidylcholine (PC) and others, or via PLC, which generates DAG, which in turn is subsequently

phosphorylated to PA by DGK (PLC/DGK action (de Jong et al. 2004); and Fig. 6). In the first case, the increase in PA levels resulting from PLD action could be explained by a PLD-specific transphosphatidylolation assay (Munnik and Laxalt 2013). However, increasing evidence points to PA accumulation in relation to the PLC/DGK action in response to pathogen effectors such as bacterial elicitors (van der Luit et al. 2000; Bargmann and Munnik 2006), specific effectors from *Pseudomonas syringae* (Anderson et al. 2006) and fungi, such as *Cladosporium fulvum* (de Jong et al. 2004) and *Botrytis cinerea* (Gonorazky et al. 2016). In this manner, PA resulting from PLC/DGK action could only be produced via PI(4)P conversion in the first period (6 h after inoculation), given that the pools of PI(4)P are higher than those of PI(4,5)P₂ at these times (data not show).

PA resulted in PI(4,5)P₂ conversion by PLC action that could only happen after a long time, either by direct turnover of PI(4,5)P₂ or via the first PI(4)P transformation to PI(4,5)P₂ by the action of a phosphatidylinositol 4-phosphate 5 kinase (PIP5K). However, the levels of LPA did not show significant changes, and/or minor changes could be generated by phospholipase A (PLA) action in one route that may involve the turnover of PA (Fig. 5). With all the data, we hypothesized that high PA accumulation was obtained by coordinated action between the PLC/DGK pathways.

Interestingly, the homologous gene for PA kinases was not found in plants or the *Capsicum* genome. Another reason why we postulated that DGK has a function relevant to the plant-host interaction is that DAG could be processed by diacylglycerol phosphatase (DGPP) via the turnover of PA to add more PA via the turnover of DAG (Fig. 6). In this way, we analyzed the transcription profile to determine the DGK genes during the infection events, and a phylogenetic test was also conducted. In an *in silico* search, at least 7 DGK homologs, *CanDGK1*, *CanDGK2*, *CanDGK3*, *CanDGK6*, *CanDGK6-L*, *CanDGK6-L2* and *CanDGK7*, were found in the *C. annuum* genome (*C. annuum* cv CM334 genome CDS). The phylogenetic assays for DGK from plants showed 3 well-defined phylogenetic groups. The first group included DGK1 and DGK2 (Fig. S3, in blue); the second included DGK3 and DGK7 (Fig. S3, in green), and DGK6, DGK6-L and DGK6-L2 were in a final group (Fig. S3, in red). According to these findings and literature review, specific genes were chosen, and mRNA levels in *C. chinense* cells infected with the consortium were determined by RT-qPCR. We investigated the expression of the genes for DGK homologs primarily in *C. chinense* (*CchDGK1* and *CchDGK3*), an unrelated gene for nonspecific PLC (*CchNPC6*) and genes related to pathogenesis, such as PR (*CchPR1a* and *CchPR5*), in infection events, where infection times below 12 h were assayed, given that at this time, the cells showed morphological changes without exhibiting damage or cell death. The *CchDGK1* and *CchDGK3* genes showed the highest increase after 1 hour from inoculation (Fig. 7). However, other unrelated genes, such as *CchNPC6*, increased as well. This observation probably indicates a general response due to the manipulations in culture. However, the expression of the DGKs increased more after 3, 6 and 12 h following inoculation when *CchNPC6* was thoroughly reduced at 3 h and completely disappeared to basal levels at 6 and 12 h after inoculation (Fig. 7). The pathogenesis-related genes *CchPR1a* and *CchPR5* increased in transcript abundance at 1 h, were reduced at 3 or 6 h and then once again increased. This pattern demonstrates that the response in the beginning was a general response, while at 12 h, there was a specific response to the microbial

consortium (Fig. 8). These data supported the concept that higher levels of PA that could be produced by the action of DGK in the phosphoinositide pathway in *C. chinense* suspension cells in response to infection events.

Discussion

The investigation of the mechanisms of interaction between plants with pathogenic microorganisms, has been pointed by different authors, however, if we want to reproduce in our study model the interactions that occur in the field, we must consider that the roots of plants interact with consortiums of microorganisms, to have a broader view of the plant-microbiome relationship. In the present work, we isolated a consortium, which was characterized by metagenomic and applied to a cellular model of *C. chinense*, that allowed us to observe the cell signaling activated by the interaction with it. The metagenomics revealed that a major microorganism, *Colletotrichum ignotum*, is present, related to the anthracnose disease in the fruits (Sharma and Shenoy 2014).

In the *in vitro* infection system established in this study, all *C. chinense* cells that grow in suspension have the same possibility of infection. Defined amounts of cs can be mixed with a standardized amount of *C. chinense* cells (Fig. 1) and the infection was followed at different times by microscopy. Shortly after inoculation, primary infection was observed with the penetration of the hyphae into the cells of *C. chinense* (Fig. 2, 3), and effects due to infection such as deterioration in the cell wall, a collapse of the plasma membrane and, this was followed by a stage where a fragmentation of the nuclei is observed (Fig. 4).

The changes observed in the main structures of the cells of *C. chinense*, such as: the cell wall, the plasma membrane and the nucleus (Fig. 4), may be due to both the infection process by cs, and the response of the cells themselves.

Damage to the cell wall may be generated due to the fact that the cs secretes proteins with protease activity and enzymes that degrade the cell wall (cellulases and pectinases), which facilitates the initial penetration and infection of the host (Fig. 4). Kim and collaborators in 2004 (Kim et al. 2004) observed nuclear modifications and structural changes of hypersensitivity in the fruit of *Capsicum annuum* cv. *Jejujaerae* (susceptible) and *Capsicum baccatum* cv. PBC80 (resistant), inoculated with the anthracnose pathogen *Colletotrichum gloeosporioides*, where a degradation of the cell wall was observed by enzymes secreted by the pathogen, in addition the separation of the plasma membrane from the cell wall, the dilatation of the endoplasmic reticulum was observed, the accumulation of dense inclusions in the vacuoles and the cytoplasmic vacuolization that accompanies the fragmentation of the cytoplasm and DNA fragmentation.

Therefore, our results suggest that cell wall damage is a characteristic of pathogen attack (cs) in the cell suspension model of *C. chinense*.

Naton and colleagues in 1996 (Naton et al. 1996), observed a reduction in cell viability and changes in the morphology of cell suspensions of parsley during infection with *P. infestans*. These changes in cells during infection are due to the formation of reactive oxygen species, mainly the highly aggressive oxygen radicals that produce lipid peroxidation (Sutherland 1991; Tzeng and De Vay 1993). The effect of cs in *C. chinense* generated an increase in cell death that became evident over time.

In this study (Fig. 4), the structural damage observed in the cells of *C. chinense* can be derived from various biochemical events, which occur primarily in the plasma membrane, for example: unsaturated fatty acids can be oxidized and eliminated from the lipid bilayer, since ROS (H_2O_2), which is generated as an initial response of cells to attack by a pathogen, can trigger the activation of lipoxygenases (Tzeng and De Vay 1993; Kulkarni et al. 1990). In the study model it can be suggested that lipases would also be participating in the process of fatty acid degradation. Therefore, these events lead to a deterioration of the plasma membrane, the cell wall and the nucleus, which would eventually result in a generalized structural collapse and intracellular metabolism, initially generated by the interaction with the consortium. In plants, PA and DGPP are well accepted as second messengers in signaling pathways and respond to biotic and abiotic stress (Hou et al. 2016). As mentioned before, high levels of PA were found when *C. chinense* suspension cells were inoculated with a microbial consortium that contains primarily *C. ignotum*. These PA levels probably provide a balance between PI(4)P and PI(4,5)P₂ as substrates and coordinate activity to PLC and DGK, where PI(4)P is enriched at short times and PI(4,5)P at late times after treatment. Many authors have reported that PA is subsequently metabolized to DGPP in response to many types of biotic or abiotic stress or plant responses, such as water deficiency (Munnik et al. 2000), fungal elicitors (van der Luit et al. 2000), osmotic stress (Meijer et al. 2001), Nod factors (den Hartog et al. 2003), and salt stress (Darwish et al. 2009).

However, it is well accepted that in plants, the phosphorylated forms of PA and DGPP have started to gain importance as signaling molecules in many stress responses (Zonia and Munnik 2006). Here, we report the presumable action of the phospholipid pathways PLC/DGK and the phospholipid-derived molecules that are the result of PI(4)P or PI(4,5)P₂ hydrolysis to form second messengers, such as PA and DGPP, which eventually invoke downstream signaling responses to infection.

When these biochemical changes were challenged relative to the DGK transcription profile during the infection events, a strong correlation between PA or DGPP accumulation and the specific expression for DGK (*CchDGK1* and *CchDGK3*) was obtained, in which the results showed the highest accumulation of transcripts at 1 to 12 h after inoculation (Fig. 7) and were consistent with marker genes related to pathogenesis (*CchPR1a* and *CchPR5*; Fig. 8) when *C. chinense* suspension cells were challenged with the microbial consortium. These results supported the hypothesis that higher DGK transcript accumulation could be related to high PA-DGPP levels in infection events between *C. chinense* suspension cells and the microbial consortium.

Recently, (Gonorazky et al. 2016) demonstrated that a PLC/DGK pathway is required to regulate defense responses to the necrotrophic pathogen *B. cinerea* by transiently silencing SIPLC2 in tomato plants.

Zhang et al. (Zhang et al. 2008) reported that the overexpression of a rice DGK in tobacco enhances the resistance to *Phytophthora parasitica* var. *nicotianae* and that the increase in the accumulation of PA confers disease resistance. In this sense, our work represents an excellent biotechnological tool to improve pepper crops from the Yucatan by conferring tolerance to the infection of pathogens related to the microbial consortium associated with the damping-off phenomenon in these regions.

Declarations

Author contribution statement

MS conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables. GR conceived and designed the experiments, analyzed the data. VG conceived and designed the experiments, performed the experiments, analyzed the data, prepared figures and/or tables. YC performed the experiments, . JM performed the experiments, analyzed the data, prepared figures and/or tables. AR conceived and designed the experiments, performed the experiments, analyzed the data. TH conceived and designed the experiments, analyzed the data, prepared figures and/or tables. All authors reviewed draft of the paper and approved the manuscript.

Acknowledgements

We would like to thank to the technical assistance from Angela Kú González and I.Q. Silvana Andrade Canto is greatly appreciated.

Funding

Our research was supported by a Consejo Nacional de Ciencia y Tecnología grant (Grant IFC 035/2015) awarded to SMTH-S and by a scholarship (166897) awarded to VMG.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

References

Aiello D, Carrieri R, Guarnaccia V, Vitale A, Lahoz E, Polizzi G (2015) Characterization and Pathogenicity of *Colletotrichum gloeosporioides* and *C. karstii* Causing Preharvest Disease on *Citrus sinensis* in Italy. Journal of Phytopathology 163(3):168-177. DOI.org/10.1111/jph.12299

- Altuzar-Molina AR, Muñoz-Sanchez JA, Vázquez-Flota FA, Monforte-González M, Racagni-Di Palma G, Hernández-Sotomayor SMT (2011) Phospholipidic signaling and vanillin production in response to salicylic acid and methyl jasmonate in *Capsicum chinense* J. cells. Plant Physiology and Biochemistry 49(2):151-158. DOI: 10.1016/j.plaphy.2010.11.005
- Andersson MX, Kourtchenko O, Dangl JL, Mackey D, Ellerström M (2006) Phospholipase-dependent signalling during the AvrRpm1- and AvrRpt2-induced disease resistance responses in *Arabidopsis thaliana*. Plant Journal 47(6):947-959. DOI: 10.1111/j.1365-313X.2006.02844.x
- Bargmann BO, Munnik T (2006) The role of phospholipase D in plant stress responses. Current Opinion in Plant Biology 9(5):515-522. DOI: 10.1016/j.pbi.2006.07.011
- Capsicum genome Kim et al (2017) New reference genome sequences of hot pepper reveal the massive evolution of plant disease-resistance genes by retroduplication. Genome Biology 18:270-280. DOI: 10.1186/s13059-017-1341-9
- Damm U, Cannon PF, Woundenberg JH, Crous PW (2012) The *Colletotrichum acutatum* species complex. Studies in Mycology 73(1):37-113. DOI: 10.3114/sim0010
- Darwish E, Testerink C, Khalil M, El-Shihy O, Munnik T (2009) Phospholipid signaling responses in salt-stressed rice leaves. Plant Cell Physiol 50(5):986-997. DOI: 10.1093/pcp/pcp051
- de Jong CF, Laxalt AM, Bargmann BO, de Wit PJ, Joosten MH, Munnik T (2004) Phosphatidic acid accumulation is an early response in the Cf-4/Avr4 interaction. Plant Journal 39(1):1-12. DOI: 10.1111/j.1365-313X.2004.02110.x
- den Hartog M, Verhoef N, Munnik T (2003) Nod factor and elicitors activate different phospholipid signaling pathways in suspension-cultured alfalfa cells. Plant Physiology 132(1):311-317. DOI: doi.org/10.1104/pp.102.017954
- Global Catalog of Microorganisms ([http://www. //gcm.wfcc.info](http://www.gcm.wfcc.info))
- Gonorazky G, Guzzo MC, Abd-El-Haliem AM, Joosten MH, Laxalt AM (2016) Silencing of the tomato phosphatidylinositol-phospholipase C2 (SIPLC2) reduces plant susceptibility to *Botrytis cinerea*. Molecular Plant Pathology 17:1354-1363. DOI: doi.org/10.1111/mpp.12365
- Gou LY, Ko WH (1993) Two widely accesible media for growth and reproduction of *phytophthora* and *pythium*. Appl Environ Microbiology 59(7):2323-2325. 0099-2240/93/072323-03\$02.00/0
- Guarnaccia V, Aiello D, Cirvilleri G, Polizzi G, Susca A, Epifani F, Perrone G (2016) Characterisation of fungal pathogens associated with stem-end rot of avocado fruit in Italy. III International Symposium on Postharvest Pathology: Using Science to Increase Food Availability. Acta Horticulturae 1144:133-139. DOI: 10.17660/ActaHortic.2016.1144.19

Hou QC, Ufer GD, Bartels D (2016) Lipid signalling in plant responses to abiotic stress. *Plant Cell and Environment* 39(5):1029-1048 DOI: 10.1111/pce.12666

Encyclopedia of Life <http://www.eol.org/>

Huang F, Chen GQ, Hou X, Fu YS, Cai L, Hyde KD, Li HY (2013) *Colletotrichum* species associated with cultivated citrus in China. *Fungal Diversity* 61(1):61-74. DOI: 10.1007/s13225-013-0232-y.

Integrated Taxonomic Information System (<https://www.itis.gov/>).

Livemap (<http://lifemap-ncbi.univ-lyon1.fr/>)

Kamoun S, Young M, Glascock CB, Tyler BM (1993) Extracellular Protein Elicitors from *Phytophthora* - Host-Specificity and Induction of Resistance to Bacterial and Fungal Phytopathogens. *Molecular Plant-Microbe Interactions* 6(1):15-25. DOI: 10.1094/MPMI-6-015

Kazan K, Lyons R (2014) Intervention of Phytohormone Pathways by Pathogen Effectors. *Plant Cell* 26(6):2285-2309. DOI: 10.1105/tpc.114.125419

Kim K-H, Yoon J-B, Park H-G, Park E W, Kim YH (2004) Structural Modifications and Programmed Cell Death of Chili Pepper Related to Resistance Responses to *Colletotrichum gloeosporioides* Infection. *Phytopathology: Genetics and Resistance* 94(12):1295-1304. DOI:10.1094/PHTO.2004.94.12.1295

Kulkarni AP, Mitra A, Chaudhuri J, Byczkowski JZ, Richards I (1990) Hydrogen peroxide: a potent activator of dioxygenase activity of soybean lipoxygenase. *Biochem. Biophys. Res. Commun* 166:417-423. DOI: 10.1016/0006-291x(90)91961-Q

Lamichhane JR, Durr C, Schwanck AA, Robin M-H, Sarthou J-P, Cellier V, Messean A, Aubertot J-N (2017) Integrated management of damping-off diseases. A review. *Agronomy for Sustainable Development*. 37(2):25p. DOI: 10.1007/s13593-017-0417-y

Laxalt AM, Munnik T (2002) Phospholipid signalling in plant defence. *Current Opinion in Plant Biology* 5(4):332-338. DOI: 10.1016/s1369-5266(02)00268-6

Meijer HJG, Arisz SA, Van Himbergen JAJ, Musgrave A, Munnik T (2001) Hyperosmotic stress rapidly generates lyso-phosphatidic acid in *Chlamydomonas*. *Plant Journal* 25(5):541-548. DOI: 10.1046/j.1365-313x.2001.00990.x

Munnik T, Testerink C (2009) Plant phospholipid signaling: "in a nutshell". *Journal of Lipid Research* 50:S260-S265. DOI: 10.1194/jlr.R800098-JLR200

Munnik T, Laxalt AM (2013) Measuring PLD activity in vivo. *Methods Mol Biol* 1009:219-231. DOI: 10.1007/978-1-62703-401-2_20

- Munnik T, Meijer HJ, Ter Riet B, Hirt H, Frank W, Bartels D, Musgrave A (2000) Hyperosmotic stress stimulates phospholipase D activity and elevates the levels of phosphatidic acid and diacylglycerol pyrophosphate. *Plant Journal* 22(2):147-154. DOI:10.1046/j.1365-313x.2000.00725.x
- Murashige T, Skoog F (1962) A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum* 15(3):473–497 DOI: 10.1111/j.1399-3054.1962.tb08052.x
- Nam MH, Park MS, Lee HD, Yu SH (2013) Taxonomic Re-evaluation of *Colletotrichum gloeosporioides* Isolated from Strawberry in Korea. *Plant Pathology Journal* 29(3):317-322. DOI: 10.5423/PPJ.NT.12.2012.0188
- Naton B, Hahlbrock K, Schmelzer E (1996) Correlation of rapid cell death with metabolic changes in fungus-infected, cultured parsley cells. *Plant Physiology* 112:433-444. DOI: 10.1104/pp.112.1.433
- Racagni-Di Palma G, Brito-Argaez L, Hernandez-Sotomayor SMT (2002) Phosphorylation of signaling phospholipids in *Coffea arabica* cells. *Plant Physiology and Biochemistry* 40(11):899-906. DOI: 10.1016/S0981-9428(02)01450-X
- Racagni G, Villasuso AL, Pasquare SJ, Giusto NM, Machado E (2008) Diacylglycerol pyrophosphate inhibits the alpha-amylase secretion stimulated by gibberellic acid in barley aleurone. *Physiol. Plant* 134:381–393 DOI: 10.1111/j.1399-3054.2008.01148.x
- Rojas EI, Rehner SA, Samuels GJ, Van Bael SA, Herre EA, Cannon P, Chen Ret al (2010) *Colletotrichum gloeosporioides* s.l. associated with Theobroma cacao and other plants in Panama: multilocus phylogenies distinguish host-associated pathogens from asymptomatic endophytes. *Mycologia* 102(6):1318-1338. DOI: 10.3852/09-244
- Sharma G, Shenoy BD (2014) *Colletotrichum fructicola* and *C. siamense* are involved in chilli anthracnose in India. *Archives of Phytopathology and Plant Protection*. Taylor & Francis, 47(10), pp. 1179–1194. DOI: 10.1080/03235408.2013.833749.
- Sharma M, Ghosh R, Tarafdar A, Telangre R (2015) An efficient method for zoospore production, infection and real-time quantification of *Phytophthora cajani* causing Phytophthora blight disease in pigeonpea under elevated atmospheric CO₂. *Bmc Plant Biology* 15:90. DOI: 10.1186/s12870-015-0470-0
- Shipton WA (1985) Zoospore Induction and Release in a Pythium Causing Equine Phycomycosis. *Transactions of the British Mycological Society* 84(Jan):147-155. DOI: 10.1016/S0007-1536(85)80228-X
- Smith PK, Kronhn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of Protein Using Bicinchoninic Acid. *Analytical Biochemistry* 150(1):76-85.

SOL Genomics Network database (<https://www.sgn.cornell.edu/>).

Sutherland MW (1991) The generation of oxygen radicals during host plant responses to infection. *Physiol Mol Plant Pathology* 39:79-93.

Testerink C, Munnik T (2011) Molecular, cellular, and physiological responses to phosphatidic acid formation in plants. *Journal Experimental Botany* 62(7):2349-2361.

Tzeng DD, De Vay JE (1993) Role of oxygen radicals in plant disease development. In: Andrews JH, Tomerup IC (eds) *Advances in plant pathology*, Vol. 10. Academic Press, London, pp 1-33.

van der Luit AH, Piatti T, van Doorn A, Musgrave A, Felix G, Boller T, Munnik T (2000) Elicitation of suspension-cultured tomato cells triggers the formation of phosphatidic acid and diacylglycerol pyrophosphate. *Plant Physiology* 123(4):1507-1515.

Weir BS, Johnston PR, Damm U (2012) The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology* 73:115-180.

www.global catalogue.com: <http://www.gcm.wfcc.info>.

Yamaguchi T, Minami E, Ueki J, Shibuya N (2005) Elicitor-induced activation of phospholipases plays an important role for the induction of defense responses in suspension-cultured rice cells. *Plant and Cell Physiology* 46(4):579-587.

Zonia L, Munnik T (2006) Cracking the Green Paradigm: Functional Coding of Phosphoinositide Signals in Plant Stress Responses. From: *Biology of Inositol s and Phosphoinositides*. Lahiri A. and Biswas B. (eds). Springer, Netherlands. pp. 207-238.

Zhang WD, Chen J, Zhang H, Song F (2008) Overexpression of a rice diacylglycerol kinase gene OsBIDK1 enhances disease resistance in transgenic tobacco. *Molecules and Cells* 26(3):258-264.

Supplemental File Legends

Supplementary Fig. S1 Relative abundance of species of fungi; only *Colletotrichum ignotum* was > 1%

Supplementary Fig. S2 Relative abundance of bacterial populations; only genera with a relative abundance >1% are shown

Supplementary Fig. S3 Phylogenetic tree of *C. chinense* DGK. The phylogeny was reconstructed based on an alignment of their predicted protein sequences from pepper (Ca), tomato (Sol), coffee (Cc) and Arabidopsis (At). The tree was made using the maximum likelihood method, testing with 1000 bootstrat replicates and displayed using MEGA 6. The numbers at the nodes are the bootstrap values (>10%) and the branch lengths from the root was displayed

Figures

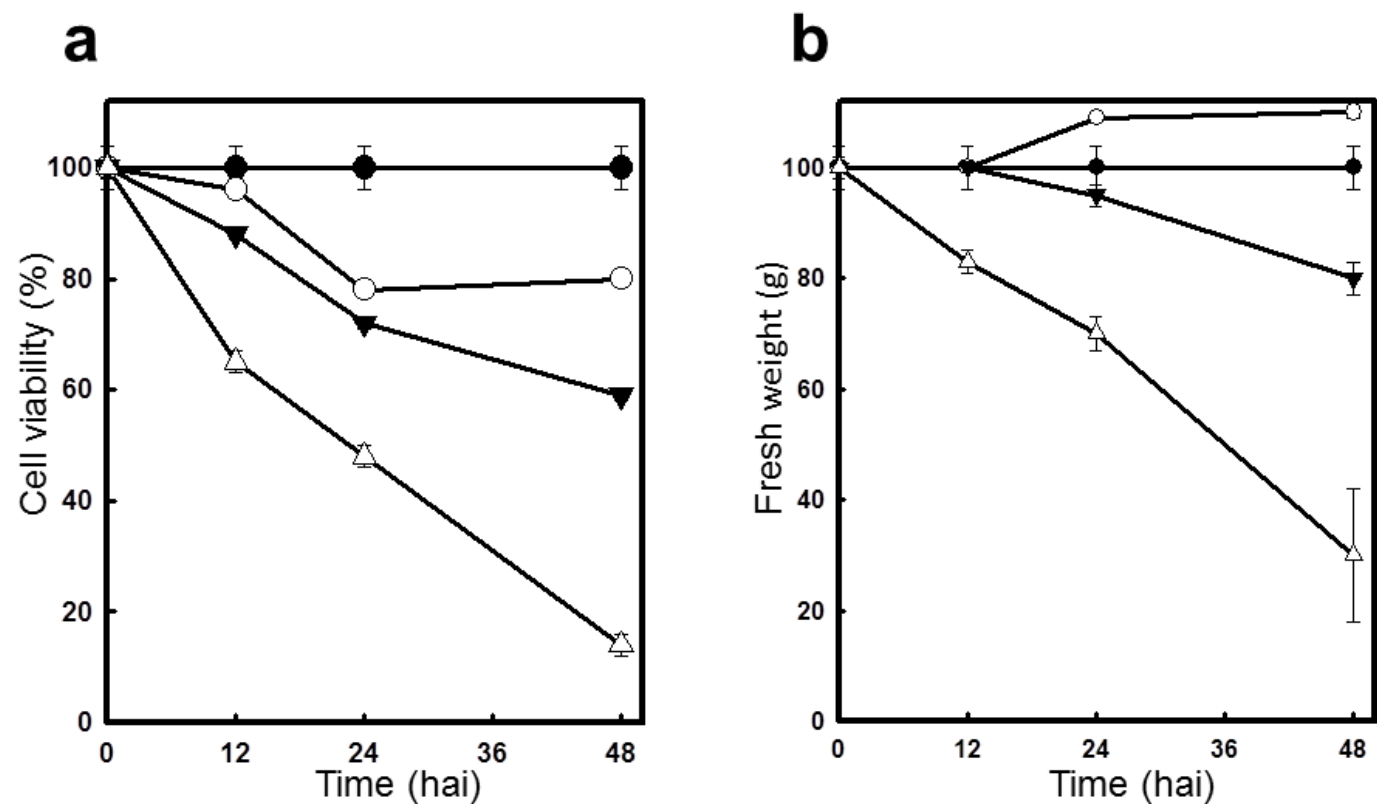


Figure 1

Evaluation of the optimal infection time using *C. chinense* cells infected with cs. A) Cells were infected with different amounts of cs (1x10⁸ = white triangles, 1x10⁴ = black triangles, 1x10¹ = white circles) from the consortium during different incubation hours after infection (hai). Cell viability was determined using MTT. B) Fresh weight of the cells after the infection treatments. Values are the means for three experiments with triplicates +/- SE

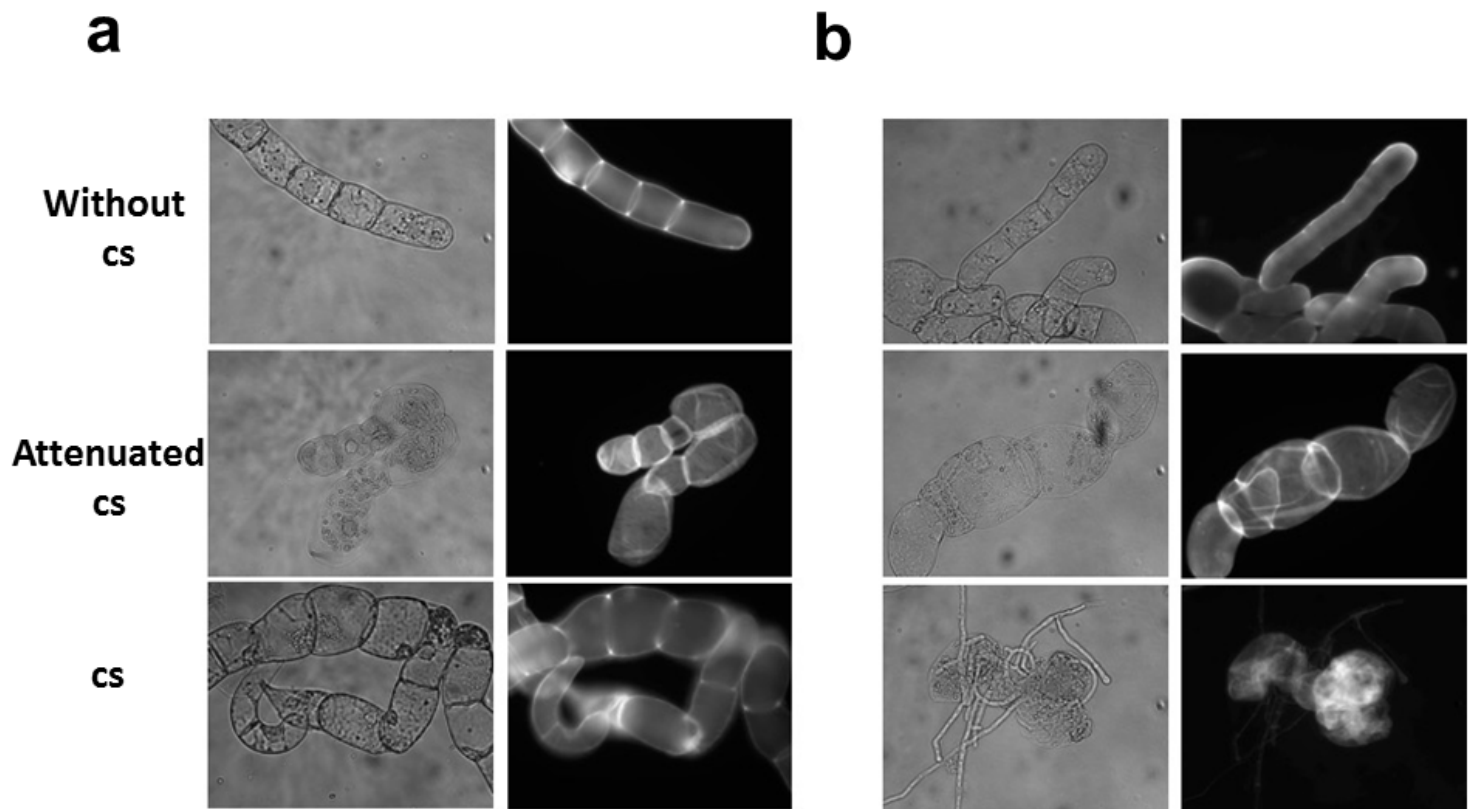


Figure 2

Morphological structure of *C. chinense* cells after infection with cs. The cells (1 g) were treated for 24 (A) and 48 hai (B) without cs, with attenuated cs and with cs (1×10^4), stained with WCF and visualized using epifluorescence microscopy. The figures are representative of three independent experiments with duplicates

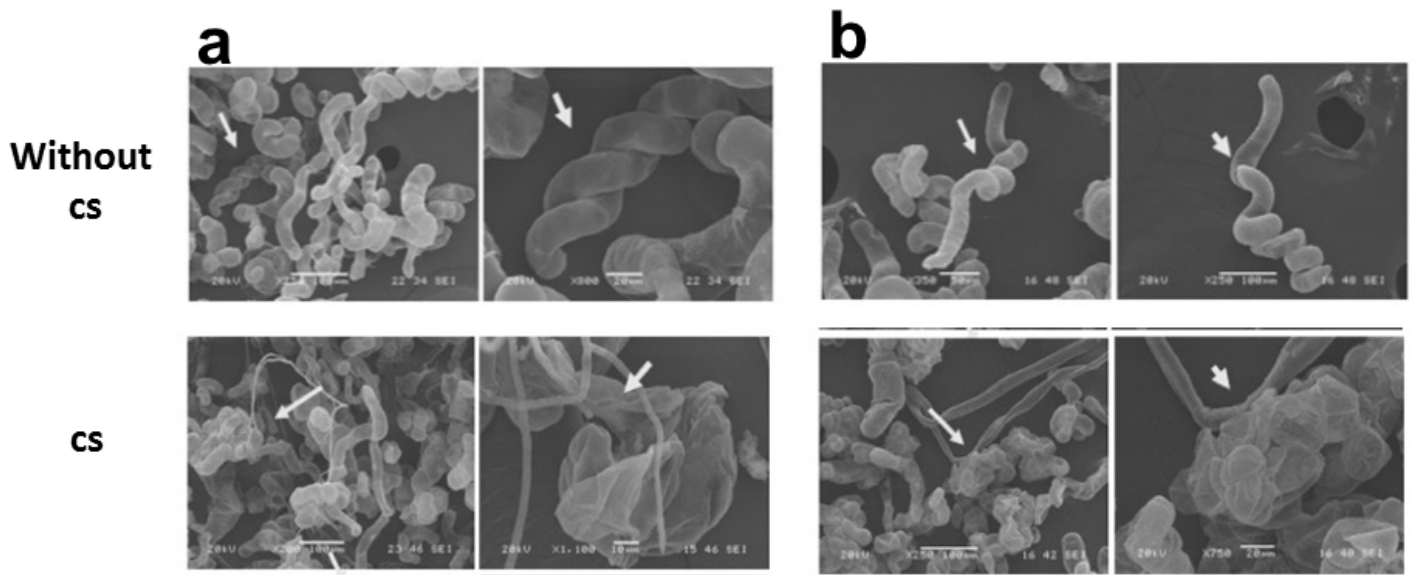


Figure 3

Morphological effects from the cs treatment. The cells were treated for 24 (A) and 48 hai (B) without and with cs (1×10^4). The cells were observed using SEM. The figures are representative of three experiments with duplicates

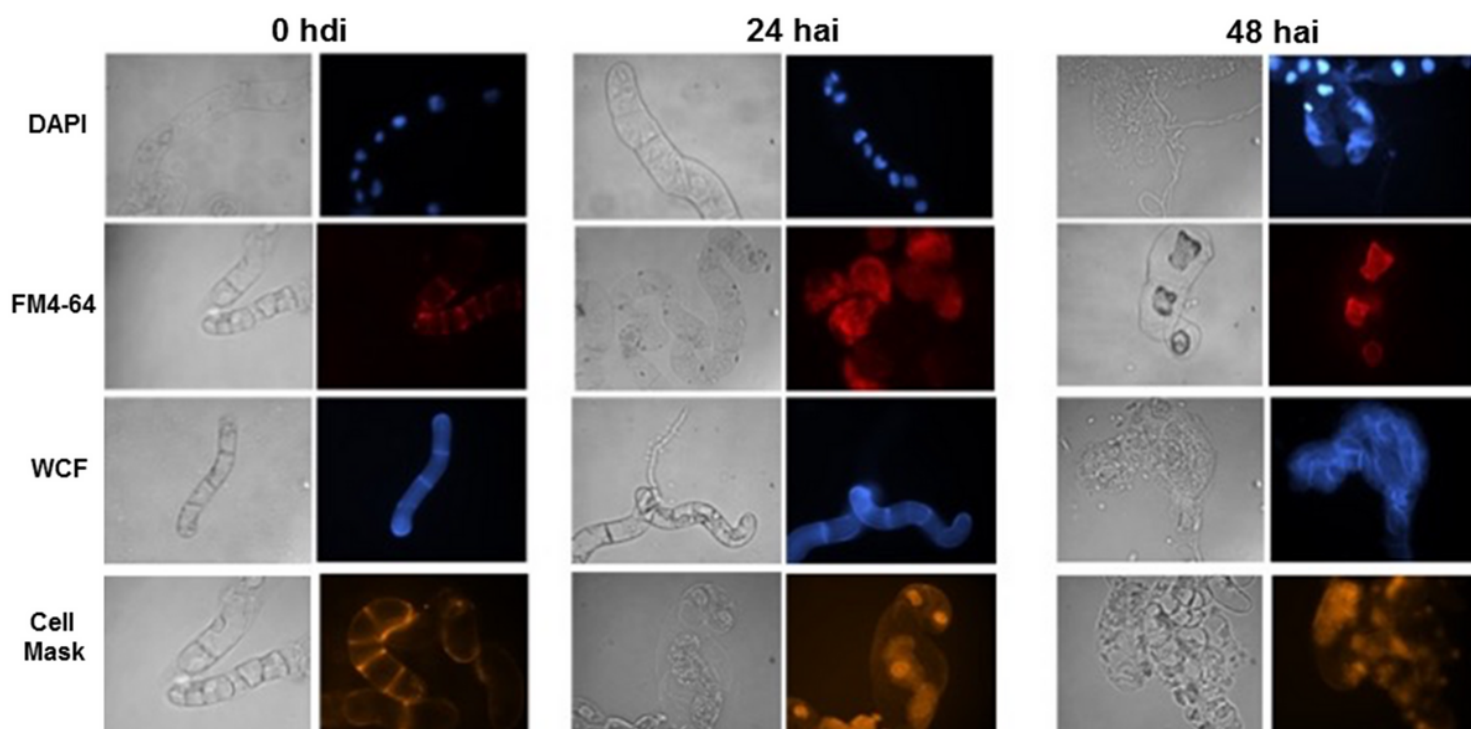


Figure 4

Cell integrity damage evaluation. The cells were treated as indicated above for 24 and 48 hai with cs. The left column shows cells with a visible field. The cells are stained with DAPI (nuclei in blue), FM4-64 (red signal), WCF (cell wall) and Cell Mask (cytoplasmic membrane). The figure is representative of three experiments with duplicates

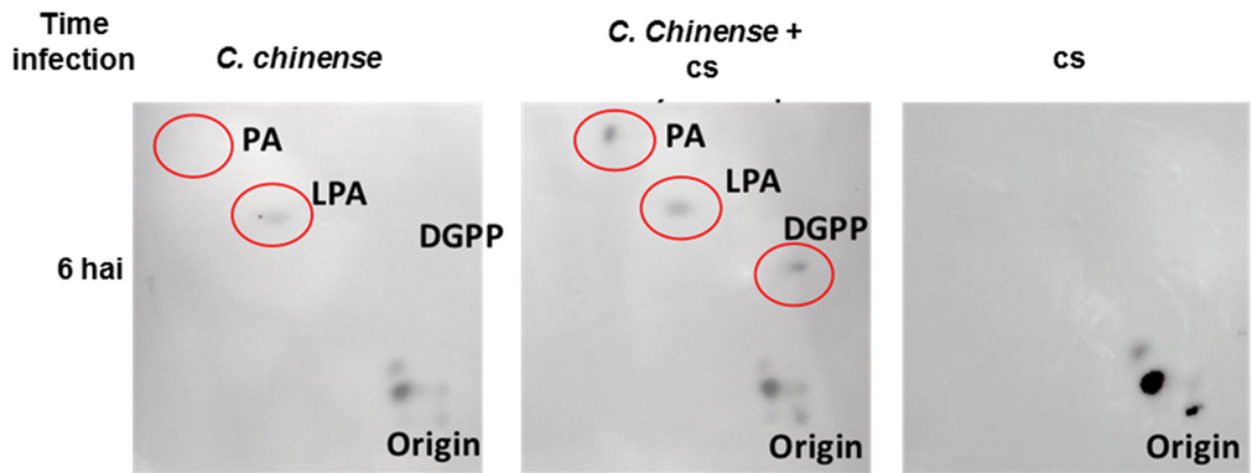


Figure 5

Detection of lipids produced by kinase activity using 2D-TLC. Lipids were extracted and subjected to alkaline TLC to separate the different phospholipid species. The *C. chinense* cell cultures were submitted to infection treatment for 6 h with cs (1×10^4) and were separated using 2D-TLC. As a control, lipids were developed from *C. chinense* cells only (C) or cs only (O). Radioactivity was detected by autoradiography. A representative of three experiments is presented

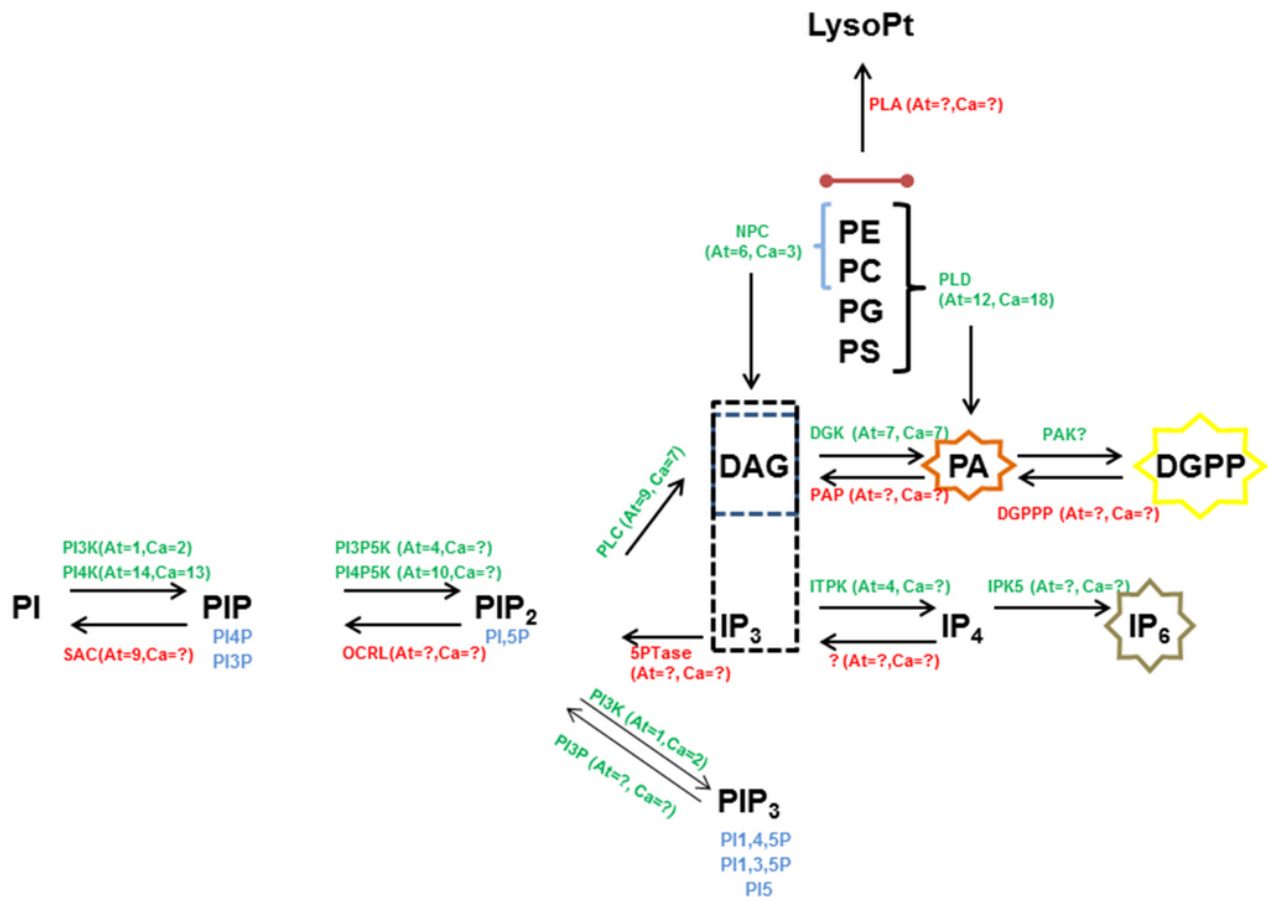


Figure 6

DGK transcription level during cs infection events. *C. chinense* cells were infected with 1×10^4 cs for different time periods, RNA was extracted and the relative expression of DGK1, DGK3 and NPC6 were analyzed through real-time quantitative PCR. (blue bar) Mock, (red bar) cs. Error bars indicate standard error

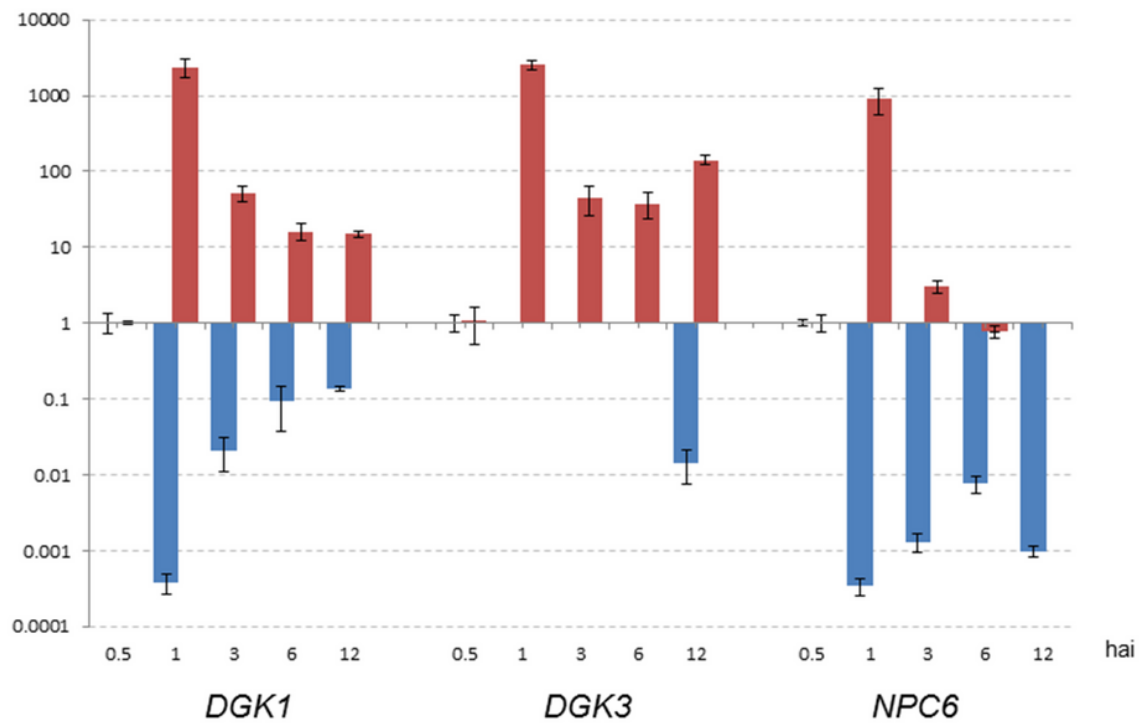


Figure 7

Pathogenesis-related transcription level during *cs* infection events. *C. chinense* cells were infected with 1×10^4 *cs* for different time periods, RNA was extracted and relative expression pathogenesis-related transcripts (PR1a and PR5) were analyzed through RT-qPCR. (blue bar) Mock, (red bar) *cs*. Error bars indicate standard error

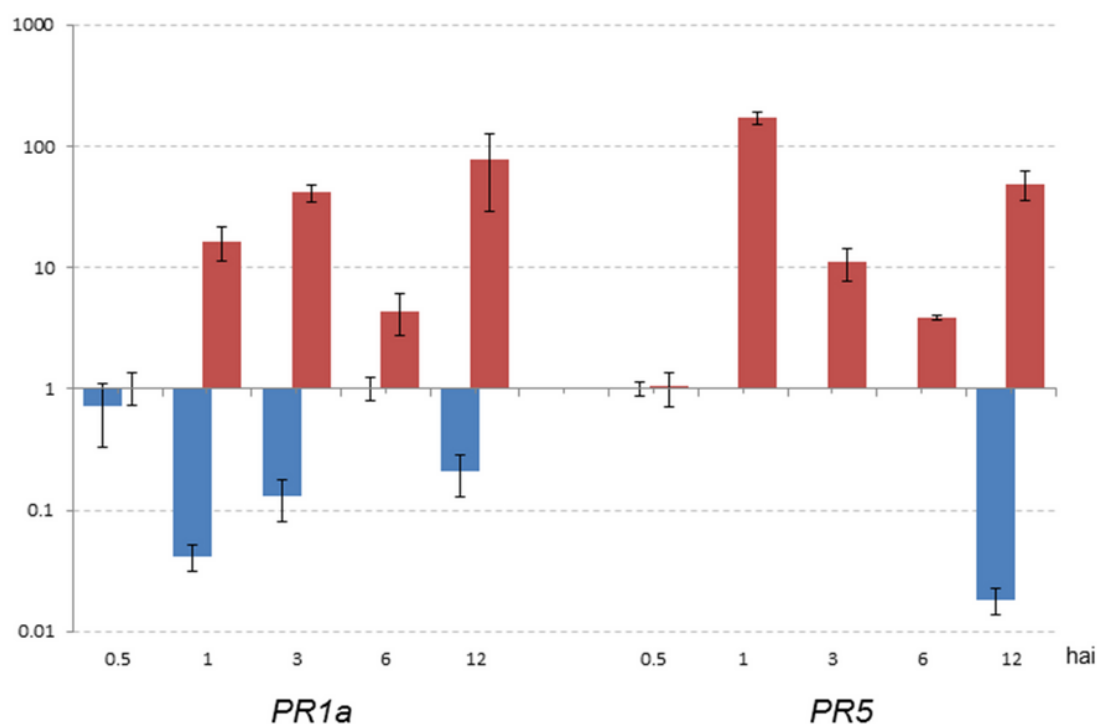


Figure 8

Production of PA and DGPP by different pathways and interconversion reactions by phosphorylation and dephosphorylation. In green is denoted phosphorylation reactions and in red dephosphorylation reactions. In other colours are denoted key enzymes in these pathways. In phosphorylation pathway: PI3K or PI4K, phosphatidylinositol 3-kinase or 4-kinase, respectively; PI4P5K, phosphatidylinositol 4-phosphate 5-kinase; PI3P5K, phosphatidylinositol 3-phosphate 5-kinase; ITPK, inositol-tetrakisphosphate 1-kinase; IPK5, inositol-pentakisphosphate 2-kinase and DGK, diacylglycerol kinase. In dephosphorylation pathway: PAP, phosphatidic acid phosphatase; 5PTase, inositol polyphosphate 5-phosphatase; PI3P phosphatidylinositol 3-phosphatase and PI5P, phosphatidylinositol 5-phosphatase. Key enzymes: PLD, phospholipase D; NPC, non-specific phospholipase C; PLC, phosphatidylinositol-specific phospholipase C and PLA, phospholipase A. PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PIP2, phosphatidylinositol bisphosphate; PIP3, phosphatidylinositol trisphosphate; DAG, diacylglycerol; PA, phosphatidic acid; DGPP, diacylglycerol pyrophosphate; IPx, inositol polyphosphates; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol and PS, phosphatidylserine

Supplementary Files

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