Solopathogenic strain formation strongly differs among Ustilaginaceae species

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Abstract

The pathogenicity of smut fungi is initiated by the fusion of two compatible saprotrophic yeasts that give rise to the formation of dikaryotic pathogenic hyphae. It has been described in the literature that complementation assays of auxotrophic yeasts of Ustilago maydis have allowed the isolation of diploid strains that are solopathogenic, i.e. pathogenic in the absence of mating. The occurrence of such strains from germinating teliospores was not investigated. We evaluated the ability of teliospores to generate solopathogenic strains in three species of smut fungi: Sporisorium reilianum f.sp. zeae, U. maydis and Moesziomyces penicilliariae. Using an approach based on the stability of pseudohyphae of solopathogenic strains, we isolated the strain SRZS1 from teliospores of S. reilianum. Microscopic observations and analyses of mating-type alleles showed that SRZS1 is monokaryotic and diploid. Inoculation tests on maize plantlets indicated that SRZS1 is infectious. The same protocol was applied to polyteliosporal isolates from M. penicilliariae, U. maydis and S. reilianum of diverse geographic origin. Surprisingly, all strains from teliospores of M. penicilliariae were solopathogenic, whereas only few solopathogenic strains were obtained from the other two species. The possible incidence of solopathogenic strain production in the biology of these species is discussed.

Introduction

Among the basidiomycetes, around 600 species are grouped in the Ustilaginaceae family. Except for Pseudozyma species, which are anamorphic yeasts parasitic in humans (Begerow et al., 2000), Ustilaginaceae are pathogens of monocotyledonous plants and cause smut diseases. The main symptom is the formation of a sorus filled with black spores: teliospores. These structures are dispersed, overwinter in soil, then germinate after karyogamy and meiosis in a basidium that generates basidiospores. Basidiospores are haploid saprotrophic yeast-form cells. To infect a host, a haploid yeast must fuse with a compatible partner to form an infectious dikaryotic hypha. Dikaryotic hyphae are unable to grow out of plant tissues. It was demonstrated on Ustilago maydis (DC) Corda that dikaryotic strains are unstable in axenic culture and revert to haploid yeasts (Trueheart & Herskowitz, 1992). Ustilaginaceae are then dimorphic fungi where the yeast to hypha switch is concomitant with the physiological transition (saprotrophic to biotrophic) upon mating control. Interestingly, strains able to switch from saprotrophic yeast to parasitic hypha without mating – so-called solopathogenic strains – have been isolated from various species (Ehrlich, 1958; Holton et al., 1968; Puhalla, 1968). By auxotrophic complementation experiments, Holliday (1974) has isolated solopathogenic strains of U. maydis that are diploid cells able to grow in axenic culture. Such strains are useful genetic tools, leading to the discovery that cell signalling transduction pathways involved in mating, virulence, dimorphism and cell cycle are intertwined processes (Banuett & Herskowitz, 1988, 1989; Kahmann & Kamper, 2004). In spite of the genetic interest of the solopathogenic strains, their incidence in the biology of Ustilaginaceae is poorly documented.

In the present study, we compare the ability of teliospores from three species of smut fungi to form solopathogenic...
yeasts: Sporisorium reilianum f.sp. zaeae (Khûn) Langdon & Fullerton, U. maydis and Moesziomyces penicillariae (Bref.) Vánky. Sporisorium reilianum f.sp. zaeae is the causal agent of maize head smut, infecting maize plantlets via the roots under field conditions (Matyac & Kommedahl, 1985; Martinez et al., 2000, 2002). Ustilago maydis, causing common smut of maize, is known to be infective on different aerial parts of corn (Agrios, 1993). Moesziomyces penicillariae is a pathogen of pearl millet, largely present in the subsahelian zone. It is an airborne pathogen spread by the wind but also by insects infecting young inflorescences (Baht, 1946; Wilson, 1995). We designed a protocol on S. reilianum to isolate solopathogenic strains based on the isolation of stable fuzzy strains from germinated teliospores. This approach was applied on the three Ustilaginaceae species to compare their frequency of formation of solopathogenic strains.

**Materials and methods**

**Fungal material**

Sori of S. reilianum f.sp. zaeae (Kühn) Landon & Fullerton were collected from seven cornfields in France (Arcis, Deux Sèvres; Bischofsheim, Bas Rhin; Buros, Pyrénées Atlantiques; Corbreuse, Essonne; Gourville, Charente; Montclar-Lauragais, Haute Garonne; Saint Ciers, Gironde, France). Two compatible haploid yeast strains, SRZN and SRZM, were isolated from a sample collected in Saint Ciers (Gironde, France). The haploid, compatible strains SRZN and SRZM were also isolated from a sample collected in Saint Ciers (Gironde). Moesziomyces penicillariae (Bref.) Vánky sori were collected in pearl millet fields in 10 areas of Senegal (Doubalampos; Kaffrine; Keur Baka; Koumpentoum; Louga; Mountogou; Mpacc; Rao; Tambacounda; Ziguinchor) (Diagne-Lèye, 2005). Ustilago maydis (DC) Corda sori were collected in corn fields at Le Vernet, Muret (Haute Garonne, France), Pau (Hautes Pyrénées, France) and Gerona (Catalunya, Spain).

**Isolation of a stable fuzzy strain of S. reilianum from the parental strains SRZN and SRZM**

The haploid, compatible strains SRZN and SRZM were inoculated in maize and the resulting teliospores were collected 6 weeks later. These teliospores were then sterilized by Chloramine T 3% for 15 min, rinsed twice with sterilized water and resuspended in water at a concentration of 500 cells mL⁻¹. A volume of 100 μL of this suspension was spread on water–agar (3%) medium and placed at 24 °C for 2 days. After 2 days at 24 °C, most of the teliospores had germinated, forming basidia and sporidia. At this time, colonies formed by 10–15 yeast cells were subcultured in liquid medium and cultured for 4–7 days (subculture 1 in Table 1). Strains showing 100% of fuzzy colonies after subculture 3 were defined as stable fuzzy strains and tested for their pathogenicity.

**Comparison of fuzzy strain production**

Using the previous protocol, the ability of U. maydis, S. reilianum and M. penicillariae to produce solopathogenic strains was compared. For each species, teliospores from the different isolates were mixed to ensure genetic diversity. One hundred sterile teliospores were analysed for each species. Cultures were performed on PDA medium with 0.5% charcoal for U. maydis, on PDA for S. reilianum and on both media by plate replication for M. penicillariae.

**Plant infection test**

The pathogenicity of the stable fuzzy strains was tested on the corresponding hosts for each species. Except for SRZS1 experiments where 40 plantlets were used, plant infection tests were carried out on 10 plantlets for each strain. For S. reilianum, artificial inoculations were performed on maize plantlets (LMZ66; Limagrain, France) using a sterile syringe to inoculate the fungus in 10-day-old plantlets. A volume of 20 μL of cell suspension (10⁶ cell mL⁻¹) was injected into the stem, near the crown. Inoculated plants were analysed 6 weeks postinoculation (w.p.i.) for PCR fungal detection on caulinar apices or for symptom observation (chlorotic spots on leaves, sorus formation). For U. maydis, foliar inoculations were performed on 3-week-old maize plantlets (LMZ66; Limagrain) and the formation of galls was observed 2 w.p.i. (Holliday, 1974). Inoculation of pearl millet (Sanyo; ICRISAT, Bamako, Mali) was carried out by infecting young floral spikelets and the symptoms were observed 5 w.p.i. (Wilson, 1995).

**Microscopic observations**

Propidium iodide (Molecular Probes) staining was used to observe nuclei with a confocal laser scanning system (SP2 SE, Leica, Germany) equipped with an upright microscope (DM 6000, Leica). Cells from young 24-h cultures were fixed in 70% ethanol, rinsed with water, treated with RNase A (65 °C, 10 min) and then incubated with propidium iodide, final concentration 0.1 μM, for 1 h before observation.
DNA analysis

DNA extractions were performed using the CTAB procedure (Gardes & Bruns, 1993). In planta PCR detection of S. reilianum was performed based on the amplification of the internal transcribed spacer (ITS) ribosomal regions using specific primers. PCR detection was performed using the primers pITS1 (5'-TCC GTA GGT AAG CCT GCG G-3') and pSRZ4 (5'-ACT CGT GAG GCC GGC CTG A-3'). PCR reactions were performed in 35 cycles (5 min, 94°C–35 cycles; 1 min, 54°C; 1 min, 72°C; 1 min, 94°C; 10 min, 72°C) and PCR products were separated by electrophoresis in 2% w/v agarose gels.

To investigate cell ploidy, we were unable to use flow cytometry as the strain SRZS1 is formed of cells grouped in pseudomycelial forms unable to be analysed in a fluorescence-activated cell sorting system. Cell ploidy of SRZS1 was investigated through the search of the two parental alleles of the compatible haploid strains SRZM and SRZN. A ‘cleaved amplified polymorphism sequence’ approach was applied. The primers pMAT9 and pMAT10 were defined on homeodomain boxes (Schirawski et al., 2005). BLAST analyses of the amplicons indicated that SRZN corresponds to matB1 and SRZM to matB2 alleles of S. reilianum as defined by Schirawski et al. (2005). PCR amplicons of haploid strains and solopathogenic isolates were digested directly without further purification with the single-endonuclease restriction enzyme, Eco 1301 (Sty I-Fermentas, France). A volume of 15 μL of digested product was mixed with 2 μL of reaction buffer and 3 μL (10 U) of restriction enzyme, and then incubated for 2 h at 37°C. Restriction fragments of amplicons were separated by electrophoresis (TAE buffer) on agarose gels.

Results

Isolation of a stable fuzzy strain of S. reilianum

Germinating teliospores were used to isolate diploid solopathogenic strains in axenic condition. Because of the mating of young sporidia formed by basidia, a major difficulty in this approach is to separate true solopathogenic strains from dikaryotic strains resulting from the fusion of compatible haploid yeasts. In order to limit the formation of dikaryotic strains, young colonies formed by 10–20 basidia from recently germinating teliospores were selected, picked up and spread on solid medium (initial culture). Colonies obtained from this first isolation mainly had a smooth surface, corresponding to colonies of haploid yeast (Fig. 1a,b). Some fuzzy colonies also appeared (Fig. 1a–c). Fuzzy colonies usually correspond to dikaryotic pseudohyphal strains produced following mating. Each fuzzy colony was subcultured in liquid medium for a week to induce the reversion of unstable dikaryotic strains to haploid yeasts. These liquid subcultures (subculture 1) were plated on solid medium to test the appearance of nonfuzzy colonies. The subcultures (subculture 1) leading to 100% fuzzy colonies on solid medium were subcultured again in liquid medium (subculture 2) for one week and afterward plated on solid medium to assess their stability. A third subculture (subculture 3) was applied as a control. Using this protocol, we isolated a stable fuzzy strain of S. reilianum, SRZS1 (Fig. 1d). In liquid medium, young cultures of SRZS1 appeared as small pellets (Fig. 1e) formed by aggregates of budding yeasts and pseudohyphae (Fig. 1f).

The cells of the strain SRZS1 are monokaryotic and diploid

The strain SRZS1 was used for further investigations. Microscopic observations of the pseudohyphae after nucleus staining with propidium iodide revealed that only one nucleus is present in the cells (Fig. 2). As the strain SRZS1 originated from the parental yeasts SRZN and SRZM, the presence of the two parental MATb alleles was determined. Two primers were designed on the conserved homeodomain boxes of the MATb loci of Ustilaginaceae. PCR amplification on DNA extracted from the parental yeasts and SRZS1 yielded an amplicon of 1334 bp (Fig. 3a). Based on sequence analysis, a StyI restriction enzyme was identified to differentially cut this region from matb1 (SRZN) and matb2 (SRZM). As observed in Fig. 3, the restriction enzyme pattern obtained with SRZS1 (line 6) corresponds to the superposition of the patterns of the two parental strains SRZM (line 3) and SRZN (line 4). The presence of the two loci in SRZS1 indicates that this monokaryotic strain is diploid.

SRZS1 is infectious, but does not form sori

The pathogenicity of SRZ1 was tested by artificial inoculations on 10-day-old maize plantlets. After 6 weeks of culture, no sori were formed on the ears. However, several typical symptoms caused by S. reilianum were observed: among the 40 infection tests, 8 plantlets were dwarf and 36 plantlets presented chlorotic spots on leaves. Microscopic observations indicated that mycelium was present in the chlorotic spots (not shown). PCR diagnosis using specific primers confirmed the presence of S. reilianum in the caulinar apex of the dwarf plants and, to a lesser extent, in leaves (Fig. 4). These results argue that the SRZS1 strain is able to grow in the plant tissue and induces some typical symptoms in maize although is unable to sporulate and form a sorus.

The capacity of three Ustilaginaceae species to form solopathogenic strains differs strongly

The protocol defined to isolate SRZS1 was applied to teliospores of M. penicillariae, S. reilianum and U. maydis.

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For each species, samples from different locations were mixed. For *M. penicillariae*, all isolates obtained in initial culture were fuzzy and remained fuzzy during subcultures. For *S. reilianum* and *U. maydis*, most of the fuzzy strains obtained in the initial culture reverted to nonfuzzy strains after subculture 1. Under our conditions, two subculture steps were necessary to obtain stable fuzzy strains. Compared with the initial number of colonies isolated from 100 germinating teliospores, *S. reilianum* showed the lowest potential to produce stable fuzzy colonies (0.15% under our conditions). *Ustilago maydis* showed an intermediate behaviour as 2.6% of the initial strains were fuzzy and stable. For *M. penicillariae* and *U. maydis*, the fuzzy strains selected were infectious and led to the formation of sori. For *S. reilianum*, we did not observe the formation of smut sori, but, as for SRZS1, the two fuzzy strains isolated were infectious as they grew in the maize tissues as defined by PCR.

**Discussion**

*Ustilago maydis* is a paradigm to illustrate the life cycle of *Ustilaginaceae* (Agrios, 1993). The cellular and molecular events involved in the transition from a haploid saprotrophic yeast form to a dikaryotic parasitic hyphal form have been extensively explored in this species (Kahmann & Kamper, 2004). However, several variations in the life cycle occur among *Ustilaginaceae* species. For instance, their systemic growth ability differs according to the plant organ infected (root, leaf or flower) (see Vánky, 1994). Among these variations, the role of solopathogenic strains was poorly investigated although such strains were considered as useful genetic tools. In the literature, solopathogenic strains of *Ustilaginaceae* were isolated from the progeny of *in vitro* mated haploid and compatible yeast strains, either wild types (Ehrlich, 1958; Puhalla, 1968) or auxotrophic mutants (Holliday, 1974; Harrison & Sherwood, 1994). Our

**Table 1.** Production of solopathogenic strains from teliospores of *Moesziomyces penicillariae*, *Sporisorium reilianum* and *Ustilago maydis*

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<th>Fuzzy strains</th>
<th>Pathogenicity</th>
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<td>Initial culture</td>
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<td><em>M. penicillariae</em></td>
<td>549&lt;sup&gt;‰&lt;/sup&gt;</td>
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<tr>
<td><em>S. reilianum</em></td>
<td>27&lt;sup&gt;k&lt;/sup&gt;</td>
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<td><em>U. maydis</em></td>
<td>61&lt;sup&gt;**&lt;/sup&gt;</td>
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<sup>1</sup>Final percentage of stable fuzzy strains $R = 100 \times (\text{number of stable fuzzy strains in subculture 3})/(\text{Total number of colonies isolated in the initial culture})$.

<sup>2</sup>Test performed with two strains for each species, 10 plants inoculated per strain.

<sup>3</sup>Evaluated on the two isolated strains of *S. reilianum* by PCR detection on caulinar apices (10 plantlets each strain).

<sup>4</sup>Total of 549 colonies analysed from 100 germinated teliospores.

<sup>5</sup>For *M. penicillariae*, only 50 strains underwent subculture 1 as all of them were filamentous in initial culture.

<sup>6</sup>Total of 1322 colonies analysed from 100 germinated teliospores.

<sup>7</sup>Total of 1239 colonies analysed from 100 germinated teliospores.
strategy was to isolate solopathogenic strains from germinating teliospores to evaluate and compare the production of such spores under control conditions by three different smut fungi. In Ustilaginaceae, dikaryotic cells are unstable and revert to haploid yeasts (Trueheart & Herskowitz, 1992), whereas solopathogenic strains are stable in axenic culture. This characteristic was used to eliminate fuzzy-dikaryotic strains from successive subcultures. Using this protocol, here we report the first isolation of a naturally occurring solopathogenic strain of S. reilianum, SRZS1. Nucleus staining revealed that SRZS1 is monokaryotic. The amplification and restriction enzyme digestion of mating type genes showed that SRZS1 has the two MATb alleles provided by the parental strains SRZM (MATb2) and SRZN (MATb1). This result is in agreement with the hypothesis that this monokaryotic strain is diploid. However, the strategy used did not allow us to exclude that the presence of the compatible allele could also be the result of a parasexual transfer leading to the

**Fig. 2.** Nucleus staining of a stable fuzzy strain of Sporisorium reilianum. Merged images of fluorescent and visible confocal observations. Nuclei were stained using propidium iodide. The fungus grew in liquid medium as a pseudo mycelium formed by monokaryotic cells.

**Fig. 3.** Restriction enzyme digestion of MATb loci of Sporisorium reilianum strains. (a) Sty1 sites and fragments deduced from MATb locus sequences on SRZM and SRZN (1334 bp); (b) Electrophoretic pattern. 1 and 5, DNA 100 bp ladder; 2, SRZM; 3, Sty1 digestion of SRZM (976 and 333 bp); 4, Sty1 digestion of SRZN (1279 bp); 6, Sty1 digestion of SRZS1. (c) Analysis of electrophoregram using IMAGEJ software. The strain SRZS1 (profile 6) shared the restriction enzyme patterns of SRZM and SRZN (profiles 3 and 4, respectively).

**Fig. 4.** In planta detection of Sporisorium reilianum SRZS1. PCR was performed with the primers pSRZ4-pITS4. Lanes 1 and 11, DNA ladder 100 bp; Lanes 2–3, DNA from noninoculated maize apex; Lanes 4–5, DNA from noninoculated maize leaves; Lanes 6–8, DNA from maize inoculated with SRZS1: a, apex; b-e, leaves; Lane 9, control detection on DNA from apex of maize inoculated with a mix of SRZN × SRZM; Lane 10, control with DNA from S. reilianum strain SRZS1.
formation of a merodiploid strain (Zeigler et al., 1997), although such a mechanism has not been observed as yet in solopathogenic strains of *U. maydis*. Using specific primers of *S. reilianum*, PCR detection of SRZS1 in caulinar apices after crown infection showed that the strain is infectious. Its pathogenicity is weak as colonization did not lead to the formation of a sorus. We obtained similar conclusions with two other solopathogenic strains isolated from a poly-teliosporal sample (Table 1): inoculated plants present symptoms (dwarf plants and/or chlorotic spots on leaves), but did not develop smutted ears. Although they are infectious, the solopathogenic strains of *S. reilianum* seem unable to perform the entire life cycle of the fungus.

We compared the ability of teliospores from *M. penicillariae, S. reilianum* and *U. maydis* to form solopathogenic cells. Surprisingly, all strains formed by the *M. penicillariae* were solopathogenic. It has already been described that monoisolates of this species can be infectious (Wilson & Bondari, 1990). Under our assay conditions, the solopathogenicity of monoisolates formed after teliospore germination is the usual cell status. This observation raises the question of a total loss of sexuality in this species and the maintenance of genetic diversity in the absence of inbreeding. It must be underlined that other fungi are known to present specific adaptations of their life cycle: *Agaricus bisporus* for instance is an amphithallic basidiomycetous species forming dikaryotic spores, although some isolates are tetrasporic (Callac et al., 2003). It would be relevant to evaluate the presence of heterothallic isolates of *M. penicillariae* in a larger sampling campaign in this species. Unlike *M. penicillariae, S. reilianum* showed a very low ability to form solopathogenic strains (0.15% of the isolated strains) and these strains are unable to sporulate and form new teliospores. It can be proposed that solopathogenic strains have few or no incidence on the life cycle of *S. reilianum*. The situation of *U. maydis* is intermediate. This species produced around 3% of solopathogenic strains under the conditions used. The solopathogenic strains tested were infectious and produced galls.

It is tempting to link the potential of *M. penicillariae* to only form solopathogenic strains with its mode of dispersal. *Moesziomyces penicillariae* is a strict aerial pathogen: infection of pearl millet occurs only via inflorescence stigmas and the disease is spread by insects or by the wind (Baht, 1946; Kousik et al., 1988). It has already been mentioned that the dispersal of dikaryotic or diploid strains forming a ‘full genetic tank’ ready to infect could be an advantage compared with the dispersal of saprotrophic haploid strains that need to mate (Piepenbring et al., 1998). Dispersal of diploid or dikaryotic strains is not rare among *Basidimycetes*: rust fungi form dikaryotic spores (ecidiospores and uredospores) that contribute to the epidemiological cycle of these diseases, allowing long-distance airborne spreading. Solopathogenicity could then be considered as an adaptive advantage for anemophilous *Ustilaginaceae* species such as *M. penicillariae*.

Our results point to the originality of the biology of *M. penicillariae* and the necessity to better characterize its life cycle. The discrepancies reported in the formation of solopathogenic strains from species to species of smut fungi illustrate the plasticity of the life cycle of basidiomycetous dimorphic fungi as already proposed (Morrow & Fraser, 2009) and stress the utility of investigating the epidemiological incidence of such strains.

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**References**


