RESEARCH LETTER

Biofilm formation by *Fusarium oxysporum* f. sp. *cucumerinum* and susceptibility to environmental stress

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Abstract

To the authors' knowledge, most studies on biofilm formation have focused on bacteria and yeasts. So far, biofilm formation by fungal plant pathogen has not been reported. In this study, the biofilm-forming capacity of *Fusarium oxysporum* f. sp. *cucumerinum* was evaluated. For biofilm quantification, a colorimetric 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium-hydroxide (XTT) reduction assay was used to observe metabolic activity. Fluorescence and confocal scanning laser microscopy revealed that the biofilms have a highly heterogeneous architecture composed of robust hyphae and extracellular polysaccharide materials. Additionally, the influence of physical factors on *F. oxysporum* biofilm formation and the susceptibility of biofilms to environmental stress was investigated. Biofilms were less susceptible to heat, cold, UV light and three fungicides than were their planktonic counterparts. Our findings may provide a novel perspective on the pathogenic mechanism associated with biofilms of *F. oxysporum* f. sp. *cucumerinum*.

Introduction

Biofilms are surface-associated communities of cells surrounded by a self-produced gelatinous matrix composed of extracellular polymeric substances (Blankenship & Mitchell, 2006; Harding *et al.*, 2009). Most microbes in nature survive as biofilms rather than as solitary cells. In fact, biofilm formation is so prevalent that it is likely to be a positively selected trait, allowing microbial cells both to survive hostile environments and to disperse to colonize new niches (Hall-Stoodley *et al.*, 2004). Some studies have reported that the microorganisms in these biofilms exhibit an altered phenotype with respect to growth rate, gene transcription, and resistance to physical, chemical and biological stresses (Martinez & Casadevall, 2007; Harding *et al.*, 2009; Mowat *et al.*, 2009). In the past few decades, biofilms have been studied in a wide range of scientific disciplines including biomedicine, water engineering, and evolutionary biology (Austin & Bergeron, 1995; Costerton *et al.*, 1999; Douglas, 2003; Martinez & Casadevall, 2007). In contrast to the extensive literature describing bacteria biofilm genesis, architecture, chemical composition, genetic regulation and antimicrobial drug resistance, little attention has been paid to fungal biofilms. Moreover, most of the reports and reviews of fungal biofilm formation have focused on yeast or yeast-like molds such as *Candida* spp. (Douglas, 2003; Imamura *et al.*, 2008), *Saccharomyces* spp. (Reynolds & Fink, 2001), *Aerobasidium pullulans* (Gorbushina & Palinska, 1999) and *Cryptococcus neoformans* (Martinez & Casadevall, 2007). Very few reports on filamentous fungal biofilms can be found besides those on *Aspergillus* (Seidler *et al.*, 2008; Mowat *et al.*, 2009), *Fusarium* and zygomycetes (Singh *et al.*, 2011; Mukherjee *et al.*, 2012), probably because filamentous fungi do not fit completely or precisely within restrictive biofilm definitions based on bacterial and yeast models. However, a set of criteria and a model describing the characteristics of filamentous fungal biofilms has been reported, and more

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medically relevant filamentous fungi have been described as biofilms (Harding *et al.*, 2009). Filamentation in fungi may be a prerequisite for robust biofilm development and virulence, and fungal biofilms perhaps represent much more than a mere biological coating (Harding *et al.*, 2009; Singh *et al.*, 2011). Moreover, biofilm formation is claimed to be involved in the pathogenesis, for example, of keratitis-associated *Fusarium* and invasive diseases caused by *Aspergillus fumigatus* (Mowat *et al.*, 2008; Mukherjee *et al.*, 2012).

Fusarium oxysporum f. sp. *cucumerinum* is a soil-borne vascular fungal pathogen on cucumber (*Cucumis sativus* L.) that causes *Fusarium* wilt, one of the most important threats to cucumber quality and productivity worldwide (Chen *et al.*, 2010; Zhao *et al.*, 2012). Many microorganisms in *Fusarium* genus have been reported to grow as biofilms, and these surface-attached communities are considered pathogenic factors. We surmise that the fungal plant pathogen *F. oxysporum* f. sp. *cucumerinum* is capable of forming biofilms, which may be an important pathogenic factor. In this study we explored biofilm development and architecture using microscopy techniques, determined the effect of various factors on biofilm formation *in vitro*, and compared the susceptibilities of mature biofilms and planktonic cells to environmental stress.

Materials and methods

Fungal strain and growth conditions

Fusarium oxysporum f. sp. *cucumerinum* strain Foc-GD was preserved by our laboratory in this study. For spore production, Foc-GD was incubated at 28 °C for 3 days in Sabouraud dextrose broth (SDB; Difco Laboratories, Detroit, MI). Following incubation, conidia were harvested and hyphae were removed by filtration through three layers of sterile gauze and washed with phosphate-buffered saline (PBS) for biofilm formation.

Biofilm formation

Fusarium oxysporum f. sp. *cucumerinum* biofilm formation was determined on the basis of the filamentous fungi forming biofilms, as reported previously (Imamura *et al.*, 2008; Pierce *et al.*, 2008). Conidia of Foc-GD were counted using a hemacytometer, and adjusted to 10^6 conidia mL^{-1} in RPMI 1640 (Mediatech, Inc., Herndon, VA). Then 200 μL of the suspension was added to the wells of 96-well, flat-bottomed polystyrene microtiter plates (Fisher, Waltham, MA) and incubated at 28 °C without shaking to allow the conidia to settle and adhere to the bottom of the plate. Following the adhesion stage, the wells containing Foc-GD biofilms were washed gently three times with PBS to remove nonadherent cells using a microtiter plate washer (Skan Washer 400; Molecular Devices, VA). Fungal cells that remained attached to the plastic surface were considered true biofilms. All assays were carried out in six wells.

Quantitative analysis by XTT reduction assay

At the end of the incubation, *F. oxysporum* f. sp. *cucumerinum* biofilms were quantified using a tetrazolium XTT [2, 3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] assay as described previously (Chandra *et al.*, 2001). Fungal mitochondrial dehydrogenase activity reduces XTT tetrazolium salt to XTT formazan, resulting in colorimetric change that correlates with cell viability (Meshulam *et al.*, 1995; Tsang *et al.*, 2012). XTT salt solution (1 mg mL^{-1} in PBS), filter-sterilized with a 0.22-μm pore size filter and fresh menadione solution (1 mM in acetone; Sigma), was prepared prior to the assay. Thereafter, 50 μL XTT solution and 4 μL menadione solution were mixed with PBS (146 μL) and transferred to each well containing pre-washed biofilms, and incubated in the dark for 3 h at 37 °C. After incubation, the colored supernatant (100 μL) was transferred to new microtiter plates and the colorimetric change was measured using a microplate reader (SpectraMax 340 tunable microplate reader; Molecular Devices) at 492 nm. In all the experiments, the same volume of heat-killed Foc-GD conidia in microtiter wells were included as negative controls.

Biofilm development

Similar to the previous description, the standardized spore suspension was inoculated on 1.5-cm^2 polystyrene strips (Fisher) and incubated at 28 °C for 2, 4, 8, 12, 24 and 48 h (Chandra *et al.*, 2001). After different incubation times, polystyrene strips with biofilms were washed twice with PBS, stained for 1.5–2 h at 30 °C in the dark with SYTO-9 (LIVE/DEAD BacLight Bacterial Viability kit; Life Technologies) according to the manufacturer's instructions and transferred to microscope slides. SYTO-9 is a green-fluorescent nucleic acid stain, generally labeling both live and dead microorganisms. Stained biofilms were examined under a fluorescence microscope (ZVS-47E microscope; Carl Zeiss, Inc., Oberkochen, Germany).

Physical factors influencing *F. oxysporum* f. sp. *cucumerinum* biofilm formation

pH

To evaluate the effect of different pH values on biofilm production by *F. oxysporum*, the standardized spore sus-

pension of Foc-GD was inoculated as described above at distinct pHs (3, 4, 5, 6, 7, 8, 9, 10) and biofilms formed over a series of time intervals (3, 6, 12, 24 and 48 h).

Temperature

The effect of temperature (4, 16, 28, 37 and 45 °C) on Foc-GD biofilm formation was examined.

Sugars

To determine the effect of sugars on *F. oxysporum* biofilm formation, the standardized spore suspension of Foc-GD was inoculated in minimal medium (20 mg mL^{-1} thiamine, 30 mM glucose, 26 mM glycine, 20 mM $MgSO_4{\cdot}7H_2O$, and 58.8 mM KH_2PO_4) containing 30 mM glucose, maltose, lactose or sucrose as sugar sources.

Susceptibility of *F. oxysporum* f. sp. *cucumerinum* to environmental stress

Susceptibility to heat

Foc-GD mature biofilms and 10^5 conidia mL^{-1} standard planktonic spores in 96-well microtiter plates were placed in a constant temperature incubator (DHP-9052/82, Shanghai) and heated to 45 °C. The metabolic activity of biofilms and planktonic cells was compared with that of unexposed cells after exposure to heat for various time intervals (30 and 60 min).

Exposure to cold

Similar to the susceptibility to heat, the susceptibility of *F. oxysporum* mature biofilms and planktonic cells to cold was determined by incubation at −20, −10 and 4 °C for 24 h.

Effect of UV light irradiation on mature biofilms

The susceptibility of *F. oxysporum* biofilms and planktonic cells to irradiation by UV light was determined by exposing fungal cells to various intervals (10, 20, 30 min) of UV light (254 nm) generated by a 30-W UV germicidal lamp (G30T8; Sankyo Denki, Japan).

Fungicide susceptibility testing

To evaluate the susceptibility of formed biofilms and planktonic cells to different fungicides, carbendazim (97%; Sigma-Aldrich), prochloraz (99.7%; Sigma-Aldrich) and propiconazole (98%; Sigma-Aldrich) were selected because they are the best choices for *Fusarium* wilt prevention and control. Briefly, stock solutions (6.4 mg mL^{-1}) of the fungicides were diluted with RPMI 1640 to working concentrations of 0.5–256 μg mL^{-1}. The Foc-GD biofilms and planktonic conidia were treated with the three fungicide solutions at 28 °C for 48 h and then inoculated on potato dextrose agar for 48 h. For all three fungicides, the minimum inhibitory concentrations (MICs) were read as the lowest concentration that produced no discernible growth. To confirm the results, metabolic activities of biofilm cells and planktonic forms were determined by XTT assay after incubation with 64 μg mL^{-1} carbendazim, prochloraz and propiconazole for 24 h. Next, the percent inhibition induced in biofilms and planktonic cells by each fungicide solution was evaluated by comparing their metabolic activities with the activity in the absence of any fungicide.

Confocal scanning laser microscopy

The architecture of *F. oxysporum* f. sp. *cucumerinum* biofilms was analyzed using confocal scanning laser microscopy (CSLM), following previously described methods (Chandra *et al.*, 2001, 2008; Jin *et al.*, 2005; Mukherjee *et al.*, 2012). Briefly, 2 mL of the standardized spore suspension was inoculated in 35-mm Petri dishes and incubated at 28 °C for 24 h. The mature biofilms were heated until death and incubated with 2 mL of PBS containing propidium iodide (PI, LIVE/DEAD BacLight Bacterial Viability kit; Life Technologies) and concanavalin A-Alexa Fluor 488 conjugate (ConA; 25 μg mL^{-1}; Life Technologies) in the dark for 1.5–2 h at 30 °C. PI is a red fluorescent nucleic acid stain and only penetrates cells with damaged membranes, and ConA binding to glucose and mannose residues of cell wall polysaccharides emits green fluorescence. After incubation with the dyes, stained biofilms were observed using a Zeiss LSM510 CSLM equipped with argon and HeNe lasers, and mounted on a Zeiss Axiovert 100 M microscope (Carl Zeiss Microscopy GmbH, Hamburg, Germany).

Statistical analysis

All experiments were performed in triplicate. Statistical analysis was performed using analysis of variance with the software SPSS 16.0 to assess the amount of biofilm formed in different conditions.

Results

Biofilm development

The morphologies of *F. oxysporum* f. sp. *cucumerinum* biofilm formation and hyphal development were photo-

graphed using a fluorescence microscope. Following initial seeding, the spore aggregation adhered to the polystyrene surface (0–2 h) and began to swell and germinate within 2–4 h. Hyphae were observed within 4–8 h of incubation. These hyphae formed hyphal networks or mycelia monolayers by 8–12 h, and then further intertwined and increased in density. The hyphal bundles 'glued' together with exopolymeric matrix by 24–48 h. After 48 h, the biofilms dispersed conidia for a new cycle (Fig. 1). This pattern resembles the kinetics of biofilm formation in *A. fumigatus* (Mowat *et al.*, 2007) and *Zygomycetes* (Singh *et al.*, 2011).

Influence of physical factors on *F. oxysporum* f. sp. *cucumerinum* biofilm formation

Fusarium oxysporum f. sp. *cucumerinum* strain Foc-GD conidia formed significantly stronger biofilms under slightly acidic and neutral pH conditions (pH 5–7) than under acidic (pH 3, 4) or alkaline (pH 8 and 10) conditions (Fig. 2a).

Figure 2b illustrates the effect of temperature on *F. oxysporum* biofilm formation. During the adhesion period and the early stage of biofilm development, the metabolic activities increased gently with increasing time. After 12 h, there was a rapid increase, which reached a plateau at 24 h and then remained high and steady. Foc-GD cells displayed similar metabolic activities and formed robust biofilms at 28 and 37 °C but did not show any significant increase of the metabolic activities and biofilm formation at 4, 16 or 45 °C.

Strain Foc-GD was able to attach and form comparable biofilms in the presence of glucose with either sugar (maltose, lactose, sucrose). In contrast, no biofilm formation was observed when the cells were grown in the presence of maltose, sucrose or lactose (Fig. 2c).

Susceptibility of *F. oxysporum* f. sp. *cucumerinum* cells in biofilms and planktonic cells

We investigated the susceptibility of biofilms formed by *F. oxysporum* f. sp. *cucumerinum* to harsh environmental factors, such as high and low temperatures, UV light and three fungicides compared with that of planktonic conidia. Figure 3a shows that the metabolic activity of biofilms was not affected by exposure to a relatively high temperature, whereas planktonic cells showed a significant reduction after being exposed to 45 °C for 30 min (62.25%) and 60 min (86.29%). Figure 3b shows that the biofilms and planktonic cells did not show significant differences at 4 °C. At −10 and −20 °C, *F. oxysporum* f. sp. *cucumerinum* biofilms were less susceptible to damage than planktonic cells. Furthermore, when *F. oxysporum* cells were irradiated for 10 min, there were no statistically

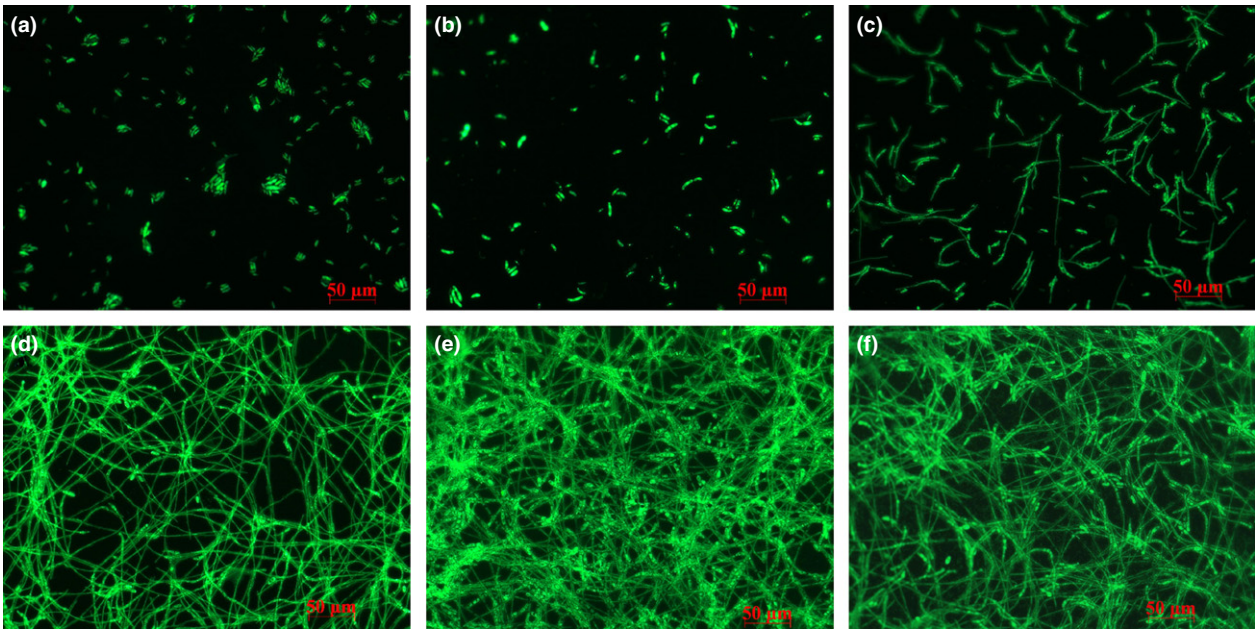

Fig. 1. *Fusarium oxysporum* f. sp. *cucumerinum* biofilms developed on polystyrene strips. The polystyrene strips were incubated at 28 °C for (a) 2 h, (b) 4 h, (c) 8 h, (d) 12 h, (e) 24 h and (f) 48 h. The images were taken using a fluorescence microscope. Scale bar: 50 μm.

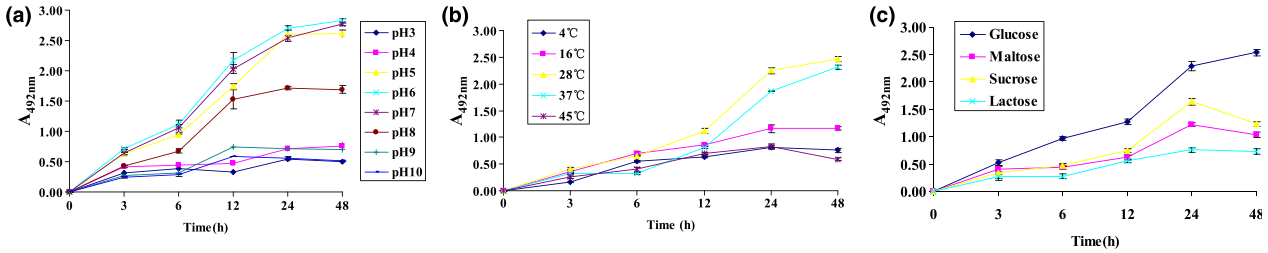

Fig. 2. *Fusarium oxysporum* f. sp. *cucumerinum* biofilm formation on polystyrene microtiter plates (a) at various pH conditions, (b) at different temperatures and (c) using different sugars as carbon source in the medium. The effect of the physical factors was evaluated by XTT reduction assay.

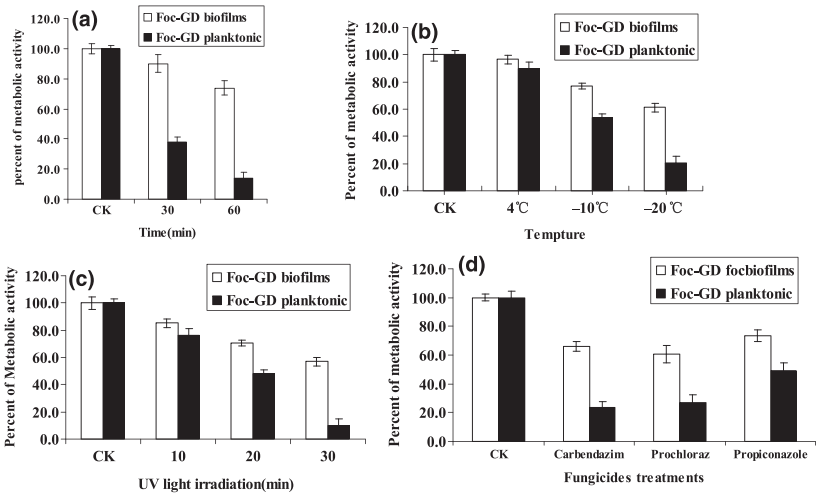

Fig. 3. Susceptibility testing of *Fusarium oxysporum* f. sp. *cucumerinum* biofilms to environmental stress. (a) The percent metabolic activity of biofilms and planktonic cells was compared with that of unexposed cells after being heated to 45 °C for 30 and 60 min. (b) The percent metabolic activity of biofilms and planktonic cells were compared with that of unexposed cells after incubation at −20, −10, and 4 °C for 24 h. (c) The percent metabolic activity of biofilms and planktonic cells was compared with that of unexposed cells after being irradiated by UV lights for 10, 20 and 30 min. (d) The percent metabolic activity of biofilms and planktonic cells was compared withthat of unexposed cells after 12 h incubation with 64 μg mL^{-1} carbendazim, prochloraz, and propiconazole, respectively.

significant differences between biofilms and planktonic cells. Conversely, after 20 min of UV light irradiation, the percent metabolic activities of planktonic cells were significantly reduced by *c.* 52.29%, but by no more than 29.18% for biofilm cells. After 30 min, the percent metabolic activities were reduced to 9.77% and 56.89%, respectively (Fig. 3c). As shown in Fig. 3d, after 24 h incubation with 64 μg mL^{-1} carbendazim, prochloraz and propiconazole, respectively, the percent metabolic activity of biofilm cells was reduced by 33.8%, 39.2% and 26.6%. In contrast, planktonic forms were reduced by 76.5%, 73.2% and 50.7%. To confirm the XTT assay, we also carried out inhibitory efficiency experiments at various different concentrations and found that the MICs were generally in agreement with XTT reduction results (Table 1). These results demonstrated that *F. oxysporum* were more resistant to these antifungal agents when grown as biofilms rather than in planktonic form.

Table 1. MIC (μg mL^{-1}) of fungicides against planktonic cells and biofilms

	Planktonic MIC (μg mL^{-1})	Biofilms MIC (μg mL^{-1})
Carbendazim	2	64
Prochloraz	8	64
Propiconazole	64	256

MIC, minimum inhibitory concentration.

CSLM of biofilms

The architecture and ultrastructure of biofilms formed by *F. oxysporum* f. sp. *cucumerinum* strain Foc-GD were

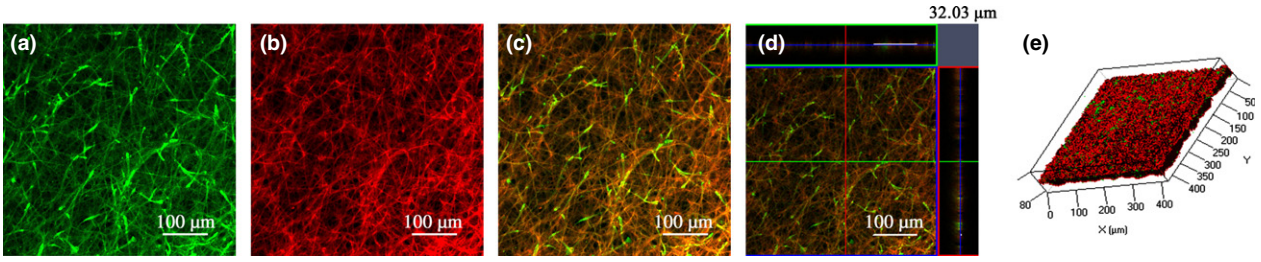

Fig. 4. CSLM images of ConA and PI stained mature *Fusarium oxysporum* f. sp. *cucumerinum* biofilms. (a) Cell wall-like polysaccharides were marked with green fluorescence. (b) The heat-killed *F. oxysporum* biofilm cells were marked with red fluorescence. (c) Dual staining biofilms. (d) The lateral views of the three-dimensional images. (e) Three-dimensional reconstruction of biofilms after dual staining. Scale bar: 100 μm.

investigated using CSLM because this technique preserved the structural integrity of the biofilms. As shown in Fig. 4, CSLM analyses showed that biofilms formed by strain Foc-GD were composed of profuse hyphae. Areas of red fluorescence represent the heat-killed *F. oxysporum* f. sp. *cucumerinum* biofilm cells (Fig. 4b), green fluorescence indicates cell wall-like polysaccharides (Fig. 4a), and yellow areas represent dual staining (Fig. 4c). Abundant polysaccharides contained in biofilms are shown in Fig. 4c. Biofilm images were displayed in three-dimensional projections (Fig. 4d and e). The side views of the three-dimensional reconstructed images show that mature biofilms consisted of a highly organized architecture (32.03 μm) with red hyphal cells interwoven with green extracellular polysaccharide materials.

Discussion

To our knowledge, biofilm formation by fungal plant pathogen has not been demonstrated previously. This is the first report of biofilm production by *F. oxysporum* f. sp. *cucumerinum in vitro* coculture. Biofilm development follows a preliminary model for filamentous fungal biofilm formation (Harding *et al.*, 2009). The standard sequence of events demonstrated in Fig. 1 is (1) spore adhesion, (2) microcolony development, (3) microcolony maturation, and (4) planktonic dispersal. For biofilm quantification, dry weight measurement and XTT reduction assay are often described in fungal biofilm research (Chandra *et al.*, 2001; Martinez & Casadevall, 2007; Imamura *et al.*, 2008). XTT reduction assay was adopted because the dry weight method was unable to distinguish between dead and living cells. Our results suggest that the method can be used reliably for quantification of *F. oxysporum* f. sp. *cucumerinum* biofilm mass. It is notable that maltose, sucrose and lactose did not stimulate biofilm maturation when strain Foc-GD was inoculated in minimal medium supplemented with these three sugars as carbon sources, respectively, which suggests that the carbon source that is available for nutrition can have an important effect on biofilm maturation. Similar to our findings, different sugars may affect fungal growth rate and the expression of proteins involved in adhesion or the production of extracellular polymeric substances (Jin *et al.*, 2004; Martinez & Casadevall, 2007). Furthermore, our data demonstrated that the temperature and pH level of the medium can have a marked influence on *F. oxysporum* f. sp. *cucumerinum* biofilm formation. In short, environmental factors may play an important role in microbial biofilm formation (Bonaventura *et al.*, 2007; Bissett *et al.*, 2008).

Both CSLM and scanning electron microscopy (SEM) were used in other studies to observe biofilm structure. However, because biofilm architecture was severely distorted by fixation and dehydration, we chose CSLM instead of SEM technology in the current study (Wood *et al.*, 2000; Seidler *et al.*, 2008). Our CSLM analyses showed that *F. oxysporum* f. sp. *cucumerinum* biofilms were composed of a homogeneous layered mesh of hyphal elements and self-produced gelatinous polysaccharides (Hall-Stoodley *et al.*, 2004; Seidler *et al.*, 2008; Singh *et al.*, 2011). This polysaccharide matrix forms a scaffold for the three-dimensional architecture of the biofilm, providing stability for the biofilm structure (Hall-Stoodley *et al.*, 2004; Flemming & Wingender, 2010).

The ability to produce a biofilm may give a fungus survival advantages in the harsh environment as well as a resistance to biocides. Our results showed that *F. oxysporum* biofilms were significantly less susceptible to stress conditions such as heat, cold, UV light and fungicides, than were the planktonic cells, which is in agreement with susceptibility tests of *Candida albicans*, *C. neoformans* and *A. fumigatus* (Chandra *et al.*, 2001; Martinez & Casadevall, 2007; Imamura *et al.*, 2008; Seidler *et al.*, 2008).

In conclusion, our results reveal that the plant pathogen *F. oxysporum* f. sp. *cucumerinum* has the ability to form biofilms under suitable external conditions such as temperature, pH and carbon source. The susceptibility testing suggests that *F. oxysporum* f. sp. *cucumerinum* biofilms possess survival advantages in harsh environments

and resistance to biocides, which may provide an excellent explanation for the resistance to drugs against *Fusarium* wilt of cucumber. Further research will be focused on the pathogenic mechanism associated with biofilms of *F. oxysporum* f. sp. *cucumerinum*.

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