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The use of GFP-transformed isolates to study infection of banana with *Fusarium oxysporum* f. sp. *cubense* race 4

Chunyu Li · Shi Chen · Cunwu Zuo · Qingming Sun · Qian Ye · Ganjun Yi · Bingzhi Huang

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Abstract *Fusarium oxysporum* f. sp. *cubense* (*Foc*) is the causal pathogen of Fusarium wilt of banana. To understand infection of banana roots by *Foc* race 4, we developed a green fluorescent protein (GFP)-tagged transformant and studied pathogenesis using fluorescence microscopy and confocal laser scanning microscopy. The transformation was efficient, and GFP expression was stable for at least six subcultures with fluorescence clearly visible in both hyphae and spores. The transformed *Foc* isolate also retained its pathogenicity and growth pattern, which was similar to that of the wild type. The study showed that: (i) *Foc* race 4 was capable of invading the epidermal cells of banana roots directly; (ii) potential invasion sites include epidermal cells of root caps and elongation zone, and natural wounds in the lateral root base; (iii) in banana roots, fungal hyphae were able to penetrate cell walls directly to grow inside and outside cells; and (iv) fungal spores were produced in the root system and rhizome. To better understand the interaction between *Foc* race 4 and bananas, nine banana cultivars were inoculated with the GFP-transformed pathogen. Root exudates from these cultivars were collected and their effect on conidia of the GFP-tagged *Foc* race 4 was determined. Our results showed that roots of the *Foc* race 4-susceptible banana plants were well colonized with the pathogen, but not those of the *Foc* race 4-resistant cultivars. Root exudates from highly resistant cultivars inhibited the germination and growth of the Fusarium wilt pathogen; those of moderately resistant cultivars reduced spore germination and hyphal growth, whereas the susceptible cultivars did not affect fungal germination and growth. The results of this work demonstrated that GFP-tagged *Foc* race 4 isolates are an effective tool to study plant–fungus interactions that could potentially be used for evaluating resistance in banana to *Foc* race 4 by means of root colonization studies. Banana root exudates could potentially also be used to identify cultivars in the Chinese Banana Germplasm Collection with resistance to the Fusarium wilt pathogen.

Keywords Banana · Green fluorescent protein (GFP) · *Fusarium oxysporum* f. sp. *cubense* · Germplasm collection

Abbreviations

Foc	*Fusarium oxysporum* f. sp. *cubense*
GFP	Green fluorescent protein

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Introduction

Fusarium oxysporum f. sp. *cubense* (*Foc*) is a soil-borne fungus causing Fusarium wilt, a disease that threatens banana production worldwide (Ploetz and Pegg 2000). The pathogen infects banana roots, colonizes and occludes the xylem vessels, and causes a reddish-brown discolouration of the rhizome and pseudostem. Leaves of infected banana plants eventually become bright yellow before they wilt and collapse around the pseudostem (Stover 1962). Diseased banana plants often die before they produce bunches, thereby reducing yields in fields affected by Fusarium wilt. Since *Foc* can survive in soil for decades, susceptible banana plants can only be grown in soil free of the pathogen (Ploetz and Pegg 2000).

Three races of *Foc* cause disease to banana. *Foc* race 1 attacks dessert bananas such as 'Gros Michel' (*Musa* spp., AAA-group), *Foc* race 2 affects 'Bluggoe' (*Musa* spp., ABB-group) and other cooking bananas, and *Foc* race 4 causes disease to Cavendish banana cultivars (*Musa* spp., AAA-group) as well as banana cultivars susceptible to *Foc* races 1 and 2 (Ploetz and Pegg 2000). In the past century, *Foc* race 1 destroyed thousands of hectares of Gros Michel bananas planted in Central America (Stover 1962). Eventually, the international banana export trade in Central America was rescued when the *Foc* race 1-susceptible 'Gros Michel' bananas were replaced with resistant Cavendish cultivars in the early 1960s. Considerable losses of Cavendish bananas to *Foc* race 4, first in the subtropics and then in the tropics (Ploetz and Pegg 2000), have raised fears that banana production internationally might be threatened by Fusarium wilt again (Pearce 2003). *Foc* race 4 has destroyed many plantations of Cavendish bananas in tropical countries such as Indonesia and Malaysia (Hwang and Ko 2004). In China, where bananas are planted in the tropics and subtropics, the disease has been reported from all banana production provinces, with outbreaks reaching epidemic proportions. To avoid further dissemination of the pathogen, quarantine measures will be introduced for the rapid detection of *Foc* race 4 using the PCR diagnostic method developed by Dita et al. (2010).

Few options exist for managing Fusarium wilt of banana in fields where the fungus is well established. Understanding the interaction between *Foc* race 4 and bananas could lead to the discovery of efficient ways to control Fusarium wilt. These include the development of resistant Cavendish cultivars and biological control of *Foc* using beneficial microorganisms that colonize the banana rhizosphere (Buddenhagen 1990; Getha and Vikineswary 2002; Nel et al. 2006; Popavath et al. 2008; Stover and Buddenhagen 1984). Studies on the infection of banana roots with *Foc* until now have focused on the *Foc* race 1-Gros Michel interaction (Beckman et al. 1961; Wardlaw 1930). In the current investigation, a green fluorescent protein (GFP)-labelled isolate of *Foc* race 4 was developed and utilized to study infection and colonization of banana plants. The use of fluorescent proteins for labelling pathogenic fungi has previously provided researchers with a powerful method to investigate infection of plants with pathogens such as *F. oxysporum* (Lagopodi et al. 2002; Maor et al. 1998). In addition, root infection of different banana cultivars with a *Foc* race 4-transformant was compared, and the effect of banana root exudates on fungal growth investigated.

Materials and methods

Plant material

Nine banana cultivars from the Chinese Banana Germplasm Collection were selected for inclusion in this study (Table 1). These include two Cavendish banana cultivars highly susceptible to *Foc* race 4 (Brazilian and Dafeng #2), and two Cavendish selections that have been improved for resistance to *Foc* race 4 by means of somaclonal variation (Wilt-resistant #1 and Wilt-resistant #5). Other banana cultivars included in the study were all non-Cavendish types representative of AA- and AAB-type dessert bananas and ABB plantains. All the plants were produced by means of tissue-culture, and plantlets with four or five leaves were selected for infection studies.

Fusarium strains and GFP transformation

An isolate of *Foc*, originally collected from the infected pseudostem of a Cavendish cv 'Brazilian' banana plant with Fusarium wilt symptoms in Panyu, Guandong Province in China, and subsequently identified as belonging to *Foc* race 4 at Stellenbosch University, was selected for GFP transformation. The

Table 1 Plant materials used in this study, their resistance to *Foc* Race 4, and the effect of root exudates on spore germination

Variety and Genotype	Cultivar	Resistance to Foc Race 4[a]	Spore Germination (%)[bc]
Musa AAB Pisang Awak	Guangfen #1	HS	20.0±0.87 A
Musa AAA Cavendish	Brazilian	HS	20.0±1.21 A
Musa AAA Cavendish	Dafeng #2	HS	16.7±0.43 B
Musa ABB	Dongguan Dajiao	R	1.0±0.58 C
Musa ABB	Zhongshan Dajiao	R	1.0±0.29 C
Musa AAA Cavendish	Wilt-resistant #1	MR	3.0±1.27 C
Musa AAA Cavendish	Wilt-resistant #5	HR	0.0D
Musa AA	Haigong	HR	0.0D
Musa AAB Pisang Awak	Guangfen #2	MR	1.0±0.43 C
Control (Water)			0.0D

[a] HS represents highly susceptibility (>60% disease incidence), MR represents moderately resistant (20–40% disease incidence), R represents resistant (10–20% disease incidence), and HR represents highly resistant (10% disease incidence).

[b] Spore germination measured 1 day after mixing 100 μl of root exudate with 100 μl of a fungal spore suspension prepared at a concentration of 10^5 conidia/ml. Figures represent the average value of the spore germination and each experiment was replicated three times.

[c] The same letters in the same column were not significantly different at $P<0.01$ by Duncan's multiple range test

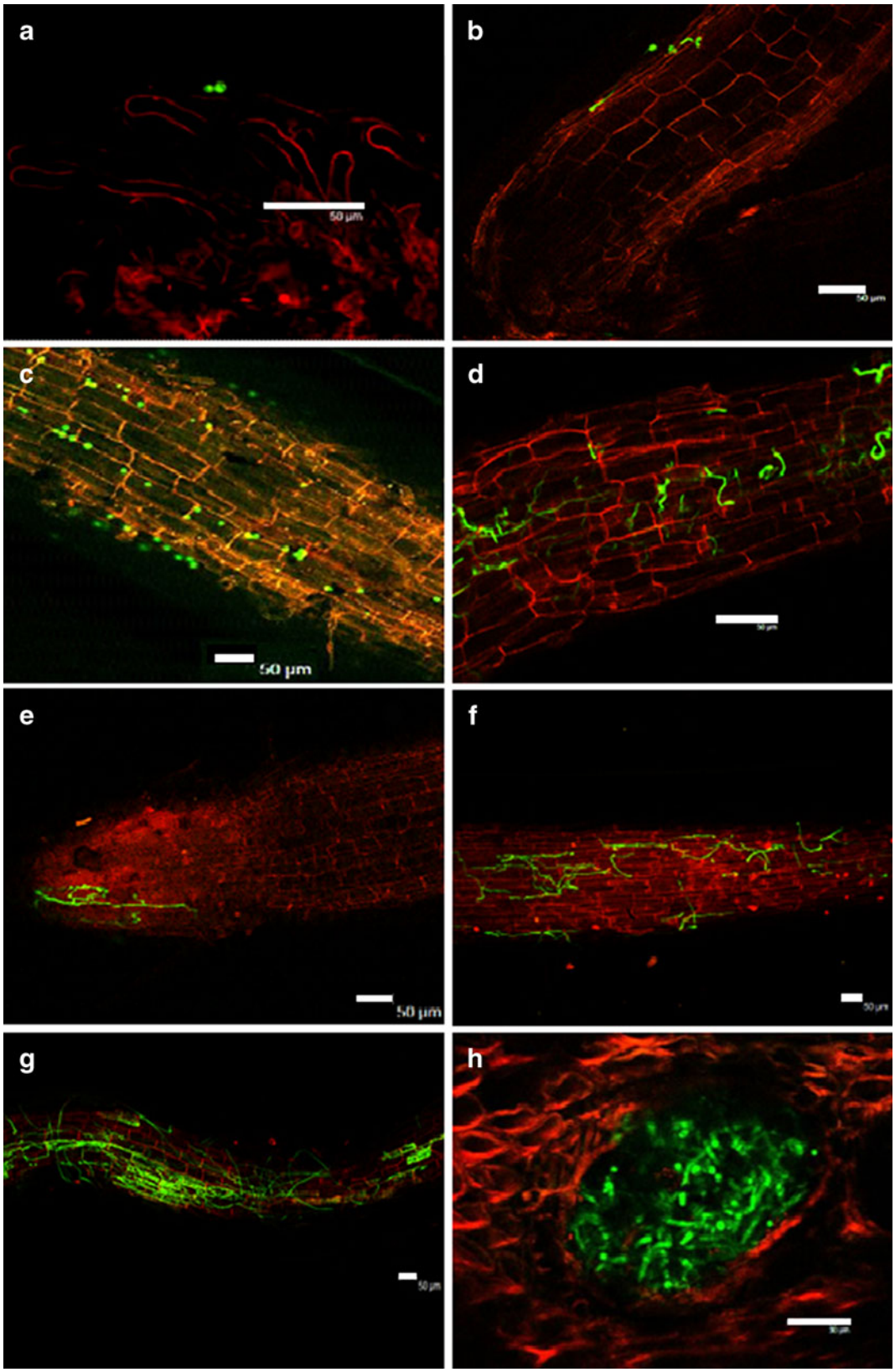
Foc isolate, CGMCCC 3.12196, was modified with the *gfp* gene using the sGFP expression vector pCT74, kindly provided by Dr. Ciuffetti (Oregon State University, USA) (Lorang et al. 2001). Fungal protoplasts of CGMCCC 3.12196 were transformed using a polyethylene glycol/$CaCl_2$-mediated transformation method as described by Liat et al. (2003). Growth characteristics and pathogenicity of GFP-expressing isolates of *Foc* were verified using the inoculation protocol described by Minhui et al. (2007). Both wild type and GFP-modified *Foc* isolates are preserved in the China General Microbiology Culture Collection Center (CGMCCC) in the Institute of Microbiology, Chinese Academy of Sciences, Beijing, China.

Plant inoculation

For artificial inoculations of banana plants, the GFP-tagged pathogen was first grown in potato dextrose broth (PDB) on a shaker (130 to 160 rpm) at 28°C for 2 days. The culture was then filtered through four layers of sterile Miracloth, and the filtrate containing microconidia was centrifuged at 4,000 × *g* for 15 min. The supernatant was discarded, and the pellet was washed four times in sterile distilled water to remove PDB medium. The fungal spores were then resuspended in sterile distilled water to a final concentration of 10^5 conidia/ml. This suspension was added to a planting medium that consisted of three parts vermiculite, one part peat, and 0.5 parts coconut coir, to obtain a soil concentration of 500 conidia/g soil. For banana cutivars resistant to *Foc*, the pathogen concentration in the soil was later increased to 1,500 conidia/g soil. Immediately after soil inoculation, 200 tissue-cultured banana plantlets of each cultivar were transplanted into the *Foc*-containing soil.

Microscopic examination

The inoculated banana plants were examined for infection and colonization by *Foc* race 4 every day for the first 7 days, and every other day thereafter, for 2 months. Five new plants of each cultivar were selected for microscopic analysis each day, and five root samples per plant were harvested to investigate six different root areas that included the root hair, root cap, meristematic zone, elongation zone, middle and basal part of the roots. For microscopic examination, the banana root tissue was prepared by first washing the roots in sterile distilled water to remove soil. The roots were then placed on a microscope slide, submerged in a water droplet, and covered with a glass cover slip. Microscopic analyses were carried out using a Leica TCS SP2 AOBS fluorescence microscope (Leica, Mannheim,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◀ **Fig. 1** Colonization of susceptible banana plantlets with a GFP-tagged isolate of *Fusarium oxysporum* f. sp. *cubense* race 4**. a** Early attachment of a few chlamydospores to root hairs of cv 'Brazilian', 3 days after inoculation. **b** Single chlamydospores attached to epidermal cells of lateral roots of cv 'Brazilian', 6 days after inoculation. **c** Considerable attachment of chlamydospores to roots of cv 'Brazilian', 7 days after inoculation. **d** Germination tubes spreading to cells adjacent to the ones where chlamydospores were attached on roots of cv 'Guangfen #1', 7 days after inoculation. **e** and **f** A network of fungal hyphae forming on root caps and elongation zone of banana roots, 11 and 15 days after inoculation of cv 'Brazilian', respectively. **g** Advanced stages of colonization of roots of cv 'Brazilian', 11 days after inoculation. **h** Fungal hyphae and spores inside vascular spaces of the rhizome, 29 days after inoculation. **a**–**g** scale bar=50 μm, **h** scale bar=30 μm

Germany) equipped with filter blocks with spectral properties matching those of the GFP (488 nm excitation, 520–540 nm long-pass emission) and root autofluorescence PMT1 (605 DF32) and PMT2.

Effect of root exudates on *Foc*

In order to elucidate the early interaction between *Foc* race 4 and banana roots, the role of root exudates on spore germination and hyphal growth was investigated. The banana plantlets were first grown in plastic cups filled with 200 ml of sterile distilled water at room temperature (Stevenson et al. 1995). Each cup contained a single plantlet, and five plantlets per cultivar were included in the experiment. After 15 days, roots were weighted and the exudate solution was collected. The exudate solution was extracted twice in a separating funnel with an equal volume of ethyl acetate (Stevenson et al. 1995). The apolar fraction in ethyl acetate was collected and evaporated to dryness with a rotary evaporator (RE-3000B, Analytical Instrument Co., Ltd. Shanghai Jinpeng), and then dissolved in methanol to give a solution equivalent to 1 g root/ml. An equal volume of water was added and this mixture was then evaporated to dryness. A second volume of water was added and the fraction was evaporated again until the concentration of sample in the solution became equivalent to 1 g root/ml. The final solution was filtered through sterilized Nalgene filters (0.45 μm) and the filtrate was collected for use as root exudates in the *Foc* bioassays.

The effect of banana root exudates on *Foc* was determined by mixing 100 μl of the root exudates with 100 μl of a fungal spore suspension, prepared at a concentration of 10^5 conidia/ml in Eppendorf tubes. The mixture was incubated at 28°C until conidial germination and fungal growth was studied. For each banana cultivar the reaction was replicated five times. The control reaction was set up by mixing *Foc* spores with sterile distilled water. After 1 and 3 days of incubation, the effect of the root exudates on *Foc* was determined by viewing spore germination and fungal growth under a fluorescence microscope, respectively. Five fields of view were chosen randomly per replicate for each treatment, and the mean germination percentage of spores was calculated and analyzed.

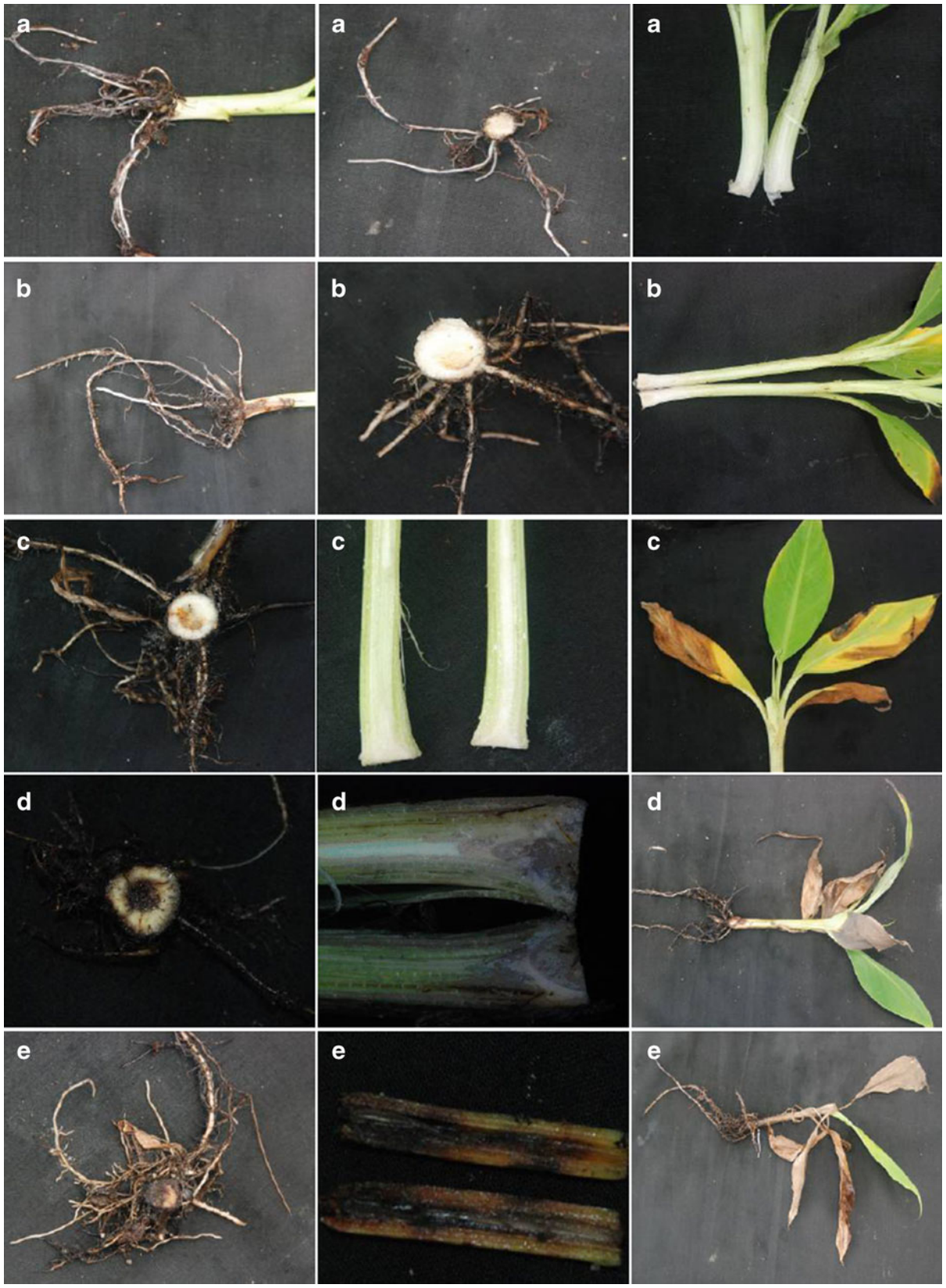
Quantification of root colonization and statistical analysis

To determine the difference in root colonization of banana cultivars by *Foc* race 4, banana plantlets were inoculated with the GFP-transformed isolate of *Foc* race 4 and examined after 0, 4, 9 and 13 days. Five plants of each cultivar were selected and five root samples per plant harvested, and the number of fungal spores that colonized the roots were counted. When spores germinated and developed into hyphae, they were still scored as single infective units (primary inoculum). The infective units attached to each of the five roots per banana plant were counted, and the average number of infective units on the roots of the different cultivars was compared. Each experiment was performed three times. Data were statistically analyzed using SAS version 8.0 software by Analysis of Variance (ANOVA), and the significance of root colonization by *Foc* race 4 was determined using Fisher's protected least significant difference (LSD) test ($P \leq 0.05$).

Results

GFP transformation

Transgenic *Foc* isolates were obtained at a rate of two GFP-transformed colonies per microgram of plasmid DNA with 40% of hygromycin B-resistant isolates expressing the GFP. GFP expression was bright and uniform in fungal spores and hyphae, but not in their vacuoles, which appeared as dark areas in the fungal cytoplasm. Green fluorescence in *Foc* remained stable after successive transfers on PDA medium with or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◀ **Fig. 2** Stages of symptom development in banana plantlets following inoculation with *Foc* race 4. **a** Roots are seldom brown, with no discolouration of the rhizome and pseudostem. No symptoms are visible on leaves. **b** Some roots become brown, and less than 25% of the inner rhizome is discoloured. Bottom leaves show brown spots or regions. **c** Roots turn brown, and between 25 and 50% of the inner rhizome is discoloured. The bottom leaves develop large brown regions. **d** The roots have become dark brown, and more than 50% of the inner rhizome is discoloured. The lower pseudostems develop internal discolouration, and most of the leaves are wilted or dead. **e** Entire inner rhizome and pseudostem are dark brown, and the entire plant has wilted

without hygromycin B. Neither virulence nor morphological characteristics (growth rate, spores, or hyphae) of the pathogen was altered by transformation with the plasmid pCT74.

Adhesion and colonization of banana roots with *Foc* race 4

The description of adhesion and colonization of banana roots with *Foc* race 4 represents several microscopic images of the same root tissue obtained from different banana plants. On the first 2 days after susceptible Cavendish banana plantlets were inoculated with *Foc* race 4, no fungal spores or hyphae were observed in or on the banana root system. On the third day, chlamydospores were occasionally found attached to root hairs (Fig. 1a) and the epidermal cell surfaces of lateral roots. Between days 3 and 6, more chlamydospores were found attached to banana root, and they began to produce germination tubes (Fig. 1b). From day 5–7, substantial colonization of banana roots with germinating *Foc* race 4 spores was observed on 'Brazilian' banana roots (Fig. 1c), while fungal hyphae began to colonize epidermal cells adjacent to the ones where the chlamydospores were attached on Guangfen #1 banana roots (Fig. 1d). At this stage, none of the banana plantlets displayed any visible internal or external Fusarium wilt symptoms (Fig. 2a).

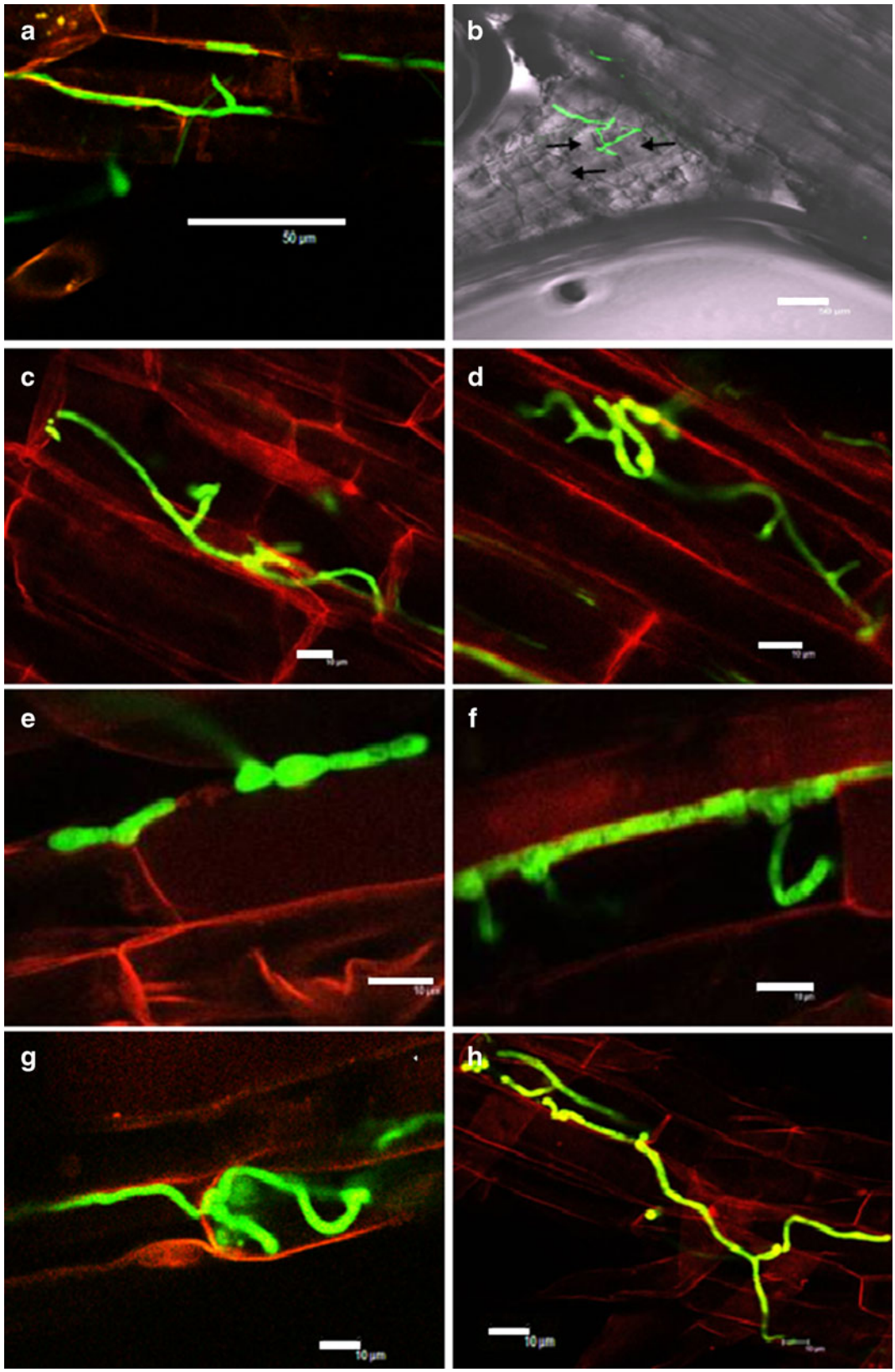
By day 11, a network of fungal hyphae was observed on the banana root cap (Fig. 1e) and the elongation zone (Fig. 1f). At this stage some banana roots became discoloured, whereas the leaves, rhizomes and pseudostems of plantlets remained unaffected by *Foc* race 4. On day 13, four plantlets out of the total examined developed small brown patches on the first set of leaves, and small brown spots developed in the rhizome. The pseudostem remained free of symptoms, but the roots were clearly brown (Fig. 2b). These symptoms indicated the early appearance of Fusarium wilt disease. Microscopic examination of banana roots on day 15 showed considerable colonization by the GFP-tagged *Foc* race 4 isolate, with fungal hyphae covering large sections of banana roots (Fig. 1g). On day 17 after transplanting, the banana roots turned brown, the rhizome developed large brown areas, and 50% of the inoculated plantlets showed symptoms of wilting (Fig. 2c). By day 25, more than 50% of the inner rhizome was discoloured and several leaves were wilted or dead (Fig. 2d). Fungal hyphae and spores have filled vascular spaces in the rhizome and the surrounding tissue became disorganized (Fig. 1h). No fungal material, however, was observed in the yellowing and wilted leaves. One month after inoculation, 70% of the plants were dead or dying (Fig. 2e).

Infection of banana roots by *Foc* race 4

Once established on root hairs, root caps (Fig. 1e) and lateral roots (Fig. 1f), *Foc* race 4 expanded its hyphal network by growing in the intercellular spaces along the junctions of root epidermal cells (Fig. 3a). In some instances, small wounds at the bases of lateral roots appeared to serve as entry points for the pathogen in the Cavendish banana cv 'Brazilian' (Fig. 3b), but in Guangfen #1, the epidermal cells of lateral roots were penetrated directly (Fig. 3c). Once inside the cells, fungal growth proceeded rapidly with the concomitant production of a network of branching hyphae (Fig. 3d).

Before epidermal cells of banana roots were penetrated, hyphae of *Foc* race 4 became swollen at the penetration sites (Fig. 3e). The swollen hyphae then entered epidermal cells through what appeared to be a narrow penetration pore by means of a constriction that returned to its normal size once inside the epidermal cell (Fig. 3f). Neither true appressoria nor penetration pegs were observed during the penetration process. From inside cells, *Foc* race 4 proceeded to infect neighbouring cells through pores in cell end plates (Fig. 3g). The fungus then grew intercellularly and intracellulary within cells of the banana root epidermis and cortex (Fig. 3h).

Almost immediately after penetrating the banana root cells, *Foc* race 4 produced thickened hyphae (Fig. 4a) and microconidia (Fig. 4b) inside the cells. The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◀ **Fig. 3** Invasion of susceptible banana roots by with a GFP-tagged isolate of *Fusarium oxysporum* f. sp. *cubense* (*Foc*). **a** Hyphal growth in intercellular spaces along junctions of root epidermal cell of cv 'Brazilian', 11 days after inoculation. **b** Hyphal growth in the vicinity of small wounds at the bases of lateral roots of cv 'Brazilian', 15 days after inoculation. **c** Direct penetration of epidermal cells of roots of cv. 'Guangfen #1', 7 days after inoculation. **d** Extensive growth of fungal hyphae inside root epidermal cells of cv. 'Guangfen #1', 7 days after inoculation. **e** Hyphal swellings developing at in intercellular spaces of epidermal cells of cv. 'Guangfen #1', 11 days after inoculation. **f** Swollen hyphae entering epidermal by making a constriction that returned to its normal size once inside epidermal cell of cv. 'Guangfen #1', 11 days after inoculation. **g** Hyphae infecting neighbouring cells through pores in cell end plates of cv. 'Guangfen #1', 11 days after infection. **h** Spread of GFP-tagged *Foc* race 4 mycelia intra- and intercellularly in roots of cv. 'Guangfen #1', 12 days after inoculation. **a** and **b** scale bar=50 μm; **c**–**h**scale bar=10 μm

thickened hyphae then developed into chlamydospores that were formed intra- (Fig. 4c) and intercellularly (Fig. 4d). No hyphae were seen attached to the chlamydospores once they were produced. By day 16, numerous chlamydospores were produced inside banana roots cells (Fig. 4e) and root hairs (Fig. 4f). In direct contrast to infection of the *Foc* race 4-susceptible banana roots with the GFP-marked pathogen, no fluorescent fungi were seen on the root caps (Fig. 4g) or roots (Fig. 4h) of resistant banana varieties at any stage.

Comparing root infection of two susceptible banana cultivars, 'Brazilian' and 'Guangfen #1', by *Foc* race 4

Foc race 4 invaded the susceptible Pisang Awak cv. 'Guangfen #1', a banana with an AAB genome, in a manner similar to that found in the Cavendish cv. 'Brazilian', which is an AAA-type banana. The invasion of banana root cells of Guangfen #1, however, appeared to be more aggressive than in 'Brazilian', in that once the fungus attached to and invaded young, tender root hairs and root caps, it rapidly spread within the epidermal cells producing microconidia, hyphal swellings and chlamydospores. The sequence of symptom development in Guangfen #1, however, was almost identical to that found in the Cavendish cv. 'Brazilian'.

Infection of resistant banana cultivars by *Foc* race 4

Six banana cultivars were selected for infection with a GFP-transformed isolate of *Foc* race 4 and proved to be moderately to highly resistant to the Fusarium wilt pathogen in the field. These include the plantain cultivars 'Dongguan Dajiao' and 'Zhongshan Dajiao' (ABB) that are resistant, the two Cavendish banana somaclonal variants 'Wilt-resistant #1' and 'Wilt-resistant #5' that are moderately resistant and resistant, respectively, a natural hybrid Fenjiao cultivar 'Guanfen #2' that is moderately resistant, and diploid banana cultivar 'Haigong' that is highly resistant to *Foc* race 4. When the roots of these banana cultivars were examined 15 and 30 days after transplanting, no GFP-transformed *Foc* race 4 spores were observed in or on their roots (Fig. 4g and f). In contrast, all three the susceptible banana cultivars included in this study (Guangfen #1, Brazilian and Dafeng #2) were infected with *Foc* race 4. Once the soil concentration of *Foc* race 4 was increased from 500 to 1,500 cfu/g soil, 30% of 'Wilt-resistant #1' banana plantlets had fungal spores attached to their roots, whereas the other resistant cultivars were still free of fungal spores and hyphae.

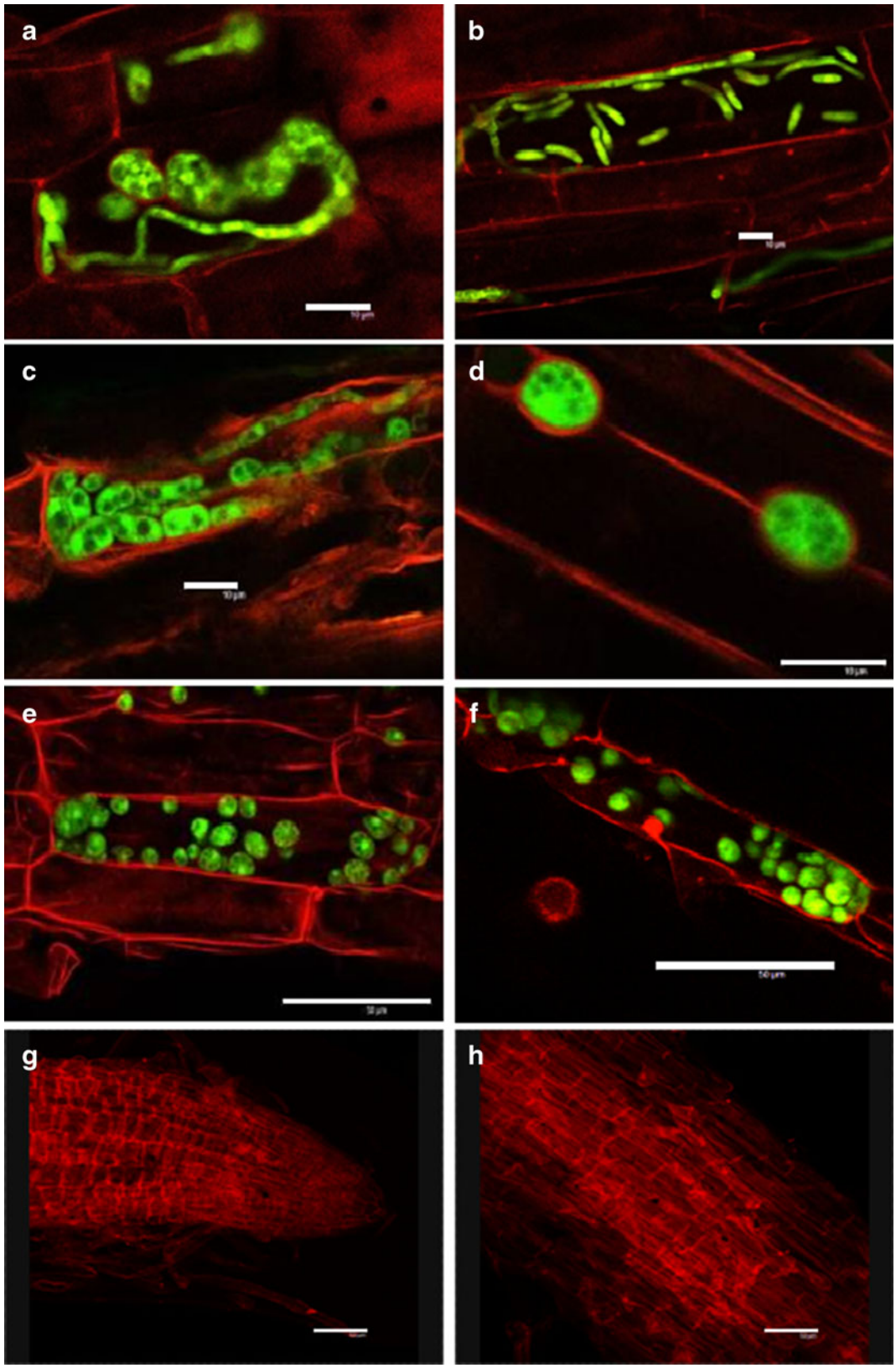
Effect of root exudates on spore germination and hyphal growth

Banana root exudates appeared to play an important role in spore germination and hyphal growth. From fluorescence microscopical observations, the effect of root exudates on *Foc* race 4 can be divided into three categories (Table 1):

1) For cultivars 'Dongguan Dajiao' (ABB), 'Zhongshan Dajiao' (ABB) and 'Guangfen #'2 (ABB) root exudates inhibited spore germination by a mean percentage of 1%, and for Wilt-resistant #1' by a mean of 3%, (Fig. 5a), 2) For cultivars 'Haigong' (AA) and 'Wilt-resistant #5' (AAA), root exudates resulted in fluorescence quenching of the pathogen, and no spores were observed (Fig. 5b), and 3) for cultivars 'Dafeng #2' (AAA), Brazilian (AAA), and 'Guangfen #1' (ABB), root exudates did not affect the germination of the pathogen, and their mean percentages of spore germination were 16.7, 20, and 20%, respectively (Table 1).

Quantification of root colonization

None of the resistant banana cultivars became colonised with *Foc* race 4, so root colonization of only the three susceptible cultivars (Guangfen #1,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◀ **Fig. 4** Establishment and sporulation of GFP-tagged isolate of *Fusarium oxysporum* f. sp. *cubense* (*Foc*) in banana roots. **a** and **b** Thickened hyphae and microconidia produced inside epidermal cells of cv. 'Guangfen #1', 11 and 12 days after inoculation. **c** and **d** Intra- and intercellular chlamydospore formation in roots of cv. 'Guangfen #1', 11 days after inoculation. **e** and **f** Prolific chlamydospore production in root cells and root hairs of cv. 'Brazilian', 16 days after inoculation. **g** and **h** No attachment of GFP-tagged *Foc* spores or hyphae on roots of the *Foc* race 4-resistant banana cvs. Wilt-resistant #5. **a**–**d** scale bar=10 μm, **e**–**h** scale bar=50 μm

Brazilian and Dafeng #2) were compared. For all cultivars more spores and hyphae were attached to the roots over time (Table 2). Too many infective units developed into hyphae for counting 13 days after inoculation, so this time point was excluded from further analysis. More infective units were associated with Guangfen #1 (AAB) compared to Brazilian (AAA) and Dafeng #2 (AAA). This indicated that Guangfen #1 (a Pisang Awak banana) is more sensitive to *Foc* race 4 than the Cavendish bananas, which was consistent with the response of these cultivars during field infection where Fusarium wilt develops faster in Guangfen #1 than in Brazilian and Dafeng #2 (data not presented).

Discussion

GFP-transformed plant pathogens can be visualized in living tissues without any processing or manipulation of samples. This property makes the technology extremely useful for the analysis of *in-planta* fungal development (Lorang et al. 2001). Using confocal microscopy and GFP-marked isolates of *Foc* race 4, we observed that fungal chlamydospores were attached to banana roots and root hairs as early as 72 h after the plantlets were planted in *Foc*-infected soil, and that plant penetration occurred through the tips and elongation zone of lateral roots, as well as through natural wounds in secondary root bases after approximately 5 days. Adhesion and colonization of the banana roots with *Foc* differed from that described for *Fusarium oxysporum* f. sp. *radicis-lycopersici* (*Forl*) on tomato roots, even though both pathogens belong to *F. oxysporum*. In tomato, *Forl* attached itself to root hairs by means of fungal mycelia, and hyphal development appeared to be faster in the crown region than at the root tips or the bases of lateral roots (Lagopodi et al. 2002). There was, however, a common mode of colonization where both the banana and tomato pathogens first attached to lateral roots and root hairs before growing along the junctions of epidermal cells. They both then penetrate roots directly through small pores at the junction of epidermal cells. Infection of tomato roots by *Forl*, however, was much faster than for *Foc* in banana, and 60-70% of tomato plantlets were killed within 6 days compared to 4 weeks in banana. The difference in root infection and disease development can most likely be attributed to the age difference of tomato and banana plants that were inoculated.

Infection of banana roots by *Foc* previously has been studied in great detail by Wardlaw (1930);

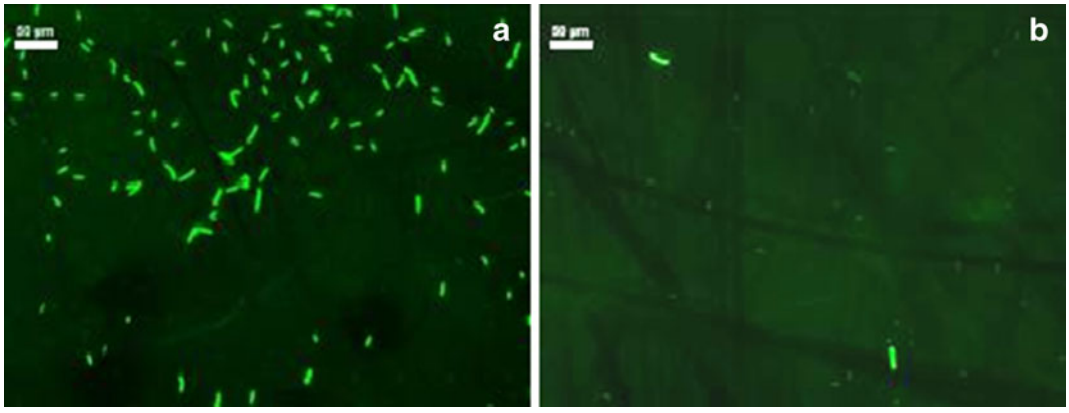

Fig. 5 Effect of banana root exudates on hyphal growth of a GFP-tagged isolate of *Fusarium oxysporum* f. sp. *cubense*. **a** *Musa* ABB cv. 'Dongguan Dajiao'; **b** *Musa* AA cv. 'Haigong'; **c** *Musa* AAA Cavendish cv. 'Brazilian'; **d** *Musa* ABB Pisang Awak cv. 'Guangfen #1'

Table 2 Quantification and statistical analysis of the root colonization by *Fusarium oxysporum* f. sp. *cubense* on three susceptible banana cultivars

Days[a]	Brazilian (AAA)	Dafeng #2 (AAA)	Guangfen #1 (AAB)
0	0	0	0
4[b]	49±12.6 B	62.3±23.5 B	89±33.1 A
9[b]	254.3±51.3 B	284±66.8 B	455±113.6 A
13[b]	562±132.7 B	439±115.9 C	763±201.8 A

[a] days after inoculation.

[b] Data in the same line are not statistically different if they share a common script. ($P \leq 0.05$).

Figures represent the average value of the spore adsorption quantity of each plant and each experiment was replicated three times

Stover and Waite (1954); Sequeira et al. (1958) and Beckman et al. (1961). In these studies, based primarily on the *Foc* race 1-Gros Michel interaction, banana suckers instead of tissue culture material were inoculated, and root infection was studied by means of light microscopy and the discolouration of root cells, rather than by using GFP-transformed fungal isolates and confocal laser scanning microscopy. Wardlaw (1930) reported that healthy roots did not become infected when inoculated with *Foc*, but that roots inoculated at the apex and young, decapitated roots became diseased, whereas older roots partially resisted penetration by the pathogen. Subsequently, Rishbeth (1957) and Sequeira et al. (1958) found that, although the pathogen did not directly invade intact main roots, it frequently invaded mature rootlets (lateral roots). In addition, Stover (1957) reported that invasion occurs at or near the root-cap of lateral roots, as was described in the current investigation for *Foc* race 4. The mode of entry, however, was not determined by Rishbeth (1955) and Stover (1957). This study, thus, demonstrated for the first time that *Foc* penetrated banana roots through small openings in the cell wall. Once inside cells, it was shown to rapidly spread from one cell to another through tiny pores in the cell wall, and to reproduce within cells. Our suggestion that infection also occurred through wounds at the base of primary roots were earlier demonstrated by Sequeira et al. (1958), who proved that both the main root and lateral rootlets could be infected through injuries. It has previously been shown that, once the fungus reached the main root base inside the lateral roots, it was well established and would eventually result in infection of the rhizome (Rishbeth 1955). We were unable to illustrate this in the current study, as entrance of the xylem vessels of lateral roots by the GFP-modified *Foc* race 4, and how it progressed in the vascular tissue of banana roots to the pseudostem, was not obvious. Direct infection of the rhizome by *Foc* was not investigated, as it was proved indisputably by Stover and Waite (1954) and Rishbeth (1957) to be unattainable.

An unexpected finding in the current study was that no *Foc* race 4 spores and hyphae were attached to the roots of resistant/tolerant banana cultivars. This finding could not be attributed to a loss in expression of the *gfp* gene, as the same GFP-modified isolate of *Foc* race 4 had been used for inoculation of both resistant and susceptible banana cultivars, and GFP fluorescence on roots of susceptible cultivars remained strong for at least 16 days. This finding, rather, can be explained by one of two possibilities: 1) the inability of *Foc* race 4 to properly attach to roots of bananas resistant to this pathogen, and its subsequent washing-off during preparation for fluorescence microscopy, and 2) the secretion of substances by roots of resistant bananas that either prevented its attachment, inhibited its germination or damaged/killed the fungus.

In 1962, Buxton (1962) noted that resistant banana cultivars can secrete material that inhibits *Foc* spore germination. The inhibitory ability diminishes as the plant ages. We confirmed the antifungal activity of root exudates in resistant cultivars to the Fusarium wilt pathogen of banana by suppressing germination of *Foc* race 4 microconidia with root exudates from resistant banana cultivars, but not from susceptible ones. This finding is of great importance, as resistance to *Foc* in banana has, in the past, primarily been considered a function of physical root structural and biochemical

barriers, such as the secretion of gums, the the formation of tyloses and the lignifications of cell walls (Beckman et al. 1961; Wardlaw 1930). Our study, for the first time, suggests that resistance to *Foc* in banana also can be contributed to by chemicals released in the root environment. Similarly, Stevenson et al. (1995) reported that exudates of Fusarium wilt-resistant chickpea roots inhibited germination and growth of *Fusarium oxysporum* f. sp. *ciceri*, and further demonstrated that the inhibition was concentration-dependent. Although we have not attempted to identify the effective components of exudates and study their emission profiles, we believe that the resistance that is present without exposure to the pathogen is constitutive, not inductive (Stevenson et al. 1995).

Banana breeding is time-consuming and expensive. The first step to evaluate new genotypes produced by breeding programs is to establish an efficient screening protocol capable of distinguishing between tolerant and susceptible genotypes. In general, new clones are evaluated in field trials. However, such trials have sometimes resulted in inconsistent results due to the disparate distribution of pathogens in the soil, and because banana varieties with different levels of resistance to *Foc* can respond unpredictably depending on soil inoculum pressure. For instance, in the current study it was demonstrated that resistant cultivars responded similarly to low and high pathogen concentrations in the soil, but that the moderately resistant cultivar 'Wilt-resistant #1' became vulnerable to pathogen colonization and infection when spore concentration in the soil was increased from 500 to 1,500 spores/ml.

Currently, no rapid small plant evaluation technique is available to banana breeding programs to rapidly distinguish between susceptible and resistant banana genotypes. Peroxidases and polyphenol oxidases have been postulated to be of some help in protecting plants against infection by root pathogens (Lagrimini and Rothstein 1987). Morpurgo et al. (1994), for instance, indicated that constitutive levels of peroxidase in SH-3362, a race 4-resistant synthetic AA hybrid produced at FHIA, were ten-fold higher than in Pisang Mas, which is a susceptible AA cultivar. This technique, however, has not yet been universally accepted. Plant tissue culture techniques have also been used to investigate the mechanisms of host–pathogen relationships and have been proposed as an aid to banana breeding programs (Beckman et al. 1961). Cell or tissue cultures are often very sensitive to direct exposure to the pathogen, causing both resistant and susceptible materials to die upon challenge. Nonspecific toxins such as fusaric acid have been used to select Fusarium wilt-resistant banana, but such toxins are not selective (Krikorian 1990). Neither meristems nor cell cultures could survive exposure to higher toxin concentrations. In contrast, at lower toxin concentrations, neither growth reduction nor mortality was observed (Krikorian 1990).

Although resistance markers can be powerful tools for plant breeders, none are currently available. Moreover, their identification is time-consuming and expensive, and they are usually specific to the analyzed cultivar. In the current study, the *gfp*-expressing isolate of *Foc* race 4 proved to consistently colonize susceptible banana genotypes and not resistant ones 15 days after application at a concentration of at 500 cfu/g. This technique, therefore, could be considered as a rapid, efficient and affordable method to separate resistant from susceptible genotypes, as roots of inoculated plantlets can easily be evaluated by fluorescence microscopy. In future studies, the effect of GFP-transformed isolates representing the different races of *Foc* should be investigated. The effect of root exudates on spore germination and hyphal growth, in addition, should be considered as a rapid method to test for Fusarium wilt resistance.

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