Root Penetration of Maize by *Ustilago maydis*

S.K. Sabbagh[1], Y. Martinez[2] and C. Roux[1]

[1]Laboratory "Cell Surface and Signaling in Plants", University Toulouse III – CNRS Joint Unit 5546, Plant Biotechnology Institute, Chemin de Borde Rouge, Auzeville, BP42617 F31326 Castanet Tolosan, France; [2]IFR40 Plant Biotechnology Institute, Chemin de Borde Rouge, Auzeville, BP42617 F31326 Castanet Tolosan, France, e-mail: roux@scsv.ups-tlse.fr

Abstract: *Ustilago maydis* is a model organism as phytopathogenic fungus: genome has been sequenced, mechanisms of pathogenicity are partly described and non pathogenic mutants are available. Although its biological cycle has been extensively studied by several authors, it's not clearly defined if root penetration is involved in pathogenesis as primo-infection site. To investigate maize root penetration by *U. maydis*, we used different microscopic approaches and PCR detection. A susceptible maize variety was inoculated via roots with different forms of the fungus: teliospores, a mix of haploid compatible strains and a solopathogenic strain. Light, confocal, and transmission electron microscopy observations showed that *U. maydis* penetrates maize roots: fungal cells are mostly present as intracellular hyphae in cortical root cells. PCR detection using primers specific to *U. maydis* allowed us to detect the fungus in stems after root inoculation. However, in spite of several attempts, root infection did not provoke any symptom on inoculated plants. Although root penetration occurs, this way of infection does not seem to participate significantly to the life cycle of the fungus.

Keywords: *Ustilago maydis;* root penetration; confocal and electron microscopy; PCR detection

Ustilago maydis (DC.) Corda is a basidiomycete fungus causing corn smut disease. The fungus forms galls (sori full of black spores) on different parts of its host plant: leaves, stem, tassels and ears. The early events of pathogenesis have been described: from mating of compatible yeasts to penetration on stem, leaves and silks (Mills & Kotzé 1981; Snetselaar & Mims 1992, 1993, 1994; Snetselaar 1993). *U. maydis* adopts two different morphologies during its life cycle. Haploid sporidia grow yeast-like and can be propagated on artificial media. After fusion of two compatible sporidia, an infectious filamentous dicaryon is generated. *Ustilaginaceae* fungi are also able to directly form diploid yeasts which could infect maize in absence of mating. For this reason, these diploid yeasts are called solopathogenic (Holliday 1974). In field condition, compatible haploid sporidia fuse on the leaf surface and the filamentous dicaryon differentiate in an appressorium-like structure that penetrates the host cell wall. From the infection site, the fungus rapidly spreads and grows intracellularly from cell to cell. In spite of these descriptions, it's not clearly defined if *U. maydis* could infect maize via roots.

The aim of this study was to investigate the ability of *Ustilago maydis* to penetrate maize roots without wounding. Roots of a sensitive maize line were inoculated. Different types of inoculum were used. First, we used teliospores as these cells are present in soil after sporulation and could be the natural inoculum in field for root infection. Secondly we used a mix of compatible haploid sporidia (FB6a × FB6b) as these forms are known to be very efficient to infect aerial parts. We investigated root penetration by using light and electron microscopy approaches. To improve our observations, we observed with a confocal microscope the penetration of a solopathogenic strain expressing the green fluorescent protein (FB11 solo-pOTEF). The ability of the fungus to grow from roots to aerial tissues was tested by PCR detection. We designed in the

ribosomal ITS regions a primer specific to *Ustilago maydis*. Lastly, the efficiency of root inoculation on symptom apparition was analysed on maize infected in greenhouse.

MATERIALS AND METHODS

Fungal strains and culture condition. Haploid strains FB6a and FB6b (Banuett & Herskowitz 1989), teliospores obtained from artificial contamination of maize with the strains FB6a × FB6b, and a diploid solopathogenic strain FBD11-pOTEF expressing the green fluorescent protein GFP (Spellig *et al.* 1996) were used in this study. All yeast strains were cultivated in liquid potato dextrose broth medium (PDB) at 24°C under shaking 100 rpm until cultures reached mid-log phase (19 h).

***Inoculation of plants with** Ustilago maydis*. Root infection was performed as described by Martinez *et al.* (2002). Seeds of a variety highly susceptible to *Ustilago maydis* (LMZ66-Limagrain, France) were surface-sterilized in 5% (w/v) chloramine T (Sigma, France) and place on PDA medium at 24°C during 4 days. According to the experiments, seeds free of microbial-contamination were transferred either in Magenta boxes containing 150 ml of M medium without glucose (Bécard & Fortin 1988) supplemented with phytagel 0.3% (Sigma, France) or to sterilized peat (1 h at 120°C). In Magenta boxes, inoculation on 1 week old maize plantlets was carried out by adding in the vicinity of roots 500 µl of cell suspension at 10^7 cells/ml. In pot, inoculation was carried out by directly pouring on seeds 1 ml of cell suspension at 10^7 cells/ml. For each experiment, the plants were grown in a night/day temperature of 18/24 ± 5°C for 3 weeks, except for symptom observation where cultivation was up to floral formation (2 months in our cultural conditions for this variety).

Microscopic observation. Infected roots and stems of 3-weeks old maize were cut into small pieces and used for microscopy analyse and PCR detection. For light microscopy, samples of root were embedded in agarose before cutting sections of 150 µm width. The sections were stained with cotton blue in lactophenol and rinsed in lactophenol for 10 min. All the specimens were mounted on a glass slide and observed using an inverted microscope. Images were acquired using a colour video camera (Hyper Had, Japan).

For TEM observations, we used the method described by Martinez *et al.* (1999). Ultrathin sections (50–60 nm in thickness) were stained with uranyl-acetate and examined using a Philips 301 transmission electron microscope at 80 KV (Philips, Eindhoven, The Netherlands).

Confocal images were acquired with a spectral confocal laser scanning system (SP2 SE, Leica, Germany) equipped with an upright microscope (DM 6000, Leica, Germany). Observations were made using 40× and 63× (HCA APO, N.A. 0.8) dry objectives. An Argon laser emitted at 488 nm was used to collect GFP in the range between 500 and 530 nm. The ray line of the He-Ne laser emitted at 543 nm was able to excite the cell wall coloured by a solution of blue evans 0.01% during 30 s and the fluorescence was collected in the range 590–710 nm. The images were acquired in the dual mode. The overlay images were then computed by projection of 20 to 30 plan-confocal images acquired in z dimension with 0.8 µm increment between two focal planes.

DNA extraction and PCR amplification. Plants inoculated in pots were used for these experiments. After root inoculation by the different inoculum forms, plants were grown during 3 weeks. Aerial parts were then collected, and caulinar apexes were isolated to avoid surface contamination by the fungus. DNA extractions from 50 mg of plant material were performed using the CTAB procedure (Gardes & Bruns 1993). DNA quality and quantity were evaluated by electrophoresis and spectrophotometry.

We designed the specific primer pUM1 on ITS1 region of *Ustilago maydis* (5′-CGGTCGGTCTGTCGAAACC-3′). PCR reactions (5 min 94°C – 35 cycles 1 min 54°C – 1 min 72°C – 1 min 94°C – 10 min 72°C) were followed by an electrophoresis of amplicons on agarose 1.5%. The presence of *Ustilago maydis* in samples is defined by the presence of a band at 680 pb.

RESULTS

Maize plantlets grown in Magenta box and pot were infected by different forms of *U. maydis*. As control, we inoculated some plants with the haploid strain Fb6b. These different strains were applied on maize roots for microscopy analysis, PCR detection and symptom observation.

Using light microscopy, we observed 3 weeks after addition of *Ustilago maydis* the presence of fungal cells in maize roots, whatever the inoculum form applied, except for the control FB6b. The

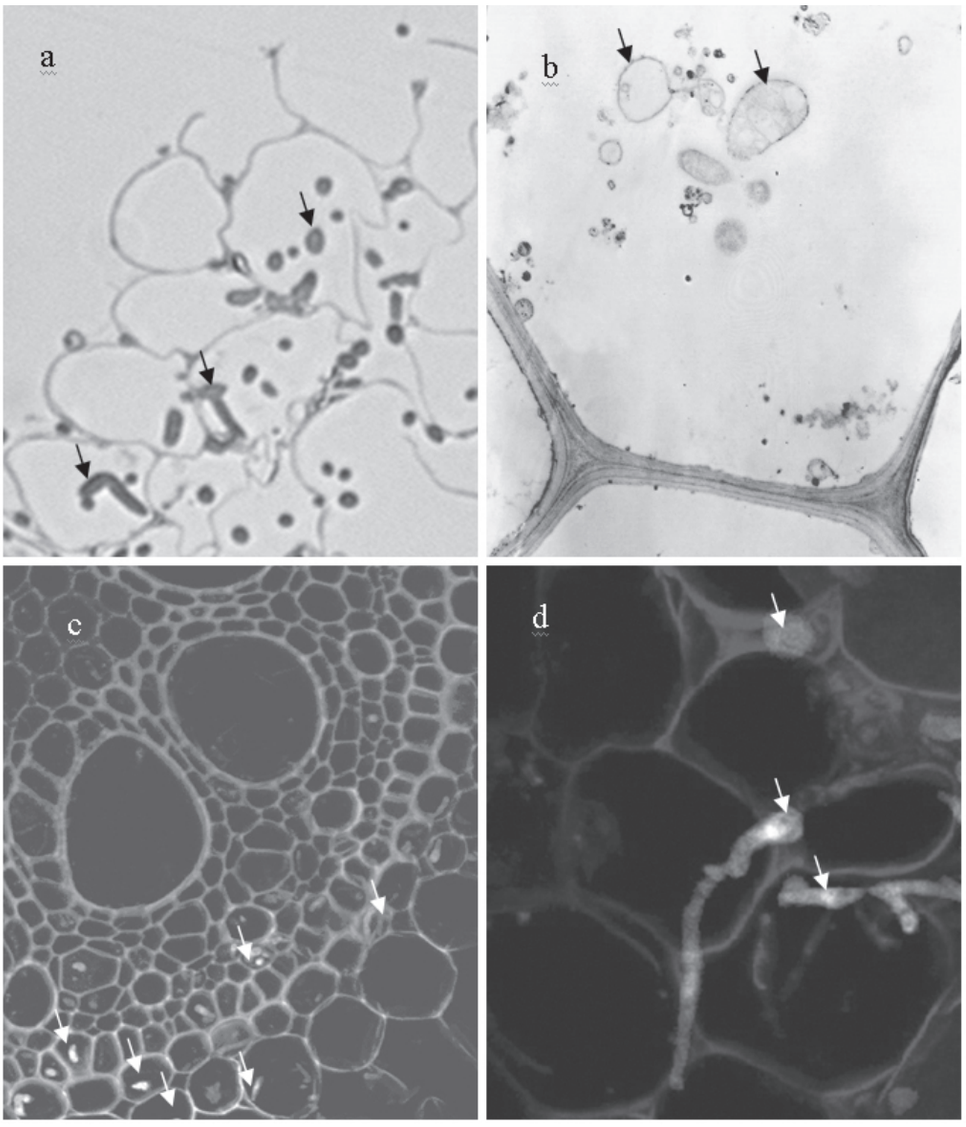

Figure 1. Observation of *Ustilago maydis* in maize roots. **a.** After cotton blue staining, the fungus appears as rounded cells or short pseudo-hyphae (black arrows) in the root cortical cells. **b.** Sections of intracellular hyphae observed by TEM. **c.** Confocal microscopy of a solopathogenic strain expressing GFP (white arrow). The fungus is mostly present in the periphery of the roots. **d.** *U. maydis* forms pseudo-hyphae growing from cell to cell (white arrow)

fungus, essentially present in cortical cells, appeared as rounded cells or part of pseudo-hyphae depending of the angle of cutting (Figure 1a). TEM observation showed that the fungus is usually intracellular, more rarely present in intercellular zone (Figure 1b).

Using the solopathogenic strain FBD11-pOTEF expressing GFP, we investigate the kinetic of development from 2 days to 3 weeks. The density of fungal cells inside root cells increase with time, but the fungus is essentially present in cortical cells and only few cells were observed in the central cylinder of root (Figure 1c). As expected, the fungus appeared in our sections as short hyphae, growing from cell to cell (Figure 1d).

In spite of our tries, we were unable to observe the fungus in the aerial part of the plantlets. To define if the fungus could migrate systemically from roots to stem, we designed a specific primer

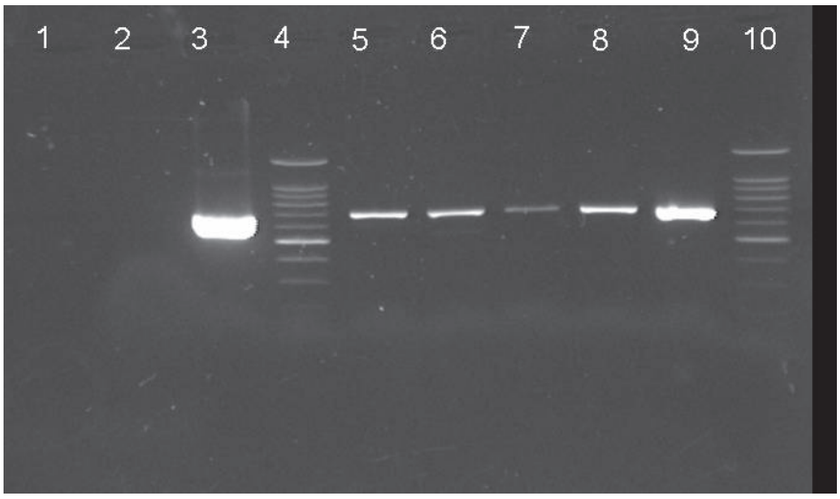

Figure 2. Electrophoresis of amplicons after PCR amplication of ITS regions using the primers pUM1-pITS4. Lane 1: maize inoculated with a haploid strain of *U. maydis* FB6b. Lane 2: non inoculated maize. Lane 3: *U. maydis* FB6b strain (680 bp). Lanes 4 and 10: 100 bp DNA ladder. Lanes 5 and 6: maize inoculated with FB6axFB6b strains. Lanes 7: maize inoculated with FBD11-pOTEF strain. Lanes 8 and 9: maize inoculated with teliospores

to detect the presence of the fungus by using PCR. This primer, pUM1, was designed on the ribosomal ITS1 region after alignment of several ITS sequences of *Ustilaginales* (not found). To prevent DNA contamination by fungal cells outside tissues, we isolated the internal caulinar apexes. We could detect the presence of the fungus in aerial part of the maize after root infection, whatever the inoculum tested, accepted with the control FB6b (Figure 2). However, about 4/5 of the plants inoculated didn't present a signal, indicating that the ability of *Ustilago maydis* to grow systemically is low.

In addition, we infected 12 plants with each form of inoculum for cultivation during 2 months in greenhouse. We did not observe any symptom on stems, leaves or floral structures.

DISCUSSION

Ustilago maydis, the pathogenic fungus causing common smut of maize, has been extensively studied for its ability to infect via aerial part of the maize. To our knowledge, there is no data about its ability to infect via roots. In this study, we showed that *U. maydis* can also penetrate maize roots in absence of wounding.

Ten days after inoculation, a dense development of the fungus at root surface was visible using a stereomicroscopy (data not shown). Different microscopic techniques allowed us to observe the process of infection. The fungus was mainly present in cortical cells, forming intracellular hyphae. These observations were validated with a mix of compatible haploid strains, a solopathogenic strain and teliospores. Control inoculation with a haploid strain did not give any result of penetration.

Although the fungus seems to migrate systemically from cell to cell, it was not possible to observe it in aerial part. As *U. maydis* develops pseudohyphae where only the apical cells are living, is not easy to find discrete structures of fungus in sections. For this reason, we used a PCR based protocol for detecting the presence of the fungus in the caulinar apex of root-inoculated maize. Our results indicated the fungus was detected in stem in 20% of plants infected via roots, 3 weeks after inoculation. We then carried out experiments of inoculation in greenhouse with the different forms of the fungus. Two months later, we didn't observe any visible symptom such as chlorotic spots on leaves, anthocyan accumulation on stems, galls or dwarf development.

Our results show that *U. maydis* could penetrate roots and migrate up to stems. This penetration did not induce symptoms on the maize variety tested. Our conclusion is that root penetration,

although possible, does not seem to participate significantly to the life cycle of the fungus, and aerial infection must be the unique way of infection of *U. maydis*.

References

Banuett F., Herskowitz I. (1989): Different a alleles of *Ustilago maydis* are necessary for maintenance of filamentous growth but not for meiosis. Proceedings of the National Academy of Sciences USA, **86**: 5878–5882.

Bécard G., Fortin J.A. (1988): Early events of vesicular-arbuscular mycorrhiza formation on Ri T-DNA transformed roots. New Phytologist (London), **108**: 211–218.

Gardes M., Bruns T.D. (1993): ITS primers with enhanced specificity for Basidiomycetes-application to the identification of mycorrhizae and rusts. Molecular Ecology, **2**: 113–118.

Holliday R. (1974): *Ustilago maydis*. Plenum Press, New York.

Martinez C., Roux C., Dargent R. (1999): Biotrophic development of *Sporisorium reilianum* f.sp. *zeae* in vegetative shoot apex of maize. Phytopathology, **89**: 247–253.

Martinez C., Roux C., Jauneau A., Dargent R. (2002): The biological cycle of *Sporisorium reilianum* f.sp. *zeae*: an overview using microscopy. Mycologia, **94**: 505–514.

Mills L.J., Kotzé J.M. (1981): Scanning electron microscopy of the germination, growth and infection of Ustilago maydis on maize. Phytopathologische Zeitschrift, **102**: 21–27.

Snetselaar K.M. (1993): Microscopic observation of *Ustilago maydis* mating interactions. Experimental Mycology, **17**: 345–355.

Snetselaar K.M., Mims C.W. (1992): Sporidial fusion and infection of maize seedlings by the smut fungus *Ustilago maydis*. Mycologia, **84**: 193–203.

Snetselaar K.M., Mims C.W. (1993): Infection of maize stigmas by *Ustilago maydis*: light and electron microscopy. Phytopathology, **83**: 843–850.

Snetselaar K.M., Mims C.W.(1994): Light and electron microscopy of *Ustilago maydis* hyphae in maize. Mycological Research, **98**: 347–355.

Spellig T., Bottin A., Kahmann R. (1996): Green fluorescent protein (GFP) as a new vital marker in the phytopathogenic fungus *Ustilago maydis*. Molecular and General Genetics (Berlin), **252**: 503–509.