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Ascochyta blight: isolation, characterization, and development of a rapid method to detect inhibitors of the chickpea fungal pathogen *Ascochyta rabiei*

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ABSTRACT

Ascochyta blight is the major disease attacking chickpea (*Cicer arietinum*) around the world. Since its first time report of isolation in Argentina in 2012, the pathogen has caused severe economic losses and has acquired a great importance. We report here the isolation of *Ascochyta rabiei* from infected chickpea beans cultivated in Santa Fe, Argentina; its identification by morphological analysis and molecular biology techniques based on internal transcribed spacer (ITS) sequence alignment, its biochemical characterization regarding the capacity to produce proteinase and phospholipase enzymes, and its antifungal susceptibility to common used antifungal agents. In order to detect new inhibitors for *A. rabiei* from natural sources, a bioautographic method was developed. From the screening method developed, we found that extracts from cultures of *Aspergillus parasiticus* are active against *A. rabiei*.

Introduction

Chickpea (*Cicer arietinum*, Fabaceae), also known as garbanzo bean, is an important edible leguminous crop which represents a significant source of dietary protein in many parts of central Asia and Africa (Gan *et al.* 2006; Harveson *et al.* 2011; Kanouni *et al.* 2011).

Chickpea is the third most important pulse crop in the world. In Argentina, it has historically been a minor crop (Knights *et al.* 2006), but due to changes in economic variables in the last decade, there has been a decrease in cultivated areas destined for winter–spring cereals such as wheat (*Triticum aestivum*, Poaceae) and, as a consequence, chickpea has emerged as an alternative crop. In 2013, Argentinean chickpea production averaged more than 53 000 tonnes, and now leads the production in South America, being considered a leader in the international market of chickpea producers (Garzón 2013; http://faostat3.fao.org/home/E).

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Among the fungal diseases that affect chickpeas around the world, one of the most serious is the Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labr. [teleomorph: *Didymella rabiei* (Kovacheski) von Arx (synonym: *Mycosphaerella rabiei* Kovacheski)], class Dothideomycetes, order Pleosporales, family Didymellaceae (Akamatsu *et al.* 2012). Ascochyta blight is a highly destructive disease in most chickpea-growing areas of the world (Nene 1982; Nene *et al.* 1991; Shahid *et al.* 2008). The pathogen grows asexually on the host plant, while its perfect stage can be recovered from wintering chickpea debris (Armstrong *et al.* 2001; Ahmad *et al.* 2014). Blight disease affects all parts of the shoot of chickpea plants, producing lesions, and shoot breakage (Pande *et al.* 2005). The disease significantly reduces chickpea seed yields and quality, and, depending on climatic conditions, yield losses for susceptible cultivars can reach 100 % (Shahid *et al.* 2008). Economic losses due to blight damage have been substantial in many regions including Australia, Canada, Latin America, southern Europe, the United States of America, and West Asia (Gan *et al.* 2006). In November 2011, Ascochyta blight was reported for the first time on commercial chickpea fields at Córdoba and Buenos Aires provinces in Argentina (Viotti *et al.* 2012).

Ascochyta rabiei is a seedborne pathogen, and infected seed can be the most important source of inoculum for long distance dispersal (Tivoli & Banniza 2007). Infection can also arise from ascospores produced on infected crop residues. Once infection has been established within the field, asexual spores cause secondary spread of the disease (Wiese *et al.* 1995).

Ascochyta blight management is the key for successful chickpea crop yields. A combination of crop management practices to minimize the disease include the selection of resistant cultivars, the use of blight-free seeds, seed dressing, crop rotation, and application of fungicides in an integrated approach (Gan *et al.* 2006; Wise *et al.* 2011).

Several fungicides proved to be effective for blight control. However, the need for repeated protective applications often makes them uneconomical (Gan *et al.* 2006). Specific formulations of metalaxyl, captan, thiabendazole, and benomyl are used on chickpea seed (Wise *et al.* 2011). On the other hand, systemic fungicides include the quinone outside inhibitors (pyraclostrobin or azoxystrobin), succinate dehydrogenase inhibitors such as boscalid and, since 2007, sterol demethylation inhibitors, such as triazoles (prothioconazole, difenoconazole, etc). The development of resistance to currently available antifungal agents as a result of repeated applications of one fungicide class, together with their acute and chronic effects on human and wildlife, are the major problems confronting their use in agriculture (Wise *et al.* 2009; Campbell *et al.* 2012). In addition, consumers are increasingly aware of the potential for contamination of food with pesticides (López-Fernández *et al.* 2012). Although significant improvements have been made in recent decades in the fungicides available for blight control, there is an urgent need for the search of new natural chemical alternatives for Ascochyta blight management (Amin & Javaid 2013).

Natural products are undoubtedly the best source of bioactive original scaffolds. They are a structurally diverse collection of molecules produced by organisms in a peculiar fashion, and their biological activities reflect the diversity of their producers and structures (Seyedsayamdost & Clardy 2014). Few investigations have been performed in order to get natural inhibitors for *A. rabiei*, and these are mainly detected by testing plant crude extracts applying *in vitro* diffusion or dilution tests, or by conidial germination or mycelial growth inhibition. For example, ethyl acetate leaf extract from *Chenopodium album* L. showed a MIC (Minimal Inhibitory Concentration) value of 1.95 mg ml^{-1} in an agar dilution test performed with the extract dissolved in the culture medium and the fungus added as a plug on the centre of each Petri dish. In another assay, the essential oils of *Thymus vulgaris* L. and *Melaleuca alternifolia* C. were tested, and the activity was reported as % MGI (% Inhibition of Mycelial Growth) regarding to the colony diameter, showing the thyme oil to have the better activity (Jabeen *et al.* 2014; Riccioni & Orzali 2011).

Here we report the isolation and full characterization of *A. rabiei* from chickpea beans cultivated in Santa Fe, Argentina, including the molecular identification, secreted phospholipase and proteinase activity, and its antifungal susceptibility profile for two commercial antifungals. In addition, we developed an effective autographic method to detect inhibitors within very complex samples such as natural extracts from plants or microorganisms, without prior separation steps, and detected that extracts from *Aspergillus parasiticus* were active on *A. rabiei*.

Material and methods

General

Sabouraud Glucose Agar (SGA) and Potato Dextrose Agar (PDA) were from Britania (Buenos Aires, Argentina). Chickpea Supplemented Medium (CSM) was formulated in our laboratory and prepared as follows: 30 % of chickpea extract (50 g of Kabuli variety chickpeas were washed, autoclaved and macerated in 200 ml sterile water for 7 d, ground and filtered through cloth), 2 % glucose, and 2 % agar (Britania, Buenos Aires).

Antimicrobial compounds: chloramphenicol was from Anedra, chlorothalonil (Rothalonil 72 SC), and difenoconazole were purified by liquid–liquid extraction and dissolved in dichloromethane and ethyl acetate at 5.6 mg ml^{-1}, respectively. Solutions were conserved at −20 °C until use.

Antifungals preparations: chlorothalonil (Rothalonil 72 SC; Rotam, Argentina) and difenoconazole (Geyser; Syngenta, Argentina) were purified by liquid/liquid extractions from emulsified products. Typically, 10–50 ml of each product were extracted with dichloromethane (3 × 20 ml), dried using Na_2SO_4 and evaporated under reduced pressure to obtain each purified compound. Purification process was followed by Thin Layer Chromatography associated with Ultraviolet detection (TLC-UV; >95 %).

Aspergillus parasiticus CCC 100-71 was purchased from CEREMIC (Colección de Cultivos CEREMIC, Facultad de Ciencias Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Argentina).

Isolation and characterization of Ascochyta rabiei

Ascochyta rabiei was isolated from infected chickpeas (*Cicer arietinum* Chañarito variety) collected in Hughes, Santa Fe, Argentina in 2012.

Whole dried chickpeas and pods were surface-treated with 95 % ethanol 1 min, sterile water 1 min, 0.5 % NaClO 1 min, and sterile water 1 min. Samples were cut aseptically into slices and placed on Petri dishes containing PDA, SGA, and CSM supplemented with chloramphenicol (0.5 g l^{-1}) and incubated at 22–24 °C under 12 h light and 12 h dark for a period up to 15 d. Isolates compatible with *A. rabiei* were subcultured in CSM and mature cultures were maintained at 4 °C until required.

Morphological identification

Strains were subcultured in CSM and PDA to ascertain their macroscopic and microscopic morphologies. Phenotypic identification was done using standard mycological procedures and comparing with the literature (Aveskamp *et al.* 2010).

Molecular identification by sequencing of internal transcribed spacer (ITS) region

Genomic DNA was extracted according to a procedure described before (Tang *et al.* 1992). Briefly, isolates were subcultured in CSM broth for 15 d at 24 °C in Petri dishes. The mycelial mat which developed on the surface of the liquid was placed on filter paper to remove excessive moisture, and then transferred to a 50 ml polypropylene tube containing eight glass beads (4 mm, Schott-Duran). The tube was immersed in liquid nitrogen for 30 s and vortexed for 30 s. A volume of 0.8 ml of extraction buffer (0.2 M Tris–HCl pH 7.6, 0.5 M NaCl, 10 mM EDTA, 1 % SDS) was added, and the mixture was gently mixed. An equal volume of phenol–chloroform (1:1) was added to the mixture, mixed and transferred to a microfuge tube and centrifuged for 15 min at 12 000 × *g*. The aqueous phase was successively re-extracted with one volume of phenol–chloroform (1:1) and chloroform. DNA was precipitated with 0.6 volume ml^{-1} of isopropanol, washed with 70 % ethanol, and resuspended in water containing 0.2 mg ml^{-1} RNAse A (Invitrogen). Genomic DNA was stored at −20 °C until use.

DNA segments comprising the ITS1 and ITS2 regions were amplified with primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) in a Veriti 96 well Thermal Cycler (Applied Biosystems). The reaction mixture contained 0.5 μM each primer (GBT oligos, Argentina), 0.2 mM each deoxynucleoside phosphate (Invitrogen), 1.5 mM $MgCl_2$, 2.5 U Taq Polymerase (Invitrogen), and 25 ng DNA in a final volume of 50 μl. The parameters were one initial cycle of 2 min at 94 °C, followed of 35 cycles of 30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C, and one final cycle of 5 min at 72 °C. The reaction products were analyzed in a 2 % agarose gel (Invitrogen) and purified by the Axyprep PCR Clean-up kit (Axygen) following manufacturer instructions. PCR products were sequenced (Macrogen, Korea) using ITS1 and ITS4 primers. Sequences were edited (Chromas Lite 2.1.1, www.technelysium.com.au) and compared with the GenBank database using the BLAST search program (http://www.ncbi.nlm.nih.gov/BLAST). Sequence similarities greater than 99 % were considered for species-level identification. Sequences generated in this study were submitted to the GenBank database under accession numbers KP643195, KP643196, and KP643197 for AR1, AR2, and AR3 strains respectively. Strain AR2 was selected for further analysis.

Bioassays

Ascochyta rabiei conidial suspensions

Colonies (14 d old) were covered with 2.5 ml of sterile distilled water supplemented with 0.1 % Tween 80 and carefully rubbed with a sterile cotton swab. The suspension was transferred to a sterile tube containing ten glass beads (4 mm, Schott-Duran), vortexed for 10 s, filtered through sterile cotton and counted in a haemocytometer chamber (Boeco, Germany). Spore suspensions were diluted in sterile distilled water to the desired inoculum concentration.

Bioautography

The methodology was essentially as described before with some modifications for *A. rabiei* (Rahalison *et al.* 1991; López & Furlán 2007). Ten microlitres of extracts or pure compounds were directly deposited as spots or bands onto silica gel plates (Merck 60 F254 TLC aluminium sheets). TLC plates were developed in appropriate solvent system when needed and allowed to air dry until complete solvent evaporation, followed by 30 min UV light sterilization. Culture medium was melted and, once warmed, a fresh suspension of spores was added to give a desired final concentration (see below) and was distributed over the TLC plate (0.1 ml cm^{-2}). After solidification, plates were incubated at 24 °C for 48 h in a humid sterile atmosphere. Clear yellowish halos representing inhibition against a blue background of the fungal growth were visualized after spraying TLC plates with a sterile 1 mg ml^{-1} MTT solution (Methylthiazolyldiphenyl-tetrazolium bromide, Merck, Germany) (López *et al.* 2007). Chlorothalonil and difenoconazole were used as positive controls.

Antifungal susceptibility testing

Broth microdilution testing was performed following the EUCAST document with minimal modifications, which included the use of Sabouraud Glucose instead of RPMI 1640 as culture medium (EUCAST Definitive document E.def 9.1, July 2008). Inoculum was prepared as described above and was used at a final concentration of 2.2×10^5 conidia ml^{-1}. The antifungal agents tested were chlorothalonil and difenoconazole dissolved in DMSO (dimethylsulfoxide, Merck, Germany). Concentrations tested ranged from 16 to 0.03 μg ml^{-1}. MIC was defined as the antifungal concentration that produced the complete visual inhibition of growth determined after 48–72 h incubation at 24 °C. Doubtful wells were observed with a light microscope (40×). Additionally, 50 μl of 1 mg ml^{-1} MTT were added to each well in order to improve visual observation of fungal growth.

Influence of plant matrix on the bioautographic detection of antifungal compounds

One hundred μg of an inactive plant extract (ethyl acetate extract from aerial parts of *Polygonum ferrugineum*, Polygonaceae) were spotted along with 0.05 or 0.10 μg difenoconazole on the same lane (0.50 cm) and compared with lanes containing the extract and difenoconazole alone. TLC was developed on hexane:ethyl acetate (10:90) and revealed under UV 254 and 365 nm. Bioautography was carried out as described above.

Screening of Aspergillus parasiticus CCC 100-71 extracts for the search of active compounds

Spores from a 14-d-old culture of *A. parasiticus* CCC 100-71 were added to 1 l Erlenmeyer flasks containing 250 ml of culture broth medium (rice broth, Sabouraud glucose broth, Czapek or potato dextrose broth) in a final concentration of 5×10^4 spores ml^{-1}. After incubation for 30 d at 28 °C in stationary mode, the fungal mycelial biomass was separated from each broth by filtration. Filtrate and biomass were subsequently extracted with ethyl acetate, and the extracts were evaporated to dryness using a rotary evaporator. Controls without fungal inoculum were performed for each culture medium and processed as described above. Dry extracts were redissolved in methanol (5 mg ml^{-1}) and were automatically applied (bandwidth 8 mm) on an aluminium chromatoplate 10 cm TLC plate (Merck 60 F254) using the ATS4 (Camag). After developing the plate (dichloromethane:ethyl acetate 80:20, distance 7 cm, ADC2, Camag), the plate was air-dried and the bioautography was performed as described above.

In vitro *determination of extracellular enzymatic activities*

A) Phospholipase: the enzymatic activity was tested in *Ascochyta rabiei* selected strain AR2 by measuring the size of the zone of precipitation around the fungal growth in a SGA medium supplemented with 1 M NaCl, 0.01 M $CaCl_2$, and 10 % sterile egg yolk (briefly, egg was previously surface-sterilized with 10 % NaClO for 10 min, and after separation of white, the yolk was transferred to a propylene tube, centrifuged for 10 min at 27 × *g*, and 10.0 ml supernatant was added to the sterilized medium, homogenized, and plated). A 25 µl suspension of 2.2×10^5 conidia ml^{-1} was applied as a spot onto the egg yolk medium and left to dry at room temperature. Plates were incubated at 25 °C for 48–72 h. Phospholipase activity was determined as the diameter in mm of each cloudy halo (the precipitate) around the producing colonies (Ibrahim *et al.* 1995). B) Proteinase: secreted proteolytic activity was measured in plates containing 0.2 % Bovine Serum Albumin (BSA), 1.17 % Yeast Carbon Base (YCB, Difco®) and 1.5 % agar (Britania, Argentina). Briefly, a stock solution containing 2 % BSA and 11.7 % YCB pH 4 was filter sterilized and then diluted into a 1.7 % agar solution which had previously been autoclaved and cooled to 55 °C (Togni *et al.* 1994). Plates were incubated for 7 d at 23 °C. Proteolytic activity was observed as a clear zone around the colonies. Plates were sprayed with a 10 % v/v HCl solution in order to denature BSA aiming to improve visualization of the clear halos and get a better contrast.

Results and discussion

Isolation and characterization of Ascochyta rabiei from Argentina

Morphological, biochemical and molecular features were considered in this study to characterize *A. rabiei* strains isolated from infected chickpeas cultivated at the central region of Argentina.

The recovery of *A. rabiei* from infected seeds was rather poor (approximately, one strain was recoverable from about 200 processed chickpeas). Temperatures above 26–27 °C resulted mostly in a complete inhibition of growth of suspected strains.

Three fungal isolates compatible with *A. rabiei* named AR1, AR2, and AR3 were recovered. In PDA plates, isolates grew as dark colonies, being grey-black on the surface and brown-black on the reverse, with regular development and characterized by the appearance of lighter concentric rings towards the outer edge of the colony (Fig 1A and B). Asexual cylindrical, broadly ellipsoidal conidia, 8–15 × 4–6 µm, were produced abundantly in dark and raised pycnidia developed on the aerial mycelium (Fig 1C and D) (Wiese *et al.* 1995).

All isolates grew slowly when the temperature was higher than 25 °C up to complete no growth, which occurred at 28 °C. Regarding culture media, only CSM allowed the recovery of *Ascochyta* isolates from infected chickpeas, meanwhile SGA and PDA were used to evaluate colonies growth from conidia and for further conidiation processes. Conidia formation was better observed in CSM and PDA media in that order, and no conidiation was observed in SGA medium. Fungal isolates were further identified as *A. rabiei* by means of ITS sequence analysis and BLAST searches performed in comparison to published sequences. To our knowledge, this is the first report of *A. rabiei* isolates fully identified by morphological and molecular methods in Argentina.

The pathogenicity of fungi can be assessed by the determination of their *in vitro* capacity to produce enzymes which allow them to infect host tissues and get nutrients *in vivo*. *A. rabiei* is a necrotrophic fungus, killing plant cells in advance of mycelial development. The infection process of *A. rabiei* on leaves and stems of resistant or susceptible chickpeas has been studied (Pande *et al.* 2005; White & Chen 2007). Some toxins like solanapyrones, hydrolytic or cell wall degrading enzymes, and enzymes involved in phytoalexin detoxification have been described as important biochemical determinants of pathogenesis for *A. rabiei* (Tenhaken & Barz 1991; Tenhaken *et al.* 1997; Hamid & Strange 2000; White & Chen 2007).

In order to complete the biochemical characterization of our isolates, we tested secreted phospholipase and proteinase activities with the egg yolk and BSA hydrolysis assay, respectively. Under the experimental conditions tested here, only proteinase activity was detected. Proteinase activity can be considered to be a pathogenicity factor during infection processes, and could be related with the aggressiveness of some blight infections. Although some information exists about the pathogenicity factors of *A. rabiei*, we know of no previous reports of proteinase and phospholipase activity on this fungal pathogen.

In vitro *antifungal susceptibility profile of Ascochyta rabiei*

Antifungal susceptibility testing is important for comparing the *in vitro* activity of new and established agents, for resistance surveillance and for epidemiological studies. Standardized methods are needed so that comparable results and

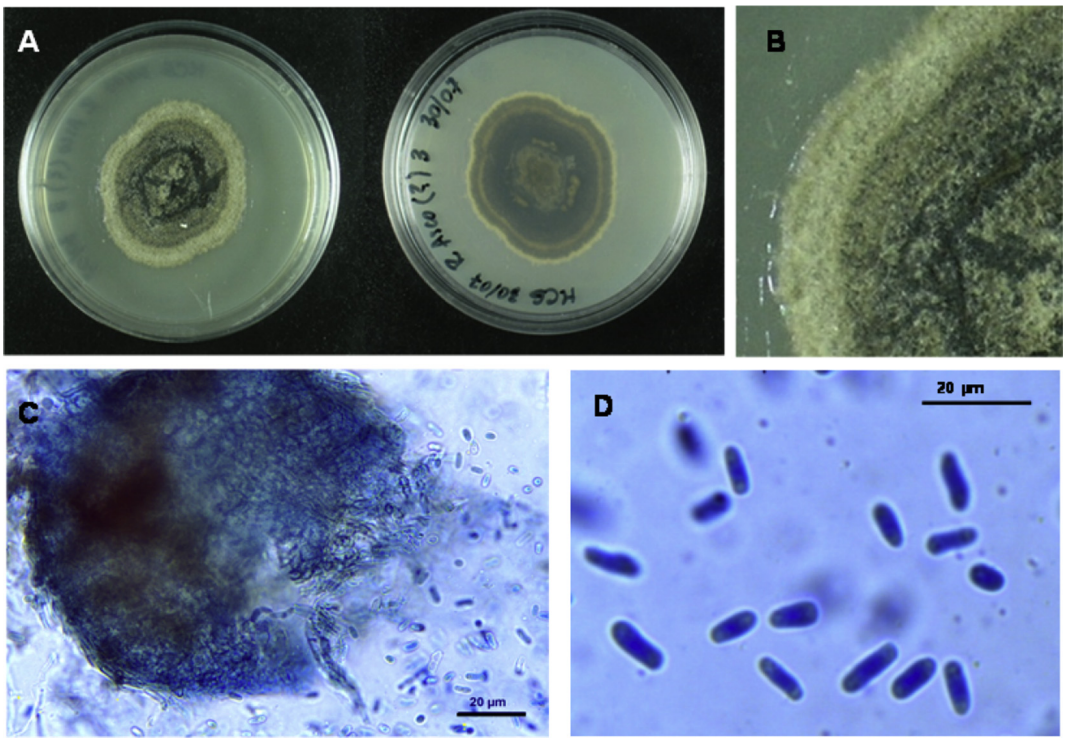

Fig 1 – Macromorphology of *A. rabiei* (A) and magnification of the colony external growth (B). Micromorphology of *A. rabiei*: direct microscopic observation of a pycnidium (C) and conidia (D) stained with cotton blue. Scale bars 20 µm.

interpretations can be achieved (EUCAST Definitive document E.def 9.1, July 2008).

In order to assess the antifungal susceptibility of *A. rabiei* isolates, a microdilution broth assay was performed following the guidelines for susceptibility testing of molds provided by the European Society of Clinical Microbiology and Infectious Diseases with some modifications (EUCAST Definitive document E.def 9.1, July 2008).

We tested chlorothalonil and difenoconazole, two commercial antifungals commonly used to treat Ascochyta blight, on strain AR2. MIC (Minimal Inhibitor Concentration) for chlorothalonil was 1–2 µg ml^{-1}, which is in agreement with previously published data (Demirci *et al.* 2003). Regarding difenoconazole, MIC was 0.06–0.03 µg ml^{-1}. When microplate wells were observed microscopically, some fungal growth was detected at concentrations greater than the difenoconazole MIC. Nevertheless, when MTT was added, no reduction to formazan was observed even after 48 h incubation, thus suggesting that no mitochondrial activity is detectable at those antifungal concentrations. These results are in agreement with previously published results (Demirci *et al.* 2003) indicating that susceptibility of the Argentinean isolate is comparable with isolates from other countries.

Although several methods have been described to determine susceptibility of *A. rabiei*, no reports were found about bioautography, which is a simple methodology to get active molecules at complex mixtures, when combined with TLC separation.

Development of a bioautographic assay to detect inhibitors of Ascochyta rabiei growth in natural complex samples

Planar chromatographic analysis combined with a biological detection method is termed bioautography (Müller *et al.* 2004). Instead of sophisticated bioassays, bioautography offers a simple, rapid and inexpensive method for chemical and biological screening and subsequent bioassay-guided isolation of compounds of interest (Hostettmann *et al.* 1997). A rapid and efficient screening method to detect inhibitors of *A. rabiei* was developed that also proved to be suitable for the localization of active molecules present even in a complex matrix like plant or microorganisms extracts.

In order to optimize assay conditions, chlorothalonil and difenoconazole were used as positive controls (see Material and methods, Bioautography). Different variables like culture medium composition, agar percentage, incubation temperature, inoculum size and addition of phenol red to the growth media were tested in order to determine the best condition for the visualization of inhibition zones.

When CSM and SGA media were tested; no differences in the visualization of the inhibition zones were observed since fungal growth was abundant enough in both media. Phenol red is usually added to the culture media as an indicator of fungal growth due to its properties as a pH indicator, improving contrast from no growth (neutral, red) to growth (acidic, yellow) zones (Saxena *et al.* 1995). The addition of phenol red (0.02 %) to CSM and SGA improved the observation and allowed an earlier visualization of the inhibition zones. For

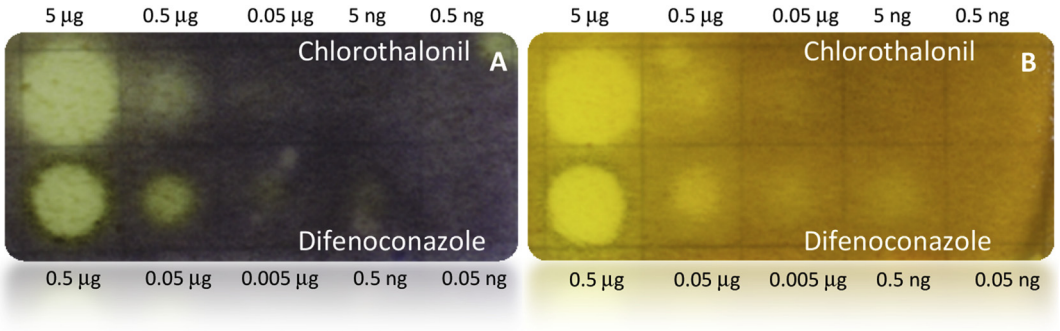

Fig 2 – Bioautographic semiquantification of the chlorothalonil and difenoconazole inhibitory effect on *A. rabiei*. Different amounts of both antifungal agents were deposited as spots. Inhibition halos were observed by spraying TLC plates with MTT solution after 48 h incubation. Other conditions were: (A) SGA medium, 0.6 % agar, and 1×10^5 conidia ml^{-1} (final inoculum concentration). (B) Idem A with the addition of 0.02 % phenol red to the culture media.

further analysis, commercial SGA medium was selected as an easier and more reproducible test condition.

After SGA plus phenol red was selected, three different agar concentrations (0.6, 1.0, and 1.5 %) were evaluated in the formulation of the culture medium. The best practical results were obtained with 0.6 % agar, allowing this condition to reduce the time consumed to achieve melting, diminishing the critical temperature of addition of the fungal inoculum to the medium and facilitating its spread over the TLC plate.

Conidial suspensions were prepared as described before using Tween 80® to help in the dissection process of conidia from fungal mycelium and special structures. Three different final concentrations of inoculum were tested: 5×10^3, 5×10^4, and 1×10^5 conidia ml^{-1}. The best results were obtained when 1×10^5 conidia ml^{-1} were assayed (Fig 2).

One of the most important issues to be solved in autographic assays applied to complex mixtures is the so-called matrix effect, defined by IUPAC (International Union of Pure and Applied Chemistry) as the combined effect of all components of a sample other than the analyte under study on the measurement of the quantity, here the antifungal activity (Guilbault & Hjelm 1989). Natural extracts can be considered to be dirty samples that are very rich in particulate matter and also contain high amounts of dissolved substances (http://www.chromatographyonline.com/node/244669?rel=canonical). The challenge is to detect active compounds that are within and interacting with the matrix. Several ways to study the influence of the matrix effect have been assayed. For example in liquid chromatography coupled with tandem mass spectrometry experiments, the postcolumn infusion of analytes in parallel with the injection of blank matrix is one of the better options when quantitative determinations are performed (Stahnke *et al.* 2012). In order to evaluate the effect of a natural (plant, fungal or bacterial) matrix in the detection of an antifungal compound, an inactive plant extract (100 µg) was tested alone, along with difenoconazole (0.05 µg), and these compared with difenoconazole alone in the bioautographic assay. No differences in shape, size or retardation factor (Rf = 0.38) were observed between inhibition halos obtained for difenoconazole alone and when spiked on the plant matrix (Fig 3). Similar results were obtained with

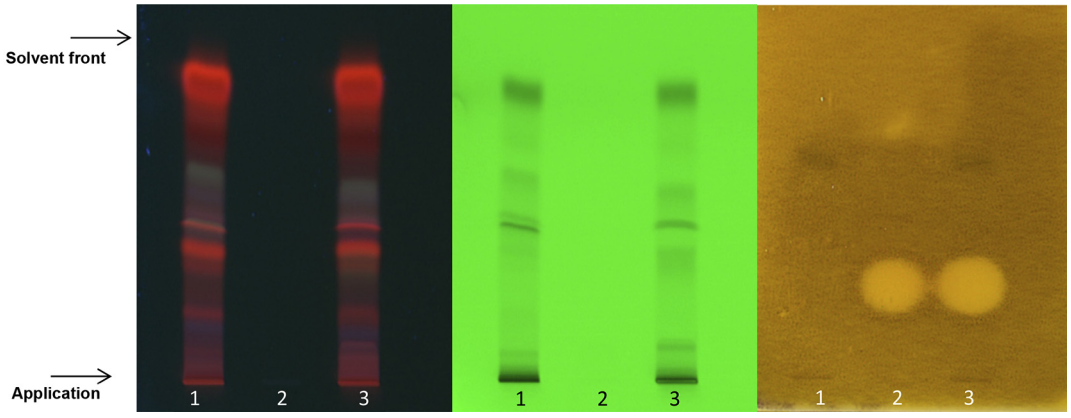

Fig 3 – Spiking experiment of inactive plant extract together with difenoconazole in comparison with each one applied alone. Left: Bioautography. Right: UV 365 nm. Application: Lane 1. 50 µg of inactive plant extract. Lane 2. 0.05 µg difenoconazole. Lane 3. 50 µg of plant extract + 0.05 µg difenoconazole. Mobile phase: hexane–ethyl acetate (10:90).

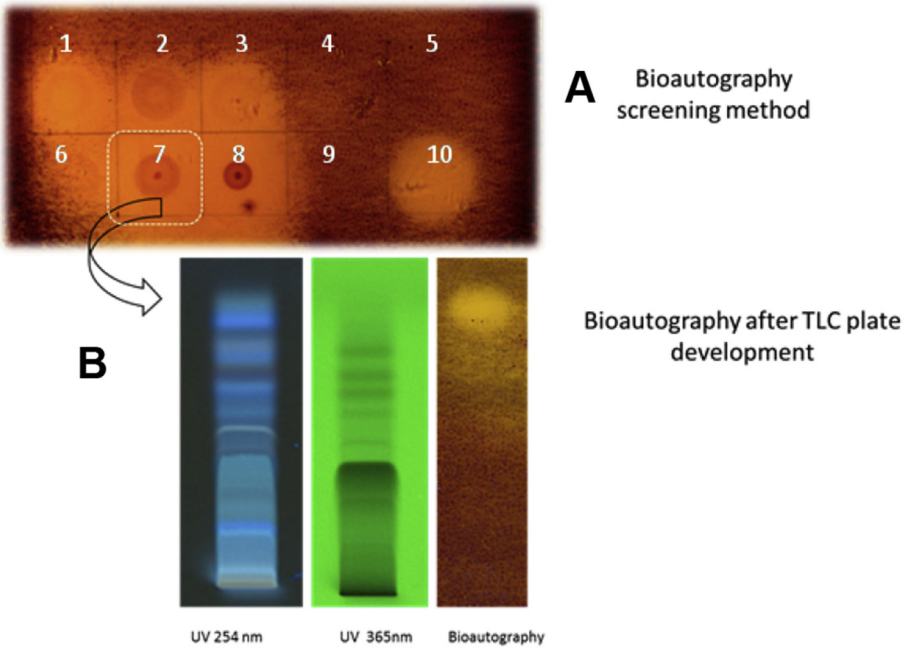

Fig 4 – Bioautography assay for the detection of *A. rabiei* inhibitors in complex samples. In a first screening, samples are applied as spots in 1.5 × 1.5 cm squares on TLC plates. Active extracts are further developed in a TLC plate using an appropriate solvent system in order to localize active/s compound/s within the mixture. (A) 1–9: *A. parasiticus* different extracts, 100 μg spot^{-1}; 10: Difenoconazole 0.05 μg spot^{-1}. (B) TLC plate after development, *A. parasiticus* ethyl acetate extract of potato dextrose broth filtrate in dichloromethane:ethyl acetate (60:40).

chlorothalonil (data not shown). These results indicate that active compounds can be detected in complex samples, even when they are minor components of them.

Fungal natural products possess numerous pharmacological uses, in particular as antimicrobial agents (Newman & Cragg 2012). In order to test the bioautographic method, organic extracts from *Aspergillus parasiticus* different cultures were evaluated at 100 μg spot^{-1} (Fig 4A). The ethyl acetate extract from the filtrate of potato dextrose broth culture (spot 4, Fig 4A) showed *A. rabiei* growth inhibition and was selected to carry out further experiments in order to localize the any active compound. After developing the TLC with an appropriate solvent system, a sole inhibition halo at Rf: 0.85 was detected (Fig 4B). Further experiments in order to confirm the activity and to obtain the isolated compound are now in progress.

Different metabolic profiles could be expected from a sole microorganism when it is grown under different culture conditions. The diversification of the biosynthesis of secondary metabolites certainly will contribute to find new natural inhibitors for *A. rabiei*.

Conclusions

Since 2011–2012 Argentina is considered as an *Ascochyta rabiei* infected country. In order to guarantee a correct infection management, it is desirable that wild strains could be isolated, unambiguously identified and their susceptibilities studied.

Three Argentinean *A. rabiei* isolates were identified, and the susceptibility of AR2 selected strain was tested applying the EUCAST standardized broth microdilution method. *A rabiei* AR2 showed proteinase but not phospholipase activity. The risk and cost of growing chickpea is high due to the threat of Ascochyta blight. Because current cultivars possess only partial resistance to the pathogen, the use of fungicides is still a major management practice. Consequently, new fungicide products to control the disease are needed. The well-established autographic method is an inexpensive and easy tool to screen natural sources; its application in the discovery process for new, more effective, lesser toxic, and environmental friendly active compounds for the Ascochyta blight management certainly will contribute for disease control.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.funbio.2015.12.002.

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