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Infection process of *Phakopsora gossypii* in cotton leavesLeonardo Araujo¹, Antonia Mirian Nogueira Moura Guerra², Paulo Geraldo Berger², Fabrício Ávila Rodrigues^{1*}¹Federal University of Viçosa – Dept. of Plant Pathology, Campus Universitário, s/n – 36570-900 – Viçosa, MG – Brazil.²Federal University of Viçosa – Dept. of Plant Science.

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ABSTRACT: Tropical rust caused by the biotrophic fungus *Phakopsora gossypii* is an emerging disease in cotton that has caused significant yield losses of crop/cotton cultivated in Brazil. Considering the current importance of tropical rust and the need to obtain additional basic information about its causal agent to better control this disease, the present study aimed to determine the infection process of *P. gossypii* in cotton leaves using scanning electron microscopy (SEM). Thirty-day-old plants were inoculated with a suspension of *P. gossypii* uredospores, and leaf fragments were collected 42 h after inoculation (hai) as well as 20, 25 and 35 days after inoculation (dai) for SEM observations. By 42 hai, the uredospores of *P. gossypii* had germinated and produced a germ tube and an appressorium that may directly penetrate the leaf cuticle. At 20 dai, closed uredia containing uredospores were observed on the abaxial leaf surface. At 25 dai, the uredia started to open and became fully open by 35 dai and contained many uredospores. By 25 dai, fungal hyphae were growing abundantly in the mesophyll next to the uredia that formed in the leaf fragments with total or partial removal of the epidermis. The results of the present study provide novel information regarding the infection process of *P. gossypii* in cotton leaves, which might be useful for the development of new and more effective strategies for tropical rust control.

Keywords: *Gossypium hirsutum*, biotrophic fungus, tropical rust, uredium

Introduction

Cotton (*Gossypium hirsutum* L.) is the most extensively cultivated fiber crop in the world and contributes to approximately 85 % of raw materials to the textile industry (Pindikur et al., 2012). The major cotton production areas are located in the Brazilian savanna and the occurrence of diseases is among the factors that greatly decrease cotton quality and annual yield (Guerra et al., 2013; Kirkpatrick and Rothrock, 2001; Pindikur et al., 2012). Tropical rust caused by the biotrophic fungus *Phakopsora gossypii* (Arthur) Hiratsuka f. is an emerging disease in cotton that has caused significant yield losses of cotton cultivated in Brazil, India and Jamaica (Guerra et al., 2013; Pindikur et al., 2012). The main symptoms of tropical rust are ferruginous pustules that are found on the abaxial leaf epidermis. On reaching maturity, the uredia easily break the epidermis to release uredospores. The first symptoms of tropical rust appear on older leaves, and plants become prematurely defoliated as the disease develops (Kirkpatrick and Rothrock, 2001; Pindikur et al., 2012).

Tropical rust control has been achieved with the use of fungicides, which greatly increase production costs because resistant cotton cultivars are not yet available to growers (Kirkpatrick and Rothrock, 2001; Pindikur et al., 2012). Considering the current importance of tropical rust and the need to obtain additional basic information about its causal agent for the development of new and effective control strategies, this study aimed to determine the infection process of *P. gossypii* in cotton leaves using scanning electron microscopy (SEM).

Materials and Methods

Cotton seeds (cv. BRS Buriti) were surface sterilized in 10 % (v/v) NaOCl for 2 min, rinsed in sterilized water for 3 min and germinated in autoclaved sand. Ten days after germination, three seedlings were transferred to plastic pots containing 5 L of a nutrient solution composed of 6.0 mM KNO₃, 1.0 mM NH₄H₂PO₄, 2.0 mM MgSO₄·7H₂O, 4.0 mM Ca(NO₃)₂, 0.30 μM CuSO₄·5H₂O, 1.30 μM ZnSO₄·7H₂O, 46.0 μM H₃BO₃, 12.6 μM MnCl₂·4H₂O, 0.1 μM (NH₄)₆Mo₇O₂₄·4H₂O, 45 μM FeSO₄·7H₂O and 25 μM ethylenediaminetetraacetic acid di-sodium salt. The nutrient solution, with aeration, was changed every three days. The electrical conductivity and pH of the nutrient solution were checked daily. The pH was maintained at ≈ 5.5 using NaOH or HCl (1 N) when needed.

Plants were inoculated with a suspension of *P. gossypii* uredospores (2.5 × 10⁵ mL⁻¹) 30 days after transplanting (V4 growth stage) (30 mL per plant). The uredospores were applied as a fine mist to the abaxial leaf blades until runoff using an atomizer (model VL Airbrush, Chicago, USA). Immediately after inoculation, the plants were transferred to a growth chamber for 24 h (temperature of 25 ± 2 °C, relative humidity of 90 ± 5 % and photon flux density at a plant canopy height of approximately 825 μmol m⁻² s⁻¹). After this period, the plants were transferred to a greenhouse (temperature of 25 ± 2 °C and relative humidity of 50 ± 5 %) and maintained there until the end of the experiment. The maximum natural photon flux density at plant canopy height was approximately 975 μmol m⁻² s⁻¹.

Leaves with tropical rust symptoms and signs of *P. gossypii* were collected and processed according to Rodrigues et al. (2014). A total of 25 to 30 leaf fragments ($\approx 25 \text{ mm}^2$) were randomly collected 42 h after inoculation (hai) as well as 20, 25 and 35 days after inoculation (dai). The leaf fragments were carefully transferred to glass vials containing 10 mL of a fixative composed of 3 % (v/v) glutaraldehyde and 2 % paraformaldehyde (v/v) in 0.1 M sodium cacodylate buffer (pH 7.2). The leaf fragments were stored at 4 °C for 10 days, washed with sodium cacodylate buffer (0.1 M) and then postfixed with 1 % (w/v) osmium tetroxide prepared in the same buffer for 1 h at room temperature. The leaf fragments were dehydrated in ethanol and subjected to critical point drying in CO₂ (model CPD 030, Hatfield, PA., USA). The abaxial epidermis of some leaf fragments collected at 25 dai was carefully removed using adhesive tape to have sight of *P. gossypii* colonization. Four specimens from each leaf fragment were mounted on aluminum stubs, sputter coated with gold (model FDU 010, Hatfield, PA, USA), examined and photographed using a LEO SEM (model 1430VP, Jena, Thuringia, Germany) operating at 10 kV and with a working distance ranging from 10 to 30 mm.

Results and Discussion

This study provides, to the best of the authors' knowledge, the first microscopic evidence of the infection process of *P. gossypii* in leaves of cotton plants. In the case of *P. gossypii* at 42 hai the uredospores germinated, produced a germ tube and an appressorium that might directly allow the fungus to penetrate the leaf cuticle (Figure 1A). According to Vittal et al. (2014), the early events of uredospore germination, appressorium formation, and penetration of *P. pachyrhizi* on the soybean leaf occurred by 24 hai. The formation of appressoria is a pivotal event that allows a number of fungi to penetrate the leaf cuticle and establish a successful infection in their hosts (Chang et al., 2014; Dean, 1997; Loehrer et al., 2014; Ludwig et al., 2014; Mendgen and Deising, 1993). Certain biotrophic and hemibiotrophic fungi, such as those causing powdery mildews and anthracnose, respectively, can directly penetrate the cuticle through the action of cutinases, but in most cases, the strong osmotic pressure exerted by the melanized or not appressoria becomes detrimental (Dean, 1997; Ludwig et al., 2014; Mendgen and Deising, 1993). Other biotrophic fungi such as those causing rust can penetrate their host indirectly by entering the leaf mesophyll through stomata (Dean, 1997; Mendgen and Deising, 1993). Edwards and Bonde (2011) observed that an appressorium of *P. pachyrhizi* penetrated the leaf of soybean plants using primarily mechanical force with the aid of digestive enzymes. Chang et al. (2014) and Loehrer et al. (2014) showed that the turgor pressure formed on the appressorium of *P. pachyrhizi* was independent of melanin biosynthesis due to the metabolic degradation of products, such as glycerol, inside the appressorium. In the present

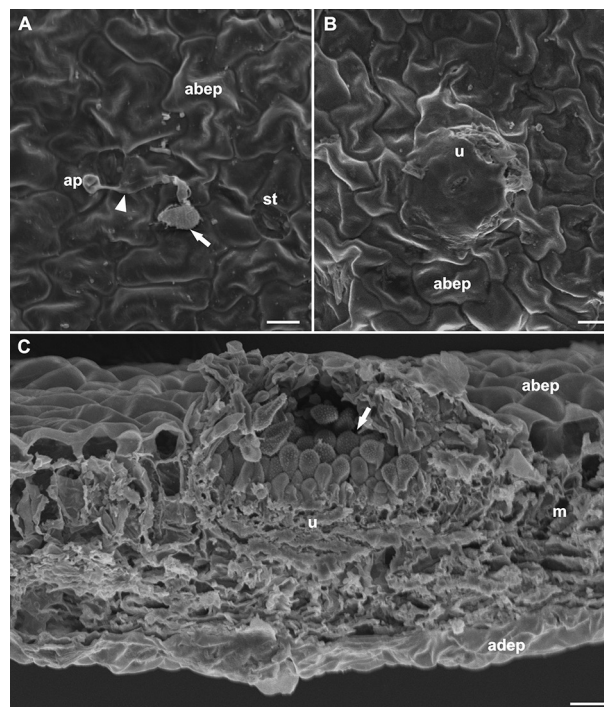


Figure 1 – Scanning electron micrographs of the abaxial leaf surface of cotton plants at 42 h (A) and 20 days (B and C) after inoculation with *Phakopsora gossypii*. (A), A uredospore (arrow) germinated, formed a germ tube (arrowhead) and produced an appressorium on the cotton leaf surface; (B), A closed uredium on the abaxial leaf surface; (C) Cross-section of a leaf fragment showing a closed uredium with uredospores (arrows). Adaxial epidermis (adep), abaxial epidermis (abep), appressorium (ap), mesophyll (m), stomata (st) and uredium (u). Scale bars = 20 μm .

study, the events of pre-penetration of *P. gossypii* (uredospore germination until appressorium formation) on cotton plants appeared very similar to those observed for the interaction soybean-*P. pachyrhizi*.

At 20 dai, closed uredia containing uredospores were observed on the abaxial leaf surface (Figure 1B and C). At 25 dai, the uredia started to open (Figure 2A, B and C) and became fully open at 35 dai (mature) when it was possible to see many uredospores (Figure 2D, E and F). According to Guerra et al. (2013), the latent period of tropical rust ranges from 20 to 22 dai on cotton plants from cv. BRS Buriti. Medice et al. (2007) reported that the uredia of *P. pachyrhizi* on soybean leaves became completely open at 36 dai. Generally, the small and closed uredia of *P. pachyrhizi* on soybean leaves were associated with reduced Asian soybean rust symptoms because they produced fewer uredospores (Cruz et al., 2012; Medice et al., 2007; Vittal et al., 2014). The results from the present study strongly indicate that control managements against tropical rust, such as fungicides should be implemented up to a maximum of 20 days after the infection took place because from this time onwards, the uredia start to open and release the first uredospores.

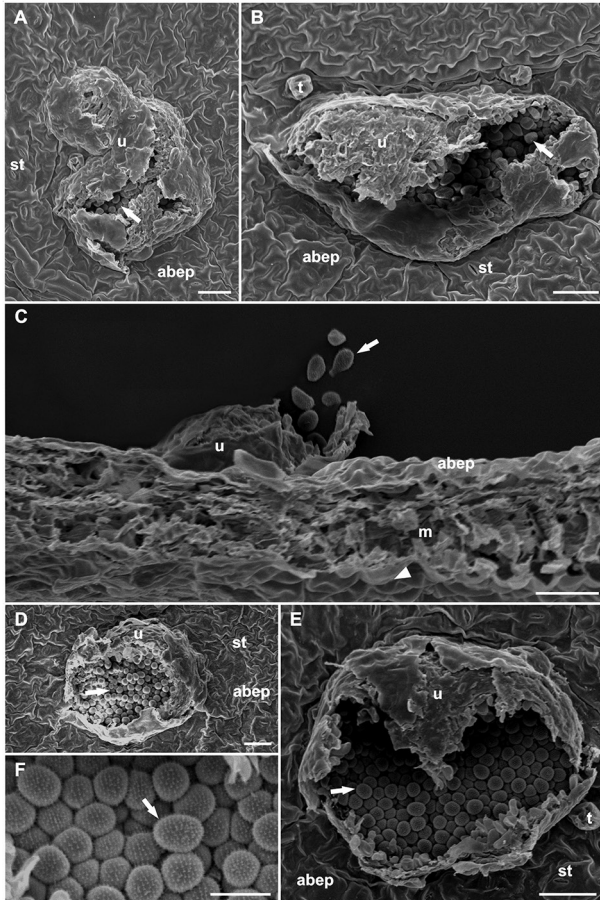


Figure 2 – Scanning electron micrographs of the abaxial leaf surface of cotton plants at 25 (A, B and C) and 35 days (D, E and F) after inoculation with *Phakopsora gossypii*. Uredia almost open (A and B) and fully opened (D and E) on the abaxial leaf surface; (C) Cross-section of a leaf fragment showing a uredium, not fully open, releasing uredospores (arrows); (F) Many uredospores (arrow) in an open uredium are shown at a higher magnification. Adaxial epidermis (arrowhead), abaxial epidermis (abep), mesophyll (m), stomata (st), trichome (t) and uredium (u). Scale bars = 50 μ m (A to E) and 20 μ m (F).

At 25 dai, the fungal hyphae grew abundantly in the mesophyll next to the uredia formed in the leaf fragments with total (Figure 3A and B) or partial (Figure 3C and D) removal of the epidermis. Based on SEM observations, Cruz et al. (2012) reported that the parenchyma cells in the soybean leaves next to the uredia of *P. pachyrhizi* were densely colonized by fungal hyphae. Vittal et al. (2014) reported that for a compatible soybean-*P. pachyrhizi* interaction, fungal hyphae need to massively colonize the mesophyll. Edwards and Bonde, (2011) used transmission electron microscopy and observed the hyphae and haustorial mother cells of *P. pachyrhizi* developed in soybean mesophyll between 14 and 21 dai. In the present study, the colonization standard of *P. gossypii* in cotton appeared similar to that of *P. pachyrhizi* in soybean.

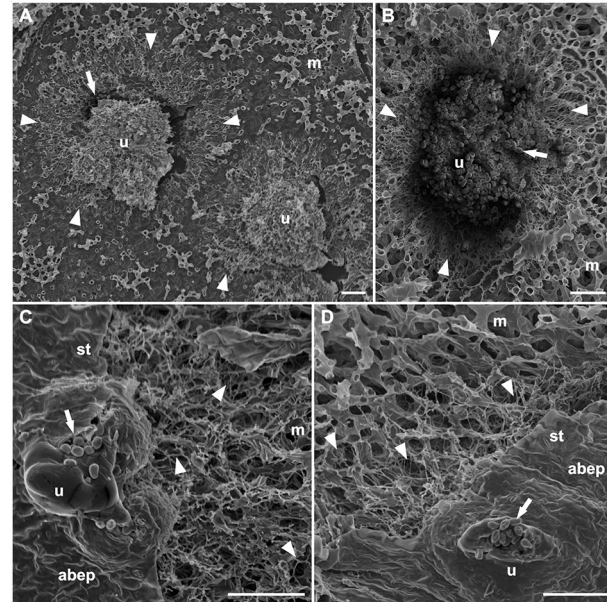


Figure 3 – Scanning electron micrographs of the abaxial leaf surface of cotton plants with total (A and B) and partial (C and D) removal of the epidermis at 25 days after inoculation with *Phakopsora gossypii*. Fungal hyphae grew abundantly (arrowheads) in the mesophyll next to the uredia. Arrows indicate uredospores in the uredia. Abaxial epidermis (abep), mesophyll (m), stomata (st) and uredium (u). Scale bars = 100 μ m.

In conclusion, the results of the present study provide novel information regarding the infection process of *P. gossypii* in cotton leaves, which might be useful in the development of new and more effective strategies for tropical rust control.

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