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Infection of *Zea mays* by Haploid Strains of *Ustilago maydis*

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Abstract

Ustilago maydis is the fungus causative of common smut in maize and teozintle. Under natural conditions, the dikaryon formed by mating of sexually compatible strains, penetrates the host plant, and induces the typical disease symptoms: chlorosis, increased synthesis of anthocyanins and formation of galls full of teliospores. In the older literature it was indicated that, under normal conditions, the haploid forms of the fungus were also capable to invade the host tissues, although the symptoms observed were almost nil.

Since previous data from our lab showed that that haploid strains of the fungus were able to infect non-natural hosts under axenic conditions, in the present communication we proceeded to determine whether haploid strains caused more serious symptoms when infecting maize plants under axenic conditions. It was observed that indeed *U. maydis* haploid strains were able to invade maize plants under both axenic or soil conditions, and induces chlorosis and increased anthocyanin formation, although, as expected, there occurrence has no formation of galls or teliospores. Production of reactive oxygen species, cell death, necrotic areas, salicylic and jasmonic acid, were higher in axenically infected plants.

These results demonstrate that haploid strains of *U. maydis* are able to infect maize plants and suggest that plants infected in non-sterile soil probably develop general resistance mechanisms, and are accordingly less sensitive to infection than plants infected under axenic conditions.

Keywords *Ustilago maydis*; Haploid strains; Maize infection; Virulence; Axenic conditions

Introduction

Ustilago maydis is a pathogenic fungus responsible for common smut in maize (*Zea mays*) and teozintle (*Zea mays* ssp. *parviglumis* or *Z. mays* ssp. *mexicana*). Its pathogenic cycle begins with the mating of two sexually compatible haploid cells, resulting in the formation of a dikaryon that invades the host (revised by [1,2]).

In modern literature it has been described that only dikaryotic, diploid and even merodiploids of *U. maydis* are infectious [1-6]. However, in the earlier literature it was described that haploid strains of *Ustilago zeae* (as *U. maydis* was initially named) were able to penetrate the host maize tissues under natural conditions. In this case the growth of haploid hyphae was very limited, almost null symptoms of infection were observed, and, as expected, no teliospores or galls were formed ([7-9]; revised in [10]). Recently, our research group described that diploids and even haploids of *U. maydis* strains have the capacity to infect non-natural hosts under axenic conditions [11-14]. Taking into consideration that *U. maydis* haploid strains have the capacity to infect Arabidopsis thaliana [12-14] and that the symptoms induced by the fungus in maize plants are extremely more severe under axenic than under in soil conditions [15], we have proceeded to analyze whether maize plants infected with haploid *U. maydis* strains under axenic or soil conditions develop symptoms of the disease.

Materials and Methods

Biological material and growth conditions

The haploid FB2 (a2b2) strain [3] of *Ustilago maydis* and *Zea mays cv* Cacahuazintle were used in this study.

The *U. maydis* strain was grown in liquid complete medium (MC [16]) at 28°C under shaking conditions (200 rpm). The cells were recovered by centrifugation at 2500 g for 10 min, washed three times with sterile distilled water (SDW), suspended in SDW, and 100 µL of a 10^7 cells/ml suspension were inoculated after 3 days post-germination (dpg), with a syringe and needle in either soil-grown maize plants or plants incubated on sterile solid MS medium [17] kept within Magenta vessels (Sigma-Aldrich V8505) connected to another one with a Coupler connector (Sigma-Aldrich C0667).

Plants were then incubated at 25°C with a 12 h of photoperiod. Control plantlets received SDW only.

Determination of symptoms in infected plants

Damage and symptoms in infected plants were observed with a stereoscope (Leica MZ-8), and photographed with a Spot digital camera (Diagnostic instruments). Whole plants were photographed with a DMC-FX12 camera (Panasonic). The biomass of the plants was determined by measurement of their dry weight.

Determination of reactive oxygen species (ROS)

Sections of infected tissue were placed in an Eppendorf tube with 500 µL of 1,2,3-dihydrorhodamine [1,2,3-DHR; Sigma-Aldrich (2.5 mg/ml in ethanol)]. Samples were incubated during 5 min under darkness, observed with an epifluorescent Leica DMRE microscope and photographed as above. Bright yellow fluorescence in the infected plant tissues indicated production of ROS [18].

Observation of cell death

Plant tissue sections were placed on a slide with 15 µl of berberine sulfate (Sigma-Aldrich; 10 µg/ml), and incubated in darkness for 5 min. Microscopic observations were performed with a Leica DMRE microscope by epifluorescence and photographed as above. Areas of plant tissue with death cells displayed a bright yellow fluorescence [19].

Determination of phytohormones

Jasmonic acid

The method proposed by [20] was followed. After 10 days post inoculation (dpi), 500 mg of wet tissue from plants were frozen and macerated with liquid N_2 in a mortar, and 1 mL of ethyl acetate and 10 µg of dihydroxyjasmonic acid (DHJA, as internal standard), were added. The samples were shaken at 4°C during 24 h and centrifuged at 13,000 g for 15 min at 4°C. The supernatant was recovered and 500 µL of ethyl acetate were added to the pellet, shaken, and centrifuged again. The two supernatants were mixed, and the solvent was evaporated with N_2 gas. For derivatization of samples, 100 µL of chloroform, 100 µL of N´N´diisopropylethylamine and 10 µL of pentafluorobenzyl bromide (PFB-Br) were added; samples were incubated at 60°C for 30 min; and the solvent was evaporated as above. The samples were suspended in 100 µL of methanol HPLC grade, and along with standard solutions of dihydroxyjasmonic acid (DHJA) they were injected into a DB-1MS UI (60 m × 60.26 × 60.5 µm) column in a gas chromatograph (Agilent Technologies 7890A GC System; Palo Alto, AC) coupled to a MSD 5973 detector.

Salicylic acid

We followed the method from [21]. After 10 dpi, 250 mg of wet plant tissue were frozen and macerated with liquid N_2. After this, 0.75 mL of 90% methanol and 5 µL of a solution containing 0.1 µg/µL of ortho-anisic acid (ortho-methoxybenzoic acid, as internal standard) were added. The samples were incubated for 24 h at 4°C, centrifuged at 13,000 rpm for 15 min, the supernatant was recovered, and 0.75 mL of methanol were added to the pellet, and centrifuged again. The supernatants were mixed and methanol was evaporated with a stream of N_2. The pellet was suspended in 0.5 mL of 5% trichloroacetic acid (TCA), centrifuged at 6000 g for 10 min, the supernatant was recovered and two volumes of ethyl acetate and cyclopentane (1:1) were added. The samples were incubated at room temperature during 10 min, the organic phase was recovered and dried with N2 gas. For the derivatization process, 80 µL of MSTFA [N-Methyl-N-(trimethylsilyl) trifluoroacetamide (Sigma-Aldrich, 394866)] and 20 µL of pyridine were added. The samples were incubated at 80°C for 1 h, and along with the standard samples they were injected into a gas chromatograph (Agilent Technologies 7890A GC System, Palo Alto, CA) with a column DB-1 MS IU (60 m × 60.26 × 60.5 µm) coupled to a MSD 5973 detector.

Results and Discussion

A quantitative determination of the symptoms of plants infected by *U. maydis* and incubated either under in soil or axenic conditions are shown in Figure 1.

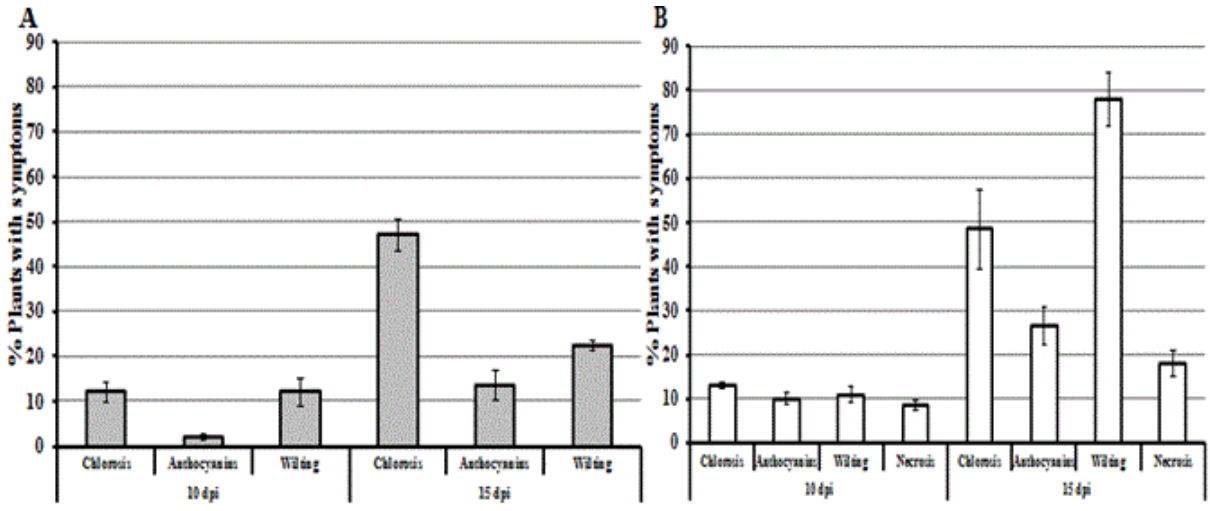

Figure 1: Quantification of symptoms in maize plants infected by haploid *U. maydis* strains. **A:** plants infected under soil conditions. **B:** plants infected under axenic conditions. Lines on each bar represent standard error values. Some symptoms quantified were observed in plants inoculated in axenic conditions only. Results of three experiments with twenty independent plants.

The results obtained showed that under soil conditions only a low percentage of infected plants (ca. 60%), showed symptoms of infection, mainly: Chlorosis, wilting and anthocyanin production (Figure 1A). On the other hand, all the plants infected under axenic conditions showed more severe symptoms of disease, including in addition

formation of areas of necrosis in their tissues, and production of ROS (Figure 1B).

It is noteworth that the haploid strain of *U. maydis*, whose diploid or dikaryotic forms are biotrophic and not necrotrophic [1,2,22,23], induced ROS production (Figures 2A-2D) and cell death (Figures 2D and 3A-3G) (symptoms of necrotic damage) in the tissues of the plants only under axenic conditions, demonstrating the virulence of the haploid form of the fungus, and suggesting that when grown in soil, but not when developed under axenic conditions, the plants develop induced resistance to infection by *U. maydis* [15,24,25] (Figures 2 and 3).

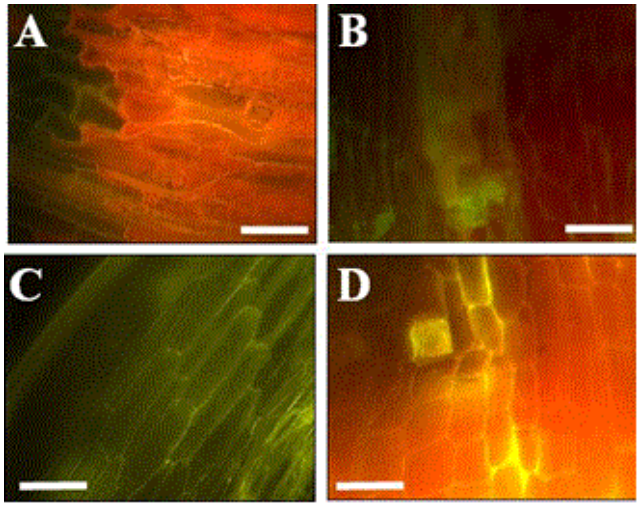

Figure 2: Representative photographs of plant tissues developing ROS production and Cell death in maize plants infected by haploid *U. maydis* strains. **B and D:** tissue of plant infected under axenic conditions and showing ROS production and Cell death areas respectively; **A and C:** plant inoculated in soil and without presence of ROS production and Cell death areas. The presence of areas with yellow fluorescence indicates areas of ROS production and cell death areas. Photographs were taken at 10 dpi. Scale bar 50 µm.

Another important difference among the plants infected in soil or under axenic conditions was the morphology of the invading fungus. In soil-grown plants, only elongated yeast-like cells were found in the chlorotic zones (Figures 3A and 3B).

On the other hand, mycelium was developed outside (Figures 3H and 3G), and inside the tissues of plants (Figure 3D) infected under axenic conditions.

In addition, after 10 days of infection, the plants inoculated under axenic conditions showed a 63.7% reduction in growth, whereas plants infected in soil showed only 29.6% growth reduction, compared to control plants [t-test student statistical analysis of three experiments with ten plants in each one showed that this was a significant difference ($p<0.01$)] .

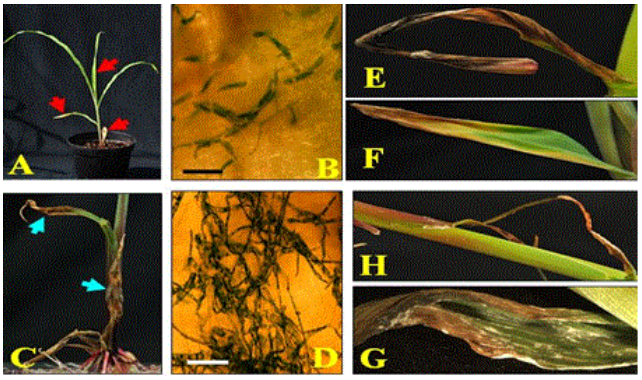

Figure 3: Photographs of maize plants infected by haploid strains of *U. maydis* at 15 dpi; **A and B:** plants infected under soil conditions, **C and D:** plants inoculated in axenic conditions; **E to G:** approach to tissue maize plants presenting symptoms of infection. Notice in A areas of chlorosis in the infected plant tissues (red arrows). Notice in B, development of elongated haploid cells within tissue of infected plants. Notice in C severe damage of infection such as areas of necrosis and development of mycelium (blue arrows); **E and F:** tissues of infected plants with necrosis and chlorosis respectively; **H and G:** development of fungal mycelium on tissues of infected plants tissues. Scale bar in B and D, 50 µm.

Interestingly, the plants infected under either axenic or soil conditions showed an increase in the production of salicylic acid (SA), compared to non-infected control plants (Figure 4A).

On the contrary, an increased production of jasmonic acid (JA) was observed only in plants infected under axenic conditions (Figure 4B).

These results demonstrate that the haploid strains of the fungus possess the ability to cause metabolic changes in the host plant, but not equally to dikaryotic strains; e.g. dikaryotic strains are known to inhibit (not increase) the synthesis of salicylic acid, or degrade it, as a characteristic of their biotrophic behavior [15,26-28]. This ability of *U. maydis* to reprogram its host has been previously described [14,15,23,26-29], and it is epigenetically regulated in the fungus [30].

In summary, in this work the ability of *U. maydis* haploid strains to infect maize plants was corroborated. In addition, it was demonstrated that virulence of the haploid strains is greater under axenic conditions, than when infection proceeds in soil-grown plants. These data suggest that i) possibly the lower susceptibility of maize plants to infection by the haploid strains of *U. maydis* under non-sterile soil conditions occurs because, contrary to sterile conditions, they develop the general mechanisms of induced resistance; and ii) maybe in nature maize plants may also be infected by *U. maydis* haploid strains, but that the symptoms of infection are very light and fail to be noticed, in contrast to the tumors developed by the infection with the sexually compatible heterokaryons.

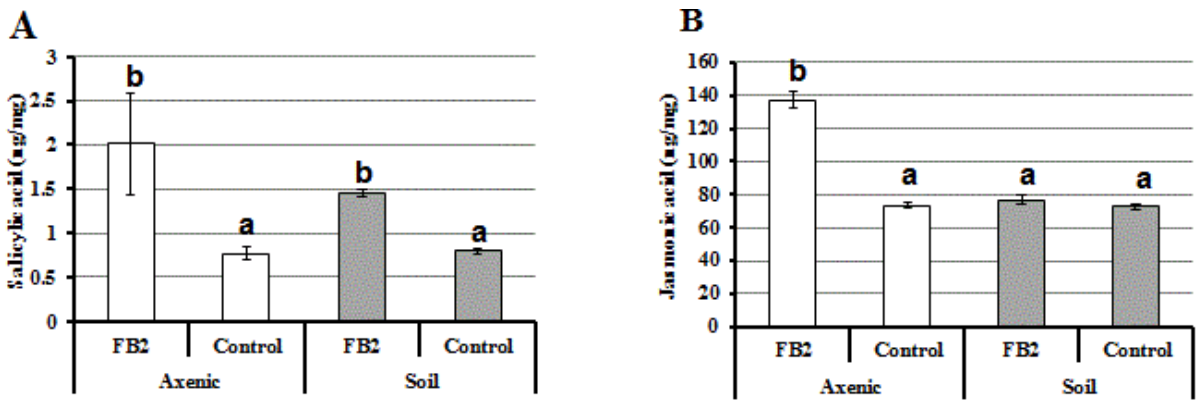

Figure 4: Production of phytohormones in maize plants infected by haploid *U. maydis* strains at 10 dpi; A: determination of salicylic acid; B: determination of jasmonic acid. Lines on each bar represent standard error values. Three different experiments were performed using three plants in each one. Two ways ANOVA, Tukey-HSD. Different letters denote significant differences.

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