UNIVERSIDAD DE CÓRDOBA

# "*IN VIVO* DETERMINATION OF INTRACELLULAR pH USING pHLUORIN PROTEINS IN *FUSARIUM OXYSPORUM*"

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El **Prof. Antonio Di Pietro** y el **Dr. David Turrá**, director y tutor, respectivamente, del trabajo titulado "***In vivo* determination of intracellular pH using pHluorin proteins in *Fusarium oxysporum***" realizado por **Antonio Serrano Salces** proponen como revisores de dicho trabajo a los profesores:

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## Resumen

El pH extracelular juega un papel clave en los niveles de fosforilación de las MAP quinasas de *Fusarium oxysporum*. Además, existen evidencias significativas de que la virulencia de distintos patógenos fúngicos se ve alterada directamente por el pH extracelular.  Actualmente se desconoce como el pH extracelular afecta al pH intracelular. En este trabajo, hemos hecho uso de una proteína fluorescente sensible al pH, la pHluorin. Dicha proteína posee la misma estructura que la GFP, pero con modificaciones que permiten medir el pH  en el interior celular como valor radiométrico a dos longitudes de excitación y una de emisión. Esta proteína ha sido ampliamente utilizada en la levadura *Saccharomyces cerevisiae*. El objetivo de este trabajo ha sido la construcción de cepas de *F. oxysporum* que expresan de forma constitutiva el gen de la pHluorin, y la optimización de las condiciones experimentales para la medida del pH intracelular. Una vez optimizada la metodología, se identificaron cambios significativos en el pH intracelular cuando la célula fue sometida a cambios drásticos de pH extracelular. Cuando las germínulas se sometieron a pH 9 se observó una subida rápida, seguida por una bajada gradual hasta alcanzar el valor homeostático entre 5.5 y 6.5. Por el contrario, cuando las germínulas se sometieron a pH 4 se detectó una bajada seguida por una subida muy lenta, siendo aún desconocido el mecanismo por el cual la homeostasis de pH es llevada a cabo. Además se observó una acidificación en presencia de glucosa, de forma opuesta a lo anteriormente descrito en levadura. Esta acidificación puede deberse a la entrada de glucosa en el interior celular, mediada por un sinporte con protones. Futuros experimentos serán necesarios para determinar los mecanismos de regulación de la homeostasis del pH intracelular cuando las germínulas se someten a pH 4 y pH 9 y su papel en la señalización y la virulencia de este patógeno.

## OBJECTIVES

1. Set up a method for *in vivo* intracellular pH measurements in *Fusarium oxysporum* using the fluorescent protein pHluorin
2. Characterization of the intracellular pH homeostasis process in *Fusarium oxysporum*
3. Monitoring of *Fusarium oxysporum* intracellular pH in response to glucose

# *IN VIVO* DETERMINATION OF INTRACELLULAR pH USING pHLUORIN PROTEINS IN *FUSARIUM OXYSPORUM*

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## Abstract

In the fungal pathogen *Fusarium oxysporum,* MAPK signalling and virulence is governed by extracellular pH. Recent results in our group revealed differential phosphorylation patterns in MAP kinases depending on extracellular pH. In this study, we set up a method to measure intracellular pH with the pH sensor protein pHluorin, previously used in yeast. We determined the effect of extracellular pH on intracellular pH, observing a homeostatic response to extracellular changes. Addition of glucose causes a rapid acidification of the intracellular pH. Future studies will address the mechanism of pH homeostasis and its effect on signalling and virulence of *F. oxysporum*.

## Introduction

The *Fusarium oxysporum* species complex is a group of filamentous ascomycete fungi that exhibit extraordinary genetic plasticity. *F. oxysporum* strains are ubiquitous in the soil and can exist as saprophytes on soil debris, colonize plant roots as endophytes, even though many strains cause some of the most destructive diseases on a diverse spectrum of hosts, including economically important crops. In recent years members of the *F. oxysporum* complex have also been reported as an emerging cause of human and animal infections on both immunocompromised and healthy individuals (Ortoneda *et al*., 2004).

Members of the *F. oxysporum* species complex produce three types of asexual spores: microconidia, macroconidia and chlamydospores (Agrios, 1997). Microconidia are single-cell dispersal structures that are abundantly produced under most conditions. Macroconidia contain three to five cells and are gradually pointed and curved toward the ends and are commonly found on the surface of dead plants killed by the pathogen. Chlamydospores are thick-walled cells generally developed through the modification of hyphal and conidial cells. Their formation is induced by aging or unfavourable environmental conditions such as low temperatures or carbon starvation and represent the principal structure for long-time survival. These spores play an important role as primary inoculum for plant root infection. In the soil *F.oxysporum* microconidia germinate in response to different signals from the plant host and differentiate into infection hyphae, which adhere to the plant roots and penetrate them directly without the need of specialized infection structures. (Di Pietro *et al*., 2001). Root penetration appears to occur predominantly through natural openings at the intercellular junctions of cortical cells, or through wounds. Once inside the root, hyphae grow inter- and intra-cellularly to invade the cortex and cross the endodermis, until they reach the xylem vessels. The fungus then uses the xylem as an avenue to colonize and cause disease in the host. (Perez-Nadales and Di Pietro, 2011).

Vascular wilt is most likely caused by a combination of pathogen activities and plant defence responses. The former include accumulation of fungal mycelium in the xylem vessels and phytotoxin production, while the latter include production of vascular gels, gums and tyloses, and vessel crushing by proliferation of adjacent parenchyma cells (Beckman, 1987). Ultimately, wilt symptoms are caused by severe water stress mainly due to vessel occlusion. Disease symptoms include wilting, chlorosis, necrosis, premature leaf loss, browning of the vascular system and stunting, which eventually will lead to plant death (Michielse and Rep, 2009). Severely infected plants wilt and die, while plants affected to a lesser degree become stunted and lose productivity.

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**Table 1**. **Common genetically encoded pH sensors used for in vivo pH measurements.** Adapted from Martiniere and coworkers, 2013.

| Constructs | **Type** | **Excitation (nm)** | **Emission (nm)** | **pH range** | **Reference** |
|---|---|---|---|---|---|
| **pHluorin,**<br>**pHluorin2*,**<br>**RaVC**,**<br>**PRpHluorin***.** | Ratiometric | 395/475 | 510 | 5.4-8.4 | (Miesenbock *et al*., 1998) |
| **Ecliptic pHluorin,**<br>**PEpHluorin*** ** | Ecliptic | 395/477 | 510 | 6,5-8 | (Miesenbock *et al*., 1998) |
| **Superecliptic pHluorin** | Ecliptic | 477 | 510 | 5.5-8.5 | (Sankaranarayanan and Ryan, 2001) |

***Eightfold brighter version of pHluorin***

*****Codon optimization for Aspergillus.***

******Codon optimization for Arabidopsis***

### *MAP kinase pathways*

Mitogen-activated protein (MAP) kinases are ubiquitous and evolutionarily conserved enzymes in eukaryotic organisms. They connect cell surface receptors to critical regulatory targets within cells that resulting in various morphogenetic processes.

MAP kinase activity is regulated through a three-tiered cascade composed of a MAP kinase, MAPK kinase (MAP2Ks), and a MAPK kinase kinase (MAP3Ks) (Hamel *et al*., 2012; Xu, 2000).

MAP kinase cascades function as key signal transducers that use protein phosphorylation/dephosphorylation cycles to channel information. Activated MAPK kinase kinases (MAP3Ks) first phosphorylate two Ser and/or Thr residues located within the activation loop of MAPK kinases (MAP2Ks). Activated MAP2Ks in turn trigger MAPK activation through dual phosphorylation of a highly conserved activation loop that possesses the hallmark motif -TXY-. Activated MAPKs can then phosphorylate downstream substrates, affecting their biochemical properties and leading to specific output responses (Hamel *et al*., 2012).

*F. oxysporum* has three known MAP kinases: *Fmk1*, *Mpk1* and *Hog1*. *F. oxysporum* mutants lacking the *fmk1* gene are impaired in multiple virulence-related processes, such as root adhesion, host penetration, and invasive growth on the living plant tissue (Di Pietro *et al*., 2001). *Fmk1* is also required for vegetative hyphal fusion, a ubiquitous developmental process in filamentous fungi (Prados Rosales and Di Pietro, 2008). In addition to this, Δ*fmk1* mutants do not show chemotropic response to nutrients such as glucose and sodium glutamate (Turrá *et al*, unpublished). The cellular mechanisms through which this conserved MAPK cascade controls such a wide array of pathogenicity functions are largely unknown.

The orthologous MAP kinases in *Saccharomyces cerevisiae*, *Fus3* and *Kss1*, are involved in cell fusion and mating, filamentous growth and nutrient responses (Hamel *et al*., 2012).

The other two MAP kinases, *Mpk1* and Hog1 are essential for cell wall integrity (Turrá *et al*, unpublished) and hyperosmotic stress response, respectively.

### *Intracellular pH sensors*

Several tools have been developed in the last decades to quantify the concentration of protons [$H^+$] inside the cell (cytosolic pH or pHin). Among these, fluorescent dyes have been the most commonly employed. One such dye is the 2´, 7´ - Bis (2–carboxyethyl) – 5 (6) - carboxyfluorescein (BCECF), which needs previous membrane permeabilization with acetomethyl esterin order to be effective (Krebs *et al*., 2010).

Nevertheless, such approaches are invasive and do not allow pH measurement in cell compartments other than the cytosol.

To overcome this problem genetically encoded pH sensors, such as pH sensitive green fluorescent protein (GFP) variants have been developed in the recent past.

The chromophore of GFP is formed by two autocatalytic reactions (cyclization and oxidation) between Ser65, Tyr66 and Gly67. It adsorbs light at two different wavelength depending upon its state of protonation: 395 nm when protonated and 475 nm when deprotonated. Most GFP variants display pH-sensitive absorption and have been used to measure pHin in living organism. However genetically engineered versions of GFP have also been obtained that display enhanced fluorescent and that therefore allow a more reliable measurement of cellular pH. Among the recently developed pH-sensitive GFP variants there are the ecliptic and superecliptic pHluorins. (Miesenbock *et al*., 1998). However, the equilibrium between the protonated and deprotonated states of these proteins is also affected by temperature and ionic strength. In addition to this the amount of light that they emit is dependent upon its own concentration.

To circumvent this issue, ratiometric pH sensors have been developed providing a pH readout independent of the probe concentration. The most popular and effective sensor is pHluorin which was generated by introducing one mutation in the GFP gene, conferring ratiometric behaviour to the protein (Miesenbock *et al*., 1998). This means that following an environmental increase in proton concentration the chromophore on one hand shows a strong increase in light emission (508 - 510 nm) when excited at a wavelength of 395 nm and on the other hand a strong decrease in light emission (508 - 510 nm) when excited at a wavelength of 475 nm, therefore generating a ratiometric measure (see Figure 1).

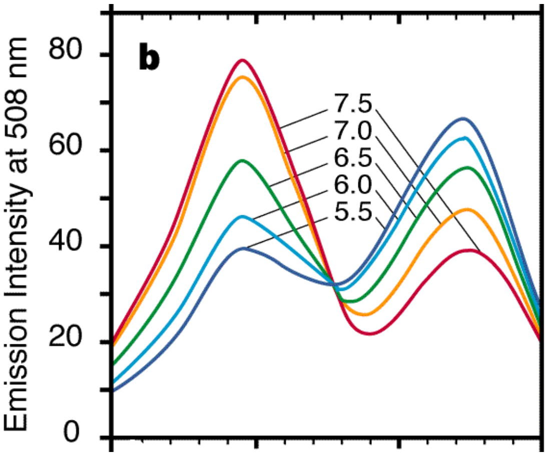

**Figure 1. Ratiometric spectrum of pHluorin proteins.** Modified from (Miesenbock *et al*., 1998).

This kind of probe allows measuring pH changes that range between 5.5 to 8.5 (see Table 1). Additional variants of pHluorin have been obtained such as the ratiometric Venus Citrine (RaVC) that has been codon optimized to be used in pHin measurement in Aspergillus hyphae. (Bagar *et al*., 2009).

Apart from pHin measurement genetically encoded pH sensors can be used to measure pH changes in specific subcellular compartments via chimeric fusion to proteins specifically resident in the organelle of interest.

For example, to determinate the intercellular membrane pH, the pHluorin gene has been joined to the Ato1p gene, a plasma membrane ammonium transporter (Pineda Rodo *et al*., 2012).

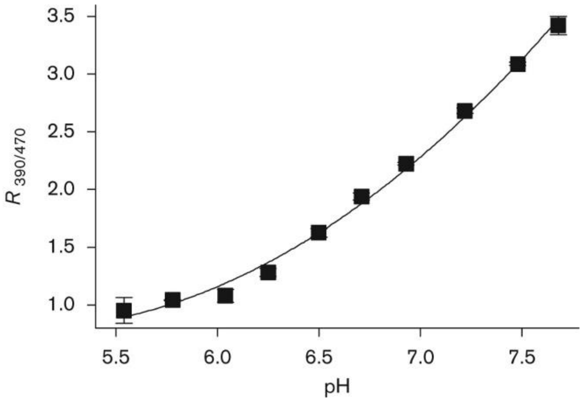

**Figure 2. pH calibration curve in *Candida glabrata*** (Ullah *et al*., 2013)

### *Regulation of intracellular pH*

It is well established that pHin and homeostasis are crucial for multiple cellular processes, such as transmembrane transport and establishment of electrochemical gradients (Brett *et al*., 2005). In addition to this, pHin also seems to be important to respond to changes in the concentration of extracellular nutrients such as glucose and acetic acid in yeast (Isom *et al*., 2013). In yeast, the presence of glucose in the extracellular medium induces an alkalinisation of pHin while glucose starvation induce acidification (Maresova *et al*., 2010).

Intracellular pH changes can activate several signalling pathways such as the cAMP-dependent protein kinase A (PKA) pathway. In presence of glucose activation of the PKA pathway occurs and pHin plays a key role as a second messenger by regulating the function of a membrane bound vacuolar ATPase (V-ATPase). (Dechant *et al*., 2010).

Other signalling components whose activation is dependent on pHin are small G proteins. It was recently been demonstrated that Gα subunits, the principal transducers of GPCR signals, can acquire different conformations and phosphorylation states depending on pHin. (Isom *et al*., 2013).

Recent results obtained in our laboratory have shown that extracellular pH (pHex) has an important role in the regulation either directly or indirectly of the phosphorylation level of the three MAPKs *Fmk1*, *Mpk1* and *Hog1* in *F.oxysporum* (Segorbe *et al*, unpublished). Two hypotheses have been proposed. In a first hypothesis specifically dedicated plasma membrane receptors could detect pHex changes and transduce the signal to upstream components of the above mentioned MAPK pathways. Alternatively, changes in pHex could determine a change in pHin by the establishment of an electrochemical gradient which could act as a second messenger that modulates phosphorylation levels of the distinct MAPK. The aim of this work was to set up a system for pHin measurement in *F.oxysporum* and to use it to detect pHin changes following pHex changes or nutrient stimulation.

# Materials and methods

**Table 2. *Fusarium oxysporum f. sp. lycopersici strains used in this study***

| **Strain** | **Background** | **Genotype** | **Reference** |
|---|---|---|---|
| 4287 (WT) | 4287 | - | J. Tello, University of Almeria, Spain |
| WT + pHluorin | 4287 | pHluorin:HYG | This study |
| WT + RaVC | 4287 | RaVC:HYG | This study |

**Table 3. *Synthetic oligonucleotides.*** All primers were designed with an annealing temperature of 62°C. Exceptions are indicated with an asterisk and correspond to a Tm of 64ºC.

| **Gene/Name** | **Sequence** |
|---|---|
| **M13F** | CGCCAGGGTTTTCCCAGTCACGAC |
| **M13R** | AGCGGATAACAATTTCACACAGGA |
| **gpda15b** | GGATCCCGAGACCTAATACAGCCCCT |
| **gpda9** | TGTGATGTCTGCTCAAGCGG |
| **pHlforcola** | *CCGCTTGAGCAGAGATCACA*AAGCTTCGGTGACGGTGCTG * |
| **pHlRevNest** | GGGACGAGGCAAGCTAAACA |
| **pHlRev** | TGTCAAGGAGGGTATTCTGGG * |

**Table 4. *Plasmid used in this study*.**

| **Plasmid** | **Origin/features** | **Reference** |
|---|---|---|
| **pGEM®-T** | Derived from plasmid pGEM®-5Zf (+), linearized with *Eco*RV and with a T added in both 3' ends. | PROMEGA |

**Table 5. Media and buffer solutions used in this work**

| Media/solutions | Ingredients and preparation |
|---|---|
| **Potato Dextrose Broth (PDB)** | Boil 200 g of peeled potatoes in 0.6 L of water for 60 min. Filter and add 20 g of glucose and deionized water up to 1 L. Sterilize by autoclaving |
| **Potato Dextrose Agar (PDA)** | Dissolve 3.9% potato dextrose agar (w/v) (Scharlau Microbiology). For culturing F. oxysporum transformants add hygromycin B (55 µg/ml), phleomycin (5.5 µg/ml) or nourseotricine (200 µg/ml) after autoclaving. |
| **Yeast extract Peptone Dextrose (YPD)** | Dissolve 10g of yeast extract and 10g of glucose in 1 L and adjust to pH 8.0 with NaOH 10 N. Add 20 ml HEPES 1M after autoclaving. |
| **Regeneration minimal medium** | Dissolve 0,15g of $MgSO_4$ x 7·H2O, 0,3g of $KH_2PO_4$, 0,15g of KCl, 0,6g of $NaNO_3$, 60g of sucrose and 6g of glucose in 0,3 L. Add oxoid agar (3g) for 0,2 L of Petri dishes (1,5% agar) and 0,5g for top Agar (0,5%) for 0,1 L. Sterilize by autoclaving. |
| **pH buffer for intracellular pH calibration** | 50 mM HEPES, 50 mM MES, 50 mM KCl, 50 mM NaCl, 0,2 M ammonium acetate, 10 mM sodium azide. Adjust to pH 5.5 with HCl, to pH 6.0 and 6.5 with NaOH 10 N and pH 7.0 and 7.5 with one pellet of caustic soda and NaOH 10 N. pH 8.0 and 8.5 needs 2 pellets of soda caustic plus NaOH 10 N. Sterilize by filtration. |
| **buffer for pH measurement** | 50 mM MES adjust to pH 6 with NaOH 10N. Sterilize by filtration. |
| **OM** | Dissolve 1.2 M of $MgSO_4$ in 180 ml of $ddH_2O$ and 0.1 M of $Na_2HPO_4$ in 100 ml of $ddH_2O$. Mix 20 ml of $Na_2HPO_4$ solution with all $MgSO_4$ solution. Adjust to pH 5.8 – 6.0 with orthophosphoric acid. Sterilize by filtration. |
| **STC** | Dissolve 14.52 g of sorbitol in 94 ml of $ddH_2O$ and sterilize by autoclaving. Mix 5 ml of 1M $CaCl_2$ solution and 1 ml of Tris-HCl 1M pH 7.5 with sorbitol solution. Sterilize by filtration. |
| **CTAB extraction buffer** | Dissolve 12.1 g Trizma base, 7.44 g EDTA; 81.8 g NaCl and 20 g Cetyltrimethylammonium bromide. Heat to 60 °C to dissolve and adjust to pH 8.0 with NaOH. Keep at 37 ºC to avoid precipitation. |
| **PEG (Polyethylene glycol)** | Dissolve 6,279 g of MOPS (Sigma) in 50 ml of $ddH_2O$. Mix MOPS solution with 30 g of PEG 4000 and keep at 50 ºC to dissolve. Adjust final volume to 50 ml with MOPS and sterilize by filtration. |
| **TEC** | Mix 500 µl of Tris-HCl 1 M pH 7.5, 100 µl of EDTA 0.5 M and 2 mL of $CaCl_2$ 1 M with 48 ml of $ddH_2O$. |
| **KSU medium** | Dissolve 1.74 g of $K_2HPO_4$, 1.42 g of sodium succinate dibasic (Sigma) and 0.3 g of Urea (Merck) in 200 ml $ddH_2O$. Adjust to pH 6.0 with NaOH. Sterilize by filtration. |

### *Escherichia coli growth conditions*

Escherichia coli strain XL1Blue was routinely grown at 37°C in Luria-Bertani medium (1% tryptone, 0.5% yeast extract, and 1% NaCl). For selection of transformant *E. coli* clones either the antibiotic ampicillin (100 µg/ml) or kanamycin (15µg/ml) was added to the media after autoclaving. To screen colonies that had been transformed either with the pGEM-T (Promega) plasmid alone or with the pGEM-T and the desired insert plasmids blue-white selection with X-gal and IPTG was carried out.

### *F. oxysporum growth conditions*

*F. oxysporum* strains were cultured either in rich PDB and YPD (see table 5) media or in nutrient limiting minimal media (MM) (see Table 5), according to specific requirements and experimental designs. For microconidia production or extraction of DNA, cultures were grown in liquid PDB at 28°C with orbital shaking at 170 rpm for 4- 5 days.

For transformant selection hygromicin B (55 µg/ml), phleomycin (5.5 µg/ml) or nourseotricine (25 mg/ml) were added to the culture medium. For preparation of desired inoculum, microconidia were obtained as a result of filtration through a nylon filter (Monodur; mesh size 10 µm) as described previously (Di Pietro and Roncero, 1998) and harvested by centrifugation at 10.000 x g for 15 min. Microconidia were counted using a Thomas Chamber and conidia concentrations were adjusted to the desired one. For long-term storage of *Fusarium* strains microconidia from 4-5 day-old cultures were collected by filtration, resuspended in 30% glycerol (v/v) and stored at -80ºC.

### *DNA isolation*

DNA samples from Fusarium mycelium were extracted by CTAB method (Torres *et al*., 1993). Briefly, mycelium were ground to a fine powder in a mortar and pestle under liquid nitrogen, and 1 ml of CTAB buffer (see Table 5) was added. Then 4 µl of β-mercaptoethanol (Merck) and 500 µl of a chloroform: octanol 24:1 (v/v) solution were added, quickly mixed with the use of a vortex and incubated at 65ºC for 30 minutes. After incubating at room temperature for 15 minutes tubes were centrifuged for 5 minutes at 10.000 x g.

The supernatant was then precipitated with 1 ml of 100% ice-cold ethanol and incubated at -20ºC for at least 10 minutes followed by centrifugation for 5 minutes at 7.500 x g and two consecutive washes with 1 ml of 75 % ethanol. Finally, the pellet was resuspended in 50-100 µl of sterile deionised water with 4 µl of RNaseA (10 mg/ml) and incubated at 37°C for 30 minutes.

### *Nucleic acid quantification*

DNA was quantified in a Nanodrop® ND-1000 spectrophotometer at 260nm and 280 nm wavelengths, respectively. In addition, the quality of the obtained DNA was monitored by electrophoresis in a 1% agarose gel (w/v).

### *PCR amplification*

For standard PCR the amplifications were performed in a termocycler using the thermostable DNA polymerase of the Roche Expand High Fidelity PCR System. Each reaction contained 10x PCR buffer, 300 nM primers, 2.5 mM of MgCl$_2$, 0.8 mM dNTPs mix and 0.05 U/µl of polymerase. Genomic DNA was added at 20 ng/µl and plasmid DNA at 2 ng/µl. PCR cycling conditions were: an initial step of denaturation (5 min, 94ºC) followed by 35 cycles of 35 s at 94ºC, 35 s at the calculated primer annealing temperature and 35 s at 72ºC (or 68ºC for templates larger than 4 Kb), and a final extension step at 72ºC (or 68ºC) for 10 minutes. PCR amplifications on DNA samples extracted from putative transformants were carried out with the Bioline Taq Polymerase system. Each PCR reaction contained 10x PCR buffer, 6 nM primers, 2 mM of MgCl$_2$, 0.6 mM dNTPs mix, 0.05 U/ µl of polymerase and 20 ng/µl of genomic DNA. PCR cycling conditions were:: an initial step of denaturation (5 min, 94ºC) followed by 35 cycles of 35 s at 94ºC, 35 s at the calculated primer annealing temperature, 35 s at 72ºC, and a final extension step at 72ºC for 10 minutes.

### *Fusion PCR*

Complementary oligodeoxyribonucleotide primers (see Table 3) and the polymerase chain reaction were used to generate DNA fragments (*Asperillus nidulans* gpdA promoter and pHluorin gene followed by the ADH5 terminator of *Saccharomyces cerevisiae*) with overlapping ends (see figure 3a). The pHluorin transformation construct was obtained by fusion PCR (see figure 3b) by fusing the overlapping fragments with the use of the gpda15b and pHlRevNest primers (see Table 3 and figure 3b).

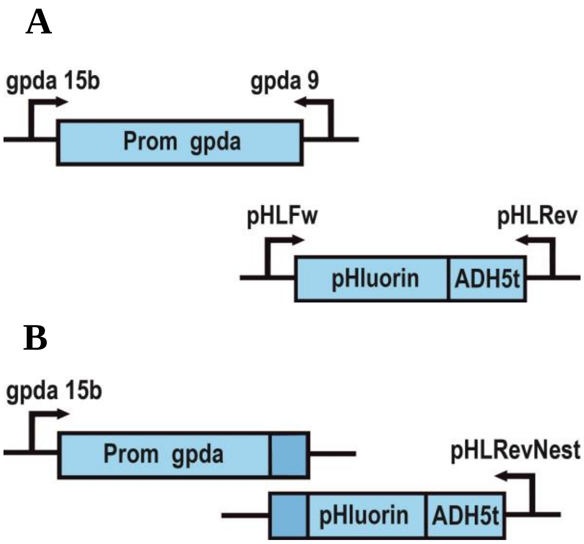

**Figure 3. Fusion PCR (A)** PCR of *gpda* promoter and *pHluorin* gene and (**B**) fusion PCR of both fragments.

### *Protoplast generation in F.oxysporum*

Protoplasts were obtained following the protocol described by (Powell and Kistler, 1990), with some modifications. Briefly, 5x10$^8$ microconidia were inoculated into 200 ml of PDB and incubated for 14 h at 28°C with 170 rpm. After, germlings were harvested by filtration with a monodur and washed first with 500 ml of ddH2O and after with 200 ml OM solution (see Table 5).

A sterile spatula was used to transfer germlings from the monodur to a sterile 50 ml Falcon tube, containing 20 ml of OM with 0.5% (w/v) Glucanex® (Novozymes) as protoplasting enzymatic mix. Protoplasts were incubated in the enzyme solution for 45 minutes at 30°C with slow shaking (60 rpm), and protoplast liberation was controlled under the microscope. When the desired quantity of protoplasts was achieved, the sample was filtered through a double layer of monodur nylon filters and washed with 400 ml of STC solution (see Table 5). The flow-through containing the protoplasts was collected in pre-chilled ice-cold 50 ml centrifuge tubes. Filtrates were centrifuged at 4°C and 1.500 x g for 15 minutes to collect protoplasts, which were carefully resuspended in 1 ml STC and counted. The protoplast suspension was adjusted to a final concentration of 2 x $10^7$ protoplasts and stored as 100 µl aliquots in Eppendorf tubes to be used directly for transformation.

For long-term storage at - 80°C, 10% of PEG (see Table 5) (v/v) and 1% DMSO (Merck) (v/v) were added.

### *F.oxysporum transformation*

Transformation was performed as described (Malardier *et al*., 1989), with slight modifications. 2-3 µg of transforming DNA was combined with 10 µl of 0.1 M aurintricarboxylic acid (ATA), a potent inhibitor of nucleases, in a final volume of 60 µl with TEC solution (see Table 5).

For cotransformation experiments, 1.5 µg of the DNA construct conferring antibiotic resistance was added. For negative transformation control, 50 µl of TEC was mixed with 10 µl of ATA in an additional tube and treated like the transformation mix in the following procedure. The 2 mixes were incubated on ice for 20 minutes in parallel with 2 tubes of 100 µl protoplasts (2x$10^7$) generated as indicated above. Next, protoplasts and DNA solutions were carefully mixed and incubated a further 20 minutes on ice. Then, 160 µl of PEG solution were added and mixed carefully, followed by 15 minute incubation at room temperature before 1 ml of STC solution was added. The tube was centrifuged for 5 minutes at 3.000 rpm to pellet protoplasts, which were resuspended in 200 µl of STC. Next, 50 µl aliquots were combined with 3 ml of top agar (see Table 5) at 45°C and spread onto plates containing 25 ml of solid regeneration minimal medium (see Table 5). Four transformation controls were done. To calculate the protoplast regeneration 10 µl of the negative control mix was diluted $10^4$ and $10^5$ with STC and spread on plates without antibiotic. A $10^5$ dilution in $H_2O$ which leads to burst of the protoplasts was used to determinate amount of conidia present in the protoplast solution, as they can reduce the transformation efficiency.

The antibiotic control was implemented by spreading the rest of the negative control (190 µl) on regenerations media containing the appropriate antibiotic. Plates were incubated at

28°C for 14-16h, 2h or 2-3h before addition of 3 ml of top agar containing 2 mg of hygromycin B, 160 µg of phleomycin or 1 mg of nourseotricine, respectively. Incubation at 28°C was prolonged for 4-5 days until transformant colonies became visible. Colonies were transferred to PDA plates with selective medium, and transformants were submitted to two consecutive rounds of single monoconidial purification on selective PDA plates.

### *Germling production in F.oxysporum*

For germling production 250 µl of spores from each strain were inoculated in 1L conical flasks containing 250 ml of PDB medium (see Table 5) supplemented with the appropriate antibiotic and incubated for 4-5 days at 28ºC 170 rpm.

Microconidia were collected by filtration through a nylon filter (Monodur; mesh size 10 µm) as described previously (Di Pietro and Roncero, 1998) and 2x$10^9$ spores were inoculated in 0.1 L of YPD medium pH 8 (see Table 5) for 3h at 28ºC 170 rpm to induce conidia germination

Germlings were finally recovered by centrifugation (10.000 x g for 15 minutes), washed twice with water and resuspended in 1 ml of water, counted and adjusted to a concentration of 1x$10^9$ germlings per ml.

### *pHin calibration curve*

Ratiometric pHluorin was used to determine intracellular pH and its changes. A calibration curve for intracellular pH measurement was performed as previously described (Maresova *et al*., 2010) with some modifications.

Germlings were resuspended in a series of calibration buffers (see Table 5) of defined pH: 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. For measuring pHin, the same amounts of cells were resuspended in 50 mM MES pH 6 (see Table 5).

We used a Flexstation microplate reader (Molecular Devices) with an emission filter at 510 nm and excitation filters at 395 and at 475 nm for all experiments. Glucose response experiments were done in a Spectrofluorometer microplate reader (Tecan) with an emission filter at 510 nm and excitation filters at 390/20 nm and at 465/30 nm. The fluorescence ratio was calculated using the software Excel (Microsoft).

On a 96-well flat-bottom PS microplate we had a calibration section (90 µl calibration buffer, three replicates of each pH) and an experimental section. We added the same amount of germlings to the calibration wells and the experimental wells ($10^9$ germlings/ml).

The Excel software calculated a calibration curve as a function of the measured values in the calibration section, relative to the pH values of the calibration buffer, which were entered into the measurement protocol manually (the data are fitted to a quadratic-degree polynomial regression).

According to the calibration curve, we calculated the pHin values from fluorescence ratios measured in the experimental part. All the data shown are the averages from two to three independent experiments.

### *pHin homeostasis experiments*

In microtiter wells, kinetic measurements were done to measure pHin in two different conditions. The cells were resuspended in 50 mM MES pH 6.0 which was the first measurement for both conditions. Then, one experimental row was adjusted to pH 9.0 with 10.5 µl of a dilution 1:80 of NaOH, and another to pH 4.0 with 15 µl of a dilution 1:100 of HCl. The data were measured for 30 minutes.

### *Glucose responses experiments*

For the pHin homeostasis experiments, a kinetic measurements was performed. Germlings were inoculated in KSU medium (see Table 5) and grown at 28 ºC, 170 rpm, during 30 minutes. Then cells were collected and counted as described previously and resuspended in microtiter wells with KSU medium. A kinetic of 30 minutes was performed after addition of 10 µl glucose (20%) into the wells.

## Results

### *Pgpda – phluorin construct*

To generate a fusion between the Aspergillus gpdA promoter and the pHluorin gene, a first PCR (1.2kb) of the pHluorin gene was carried out with pHlforcola and pHlRev primers (see Table 3, figure 3, 4), generating an overlapping 3' end complementary to the gpda9 primer. In a second PCR the gpdA promoter (2kb) was amplified with the primers M13F and gpda9 (see Table 3, figure 3, 4).

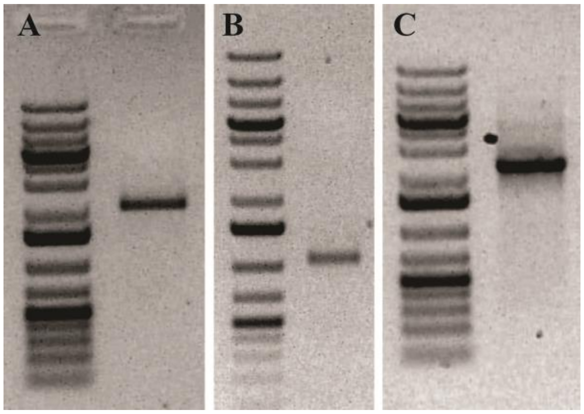

**Figure 4. Gels performed for fusion PCR.** (**A**) Lane 1: GeneRuler 1kb DNA ladder, lane 2: gpdA fragment (2kb). (**B**). Lane 1: as gel A. Lane 2: pHluorin fragment (1,2kb). (**C**). Lane 1: as gel A. Lane 2: Fusion PCR (2,1kb).

The fusion PCR (2,1kb) was carried out with gpda15b and pHlRevNest, generating a construct containing the gpdA promoter fused to the pHluorin gene (see Figure 3, 4). This construct was cloned in pGEM-T (see Table 4) and overexpressed in *E.coli* DH5α.

### *Generation of transformants in F.oxysporum*

The gpdA – pHluorin construct and the hygromycin cassette were used to cotransform protoplast from the wild-type strain of *F.oxysporum.* The transformation was performed with 6 µg of gpdA – pHluorin construct and 3 µg of hygromycin cassette. In the case of the RaVC transformation cassette, a single PCR with the primers M13F and M13R (see Table 3) was

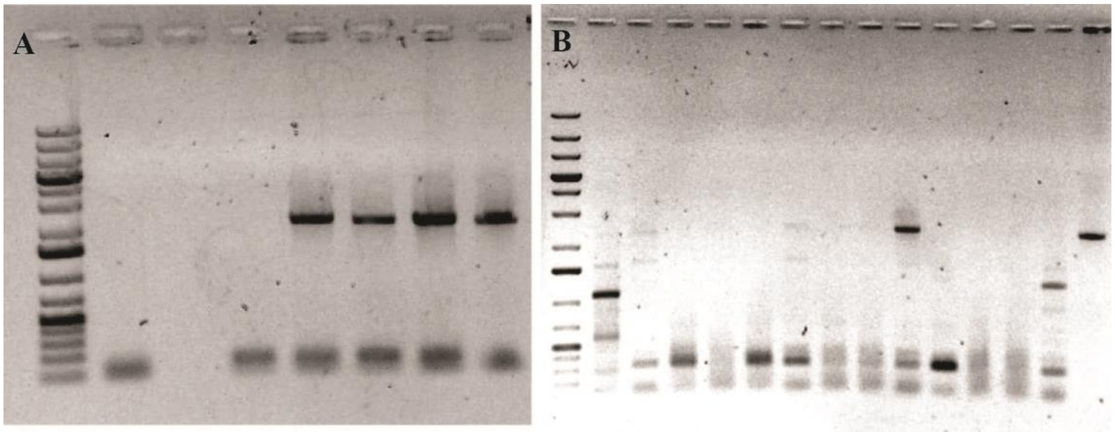

**Figure 5. PCR analysis to study transgene integration in F. oxysporum transformed isolates obtained by protoplast transformation.** (**A**) Correspond to pHluorin PCR with primers gpda15b and pHlNestRev (see Table 3). Lane 1: GeneRuler 1kb DNA ladder, lane 2: wild-type DNA, lane 3-6: transformants isolated. T.9, T12 and T.15 respectively. Lane 7: pHluorin plasmid DNA (C+). (**B**) Correspond to RaVC PCR with M13F and M13R (see Table 3). Lane 1: GeneRuler 1kb DNA ladder, lane 2: Wild-type DNA, lane 3-14: transformants isolated. Lane 15: RaVC plasmid DNA.

performed (data not shown). This amplicon (6 µg) together with the hygromycin cassette (3 µg) was used to cotransform protoplast from the wild-type strain of *F.oxysporum*. Three and one transformants that had integrated pHluorin (T.9, T.12 and T15) or the RaVC (T.13) gene, respectively, were obtained as confirmed by PCR amplification from genomic DNAs of the entire construct with the primer pairs gpda15b/pHlNestRev for pHluorin PCR and M13F/M13R for RaVC PCR (see Table 3, Figure 5). Subsequently we checked the fluorescence intensity in the transformed strains with confocal microscopy (see Figure 6) as previously described (Pineda Rodo *et al.*, 2012).

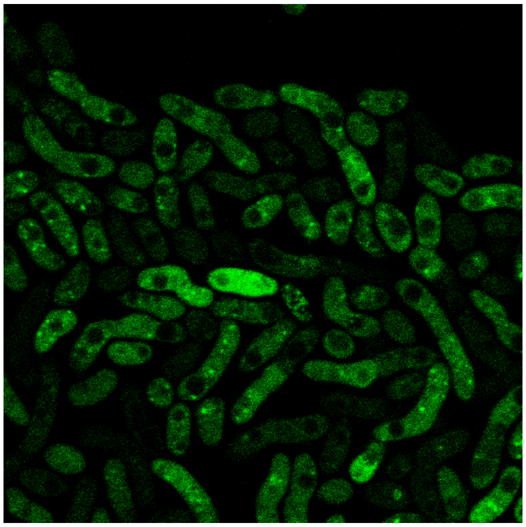

**Figure 6. Image obtained with confocal microscopy with GFP settings.**

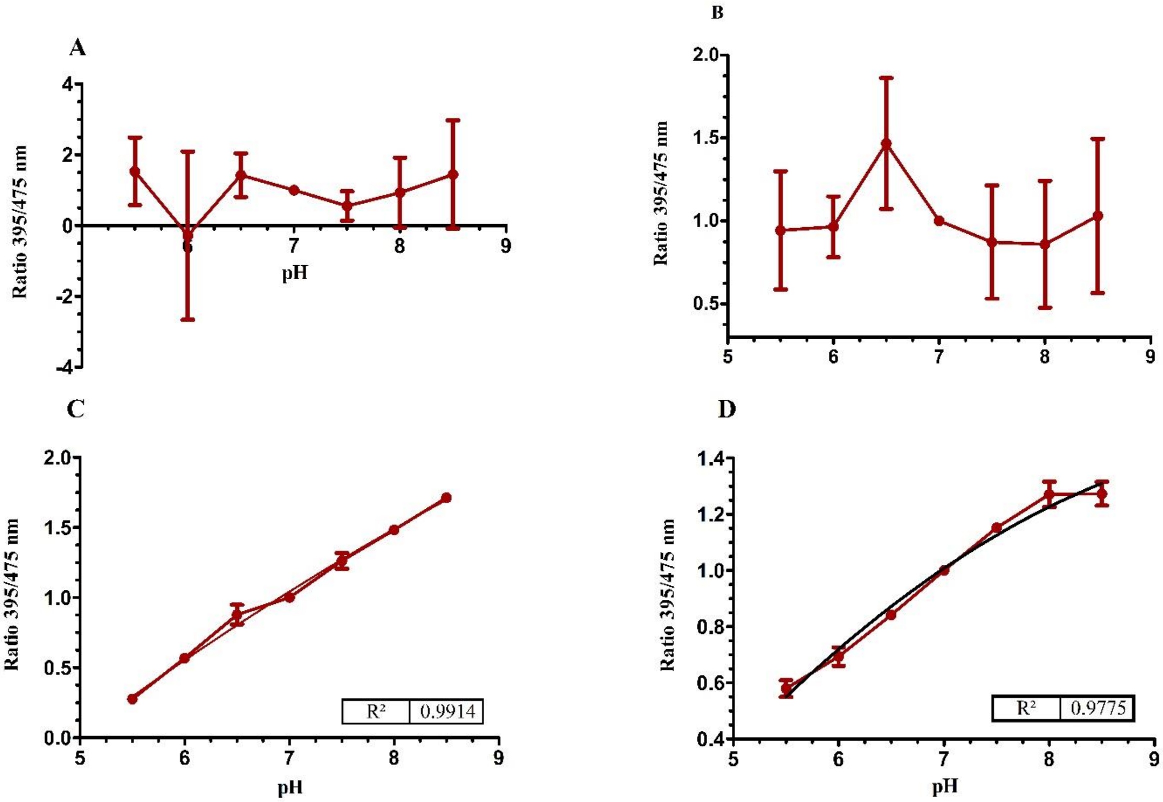

**Figure 7. Optimization of calibration curve using different germination times in pHluorin expressing *F.oxysporum* strain T.9.** (**A**) Spores not germinated. (**B**) Germination 1 hour. (**C**) Germination 2 hours. (**D**) Germination 3 hours.

### *Optimization of the pHin calibration curve*

*F.oxysporum*, as most filamentous fungi shows a high degree of autofluorescence when excited with ultraviolet light (Rath *et al*., 2014). To overcome this problem when measuring the fluorescence emitted by cells of the WT + pHluorin or WT + RAVC strains, we always subtracted the autofluorescence of the wild type strain (used as a negative control) grown in the same environmental conditions. In addition, in order to minimize fungal autofluorescence we tested conidia germinated for different periods of time (see Figure 7). The time of germination that provided the most reproducible and similar data to previously described pHin calibration curves in *S. cerevisiae* and human cells were 2 and 3 hours (Figure 7d). Conidia germinated for less than two hours failed to show a pH-dependent variation in the ratio of emitted fluorescence at 395/475 nm (Figure 7a,b).

### *pH homeostasis in F.oxysporum*

As commented previously, precious studies in *F.oxysporum* revealed alterations in MAP kinase phosphorylation in response to extracellular pH

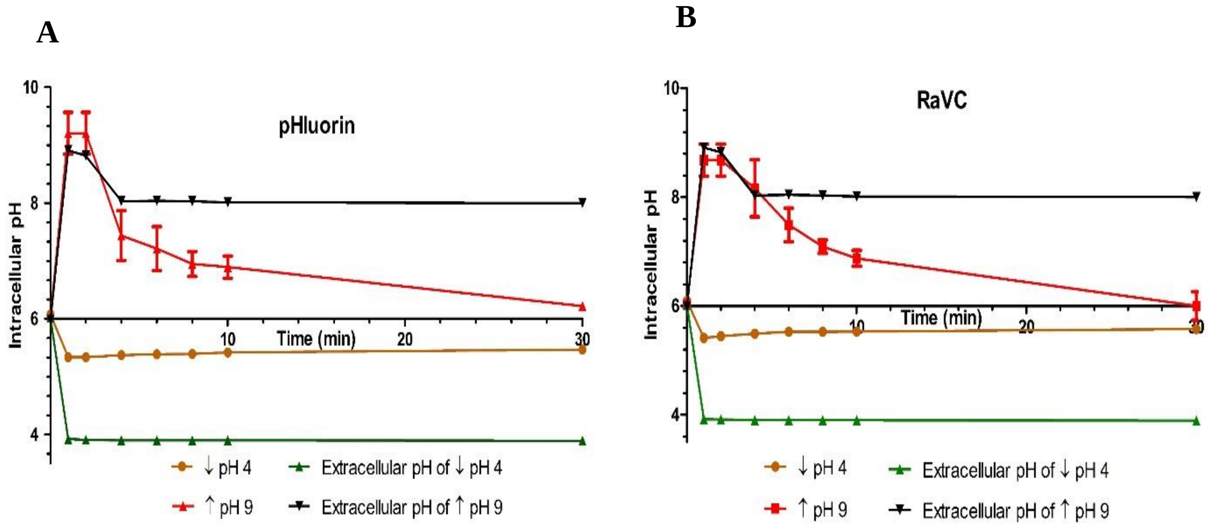

**Figure 8. pH homeostasis in *Fusarium oxysporum*.** (**A, B**) Measurement of pHin and pHex in the pHluorin transformed strain (**A**) and the RaVC transformed strain (**B**). Both strains were pregerminated for 3 hours in YPD medium (see Table 5) and pHin and pHex measured as described in *materials and methods* and plotted over time.

changes (Segorbe *et al*, unpublished). However, it remains to be determined how pHex affects pHin values. For this purpose, germlings were grown in buffer optimized for pH measurement (see Table 5) and treated either with NaOH or HCl.

The addition of NaOH produced a rapid and simultaneous increase of pHex and pHin approximately to pH 9.0. While pHex decreased to pH 8.0 in 3 minutes and then remained stable during the remaining time of the experiment. pHin was gradually decreased to the original pH 6.1 over 30 minutes. Addition of HCl produced an immediate decrease of pHex and pHin within only 1 minute of treatment. While pHex decreased to pH 4 and remained constant over time. pHin decreased until pH 5.3, showing a slowly increase to pH 5.7 over the 30 minute (see Figure 8). Experiments were performed both with pHluorin and RaVC strains showing very similar results independently of the fluorescent protein used (see Figure 8).

### *pHin changes in response to glucose addition*

Although is not well clarified the mechanism by which glucose induces a change in pHin, has been reported in *Saccharomyces cerevisiae* (Isom *et al*., 2013; Maresova *et al*., 2010; Orij *et al*., 2011) and *Aspergillus niger* (Hesse *et al*., 2002), among others.

Considering this information, we aimed to determine whether addition of the glucose to the medium could affect pHin in *F.oxysporum*.

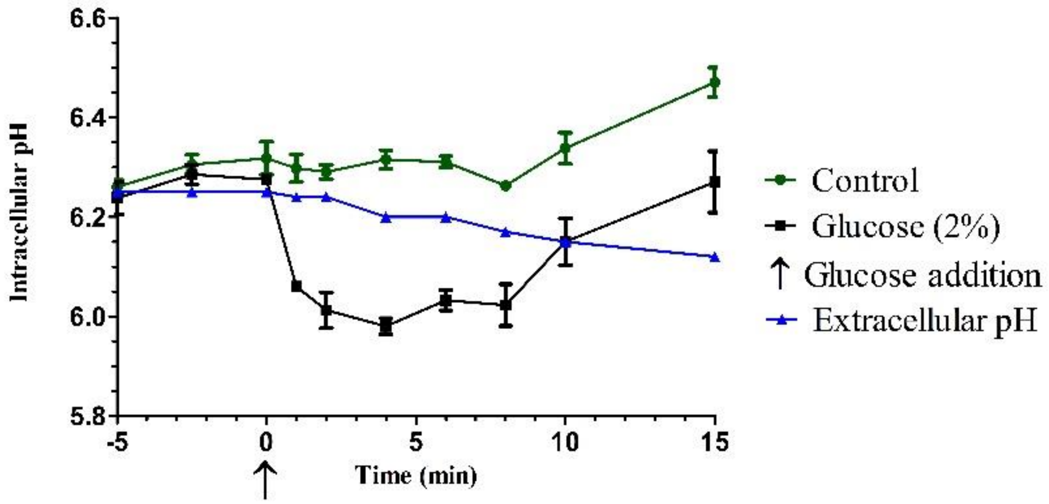

**Figure 9. pHin acidification in response to glucose addition in pHluorin transformed *F.oxysporum* strain T.9.** pH responses in KSU medium (1:5 dilution). Control correspond to cells without glucose addition. Glucose was added at time 0 (↑). Blue line represents values of extracellular pH in wells where glucose was added.

Germlings were grown in KSU medium (see Table 5) and glucose was added to 2% (w/ v) of final concentration (see Figure 9). Control germlings were maintained in KSU medium without glucose addition. The addition of glucose produced a rapid acidification of pHin from 6.4 to 6.0 approximately, while addition of water did not produce any significant pH changes.

The acidification was maintained after 10 minutes, after which pHin of control and treated cells increased. pHex was also quantified in treated cells, showing a slow decrease by 0.15 units. Data were recorded for 15 minutes.

## Discussion

pHin plays an important role in the physiology of yeast as it regulates a variety of cellular processes which are essential for proliferation and survival (Orij *et al*., 2011). In the human pathogenic hemiascomycete *Candida albicans*, germ tube formation requires of pHin alkalinisation (Kaur *et al*., 1988).

In addition, pHin alkalinisation promotes differentiation and cell cycle progression, while acidification induces apoptosis in *S.cerevisiae* (Orij *et al*., 2011).

In mammalian cells there is evidences that pHin is a clock for cell cycle, because tubulin net charge depends strongly on pH, which might be critical for microtubule (MT) dynamics during mitosis (Gagliardi and Shain, 2013). In the same way, important cell regulators such as Gα proteins, are high regulated by pHin promoting different response to the same stimulus (Isom *et* *al*., 2013). There is also evidences that pHin plays an essential role as PKA signalling of glucose metabolism (Dechant *et al*., 2010).

In view of the above, pHin is now considered as a second messenger in signalling pathways, being as important as $Ca^{2+}$ signalling for the cell. Here tried to understand the behaviour of pHin in the normal cell processes of *F.oxysporum*.

### *pHluorin expression is functional in F.oxysporum*

A growing body of evidence suggests that environmental pH governs fungal virulence. (Alkan *et al*., 2008; Bignell *et al*., 2005; Caracuel *et al*., 2003). In *F.oxysporum* ambient pH modulates both fungal physiology and pathological behaviour (Segorbe *et al*, unpublished).

Here we demonstrate, for the first time, that pHluorin, a genetically encoded pH sensor, can be efficiently used as a probe for *in vivo* pHin measurement in *F.oxysporum.* To obtain a pHin calibration curve we used a pH calibration buffer (pH buffer for intracellular pH calibration, see Table 5).

Because this solution allows a great buffering capacity throughout a wide range of pHs (5.5 to 8.5) it provides the possibility to measure pHin of *F.oxysporum* cells even when submitted to extreme environmental pH changes. For intracellular pH calibration, we supplemented the buffer with sodium azide, a specific inhibitor of membrane ATPase to abolish cell homeostasis and to ensure proper equilibration of intracellular and extracellular pHs (Brett *et al*., 2005; Noumi *et al*., 1987).

### *F.oxysporum shows homeostatic regulation of pHin*

Like any living organism fungi are exposed to changes in the extracellular environment. Therefore they have evolved mechanisms to prevent or modify changes caused by the extracellular pH in the cell. Here we tested the ability of *F.oxysporum* to maintain a stable pHin in different extracellular environments through a wide pH range.

This is the first time that changes of pHin in response to extracellular pH (pHex) have been investigated in fungi. The results reveals clues to mechanisms of pH regulation and homeostasis (see Figure 8).

Surprisingly, pHin was dramatically modified when either NaOH or HCl was added. However, in response to perturbations of pHex *F.oxysporum* rapidly (30 minutes) adjusted pHin.

Moreover, we noted that pHin decreased below 5.3 (see Figure 8). This results might be explained by the polarization of the plasma membrane which closes the proton channels and break the entrance of protons inside the cell (Cyert and Philpott, 2013)

At present, we do not have enough data to conclude understand the cellular events occurring in response to pHin alkalinisation and acidification. In future experiments, we would like to determine the role membrane $H^+$-ATPase in the homeostatic process. Moreover, to discard an artefact the pHin measurements should be repeated with another technique such as flow cytometry or confocal microscopy.

### *F.oxysporum induces pHin acidification in the presence of glucose*

Glucose is used by most fungi as a source of carbon and energy in different metabolic processes. Glucose must be transported into the cell. To this aim, the cells must detect the presence of the nutrients, generating a signalling cascade to respond to this stimulus.

Here we observed an acidification of pHin in response to the addition of glucose (see Figure 9) in contrast with reported in *Saccharomyces cerevisiae* (Dechant *et al*., 2010; Isom *et al*., 2013; Orij *et al*., 2011*)* and *Aspergillus niger* (Hesse *et al*., 2002).

Both showed an alkalinisation of pHin upon addition of glucose to the extracellular medium.

In *S.cerevisiae, w*hen glucose is in the medium, the cells take up via cotransport with protons. This transport produces a rapid acidification of pHin in approximately 30 seconds, followed by an alkalinisation (Cyert and Philpott, 2013). The metabolism of glucose (glycolysis) produces an increase of intracellular proton concentration (2 protons for each molecules of glucose) leading to an electrophysiological gradient of proton concentration between the intracellular and extracellular medium (Orij *et al*., 2011).

Addition of glucose leads to the activation of the plasma membrane P-ATPase Pma1p (Ambesi *et al*., 2000). This proton pump exports protons to the extracellular medium, creating ATP in each reaction. This last step produces the alkalinisation of pHin until the end of glycolysis (Cyert and Philpott, 2013; Orij *et al*., 2011).

Although we need to perform more experiments, a possible explanation of the observed pHin acidification in glucose transport (symport with protons) without subsequent pHin increase because glucose it is not rapidly metabolized in the medium used in the experiment (KSU, see Table 5).

In future goals, we would like to test the effect of glucose addition in germlings growth in different medium to determine if glucose is metabolized. Furthermore, we would like to check the effect of 2-deoxyglucose, an analogue of glucose that cannot be metabolized. This compound is transported into the cell, but not recognized by the enzymes of the glycolytic pathway. With these experiments we should clarify the role of the metabolic pathways in response to glucose addition and pHin.

## Conclusions

1. pHluorin can be used to measure pHin in Fusarium oxysporum.
2. Extracellular changes in $H^+$ concentration cause a rapid and strong modification of pHin.
3. A unknown homeostasis mechanism quickly adjust pHin in response to pHex perturbations
4. An unknown mechanism prevent changes of pHin below pH 5.3.
5. *F.oxysporum* responds to addition of glucose with a rapid acidification of pHin.

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