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Colletotrichum gloeosporioides can overgrow *Colletotrichum kahawae* on green coffee berries first inoculated with *C. kahawae*

Zhenjia Chen[1,2,3,*], Jingsi Liang[1,2,3] & C.J. Rodrigues Jr.[1]
[1]*Centro de Investigação das Ferrugens do Cafeeiro (CIFC), Instituto de Investigação Científica Tropical, Quinta do Marquês, 2784-505 Oeiras, Portugal*
[2]*Plant and Environmental Protection Institute, Chinese Academy of Tropical Agricultural Sciences, Hainan, China*
[3]*Instituto de Tecnologia Química e Biológica (ITQB), Universidade Nova de Lisboa, Oeiras, Portugal*
**Author for correspondence (Fax: +351-214423023; E-mail: chenzhenjia@yahoo.com)*

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Abstract

Colletotrichum gloeosporioides is a weak pathogen of coffee that infects ripe berries at dark red stage causing necrotic lesions, but only penetrates up to the second superficial layers of the pericarp at the rose and pink stages. *C. kahawae*, the causal agent of coffee berry disease (CBD) and responsible for 70–80% of crop loss, infects berries at any stage of development. When green berries are first inoculated with *C. kahawae* and then at 2, 72 or 96 h later with *C. gloeosporioides*, the necrotic lesions were significantly larger than in the controls, and were much more evident when the berries were incubated at the optimum growth temperature of 28 °C for *C. gloeosporioides*. Isolations from the lesions induced by the first inoculations with *C. kahawae* followed by inoculation with *C. gloeosporioides* revealed that all or most of the time the recovered isolates of the latter. Thus, *C. gloeosporioides* can overwhelm *C. kahawae* under conditions of higher environmental temperature and humidity and may enhance the CBD infection process under field conditions.

Introduction

Colletotrichum kahawae Waller & Bridge and *C. gloeosporioides* Penz are two fungi that infect coffee. The former infects coffee berries at any stage of development and is responsible for coffee berry disease or CBD, inducing high losses (70–80%) when no control measures are applied. The latter is a weak or saprophytic fungus infecting only ripe coffee berries without causing economic losses. However, under special occasions, for instance, of high temperature such as heat shock (Chen *et al.* 2003) or of high temperature and heavy rain (Wang & Lai 1994), this fungus apparently becomes more deep-seated and causes some necrosis. Die-back associated with *C. gloeosporioides* infections has been reported from most coffee growing countries of the world (Firman & Waller 1977).

C. gloeosporioides conidia from coffee germinate and produce appressoria on green coffee leaves and berries like *C. kahawae* but, on the contrary, no penetration takes place (Chen *et al.* 2003). In the field, from CBD infected berries, very often some saprophytic species such as *C. gloeosporioides* and *C. acutatum* are also isolated (Waller *et al.* 1993). The CBD fungus itself is highly variable when maintained *in vitro* culture, in terms of colony colour, acervulus formation and other characteristics, and sometimes confusion arises as to distinguish it from *C. gloeosporioides* (Waller *et al.* 1993). *C. gloeosporioides* has been sometimes isolated

at Coffee Rust Research Center (CIFC) and elsewhere from coffee berry samples supposedly infected by *C. kahawae*. This fact raises the possibility of *C. gloeosporioides*, in some environmental conditions, to establish itself on *C. kahawae* lesions and colonize them by overgrowing. In this study we provide experimental evidence of the capacity of *C. gloeosporioides* to overgrow on *C. kahawae* lesions under laboratory conditions.

Materials and methods

Fungus isolates

Single spore isolates of *C. gloeosporioides* Ch27 (China) and *C. kahawae* Z1 (Zimbabwe) were grown on potato/dextrose agar (PDA) plates in the dark at 22 °C. Conidial suspensions of 2×10^6 ml^{-1} were prepared from 7–10-day old cultures.

Inoculation, re-isolation and cross section

Four-Five months old detached green berries of *Coffea arabica* var. Caturra were inoculated first with a 15 μl conidial suspension of *C. kahawae* Z1, then, at 2, 72 or 96 h later with identical conidial suspension of *C. gloeosporioides* Ch27. The berries were put in trays on the top of an alveolated rigid plastic net lined on the bottom with wet sponge. The trays were covered with a humid plastic bag (Chen *et al.* 2004). In one case, the berries were incubated at 22 °C, the optimum growth temperature for *C. kahawae*, while in the other they were incubated at 22 °C for 72 h and then at 28 °C, the optimum growth temperature for *C. gloeosporioides*. The diameters of the necrotic lesions were measured for comparison and the inoculated berries were randomly selected for fungus re-isolation. A piece of tissue from the edge of the lesions was cut off and inoculated on PDA plates for conidia production. Conidial suspensions were then inoculated on green berries for the symptom evaluation. Since only *C. kahawae* can infect green berries to cause necrotic lesions, the final identification of the fungal species was based on percentage of infections with necrotic lesions. To evaluate fungal post-penetration stages, cross sections of berry fragments were made by following the method described by Chen *et al.* (2004). Individual experiments were repeated twice.

Statistical analysis

The diameters of the necrotic lesions induced by inoculation with *C. kahawae* alone or together with *C. gloeosporioides* were submitted to Tukey's multiple range analysis using $p \leq 0.05$ significance for comparison.

Results and discussion

Lesion diameters induced on berries inoculated first with C. kahawae *then with* C. gloeosporioides, *incubated at 22* °C

Green coffee berries inoculated with both fungi and incubated at 22 °C showed significant larger lesions than *C. kahawae* Z1 alone (Table 1). The first difference started at the 8th day on the berries inoculated with *C. gloeosporioides* 96 h after *C. kahawae*. This result indicates that *C. gloeosporioides*, at this temperature, participated slowly in the infection process, and that

Table 1. Diameter (mm) of necrotic lesions on infected green berries, inoculated first with *Colletochum kahawae* Z1 and then with *C. gloeosporioides* Ch27 at 2, 72 or 96 h later, incubated at 22 °C.

Days after inoculation	Control of Z1	Z1 at 2 h + Ch27	Z1 at 72 h + Ch27	Z1 at 96 h + Ch27
4	2.9 ± 0.8a	2.9 ± 0.8a	2.9 ± 0.8a	2.9 ± 0.9a
6	4.9 ± 1.0a	4.9 ± 0.9a	4.7 ± 1.0a	4.5 ± 0.8a
8	7.1 ± 2.0a	7.5 ± 1.1a	7.3 ± 1.4a	8.9 ± 1.5b
9	11.0 ± 1.6a	12.5 ± 2.1b	12.1 ± 3.0ab	12.9 ± 2.6b

Green berries inoculated with Ch27 showed no necrotic lesions. Values are the average of three replicates with a total of 60 infected sites measured ± standard deviation. In each line values followed by the same letter are not significantly different. Tukey's multiple range analysis with 95% confidence.

the best time for this participation was, in fact, at 96 h after the inoculation with *C. kahawae*, i.e. when the berries presented already the first necrotic symptoms.

Lesion diameters induced on berries inoculated first with C. kahawae *then with* C. gloeosporioides, *incubated at 22 °C for 72 h, then at 28 °C*

Table 2 shows that significant differences from the control (*C. kahawae* alone) started at the 6th day after the first inoculation in all cases. At the 8th and 9th day, significant larger lesions were found between berries inoculated with Ch27 at 72 or 96 h and the berries inoculated with this fungus at 2 h. These results indicate that temperature played an important role on berries colonization by the saprophytic fungus *C. gloeosporioides*. Together with the data presented in Table 1, they suggest that commensal behaviour of *C. gloeosporioides* is somehow dependent upon the initial infection process of *C. kahawae* and environmental conditions.

Fungus isolation from berries inoculated first with C. kahawae *followed by* C. gloeosporioides

Berries inoculated first with *C. kahawae* Z1, then with *C. gloeosporioides* Ch27 showed 87.5% of Ch27 and 12.5% of Z1 when they were incubated at 22 °C (Table 3). One hundred percent of Ch27 and 0% of Z1 were obtained from berries inoculated in identical conditions, but incubated at 22 °C for only 72 h then at 28 °C. These results reveal that Ch27 can take over the growth on berries first colonized by Z1, thus explaining why in many cases only *C. gloeosporioides* is isolated from the berries supposedly infected by CBD.

Symptom expression

The initial penetration of the green coffee berry cuticle by *C. kahawae* is at about 19 h, with first necrotic symptoms at 4–5 days, and complete necrosis within 9–10 days after inoculation (Chen *et al.* 2003). Inoculation of *C. gloeosporioides* on green berries does not lead to any penetration (Chen *et al.* 2003). Inoculation of *C. gloeosporioides* on ripe berries produced a percentage of infections with necrotic lesions somehow variable, probably due to difficulties in obtaining berries with the same degree of maturation. In fact, when the berries were not fully ripe, i.e. not darkish red and soft, the developed lesions were only restricted to the inoculated area and did not produce reproductive structures (Figure 1a). Histological sections on these fruits

Table 2. Diameter (mm) of necrotic lesions on green berries, inoculated first with *Colletotrichum kahawae* Z1 and then with *C. gloeosporioides* Ch27 at 2, 72 or 96 h later, incubated at 22 °C for 72 h, then at 28 °C.

Days after inoculation	Control of Z1	Z1 at 2 h + Ch27	Z1 at 72 h + Ch27	Z1 at 96 h + Ch27
4	3.1 ± 0.8a	3.5 ± 0.8a	3.5 ± 0.9a	3.2 ± 0.8a
6	3.6 ± 1.0a	6.2 ± 1.5b	6.6 ± 1.5b	6.3 ± 1.4b
8	4.6 ± 1.1a	8.3 ± 1.9b	11.0 ± 2.3c	11.9 ± 2.2c
9	7.5 ± 1.6a	11.2 ± 2.0b	15.9 ± 3.0c	17.3 ± 2.1c

Green berries inoculated with Ch27 showed no necrotic lesions. Values are the average of three replicates with a total of 60 infected berries measured ± standard deviation. In each line values followed by the same letter are not significantly different. Tukey's multiple range analysis with 99% confidence.

Table 3. Number of times (%) that *Colletotrichum kahawae* Z1 and *C. gloeosporioides* Ch27 were isolated from green berries inoculated first with Z1 and then with Ch27, 8 days after the first inoculation and in the conditions expressed.

Isolates	Control of Ch27	Control of Z1	Z1 + Ch27 at 22 °C	Z1 + Ch27 at 22 °C for 72 h, then at 28 °C
Z1	0	100	12.5	0
Ch27	0	0	87.5	100

In each column, 48 infected berries were used for fungus re-isolation. Conidial suspensions obtained from each berry were inoculated on 12 green berries for the fungus identification. No penetration occurs on green berries by Ch27.

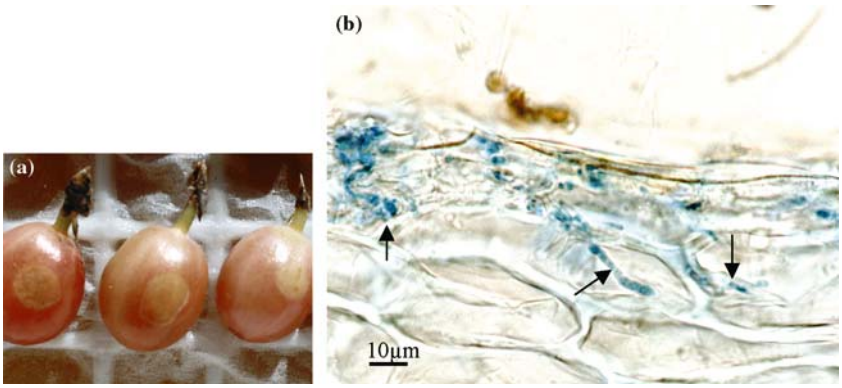

Fig. 1. Ripe berries at pink colour stage inoculated with *Colletotrichum gloeosporioides* Ch27. Symptoms, 30 days later, did not develop further (a). Hyphal length (b, arrowheads) restricted only to the second layer of the pericarp cells.

Fig. 2. Different symptoms induced by *Colletotrichum gloeosporioides* (a and b), *C. kahawae* (c) and by inoculation first with *C. kahawae* then with *C. gloeosporioides* at 2 h (d) or 96 h later (e). Note that *C. gloeosporioides* produced white mycelia on dark red berries (a) and that on green berries no lesions were produced (b). Green berries inoculated with *C. kahawae* showed sunken lesions (c), and when inoculated with both fungi showed visible white mycelia (d and e).

showed a limited hyphal length, reaching only the 2nd layer of the pericarp (Figure 1b). When the berries were fully ripe, total destruction occurred at 7–9 days after inoculation. Green berries inoculated first with *C. kahawae* and then with *C. gloeosporioides* showed necrotic lesions much faster than with *C. kahawae* alone, thus indicating that *C. gloeosporioides* contributed to the infection process (Figure 2). The visible white mycelia produced on the berries surface of these combined inoculation is due to the saprophytic behaviour of *C. gloeosporioides* (Figure 2), since the berries inoculated with *C. kahawae* alone always produced sunken lesions without visible mycelium. The only recovered isolate Ch27 obtained from the berries showing visible white mycelium clearly supports this observation. Further work should be carried out to see if the absence of any enzymes and/or the presence of fungal inhibitors would be responsible for the non-pathogenicity of *C. gloeosporioides* on the green berries.

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