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Selection of aggressive pathogenic and solopathogenic strains of *Ustilago maydis* to improve Huitlacoche production

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ABSTRACT

Ustilago maydis is a basidiomycete known as the causative agent of 'common smut', worldwide disease of maize that is recognized by the galls it forms, which have considerable potential as a gourmet food. Results of infection are quite variable, even under optimal greenhouse conditions. In order to find pathogenic strains able to be used as a highly infective and stable inoculum for the successful production of galls either in greenhouses or in the field, ears with gall symptoms containing teliospores were recovered from maize plants. The teliospores were suspended in water and plated on nutrient-rich medium. Twenty-six colonies developed, containing three types of yeast-like colonies: saprotrophic, pathogenic, and solopathogenic. DAPI staining confirmed the presence of solopathogenic strains with diploid sporidia. Groups of different mating types were found when pairs of the 26 strains were arranged resembling partial-diallel combinations. Amplification of the partial *b* locus revealed that the strains found harbor the alleles *b*3 and *b*4, allowing the formation in dikaryotic strains of heterodimeric regulatory proteins associated with fungal development and pathogenicity. In this study, we isolated compatible haploid and solopathogenic diploid strains for their high capacity for inducing smut.

Keywords: Basidiomycete, maize galls, mating types, molecular diagnosis, smut

Introduction

The heterothallic basidiomycete, *Ustilago maydis*, is a fungal pathogen of maize (*Zea mays*) causing 'common smut' disease (Christensen 1963). Since the mid eighteenth century, studies have shown that its life cycle, mechanisms of dissemination and the availability of resistant maize varieties (Shutleff 1980) generally result in only mild grain yield losses (*c*. 2 %) under field conditions due to this pathogen. Although, these losses are small in fractional terms, and can be argued that their commercial value is significant where production levels are especially high as in the USA and China that produce 313 and 192 million tonnes, respectively (FAOSTAT 2013). Maize smut is thus a common disease worldwide, with a significant economic impact on corn sector (Djamei & Kahmann 2012). *U. maydis* is also a good model for fungal genetics, and it is considered one of the topten pathogens in molecular plant pathology (Dean *et al*. 2012).

Mexico is a centre of origin of maize, and so encompasses a very broad genetic diversity. Moreover, the association

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between *U. maydis* and *Zea mays* and its ancestor, the teosinte, *Zea mays* sp. *parviglumis* is well known, as they have co-evolved since pre-Hispanic times (García-Pedrajas *et al*. 2010; Kretschmer *et al.* 2012).

Since ancient times, Mexican farmers have harvested the galls of the infected ears to prepare a popular, traditional dish named 'huitlacoche' (Allen *et al*. 2011; Dean *et al*. 2012). Because in the USA and some countries of the EU can be considered this high-protein fungus in the preparation of exotic, gourmet dishes in up-market restaurants (Tracy *et al*. 2007), the commercial potential of developing crop systems enabling production of large quantities of *U. maydis* galls is increasingly being recognised.

Intensive artificial production of *U. maydis* requires a high level of compatibility between the fungus and the host, ideally matching a virulent fungal strain with a highly-susceptible host variety in favourable environmental conditions (Banuett 1995). In order to obtain a large number of galls in the field, conventional method of artificial inoculation by injection of the ear with an inoculum containing a mixture of teliospores (a conservative diploid structure of basidiomycetes) is frequently used. These are germinated on potato dextrose agar (PDA) medium to form haploid sporidia, which are placed in a flask and diluted to 10^6 sporidia mL^{-1}. Optimally, the galls are collected from several regions to increase the probability of including sporidia of a range of mating types to create a highly-infective inoculum (Snetselaars & Mims 1993).

However, in field experiments this method produces erratic results in terms of the incidence and severity of fungal infection. Results are even more variable when this technique is applied on different maize genotypes and or in a range of environments. Indeed, results are quite variable even under optimised greenhouse conditions with disease incidence and severity varying from 1 to 53 % (Madrigal-Rodríguez *et al.* 2010). To obtain an infective inoculum capable of producing consistent gall formation in a range of maize cultivars, it is necessary to understand the biology and life cycle of the disease. We know that *U. maydis* forms three cell types during its life cycle: (i), haploid, unicellular, yeast-like sporidia, (ii) dikaryotic, filamentous hyphal cells resulting from crosses between two different haploid mycelia, and (iii) diploid teliospores (Schulz *et al*. 1990). However, it is important to consider that haploid cells are saprotrophic and nonpathogenic, in contrast to the dikaryotic forms that are pathogenic. The latter are not cultivable in artificial media but it is the most frequent form found in infected host plants. Considering its heterothallic characteristic, mating between haploid cells is controlled by two genetic loci, the *a* locus with two alleles controls cell fusion, and the *b* locus which is multiallelic with at least 25 alleles (Kahmann & Kämper 2004). The last controls events after fusion including fungus development within the maize plant. Each combination of *b* alleles can be distinguished by a homozygous or heterozygous stage, which influences the pathogenicity, sexual development and cell morphology (Romeis *et al*. 2000). The *b* locus presents an excellent opportunity for studying the mechanism of self-*versus* non-self-recognition involving a multiallelic gene in a relatively simple organism (Kronstad & Leong 1989). Also, in the absence of pathogenic mating, yeast-like colonies can give rise to diploid 'solopathogenic' hyphae (Banuett & Herskowitz 1989). It has been estimated that approximately 2.6 % of sporidia possess this feature (Sabbagh *et al*. 2010). Based on this, a way to produce virulent inoculum is via solopathogenic hyphae isolated from yeast-like sporidia or from dikaryotic mycelia formed by mating two haploid hyphae. In nature, a 10-cm long gall contains about 200 trillion of teliospores (Madrigal-Rodríguez *et al*. 2010), each with the capacity to produce cells of a unique genotype, offer the opportunity to isolate pathogenic strains *in vitro* using the fuzz reaction (Banuett & Herskowitz 1994; Chew *et al.* 2008).

The aim of this study was to determine whether it is possible to isolate teliospores from maize ears infected with *U. maydis* under field conditions in Mexico, with compatible sporidia or solopathogenic strains able to produce a highly infective inoculum. For this, optical microscopy was used alongside partial-diallel type arrangements of paired strains and phylogenetic identification methods using PCR amplification of the *b* locus in *U. maydis* strains obtained from galls, recovered from landrace maize fields adapted to the Mexican Highlands.

Materials and methods

Experimental material

Twenty ears with gall symptoms were collected during the spring-summer growing season in 2010-2011, from experimental maize plots located at Montecillo, State of México, in the Mexican Highlands (2250 m, altitude). The galls came from two open-pollinated experimental maize varieties, Montecillo and Santos. Maize kernels containing teliospores were separated from the ears (Fig. 1).

Isolation of teliospores

From each gall, 10 mg of teliospore material was weighed and placed in an eppendorf tube containing 1 mL of sterile distilled water and 2 µL of Tween 20. The tubes were shaken gently until the teliospores were observed to be well dispersed in the solution. Each tube was serially diluted from 10^{-1} to 10^{-10}, and each dilution step was plated in triplicate on Petri plates containing PDA medium (Bioxon, USA).

After 48 h incubation at room temperature (c. 20 °C) yeast-like colonies were observed. A total of 26 colonies were selected on the criterion that each isolate was the result of

Figure 1. Maize ears showing gall symptoms in Montecillo-Mexico during the spring-summer growing season 2010-2011. These galls came from ´Santos´, an open-pollinated experimental maize variety.

a teliospore germination. These were identified as 1 to 15 (from Montecillo) and 16 to 26 (from Santos).

Detection of pathogenic strains

Colonies developed on PDA medium supplemented with 5 % activated charcoal powder (PDA+AC) (Merck, Germany) were assessed individually and their growth features were scored for eight days according Martínez-Espinoza *et al.* (1997).

An initial evaluation was made after incubation at room temperature for 48 h. All strains were classified according to their growth as: (-) a yeast-like colony of rough or smooth appearance but without a surrounding mycelium, (+) a yeast-like colony with sparsely-developed mycelium around the colony, and (++) a yeast-like colony surrounded by a dense, cottony mycelium. Strains were considered pathogenic when sparse or cottony mycelia grew around the colony.

Selection of solopathogenic strains

All strains showing a growth of sparse or cottony mycelium were transferred by four or five successive streaks to the PDA+AC in which pathogenic strains were selected. In this study, strains were considered solopathogenic when the cottony mycelia were unaltered after successive subcultures (Sabbagh *et al.* 2010).

Partial-diallel arrangement of strains

The 26 strains were plated in pairs on Petri plates containing PDA+AC medium resembling a partial-diallel arrangement (Rowell & DeVay 1954) (Tab. 1), so that mating type compatibility could be observed by the growth mycelium. This positive phenotype is termed fuzz reaction (Martínez-Espinoza *et al.* 1997).

In this test, dikaryotic mycelium formed *in vitro* by mating between two compatible haploid hyphae, may give rise to white colonies with sparse or cottony appearances. Strains that do not mate directly develop grayish, yeast-like colonies indicating incompatibility between hyphae grown in the same medium (Brefort *et al.* 2009).

DAPI staining

To confirm the presence of diploid sporidia and clamp-connection in solopathogenic strains, 1 mL of sterile distilled water was added to each plate to detach the sparse mycelium. To stain the DNA, a drop of this suspension was placed on a slide with 0.5 μg mL^{-1} of the reagent 4’, 6-diamino-2-phenylindole (DAPI) in phosphate-buffered saline (pH 7.2) for 20 min at 60 °C and subsequently washed with the same buffer (Dann *et al.* 1971). After 20 min, samples

Table 1. Morphology of 26 colonies from *Ustilago maydis* in partial-diallelic arrangement evaluated on PDA medium supplemented with a 5 % carbon source.

CPO strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1		++	++	+-	++	+-	+-	++	**++**	+-	**++**	**++**	++	++	+-	+-	++	++	++	+-	++	++	**++**	++	++	++
2			++	+-	++	+-	+-	++	++	+-	++	++	++	++	+-	+-	++	++	++	+-	++	**++**	++	++	++	++
3				+-	++	+-	+-	++	++	+-	++	++	++	**++**	+-	+-	++	++	++	+-	++	++	++	++	++	++
4					-+	--	--	-+	-+	--	-+	-+	-+	-+	--	--	-+	-+	-+	--	-+	-+	-+	-+	-+	-+
5						+-	+-	++	**++**	+-	++	++	++	**++**	+-	+-	++	++	**++**	+-	++	++	++	++	++	++
6							--	-+	-+	--	-+	-+	-+	-+	--	--	-+	-+	-+	--	-+	-+	-+	-+	-+	-+
7								-+	-+	--	-+	-+	-+	-+	--	+-	-+	-+	-+	--	-+	-+	-+	-+	-+	-+
8									**++**	+-	++	++	**++**	++	+-	+-	**++**	++	++	+-	++	++	++	++	++	++
9										+-	++	++	++	++	+-	+-	**++**	++	++	+-	++	++	++	++	++	++
10											--	-+	-+	-+	--	--	-+	-+	-+	--	-+	-+	-+	-+	-+	-+
11												++	++	++	+-	+-	++	**++**	++	+-	++	++	++	++	++	++
12													++	++	+-	+-	++	++	++	+-	++	++	++	++	++	++
13														++	+-	+-	++	++	++	+-	++	++	++	++	++	++
14															+-	+-	++	++	++	+-	++	++	++	++	++	++
15																--	-+	-+	-+	--	-+	-+	-+	-+	-+	-+
16																	-+	-+	-+	--	-+	-+	-+	-+	-+	-+
17																		++	++	+-	++	++	++	++	++	++
18																			++	+-	++	++	++	++	++	**++**
19																				+-	++	++	++	++	++	++
20																					-+	-+	-+	-+	-+	-+
21																						++	++	++	++	++
22																							++	++	++	++
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24																									++	++
25																										++

[+]presence of mycelia; [−]absence of a surrounding mycelium; [++]compatible mating type. Solopathogenic strains (sign in bold).

were observed under a fluorescence microscope with mercury short arc lamps HBO 50 W at a wavelength of 350 nanometers (Axiolab, Carl Zeiss, Germany). The infective potential of each solopathogenic strain was corroborated in maize seedlings in a greenhouse and in the field studies (unpubl.res.).

DNA extraction

Total DNA was extracted from saprotrophic, pathogenic and solopathogenic representative strains by addition of 1 mL of CTAB 2 % (Doyle & Doyle 1990). Subsequently, 600μL of the solution was recovered from the Petri plate and transferred to a 2 μL eppendorf tube to continue DNA extraction, including a washing with 3 M sodium acetate. The DNA pellet from each tube was re-suspended in 50 μL of TE buffer (10 mM Tris-HCl, pH 8, 1 mM EDTA). The quality of the DNA was verified by electrophoresis in a 1.5% agarose gel in 1x TAE buffer (Tris Acetate EDTA) run at 87 V cm^{-1} for 1 h. The gel was stained with Gel Red (Biotium, Ca, USA), and the bands were visualised using an Infinity 1000/26MX Xpress (Vilber Lourmat, Germany) gel documentation system. The DNA concentration was quantified using a Nanodrop 2000C spectrophotometer Lambda BIO 10 (Thermo Scientific, USA), and the samples were diluted to 20 ng μL^{-1} for amplification of the *b* alleles.

PCR amplification and sequencing

Specific primers *bE4* (5´-CTCGAGGTTCATCAGCTCA-3´) and *bE8* (5´-GCTGAGTTCTGGAGTCG-3´) to amplify *b*E locus to determinate the type of regulatory protein were used (Albert & Schenck 1996). A PCR master mix was prepared in a final volume of 25 μL containing 1x taq DNA polymerase buffer, 0.8 μM deoxynucleotides (0.2 mM each), 100 ng DNA, 10 *p*mol of each primer, and 2 Units of *Taq* DNA polymerase (Promega, USA).

PCR amplifications were performed with an initial denaturation at 95 °C for 2 min; 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min; followed by a final extension at 72 °C for 10 min. PCR reactions were carried out in a Peltier Thermal Cycler PTC-200 (Bio-Rad, USA), and the PCR products were verified by loading 5 µL in a 1.5% agarose electrophoresis gel, which was stained as described above. The remaining amplified PCR products were purified using ExoSAP-IT (Affymetrix, Santa Clara CA, USA) following the manufacturer's instructions.

Sequencing reactions were prepared with big dye terminator v3.0 (Applied BioSystems, Ca, USA), and fragments were resolved in an automated Genetic Analyser model 3730 DNA (Applied BioSystems, Ca, USA). To avoid misreadings, the PCR products were sequenced in both directions. Sequences corresponding to regulatory protein from new *U. maydis* isolates were deposit in GenBank from the National Center for Biotechnology Information (NCBI) under accession number KF647221-KF647225 and KF689679-KF689685. For cases in which multiple isolates had identical sequences, only one accession number per sequence was deposited, representing the common sequence of those isolates.

Analysis of b-locus diversity

Sequences corresponding to both strands of the *b*E gene were assembled and edited using BIOEDIT v7.2.0 software (Hall 1999), and a consensus sequence of each isolate was created and compared with GenBank from NCBI database through BLASTN 2.2.19 option (Zhang *et al*. 2000).

For evolutionary analysis, two different methods were performed. In the first one, all consensus sequences were compiled into a single file (FASTA format) to build a nucleotide sequence alignment with the profile mode of ClustalW 1.8.1 (Thompson *et al*. 1994). Phylogenetic reconstruction was performed with *b*E sequences using the statistical method of Maximum Parsimony (MP) included in the MEGA 6 software (Tamura *et al*. 2013). To determine the confidence values for clades within the resulting tree, a bootstrap analysis was assessed with 1000 replicates (Felsenstein 1985). Data subset was conducted using the close-neighbour interchange (CNI) on random trees, with MP search option (level = 1) and initial tree by random addition (10 reps), and gaps/missing data were considered a complete deletion. The accession numbers of *U. maydis* deposited in the GenBank – NCBI database corresponding to *b*1, *b*2, *b*3 and *b*4, are different allele of the same gene, they were downloaded and included as reference sequences along with the sequences obtained in this study. *Sporisorium reilianum* (accession number EU167575) was designated as out group.

In the second one, Bayesian inference was calculated using MrBayes v3.2.2 (Ronquist & Huelsenbeck 2003) with Markov Chain Monte Carlo (MCMC) algorithm to generate trees with Bayesian posterior probabilities. Three heated chains and one cold chain with 200,000 generations were run to reach a P value lower than 0.01 with two parallel searches considering a sample frequency of 1 in 100. To obtain the consensus tree, we used the most complex model of evolution available for nucleotides (GTR + gamma distribution + invariant positions). The first 25 % of trees were discarded as the burn-in phase of each analysis and posterior probabilities was determined for the remaining trees. The consensus tree was visualized with FigTree v1.4 (Rambaut & Drummond 2010) and export to Mega 6 software for final print.

Results

Teliospore germination

A mass of sooty appearance of melanized teliospores was collected from inside galls produced from infected maize kernels. Diploid teliospores placed on PDA medium germinated after three days, and yeast-like colonies were observed. Galls of *U. maydis* collected from maize fields are associated with a complex of yeast-like colonies of similar colour and consistency, among these are: *Candida railenensis*, *C. quercitrusa* and *Pichia guilliermondii*. For this reason phylogenetic identification was performed with all strains obtained from teliospores germinated *in vitro*, to be sure that analysis was carried out with *U. maydis* strains.

Microscopic observation

When yeast type colonies cultured on PDA were transferred in PDA+AC medium, three types of colonies development were observed after 72 h. The first colony type was formed by saprotrophic strains with yeast-like colonies with no mycelium. These were bright or opaque in appearance, cream or pale yellow in colour and had either a smooth or rough surface (Fig. 2A). The second colony type was characterised as pathogenic strains because their yeast-like colonies were white and had rough surfaces with sparse mycelium (Fig. 2B). In the third colony type, the pathogenic, yeast-like colonies developed a cottony mycelium. These colonies were initially white but became brown after 72 h. All strains of this type retained the same morphology after five subcultures (Fig. 2C).

Detection of compatibility group

The partial-diallel strain arrangement allowed determination *in vitro* of compatible and incompatible matings, as well as saprotrophic and pathogenic combination (Fig. 3). The growth of mycelial fuzz was evident only when sexually compatible hyphae fused (Tab. 1).

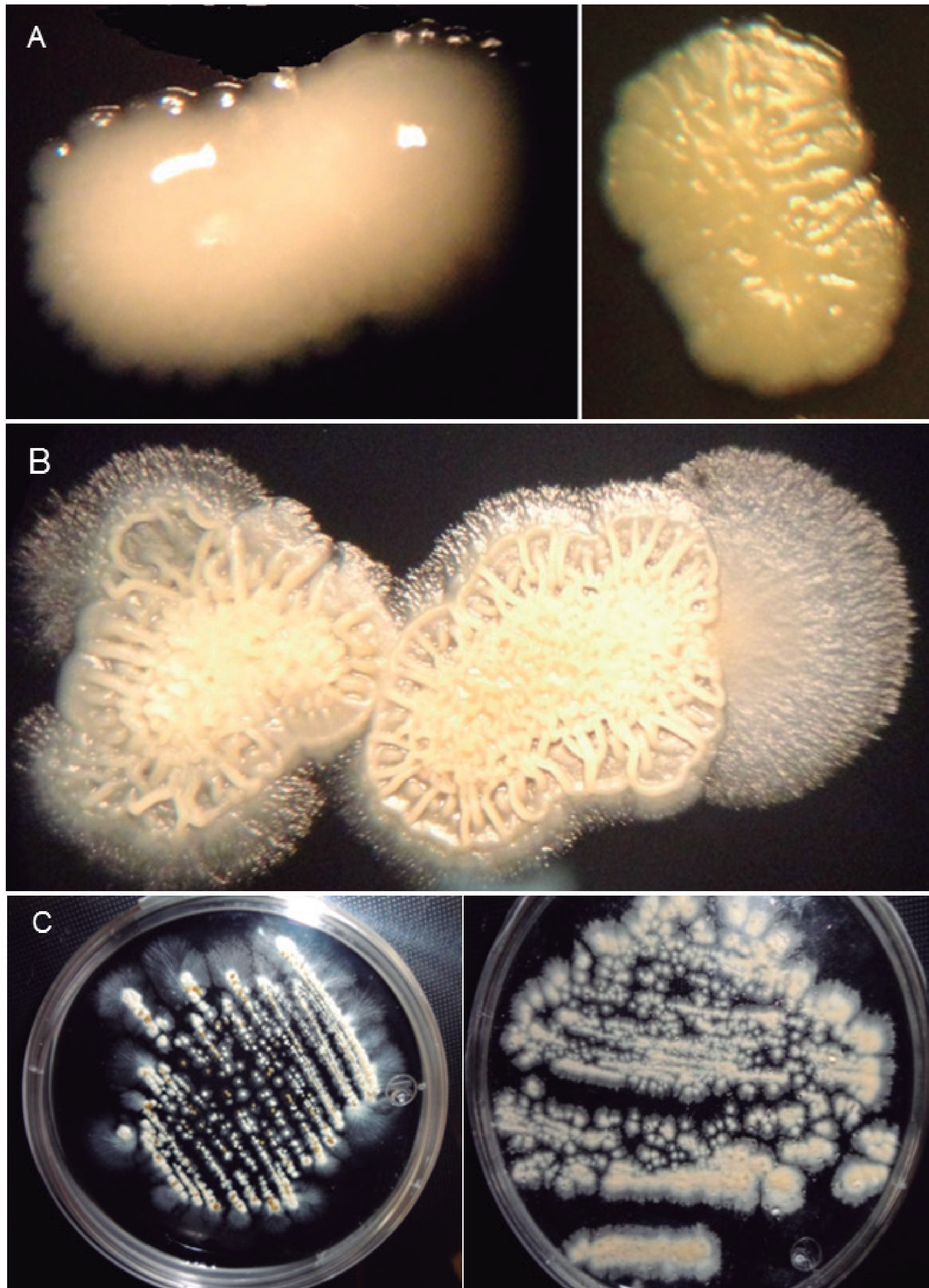

Figure 2. Yeast-like colonies of *Ustilago maydis* collected from landrace maize in the Mexican Highlands, grown on a PDA medium supplemented with a 5% carbon source. Panel A: yeast-like colony with no mycelium, bright or opaque in appearance, cream or pale-yellow in colour and having a rough or smooth surface. Panel B: yeast-like colony, white and with a rough surface, and an obvious surrounding filamentous mycelium. Panel C: yeast-like, white colony developing cottony mycelium but becoming brown after 72 h, that retained their properties after five re-streaks under *in vitro* conditions.

The group of 26 strains in 325 pairs of combinations were characterised individually (Tab. 1). When grown on a PDA+AC, all showed a yeast-like colony with a smooth or rough surface. When the strains were placed in pairs, most produced rough-textured colonies with filamentous mycelia at the periphery. In Table 1, '+' indicates when a peripheral mycelium was present and '–' when absent.

Analysis of the mating behaviour of the 26 colonies

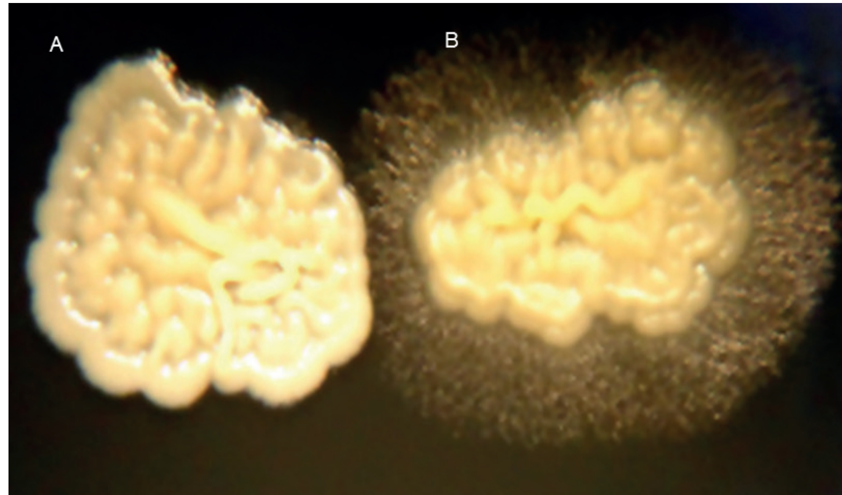

Figure 3. Colonies of *Ustilago maydis* developed on PDA medium supplemented with a 5% carbon source. A: Saprotrophic colony showed a rough surface. B: Colony with a rough surface developing a peripheral mycelium.

(Tab. 2) indicates that CPO-1 strain in combinations with others resulted in the detection of four solopathogenic strains; they showed rough colonies and peripheral mycelia in all cells. In two strains (CPO-5 and CPO-8) there were three; in five strains (CPO-2, CPO-3, CPO-9, CPO-11 and CPO-18) there was only one. Growth showed differences in the size of colonies, as well as repeated streaks of each colony. Fifteen strains were identified as solopathogenic (signed in bold, Tab. 1).

DAPI staining

Sporidia of *Ustilago maydis* having one nucleus was visualised by DAPI staining (Fig. 4). On the other hand, the dikaryotic growth stage was also evident in Petri plates, plated under laboratory conditions in pathogenic strains. Solopathogenic diploid sporidia are not a product of the fusion of two compatible haploid hyphae but are formed during teliospore germination. The nucleus migrates to the developing pro-mycelium centre, where the first meiotic division occurs. Usually, but not always, four cells remains in the pro-mycelium, and each gives rise to a single primary sporidium. While this description of the germination and the formation of sporidia is classic, it is not the most common process. A single pro-mycelium can lead to five or seven primary sporidia and some of these can be diploid (Sabbagh *et al*. 2010). These diploid sporidia give rise to solopathogenic strains.

Amplification of the b-locus diversity

PCR amplification with primers to identify a partial sequence of the *b*E locus of *U. maydis* was successful. A 480 bp fragment was clearly observed by agarose electrophoresis gel.

Twelve representative sequences corresponding to amplification of the partial *b* locus were used to establish the type of regulatory protein and phylogenetic reconstruction. The resulting consensus bootstrap tree performed with maximum parsimony method and Bayesian inference show that the strains clearly fell into two clades. In maximum parsimony method Clade I: the strains CPO-5af, CPO-5bf, CPO-15af, CPO-15bf, CPO-20af, CPO-20bf, CPO23af, CPO-23bf, CPO-24af and CPO-24bf were grouped with GenBank accession number M58556, belonging to *U. maydis b*4 protein. In Clade II, the strains CPO-9af and CPO-9bf were grouped with GenBank accession number M58555 belonging to *U. maydis b*3 protein. Both accessions corresponded to sequences obtained from RK32 and RK138 respectively selected as meiotic segregants from *U. maydis* teliospores collected in the Bonn area in Germany (Schulz *et al*. 1990). No single sequences matched with accession number M58553 *U. maydis b*1 protein or with M58554 *U. maydis b*2 protein (Fig. 5). In the same way, Bayesian inference grouped all the strains into two clades showing consistency in both tree, p value after 200,000 generations was 0.008.

Discussion

Although *U. maydis* is widely studied, few works deal on the diversity of Mexican or Mesoamerican strains. We here report an original work to isolate aggressive solopathogenic strains suitable for artificial inoculation in this part of the world where maize is the main crop. In this research, we present the results of attempts to isolate teliospores collected from galls formed in ears of field-grown, landrace maize adapted to the Mexican Highlands, and their ability to form the dikaryotic filamentous mycelia required to initiate the pathogenic process, and the existence of solopathogenic strains.

Table 2. Light microscope study of the morphologies of 26 *Ustilago maydis* strains and their partial-diallelic arrangements evaluated on PDA medium supplemented with a 5 % carbon source.

CPO Strain	Yeast-like colony [a]		Number of mating pairs with solopathogenic strain
	Smooth surface without mycelia [-]	Rough surface and weak peripheral mycelium [+]	
1	0	25	[1x9], [1x11], [1x12],[1x23]
2	0	25	[2x22]
3	0	25	[3x14]
4	25	0	0
5	0	25	[5x9], [5x14], [5x19]
6	25	0	0
7	25	0	0
8	0	25	[8x9], [8x13],[8x17]
9	0	25	[9x17]
10	25	0	0
11	0	25	[11x18]
12	0	25	0
13	0	25	0
14	0	25	0
15	25	0	0
16	25	0	0
17	0	25	0
18	0	25	[18x26]
19	0	25	0
20	25	0	0
21	0	25	0
22	0	25	0
23	0	25	0
24	0	25	0
25	0	25	0
26	0	25	0

aTwenty-six strains of *Ustilago maydis* evaluated in 25 partial-diallelic combinations.

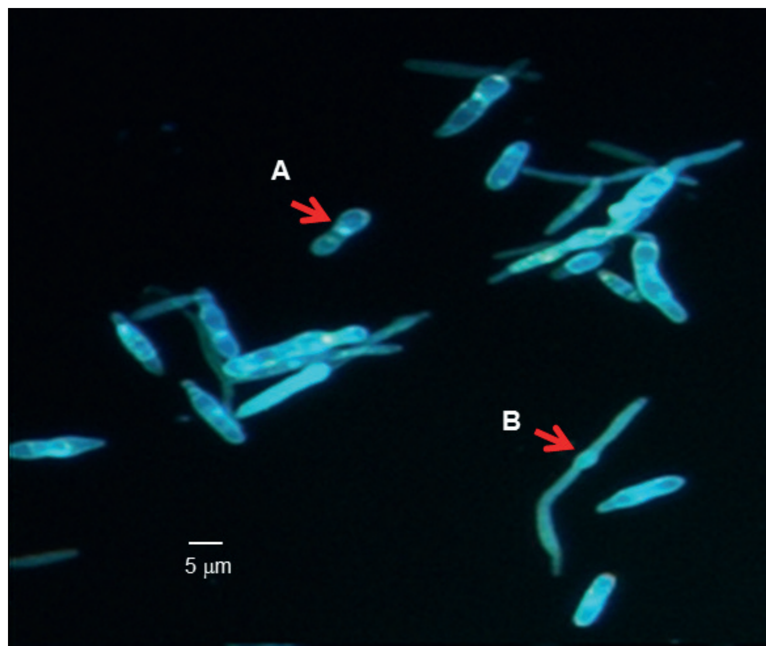

Figure 4. DAPI staining of *Ustilago maydis* CPO-23 solopathogenic strain observed with fluorescence microscopy. A: diploid sporidia and B: clamp-connection structure. Scale bar. 5 mm

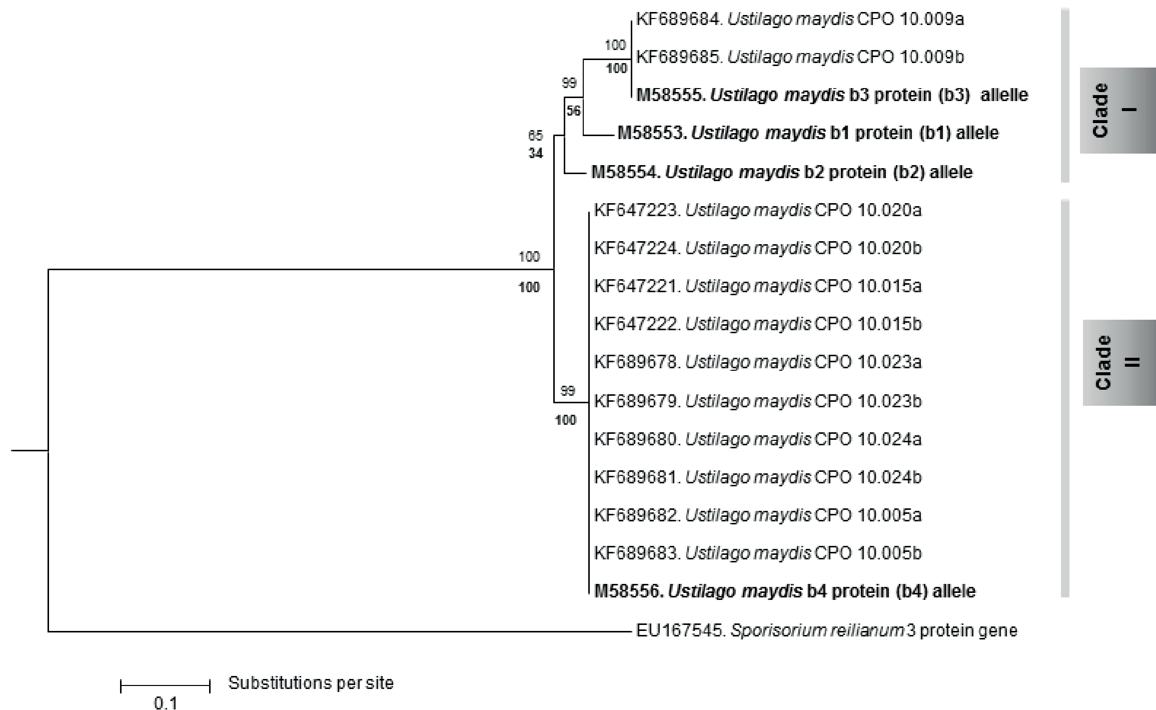

Figure 5. Phylogenetic consensus tree based on Bayesian inference, showing the relationship between *Ustilago maydis* sequences of partial *b* locus obtained of teliospores isolated from galls in Mexican Highland field and the four alleles reported previously of the same locus. The bootstrap confidence value is displayed in each cluster and for maximum parsimony method is shown in bold.

Yeast-like colonies obtained by germinating teliospores, were grown on an artificial medium supplemented with a carbon source. These colonies have different forms and various colours. In some strains, there was a peripheral growth of filamentous mycelium, in line with studies on fungal morphology, physiology and ultrastructure performed in other parts of the world where maize is grown (Kahmann & Kämper 2004). As known for *U. maydis*, we obtained three types of stages: i) diploid teliospores, conservative pathogenic structures that form inside galls, germinate outside the host to produce septate basidia on which haploid basidiospores (sporidia) are borne, ii) saprotrophic mycelia arise from germinated haploid unicellular sporidia that divide by budding, to form yeast-like colonies, and iii) filamentous dikaryotic hyphae, formed as the product of sexual reproduction between compatible haploid cells colonise the host plant without inducing defence responses (Mueller *et al.* 2013) and induce the formation of galls (sori). While formation and development of the dikaryon is restricted to maize tissue, the haploid mycelia and diploid mycelia from solopathogenic strains can grow well on artificial media.

Meanwhile, the *b* locus regulates the combination of any two different alleles that triggers the pathogenicity response, but not when the alleles are identical. Resulting from these combinations, it can be observed the development of yeast-like colonies taking one of three forms: a colony with no surrounding mycelium, a colony with a sparse surrounding mycelium or a colony surrounded by a dense, cottony mycelium in the case of solopathogenic strains (Fig. 2C). Light microscope studies of the morphology of these colonies revealed the presence in the mycelia of specialised structures known as clamp connections. These are one of the most characteristic features of basidiomycetes. DAPI staining confirmed the presence of haploid sporidia that give rise to solopathogenic strains that form stable colonies through successive re-streaks on artificial media. Later, amplification and sequencing by chain termination (Sanger sequencing) of the partial *b*E gene were carried out because phylogenetic identification of the Ustilaginales using large-subunit, nuclear ribosomal DNA sequences does provide ambiguous identification either among or within species (Stoll *et al.* 2003). Our results obtained using *b*E sequencing were particularly interesting because they revealed only the presence of alleles that encode *b*3 protein (B3) and *b*4 protein (B4) (Fig. 5). These alleles were first reported by Schulz *et al.* (1990), as functionally distinct from *b*1, *b*2, and each other protein regulator that encodes for polypeptides containing a homeodomain-related motif.

We infer that strains collected from *U. maydis* galls in the Mexican Highlands are closely related to the strains described by Schultz *et al.* (1990). The combination *a*1/*b*3 and *a*1/*b*4 alleles are the most common in this area and

they are not efficient because a1/a2 is necessary. However, it is necessary to collect galls from other areas of Mexico to determinate (i) whether a1/b1 or a1/b2 alleles are present elsewhere in Mexico and (ii) to identify the role of these alleles in the pathogenicity process because both genes are considered to regulatory proteins related with development and pathogenicity.

In summary, our study has identified strains of *U. maydis* that develop yeast-like colonies with mycelia haploid or with solopathogenic properties, thus creating the underlying knowledge base required to begin the development of effective commercial systems for the production of *U. maydis* galls worldwide.

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