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Biofilm Morphologies of Plant Pathogenic Fungi

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ABSTRACT

Microbial biofilms often complicate management of bacterial and fungal diseases. While much is known about biofilms formed by bacteria and yeasts, few descriptions of biofilms formed by filamentous fungi are available. A novel biofilm culture technology, the BEST Assay™, was used to culture biofilms of plant pathogenic fungi *in vitro*. Biofilm growth was characterized at 6-, 12-, 24-, and 48 h via scanning electron microscopy and compared with fungal growth seen *in planta*. Descriptions of the *in vitro* and *in planta* biofilm morphologies of *Fusarium* sp. *Verticillium dahliae,* and *Botrytis cinerea* are presented. The surface-associated growth of these plant pathogenic fungi is consistent with criteria for biofilm morphology indicating that filamentous fungi likely do form biofilms on host plant surfaces.

Keywords: biofilm development, filamentous fungal biofilms, plant-associated biofilms

INTRODUCTION

Biofilm research has led to many important discoveries in environmental, medical and industrial microbiology. A biofilm can be simply described as an assemblage of microorganisms within a self-produced polymeric matrix and growing in association with a biotic or abiotic surface. Additional characteristics have been associated with biofilm formation, such as in gene expression and resultant phenotypic changes. The most commonly described phenotype ascribed to biofilms is an increased tolerance to chemical or antibiotic treatments (Costerton *et al.* 1999; Ceri *et al.* 2001; Olson *et al.* 2002).

Bacteria and yeast biofilms that have a role human diseases have been most extensively studied, however microbes associated with plant tissues also form biofilms (Marques *et al.* 2002; Dow *et al.* 2003; Morris and Monier 2003; De Boer *et al.* 2004; Harrison *et al.* 2005a) and biofilm formation has been suggested to play a role in attachment (Matthysse *et al.* 2005), colonization (Marques *et al.* 2002),

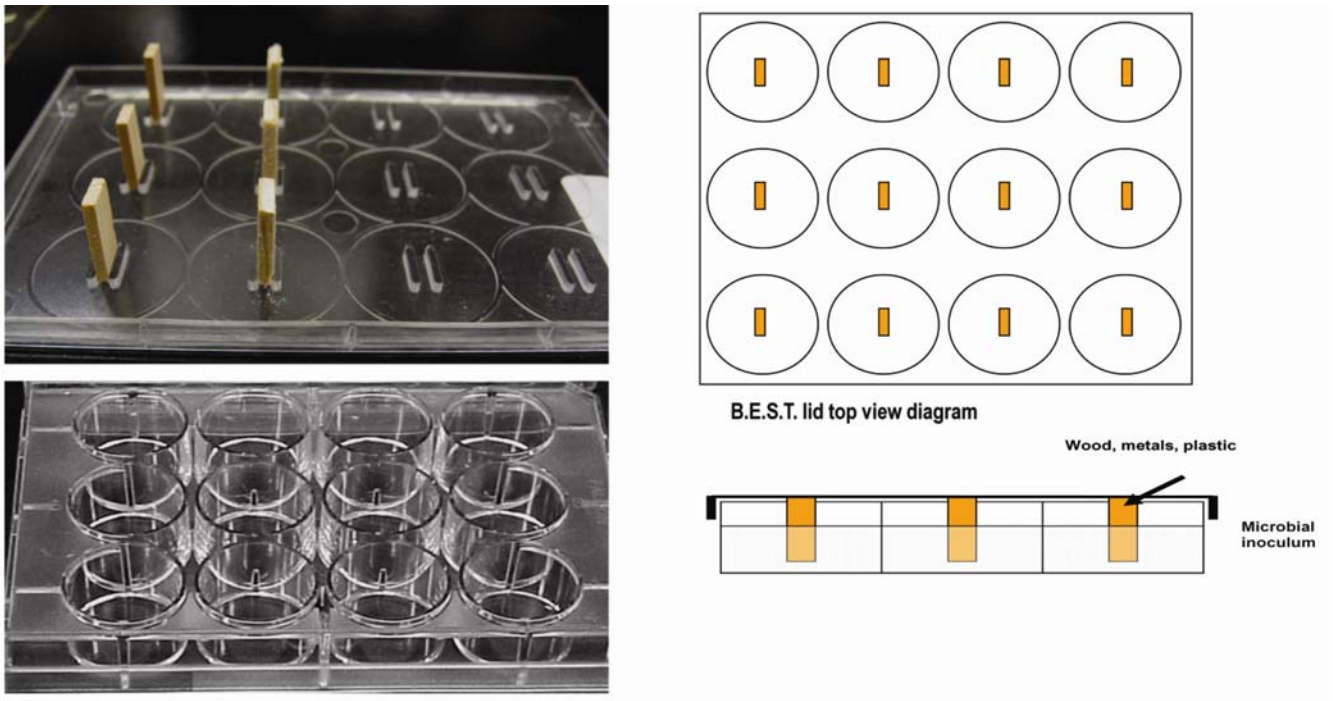

Fig. 1 BEST Assay™ plate.

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pathogenicity (Walker *et al.* 2004), survival in harsh conditions (Monier and Lindow 2003), dispersal and virulence (Dow *et al.* 2003), vector transmission (Newman *et al.* 2004), and resistance to host defense mechanisms (de Souza *et al.* 2005). To date there have been sufficient published works on plant-associated biofilms to warrant several excellent reviews on the subject (Ramey *et al.* 2004; Danhorn and Fuqua 2007; Rudrappa *et al.* 2008).

Although biofilms are commonly found in natural settings, standard laboratory culture techniques, such as broth cultures and semi-solid agar gels, rarely encourage biofilm formation and are therefore frequently unsuitable for growing biofilms, particularly for axenically cultured isolates in replicated experiments. Methods developed to culture biofilms include continuous flow-through systems, such as the Robbins device (McCoy and Costerton 1982), modified Robbins devices (Nickel *et al.* 1985; Ramage *et al.* 2008), drip flow (Goeres *et al.* 2009) and others (Ramey and Parsek 2005) as well as static culture methods (Merritt *et al.* 2005). While each method successfully forms biofilms, not all *in vitro* biofilms are equal in their responses to treatments. The key is to select methods and technologies that encourage biofilm formation and best approximate natural conditions (Buckingham-Meyer 2007). In some cases, biofilms formed in continuous flow reactors are more desirable to static culture biofilms, however, flow through systems are not easily expandable for experiments with numerous replicates or treatments. A major breakthrough in biofilm cultivation techniques was the development of the MBEC, and BEST, Assays™ (Ceri *et al.* 2001; Marques *et al.* 2006; Harding *et al.* 2009a). These static plate technologies encourage formation of microbial biofilms under liquid shear force in a multi-well plate format. Individual biofilms are formed on pegs that extend from the lid of the plate into each of the wells of the base (**Fig. 1**). As such, the lid, with biofilms attached, can easily be transferred to serial treatments. These high throughput plate assays have been employed in the study of biofilm formation, and treatment, of human-pathogenic bacteria and yeast (Harrison *et al.* 2004, 2005b, 2006a, 2006b; Davies *et al.* 2007; Harrison *et al.* 2007). The MBEC Assay™ allows up to 96 biofilms per plate on plastic or coated plastic surfaces in a standard 96-well plate format and is ideal for high throughput efficacy testing or mutant screening (Ceri *et al*. 1999). The BEST Assay™ is extremely versatile allowing for microbial growth on a wide array of surfaces, and can be used in 6-, 12-, 24-, 48-, or 96-well plate formats, depending on the size, shape and composition of the material used as a substrate for biofilm formation.

It has been suggested that filamentous fungi are capable of meeting basic criteria for biofilm development (Harding *et al.* 2009b). However, very few descriptions of biofilms formed by filamentous fungi exist (Goncalves *et al.* 2006; Villena and Gutierrez-Correa 2007; Imamura *et al.* 2008; Mowat *et al.* 2008; Villena *et al.* 2010). Since the majority of plant diseases are caused by filamentous fungi, we desired to know if morphologies of surface-associated growth by plant pathogenic fungi could fit with existing biofilm definitions. Specifically, does their growth on surfaces *in planta* and *in vitro* display typical structural features of filamentous fungal biofilms, such as aggregated growth (hyphal layering, hyphal bundling), and evidence of an exopolymeric matrix? This question was addressed by characterizing *in vitro* growth of three filamentous fungi on balsa wood pegs in the BEST Assay™ at 6-, 12-, 24-, and 48 h via scanning electron microscopy, and compared to growth *in planta.*

MATERIALS AND METHODS

Isolation of fungi

Diseased potato and tomato plants were collected from commercial fields or greenhouses respectively, placed in plastic bags with moist paper towels, and transported in a cooler to the laboratory. Potato stems with discoloured vascular tissues were considered to have symptoms of verticillium wilt caused by *V. dahliae* and/or fusarium wilt caused by *Fusarium* sp. Symptoms of grey mold on tomato were darkened, sporulating stem lesions. Small (1-cm × 1-cm) sections of symptomatic tissues were excised with an ethanol-flamed scalpel and placed in 1% sodium hypochlorite (Ultra Liquid Bleach, Wal Mart Canada) for 1-min, rinsed in sterile water and then aseptically placed onto acidified PDA agar (PDA-A; Oxoid Ltd., UK). Fungi growing out of the infested tissues were isolated and purified by sub-culturing to fresh PDA-A plates with ethanol-flamed forceps and aseptic technique. The fungi were identified using morphological characteristics.

BEST Assay™

The BEST Assay™ was performed as previously described (Marques *et al*. 2006; Harding *et al.* 2009). Briefly, the inoculum was prepared by scraping mycelia from a sporulating culture growing on an agar plate, and homogenizing (2 × 30-sec) in sterile phosphate buffered saline in a Waring blender. Homogenate was filtered through two layers of cheesecloth and adjusted to an estimated 10^5 cfu/mL using a haemacytometer. Cultures were allowed to recover for 1 h at room temperature after which 5 mL were placed into each well of a 12-well plate. A BEST Assay™ lid, with 12 wood pegs attached (**Fig. 1**), was used to cover the 12-well plate base. Plates were incubated on rotary shaker at ~75 rpm and at room temperature for 72 h. Pegs were sampled at 6-, 12-, 24-, 48- and 72 h.

Scanning electron microscopy

All samples were prepared for SEM using chemical fixation. Briefly, colonized wood pegs from the BEST Assay™ were collected and air-dried in a fume hood for 30 min in an open Petri dish. Primary fixation was done in a 7-mL scintillation vial containing 2-5 mL of primary fixative [3% glutaraldehyde (Electron Microscopy Sciences, USA) +1.6% paraformaldehyde (Electron Microscopy Sciences, USA) in 0.1 M Na Cacodylate buffer pH = 7.5 (Electron Microscopy Sciences, USA)], or enough to completely cover all tissues. Vials were capped and incubated at room temperature in a fume hood for at least two hours. Pegs were then removed from vials and air-dried in an open Petri dish in a fume hood overnight.

Infested plant tissues were excised with a scalpel, placed in a 7-mL scintillation vial and covered with primary fixative (as previously described) and incubated in a fume hood at room temperature at least two hours. Tissues were rinsed three times (15 min each) in 0.1 M Na Cacodylate buffer (Electron Microscopy Sciences, USA). After rinsing, 1-3 mL (or just enough to cover plant tissues) of 1% osmium tetroxide (Electron Microscopy Sciences, USA) were added to each vial. Samples in osmium tetroxide were incubated in a fume hood at room temperature for at least 1 hr. Rinsing with buffer was repeated (as above) and samples were dehydrated through a graded ethanol (Fisher Scientific, Canada) series with 10 min rinses each in ethanol at concentrations of 35%, 50%, 70%, 85%, 95% and two rinses in absolute ethanol). If samples were stored overnight they were immersed in 35% EtOH and kept at 4°C.

All samples, both *in vitro* and *in planta*, were critical point dried, mounted onto aluminum stubs, sputter coated, and scanned in a Philips C-60 ESEM.

RESULTS AND DISCUSSION

Fungal biofilms in BEST Assay™

Subsequent to contact with the wood pegs fungal spores became attached to the pegs and germinated. After 6 h, spore attachment, germination, germling extension and hyphal development across the surface of the wood was observed (**Fig. 2A, 2E, 2I**). Spores and germlings of some filamentous fungi are known to secrete extracellular polymeric substances or 'mucilage' to assist in adhesion (Hamer *et al.* 1998; Braun and Howard 1994).

By 12 h, hyphae began to grow and branch to form a

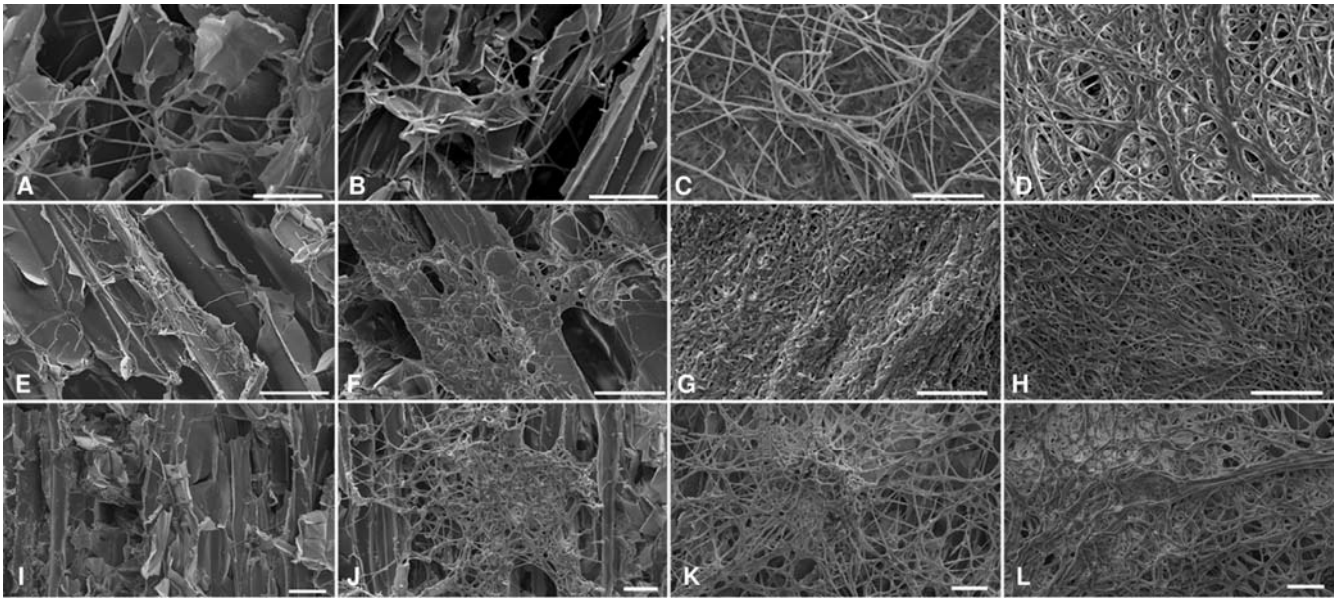

Fig. 2 Fungal biofilms cultured on balsa wood surfaces sampled at 6-, 12-, 24- and 48-h. (**A-D**) *Fusarium* hyphal growth after (**A**) 6-h. (**B**) Hyphal layering and bundling after 12-h. (**C**) Mature biofilm is seen after 24-h. Extensive layering of hyphae within EPS is seen associated with the biofilm. (**D**) Mature *Fusarium* biofilm seen after 48-h of incubation on balsa wood. Note the macrocolony morphology with hyphal bundles, layering and extensive EPS. (**E-H**) *Verticillium* biofilm on balsa wood surfaces. (**E**) Hyphal growth after 6-h. (**F**) Hyphal colonization, bundling and layering after 12-h. (**G**) A mature biofilm is seen beneath a secondary colony forming on the biofilm surface after 24-h. Extensive EPS is seen associated with the biofilm. (**H**) Mature *Verticillium* biofilm seen after 48-h of incubation on balsa wood. Note the three-dimensional nature of the macrocolony, hyphal bundles, extensive EPS and empty spaces in the matrix allowing liquid channeling. (**I-L**) *Botrytis* biofilms on balsa wood surfaces. (**I**) Hyphal growth after 6-h. (**J**) Hyphal colonization, bundling and layering after 12-hours. (**K**) A mature biofilm with many hyphal layers is seen after 24-h. (**L**) Mature *Fusarium* biofilm seen after 48-h of incubation on balsa wood. Note the hyphal bundles, extensive EPS and empty spaces in the matrix allowing liquid channeling. Scale bars = 50 μm.

monolayer or microcolony at which time hyphae appeared intertwined and the remains of extracellular polymeric substances (EPS) were visible (**Fig. 2B, 2F, 2J**). It is important to note that a polymeric matrix would be desiccated and collapsed after chemical fixation, dehydration and drying. Well-preserved EPS of fungal biofilm have been visualized using cryofixation protocols in preparation for SEM and/or cryo-SEM imaging (Beauvais *et al.* 2007; Villena and Gutierrez-Correa 2007; Villena *et al.* 2010). The artifacts that remain from the EPS after chemical fixation have also been described for fungal biofilms (Elvers *et al.* 2001; Mowat *et al.* 2008), and are very similar in appearance to those observed in this study.

At 24 h, biofilm development had progressed and hyphae were more frequently observed to be intertwined and beginning to form layered and/or aggregated clusters, often bundled together with more abundant EPS (**Fig. 2C, 2G, 2K**). Finally, in the latter stages of biofilm development (48 h), large cables of bundled hyphae and deeply layered sheets of intertwined hyphae punctuated by channels were observed within abundant EPS (**Fig. 2D, 2H, 2L**). During this final stage, sporulation was occasionally observed (not shown). These results are consistent with biofilm development models for filamentous fungi (Harding *et al.* 2009b). Specifically, evidence for all six stages of biofilm development were observed, namely; (I-II) adsorption and active attachment of spores, (III) microcolony formation with hyphal branching and surface colonization, (IV) hyphal-hyphal adhesion, layering and bundling permeated by channels and encased in abundant EPS material, (V) maturation and formation of reproductive structures, (VI) potential dispersal via spore production.

Fungal biofilms *in planta*

When diseased plant tissues, heavily infested with filamentous fungal growth, were examined, fungal morphologies observed were similar to mature biofilms seen on the BEST Assay™ wood pegs after 48 h. Fungal hyphae *in planta* had undergone bundling, layering and appeared to have produced abundant extracellular matrix (**Fig. 3**). For example, *Fusarium* sp. growth in potato showed hyphal layering and remains of what appeared to be abundant EPS (**Fig. 3B, 3C**). This was comparable to *Fusarium* sp. growth in the BEST Assay™ after 12 to 24 h (**Fig. 2B, 2C**). *Botrytis cinerea* growing on tomato stem was heavily layered, with large bundles of hyphae punctuated by open channels and significant exopolymeric matrix (**Fig. 3E, 3F**), similar to the growth of *B. cinerea* in the BEST Assay™ after 24 to 48 h (**Fig. 2K, 2L**).

CONCLUSIONS

The BEST Assay™ allows for consistent and rapid formation of surface-associated fungal growth under liquid shear force biofilms in a high throughput, multi well plate format. Additionally, the *in vitro* biofilms had high morphological similarity to those of *in planta* biofilms, indicating that fungal growth using the BEST Assay™ may accurately represent growth on or within host tissues. Although surface-associated growth by filamentous fungi has rarely been described as biofilm, we found that the morphologies of three filamentous fungi resembled biofilms when growing on surfaces within host plants and on wood pegs in the BEST Assay™ plates. For example, the fungal cells form a surface-associated community that appeared to been encased in a polymeric matrix. Stages and appearances of surface-associated fungal growth were consistent with microbial biofilm criteria and models indicating that some filamentous fungi appear capable of forming biofilms. Perhaps plant pathogenic fungi, growing as biofilms, display increased tolerances to chemical and physical treatments, similar to phenotypes seen in bacteria and yeast. If so, laboratory testing and development of plant disease management strategies should be done using biofilm cultures, such as those formed in the MBEC Assay™ and BEST Assay™, in order to more closely approximate efficacy of treatments in actual disease management surfaces and conditions.

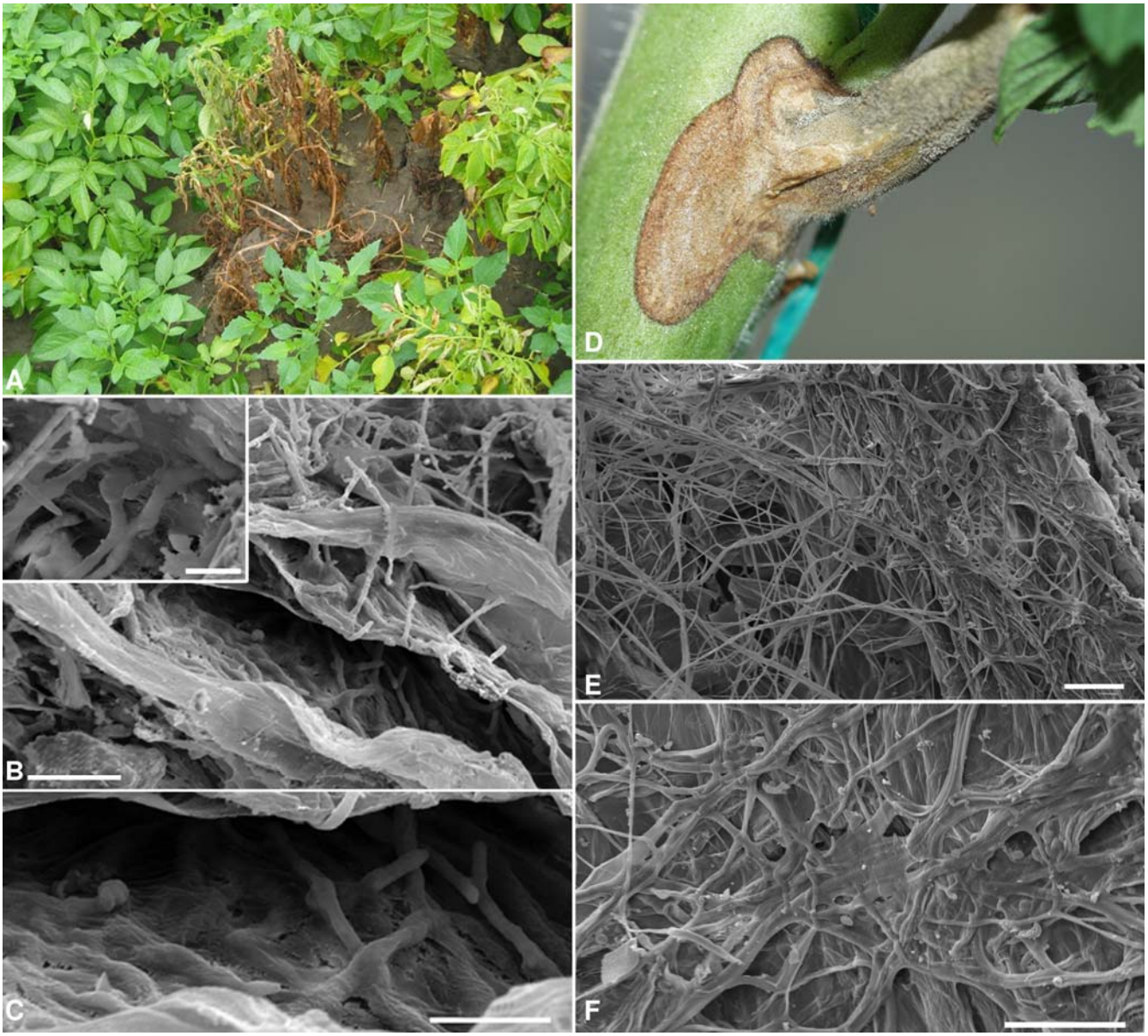

Fig. 3 Photographs and scanning electron micrographs of *Fusarium sp.* in potato stem tissues and *Botrytis cinerea* on tomato stem tissues. (**A**) Wilting symptoms in a potato field characteristic of a fungal wilt disease. (**B**) Fungal hyphae appressed to the host surface or projecting sub-aerially and attached to host vascular tissues (inset). Scale bar = 20 μm (5 μm – inset). (**C**) Hyphae embedded in remains of extracellular matrix. Compare with *Fusarium* bioiflms grown *in vitro* using the BEST Assay ™ (**Fig. 2C, 2D**). Scale bar = 10 μm. (**D**) *Botrytis cinerea* canker sporulating on tomato stem. (E-F) *Botrytis* biofilms on tomato stem show layering and bundling of hyphae and abundant. Compare with *B. cinerea* biofilms grown *in vitro* using the BEST Assay™ (**Fig. 2K, 2L**) Scale bars = 50 μm.

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