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Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil

Infection Process of *Cercospora coffeicola* on Coffee Leaf

André Gomes Coelho Souza, Fabrício Ávila Rodrigues, Luiz Antônio Maffia and Eduardo Seiti Gomide Mizubuti

Authors' address: Viçosa Federal University, Department of Plant Pathology, Viçosa, Minas Gerais State 36570-000, Brazil (correspondence to F.Á. Rodrigues. E-mail: fabricio@ufv.br)

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Abstract

Brown eye spot, caused by *Cercospora coffeicola*, is an important disease of coffee. Both adaxial and abaxial leaf surfaces were inoculated with a conidial suspension of *C. coffeicola*. Samples were collected from 4 to 168 h after inoculation and then again at 35 days. Germinated conidia showed positive tropism to stomata where attempted penetrations occurred. Appressoria were not observed. After penetration, *C. coffeicola* colonized the lacunous parenchyma both inter and intracellularly. Sporulation occurred through or around the stomata. Results from this study provide new insights into the infection process of *C. coffeicola* on coffee leaf.

Introduction

Coffee (*Coffea arabica* L.) is largely cultivated in Brazil, and it represents a strategic commodity to the Brazilian economy. Diseases are considered to play a significant role in reducing coffee production in Brazil. Epidemics of brown eye spot caused by *Cercospora coffeicola* Berk. & Cooke, are often reported in many coffee growing areas in Brazil. Brown eye spot is one of the oldest coffee diseases reported and was first discovered in 1881 in Jamaica (Cooke 1881). In Brazil, this disease was first reported in 1901 on coffee trees in the cities of Campinas and Araraquara in the state of São Paulo (Noack 1901). However, the first record of severe epidemics in Brazil was in 1971 in the state of Espírito Santo (Carvalho and Chalfoun 1998).

Brown eye spot may occur under both nursery and field conditions and is often considered to be more severe on plants suffering stress caused by water and/or nutrient deficiency (Fernández-Borrero et al. 1966; López-Duque and Fernández-Borrero 1969). Yield losses may reach up to 50% due to leaf drop and berry damage (Fernández-Borrero et al. 1982). In the past few years, concerns were raised due to an increase in brown eye spot severity in Brazil (Martins et al. 2008). The increase in brown eye spot intensity was mainly attributed to several factors such as the new coffee growing areas in the Cerrados region, the release of new coffee cultivars, the changes in cultural practices and alteration of the climate conditions (Juliatti et al. 2000).

The fungus infects leaves and berries. Numerous lesions appear on leaves that coalesce producing large necrotic areas. Diseased leaves fall early in the season. Round brown-purple spots are formed on berries. The lesions coalesce and become darkened, giving the berries a dried appearance. Furthermore, maturation is accelerated leading to premature berry drop and reduction in their quality (Castaño 1956). As the fungus can affect both coffee leaves and berries and that there have been several recent outbreaks of brown eye spot, the Brazilian coffee industry warrants more studies to derive effective control methods. So far, disease management is heavily based on the use of fungicides sprays, and there are no current resistant cultivars to brown eye spot. Studies on host resistance and pathogen variability do require a basic knowledge of the pathogen's life cycle, especially of its mode of infection.

The genus *Cercospora* shows wide variation in the infection process, and even the same species shows different pattern on different hosts. *Cercospora moricola* on mulberry and *Cercospora henningsii* on cassava form several germ tubes with or without appressoria formation (Gupta et al. 1995; Babu et al. 2007). On cassava leaves, the germ tubes of *C. henningsii* got branched and made multiple penetrations (Babu et al. 2009). Penetration may be only through epidermis as in *C. henningsii* on cassava leaf (Babu et al. 2009), through epidermis and stomata as in *Cercospora arachidicola* on peanuts (Smith et al. 1992) or only through stomata as in *C. moricola* on mulberry (Gupta et al. 1995), *Cercospora beticola* on sugarbeet (Rathaiah 1976, 1977) and *C. caricis* on purple nutsedge (Borges Neto et al. 1998).

The available information about the penetration and infection stages of *C. coffeicola* on coffee leaves is scant and somewhat contradictory. Penetration of the leaf tissue by *C. coffeicola* was reported to occur through the stomata (Echandi 1959; Siddiqi 1979) or directly (Castaño 1956). It has been reported that fungal colonization in leaves can be inter and intracellularly or strictly intracellularly (Castaño 1956; Siddiqi 1979). The present study aimed to determine the events of *C. coffeicola* penetration, colonization and sporulation on coffee leaves by light and scanning electron microscopy.

Materials and Methods

Fungus growth and conidia production

Cercospora coffeicola was directly isolated from diseased leaves and grown on Petri dishes containing potato-dextrose-agar (PDA) medium. The technique of drying the mycelial mass was used to induce conidial production (Souza et al. 2005). Three mycelial disks (0.5 mm in diameter) taken from the border of a fungus colony were transferred to 10 ml of V8 medium (200 ml V8® plus 800 ml of distilled water) in 25-ml Erlenmeyers that were kept continuously agitated (120 rpm) at 25°C. After 4 days, the content of each Erlenmeyer was poured into Petri dishes containing water–agar at 1.5%. The dishes were kept open at 40 cm distance from white fluorescent and 40 W grow lux lamps, distributed alternately to provide light intensity of 165.3 μmol/s/m^2. Incubation conditions were 12-h photoperiod at 25°C. After dehydration of the culture medium (approximately 4 days of incubation), 10 ml of distilled water were added to each Petri dish, the fungal colony was scratched with a glass rod, and the suspension was filtered through one layer of cheesecloth. Conidial concentration was adjusted to 2×10^4 conidia/ml with a haemocytometer.

Plant inoculation with *C. coffeicola*

Conidial suspension was sprayed on two leaves of each of 32 coffee plants (cv. 'Catuaí Vermelho', 6 months-old) with a DeVilbiss sprayer and grown under greenhouse conditions. Thirty-two leaves were inoculated on the upper surface and the other 32 on the lower surface. Out of the 64 leaves, 44 were carefully detached from the plants and kept with the inoculated surface facing up inside plastic boxes (11 cm length × 11 cm width × 3 cm height) with wet sponges. Two leaves, one inoculated on the upper surface and one inoculated on the lower surface, were set in a plastic box, which was closed and kept at 25°C with 90 ± 5% relative humidity (RH), and continuous light (40 W grow lux lamps distributed alternately to provide light intensity of 165.3 μmol/s/m^2). The remaining inoculated leaves were left attached to the plants that were kept in a dew chamber under the same conditions described earlier. After 12-h incubation, the boxes and the plants were transferred to greenhouse at 25 ± 3°C at 70 ± 5% RH and natural light (≈385.09 μmol/s/m^2).

Light microscopy

Thirty-five days after inoculation, four leaf samples (≈25 mm^2) were carefully collected from the lesions border with abundant fungal sporulation and transferred to glass vials containing 15 ml of a 50% (v/v) active chlorine 2.5% solution. After 24 h, leaf samples were transferred to glass slides containing a drop of lactophenol cotton blue stain. The slides were observed under a light microscope (Carl Zeiss Axio Imager A1, Göttingen, Germany) using the differential interference contrast technique.

Scanning electron microscopy

Four leaves from the two boxes were collected at 4, 6, 8, 12, 24, 36, 48, 72, 96 and 168 h after inoculation (hai). Leaf samples kept on the plants were collected only at 35 days after inoculation (dai) when symptoms and fungal sporulation on lesions became evident. A total of 20 leaf pieces (≈2.5 cm^2 in size) per each sampling time were randomly taken from the leaf blade and carefully transferred to glass vials containing 15 ml of fixative (2.5% v/v glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2). Samples were stored at 4°C for 10 days and then carefully washed with sodium cacodylate buffer (0.1 M), dehydrated in a graded ethanol series and critical point dried in CO_2 (Bal-tec, model CPD 030; Electron Mycroscopy Sciences, Hatfield, PA, USA). Four specimens from each sample were mounted on aluminium stubs, sputter coated with gold (Balzers Union, model FDU 010; Electron Mycroscopy Sciences, Hatfield, PA, USA), examined and photographed with a LEO scanning electron microscope (SEM) (model 1430 VP) operating at 10 kV and with working distance ranging from 10 to 30 mm. For each treatment, one stub with four specimens was examined by SEM. Leaf samples collected at 35 dai were carefully fractured with a scalpel and examined under the SEM. The identity of the pathogen on the lesions was confirmed by removing conidia and transferring them to Petri dishes containing PDA. After 7 days, the colonies growing on the media were confirmed to be *C. coffeicola* based on the morphological characteristics reported by Echandi (1959).

Results

Conidial germination and fungus penetration

A total of 20 conidia from each sampling time were observed. Conidial germination did not follow a pattern. After 4 hai, each conidium formed, on average, three germ tubes on the adaxial and abaxial leaf surfaces (Fig. 1a,b). Germ tubes were formed by different conidial cells and, occasionally, the germination tended to be bipolar (Fig. 1a,b). Conidial germination on the adaxial leaf surface started 4 h later than on abaxial surface. On leaf surfaces examined at 6, 8, 12 and 24 hai, conidia formed germ tubes, and fungal growth occurred predominantly towards the stomatal opening (Fig. 1c,d). Some germ tubes grew towards the stomata, but did not penetrate them. On some occasions,

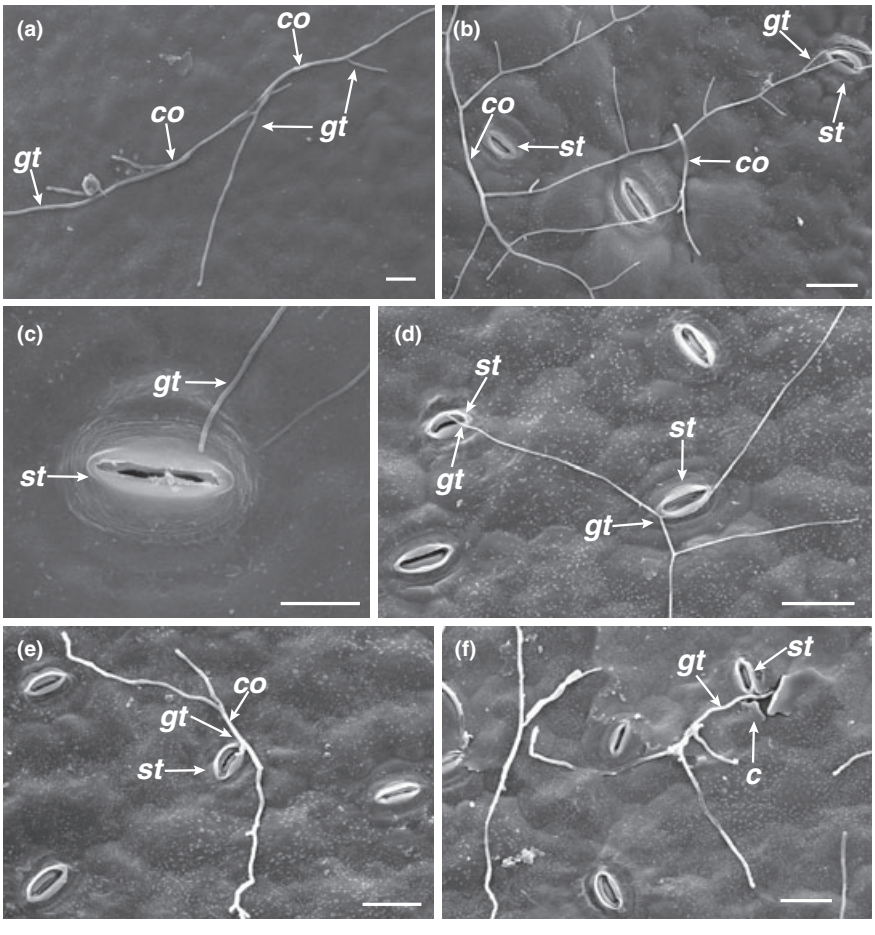

Fig. 1 Scanning electron micrographs of coffee leaves inoculated with *Cercospora coffeicola*. (a) growth of two germ tubes from a bipolar-germinated conidium on the adaxial leaf surface 4 h after inoculation (hai) (Bar = 10 μm); (b) conidia producing germ tubes that crossed stomata without penetration 4 hai (Bar = 20 μm); (c) the tip of a germ tube grows in the direction of the stomatal opening. (Bar = 10 μm); (d) a germ tube passed over a stomatal opening and another one surrounded the ridge of a stoma without penetration (Bar = 20 μm); (e) penetration through a stoma (Bar = 20 μm); (f) *C. coffeicola* penetration through cracks on the leaf epidermis (Bar = 20 μm). c, cracks; co, conidium; gt, germ tube; st, stomata

germ tubes passed over the guard cells or surrounded them (Fig. 1b–d). Penetration was observed 36 hai. The germ tubes penetrated mostly through the stomata (Fig. 1e,f) or sometimes through cracks found on the leaf epidermis (Fig. 1f). Neither appressorium formation nor direct penetration was observed for all infection sites examined.

Fungus colonization and sporulation

At 35 dai, profuse hyphal growth of *C. coffeicola* was observed underneath the substomatic chamber from where conidiophores were formed (Fig. 2a). Intracellular and intercellular hyphae were also present under the epidermis at the lacunous parenchyma (Fig. 2b). Conidiophores emerged singly or in groups (fascicles) through or around the stomata (Fig. 3a–c). Conidiogenic cells were formed on the tips of the conidiophores (Fig. 3a). Conidiophores and conidia were more dense on the abaxial leaf surface (Fig. 3a).

Brown eye spot symptoms on coffee leaf blades

Disease symptoms started to develop on the adaxial leaf surface by the appearance of several round brown spots surrounded by yellow halos. The brown spots enlarged and became necrotic. A mature lesion typically had a white centre with a middle brown ring and an outer yellow ring (Fig. 4a,b). Conidiophores bearing conidia were observed on the lesions (Fig. 4c). Conidia of *C. coffecicola* were hyaline, acicular to obclavate, nearly straight, truncate to subtruncate on their base, with acute tip and multiseptate.

Discussion

The germination process of *C. coffeicola* has not been fully described in the literature. The present study showed that the conidia germinated mostly from the tip and/or basal cells and less frequently from the middle cells. Each conidium produced one to several germ tubes, and appressoria were not developed on the leaf surface. The formation of several germ tubes has been observed in *C. moricola* on mulberry (Gupta et al. 1995) and in *C. henningsii* on cassava (Babu et al. 2007) with or without apprressorium formation. Germ tubes of *C. coffeicola* branched in many different directions. On cassava leaves, the germ tubes of *C. henningsii* got branched and made multiple penetrations contributing to the higher aggressiveness of the pathogen (Babu et al. 2009).

The proximity of conidia of *Cercospora* spp. to somata may or may not affect their germination (Rathaiah 1977; Gupta et al.1995; Babu et al. 2007). In the present study, majority of the germ tubes present on the abaxial leaf surface tended to be directed towards the stomata while on the adaxial leaf surfaces their growth occurred randomly. The germ tubes tend to enter the nearest stoma, but sometimes the penetration

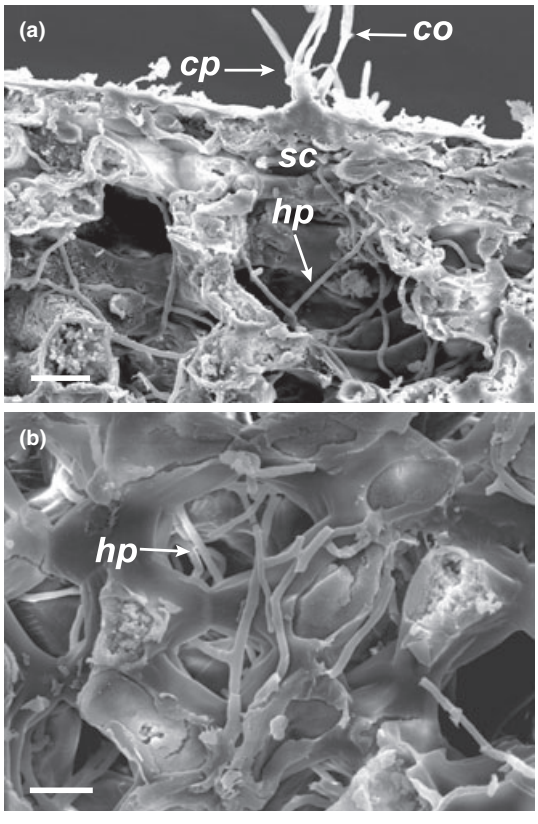

Fig. 2 Scanning electron micrographs of fractured coffee leaf samples at 35 days after inoculation with *Cercospora coffeicola*. (a) profuse hyphal growth underneath the substomatic chamber from where conidiophores emerged (Bar = 20 μm); (b) intracellular and intercellular hyphae in the parenchyma (Bar = 10 μm). co, conidium; sc, substomatic chamber, cp, conidiophore; hp, hyphae

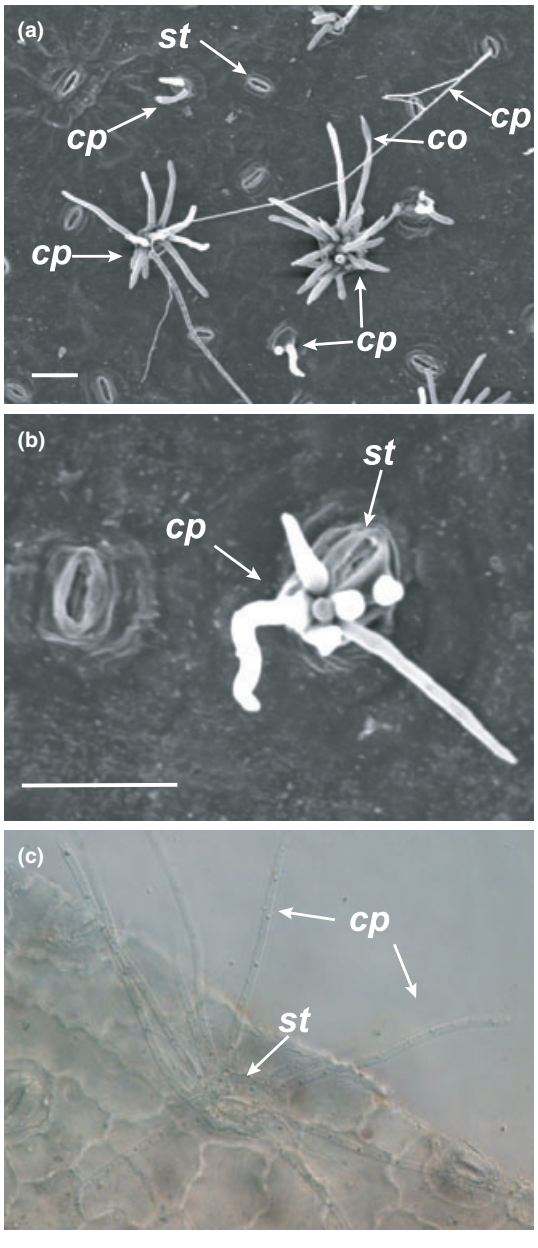

Fig. 3 Scanning electron micrographs (a and b) and differential interference contrast microscopy (c) of conidiophores emerging singly or in fascicles through or around stomata on the abaxial surface of coffee leaves at 35 days after inoculation with *Cercospora coffeicola* (a and b = bars are 20 μm; c = 400×). co, conidium; cp, conidiophore; st, stomata

occurs through a stoma situated farther. A similar pattern is reported in many other species of *Cercospora* (Dedecca 1957; Gupta et al. 1995; Rathaiah 1976). For other species of *Cercospora*, germ tube growth was extensive and random, and stomata were not necessarily penetrated by the hyphae that passed immediately beside or across from them (Echandi 1959; Rathaiah 1977; Siddiqi 1979; Babu et al. 2007). The microenvironment around the conidia and their growing germ tubes may be crucial to guarantee further growth on the leaf surface. Chemical signals such as sugars, phenolic compounds, volatile metabolites, and physical signals such as stomata and cuticle topography, may affect fungal growth on the host leaf surfaces (Dean 1997).

The penetration of *C. coffeicola* took place through the open stomata or cracks found in the epicuticular wax layer. No evidence of direct penetration was found in the present study which is in conformity with the reports of Echandi (1959) and Siddiqi (1979). However, Castaño (1956) found that penetration can occur both directly or through the stomata. As reported by Echandi (1959), no attempts of *C. coffeicola* penetration occurred on the adaxial coffee leaf surface where stomata are not present (Dedecca 1957). Penetration may be only through epidermis as in *C. henningsii* on cassava leaf (Babu et al. 2009), through epidermis and stomata as in *C. arachidicola* on peanuts (Smith et al. 1992) or only through stomata as in *C. moricola* on mulberry (Gupta et al. 1995) and *C. beticola* on sugarbeet (Rathaiah 1976).

After penetration, *C. coffeicola* colonized the substomatal chambers and invaded the adjoining tissues. Two patterns of colonization are reported in *C. coffeicola*: strictly intracellular (Echandi 1959) and both inter and intracellular (Castaño 1956). In the present study, *C. coffeicola* colonized the leaf tissue both inter and intracellularly.

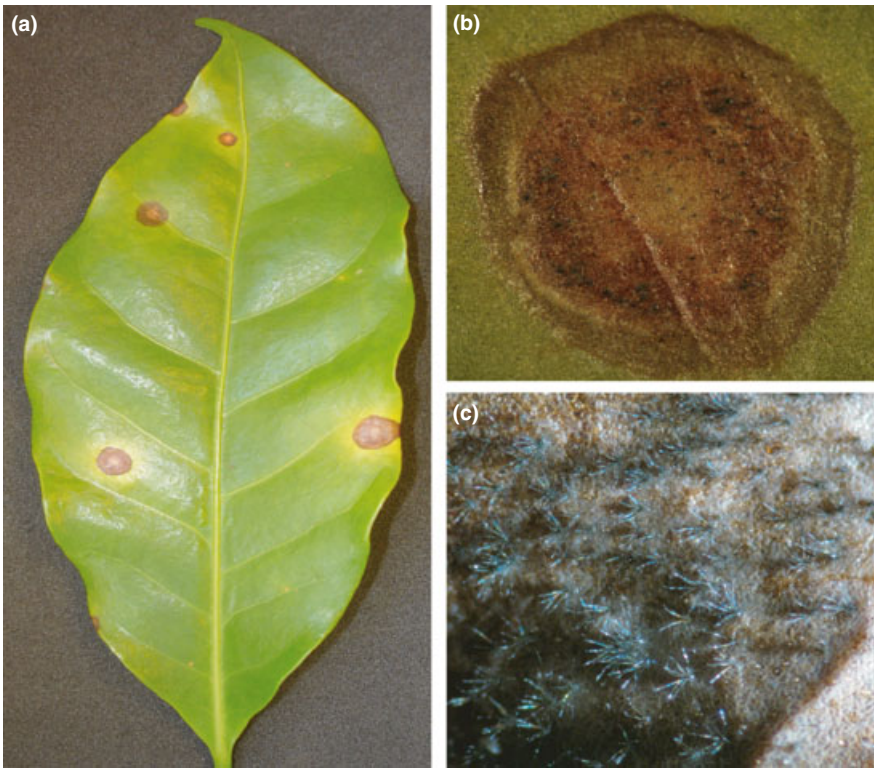

Fig. 4 Brown eye spot on coffee leaf caused by *Cercospora coffeicola*. (a) brown round spots surrounded by yellow halos, (b) close-up of a lesion showing intense necrotic tissue at 35 days after inoculation, (c) fungal sporulation on the necrotic leaf tissue

Considering the importance of brown eye spot to coffee production in Brazil and the lack of information in the literature regarding the infection process of *C. coffeicola*, the results from the present study offer novel information for a better understanding of the fungal life cycle that may help for evolving more effective disease control strategies. The study revealed that only the conidia deposited on the abaxial leaf surface were capable of penetration leading to disease development. It gives insight into the need of efficient fungicide application methods and agents of biological control targeting the lower surface of coffee leaves for an effective brown eye spot disease control.

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