Plant-associated fungal biofilms—knowns and unknowns

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One sentence summary: The knowns and unknowns of plant-associated fungal biofilms are highlighted, and precisely, viewpoints are given on the relationship between biofilms and the benefits, disease potential, secondary metabolite production and climate change.

ABSTRACT

Nearly all microbes, including fungi, grow firmly attached to surfaces as a biofilm. Yet, attention toward fungal interactions with plants and the environment is dedicated to free-floating (planktonic) cells. Fungal biofilms are generally thought to configure interactions across and among plant populations. Despite this, plant fungal biofilm research lags far behind the research on biofilms of medically important fungi. The deficit in noticing and exploring this research avenue could limit disease management and plant improvement programs. Here, we provide the current state of knowledge of fungal biofilms and the different pivotal ecological roles they impart in the context of disease, through leveraging evidence across medically important fungi, secondary metabolite production, plant beneficial functions and climate change. We also provide views on several important information gaps potentially hampering plant fungal biofilm research, and propose a way forward to address these gaps.

Keywords: cell communities, filamentous fungi, infection, mycorrhizae, pathogen

INTRODUCTION

Many microbes, including fungi, predominantly grow as crowded cell communities known as biofilms—the matrix-bound and architecturally complex community used to exploit new environmental opportunities. Typically, biofilms grow attached to either biotic or abiotic surfaces and exhibit properties that are distinct from those of free-floating (planktonic) cells (Harding *et al.* 2017). Biofilms are not exclusively formed in a laboratory setting where they are often studied. However, they can develop in natural habitats as a product of one or more of the partner microbes (e.g. bacteria, protozoa and fungi) in a syntrophic consortium (Lohse *et al.* 2018).

Microbial biofilms were first observed in 1683 by van Leeuwenhoek on materials from his own mouth and in the 1860s described from bacteria by Louis Pasteur (Høiby 2014, 2017). Toward the late 1960s, many biofilm traits were discovered, including communication between cells, which was attributed to quorum sensing in the marine bacterium *Vibrio fischeri* (Kempner and Hanson 1968). These early observations inaugurated a contemporary and inclusive meaning of microbial biofilms, clearly making reference to coating of surfaces by a prokaryotic and/or eukaryotic cell mass that is embedded in a self-produced sticky matrix of extracellular polymeric substance (EPS) (Costerton *et al*. 1995, 1999; Ramey *et al.* 2004; Soll and Daniels 2016; Azeredo *et al.* 2017).

Fungal biofilms are implicated in diseases of animals and plants, yet great emphasis is put on bacteria and yeast biofilms (for reviews, see Ramey *et al.* 2004; Fanning and Mitchell 2012). This is in light of the overwhelming evidence that many species of fungi are increasingly becoming a global food security, wildlife, and human health concern (Dean *et al.* 2012; Savary *et al.* 2019; Fisher *et al.* 2020; Fones *et al.* 2020). For example, pathogenic species of fungi account for substantial yield losses in major calorie and commodity crops, such as wheat, rice, sugarcane, maize, potato, and soybean globally (Savary *et al.* 2019; Fones *et al.* 2020). This emphasizes the need to fortify current intervention strategies to effectively curb the spread of fungal infections, to many of which the influence of biofilms goes largely unnoticed. On the other hand, fewer studies report on biofilms from fungi existing in mutualistic arrangements with plants and other associated microbes even though the significant impact of these microbes on both plant growth and development in the rhizosphere has been reported (Martin *et al.* 2016; van der Heijden 2016; van der Heijden and Hartmann 2016).

The overarching theme is that the dearth of research on plant-associated fungal biofilms suggests that the scope of the influence to which these consortia of microbial cells underlie plant health is generally less acknowledged. Therefore, the current paper explores the 'knowns and unknowns' of fungal biofilms, and places great emphasis on fungi that form pathogenic and beneficial interactions with plants. In addition, a few illustrative examples of how biofilm formation in these organisms almost always explains their survival in diverse host environments are discussed in relation to secondary metabolite production, ecological niche fitness and climate change. Lastly, we present some challenges facing the fungal biofilm research field as it relates to plant-associated fungi, and propose some solutions to overcome those challenges.

FUNGAL BIOFILMS AND ASSOCIATION WITH DISEASE

Ecological preferences (e.g. aquatic sites or rhizosphere) and site-specific conditions are likely to influence the formation of biofilms and their morphological or anatomical features. However, the basic process of biofilm formation appears to be conserved among fungal species (Table 1). Drawing from other biofilm models (Donlan 2001; Blankenship and Mitchell 2006; Mowat *et al.* 2009), Harding *et al.* (2009) have presented the general process of filamentous fungal biofilm development, which serves as a useful tool for studying other fungi for which biofilm formation was not previously examined. In subsequent studies, Harding and co-workers compared plant fungal biofilms *in vitro* and *in planta* (Harding *et al.* 2010), in order to update a previously described six-stage model (Harding *et al*. 2009, 2017). Based on these studies, it is clear that the biofilm developmental process is closely similar to

that of yeasts and bacteria. In short, this entails attachment to a surface, colonization and growth (accompanied by EPS production), and maturation and detachment from EPS. This last phase generates loose asexual fungal cells (mainly conidia) that are able to seed new sites to restart the biofilm cycle (Fig. 1A, biofilm model).

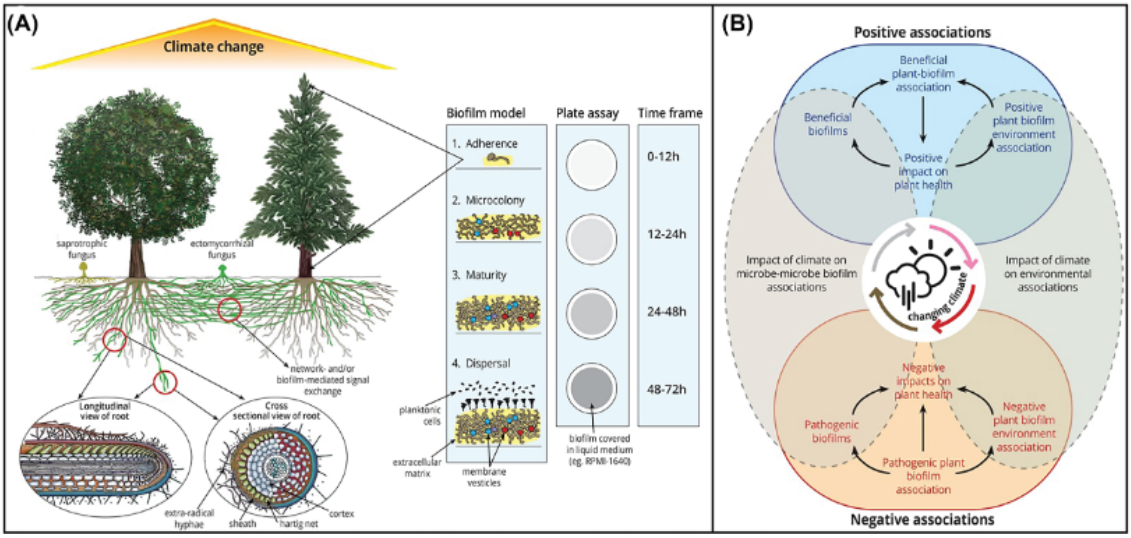

Figure 1. A model depicting plant-associated fungal biofilms and their complex interaction with climate change. **(A)** Biofilms formed on various plant surfaces, including on root tissues, can be studied following a typical 'biofilm model' (adherence → microcolony → maturity → dispersal). The reader is directed to Harding *et al.* (2017) for a more detailed description of this model. Whether this model holds true for biofilm formation in the rhizosphere is not yet clear. *In vitro* biofilm formation using a simple 'plate assay' and in liquid growth medium such as Roswell Park Memorial Institute (RPMI) 1640 (or potato/sabouraud dextrose broth with 2% glucose) can take up to 72 h to reach maturity and dispersal. **(B)** Theoretical schematic diagram showing the roles of climate and environment in influencing associations of beneficial and pathogenic fungal biofilms with plants. These associations are likely complex and, in combination with a changing climate, can positively or negatively influence plant health ('positive associations' and 'negative associations', respectively).

Fungal biofilms can colonize different anatomical parts of the plant (e.g. roots, stems and leaves) (Fig. 1A). This ability has largely been examined using biofilms formed on wood pegs (Harding *et al*. 2010, 2017, 2019). These studies have been used to identify biofilms formed by species from a diversity of plant damaging fungal genera, including *Botrytis*, *Didymella*, *Fusarium* and *Verticillium*. Experimental work from *Fusarium oxysporum* f. sp. *cucumerinum* further demonstrated that the biofilms of fungi exhibit tolerances to physical challenges including high temperatures and irradiation (Table 1), to which typically planktonic cells are vulnerable (Li *et al*. 2014, 2015). These studies fundamentally address the question of environmental adaptation mediated by fungal biofilms, which are important aspects relevant to fitness and pathogenicity in relation to plant health.

Table 1. Examples of filamentous fungi of plants with biofilm-related impacts.

Fungus	Reported biofilm-related functions/traits	Associated plant disease or benefit	Reference
Agaricus bisporus	Support for beneficial bacterial biofilm	Nutrient acquisition	Kjeldgaard *et al.* (2019)
Aspergillus niger	Support for beneficial bacterial biofilm	Black mold	Kjeldgaard *et al.* (2019)
Aspergillus niger	Biofilms reported on onion bulbs and found to be sensitive to mandarin essential oil	Black mold	Abdel-Aziz *et al.* (2019)
Aspergillus ustus	Support for beneficial bacterial biofilm	Promotes plant growth	Miquel Guennoc *et al.* (2018)
Botrytis cinerea	Biofilms reported to form on tomato stems	Grey mold disease	Harding *et al.* (2010)
Didymella bryoniae	Biofilms found colonizing wood pegs	Gummy stem blight	Harding *et al.* (2017)
Fusarium oxysporum	Stress-tolerant biofilm under laboratory conditions	Fusarium wilt	Li *et al.* (2014)
Fusarium sp.	Biofilms reported to form on potato stem tissue	A range of diseases	Harding *et al.* (2010)
Fusarium verticillioides	Biofilm reduced by fungicides	Mycotoxin contamination	Miguel *et al.* (2015)
Hebeloma cylindrosporum	Support for beneficial bacterial biofilm	Nutrient acquisition	Miquel Guennoc *et al.* (2018)
Laccaria bicolor	Support for beneficial bacterial biofilm, poplar	Nutrient acquisition	Miquel Guennoc *et al.* (2018)
Magnaporthe oryzae	Germling attachment on rice surfaces promotes infection	Blast on rice and wheat	Geoghegan and Gurr (2016)
Penicillium funiculosum	Support for beneficial bacterial biofilm	Fruitlet core rot	Miquel Guennoc *et al.* (2018)
Penicillium spp.	Mycorrhizae with soybean	Mycotoxin contamination	Jayasinghearachchi and Seneviratne (2004)
Phanerochaete chrysosporium	Support for beneficial bacterial biofilm	White rot	Miquel Guennoc *et al.* (2018)
Piloderma croceum	Support for beneficial bacterial biofilm	Nutrient acquisition	Miquel Guennoc *et al.* (2018)
Pleurotus ostreatus	Mycorrhizae with tomato	Nutrient acquisition	Jayasinghearachchi and Seneviratne (2004)
Rhizoglomus irregulare	Promotes nutrient uptake	Nutrient acquisition	Taktek *et al.* (2017)
Sclerotinia sclerotiorum	Biofilms found colonizing cellulose-coated plastic pegs	White mold	Harding *et al.* (2019)
Thelephora terrestris	Support for beneficial bacterial biofilm	Nutrient acquisition	Miquel Guennoc *et al.* (2018)
Tuber melanosporum	Support for beneficial bacterial biofilm	Nutrient acquisition	Miquel Guennoc *et al.* (2018)
Verticillium dahliae	Colonization of wood pegs	Verticillium wilt	Harding *et al.* (2010)

In the filamentous rice blast fungus *Magnaporthe oryzae*, chitosan (a partially deacetylated form of chitin) was found to mediate the adhesion of germlings (spores with a germination spike) to host plant surfaces, leading to differentiation of these spores into appressoria (Geoghegan and Gurr 2016). While chitosan is known to increase virulence in soil-borne fungal pathogens (Gao *et al.* 2019), adhesive germlings are probably characteristic of planktonic forms in filamentous fungi (e.g. asexual and sexual spores, hyphal fragments and sporangia) that initiate biofilm formation (Harding *et al.* 2009). Chitosan has also been reported to display biofilm stimulating properties in cyanobacteria (Kaushik *et al.* 2016), hinting at the likelihood that this compound can stimulate fungal biofilms. This is not farfetched given that chitosan is an essential component of the fungal cell wall, remodeling of which can influence biofilm formation. Recently, chitosan accumulation was reported to regulate conidiation and *N*-acetyl-glucosamine metabolism in the cell wall of *Aspergillus fumigatus* (Mouyna *et al.* 2020). Conidia are the key planktonic cells that initiate biofilm formation in filamentous fungi (Harding *et al.* 2009). Similarly, *N*-acetyl-glucosamine is a potent, indirect, inducer of fungal biofilms by inducing hyphal formation and expression of proteins required for adhesion (adhesins)

that promote biofilm formation (Naseem and Konopka 2015; Min, Naseem and Konopka 2020). Based on a recent report, it is possible that plant-derived *N*-acetyl-glucosamine (potentially through the *no perception 1* or NOPE1 protein) can mediate hyphal root colonization by biofilms of mycorrhizae in rice and maize (Nadal *et al.* 2017).

Purely from a fungal disease perspective, extensive knowledge of fungal biofilms is available in pathogenic species of clinical importance (Blankenship and Mitchell 2006; Nobile and Johnson 2015; Soll and Daniels 2016). In fact, these biofilms represent key sources of nosocomial infections that are tough to treat with commonly applied antimicrobial drugs. Examples include biofilms containing *Aspergillus* species, *F. solani* and *F. oxysporum* that are associated with severe infections of the cornea (Sharma 2017). Specifically in *A. fumigatus*, biofilm formation is implicated in invasive pulmonary aspergillosis and aspergilloma in immunocompromised patients, and in the resistance of this pathogen to antifungal drugs (Kaur and Singh 2014). There are numerous similar examples from the literature highlighting the impact of biofilm formation on fungal infections and diseases (e.g. Nobile and Johnson 2015; Soll and Daniels 2016; Kirchhoff *et al.* 2017). Since fungal pathogens commonly exploit biofilm formation for infecting humans, it is likely that many fungi also use biofilms to promote disease in their plant hosts (Table 1).

Although the link between filamentous fungal pathogen biofilms and disease has not been thoroughly examined in the plant pathology domain, a few examples where biofilm formation possibly impacts plant infections have come to light. A recent analysis using scanning and fluorescence microscopy showed that an isolate of *Aspergillus niger* forms biofilms that are probably partly responsible for the infection observed on onion bulbs (Abdel-Aziz, Emam and Elsherbiny 2019). These biofilms were observed as hyphal bundles coupled to exopolymeric substances (i.e. EPS) after 7 days. Similar examples where disease causing plant fungal pathogens form biofilms, albeit *in vitro* or *in planta*, were reported in the last decade or so (Harding *et al.* 2010; Li *et al*. 2014, 2015; Miguel *et al.* 2015, 2017, 2019 and references therein), and these are in addition to other filamentous eukaryotic pathogens of plants, including the oomycete *Phytophthora parasitica* (Theodorakopoulos *et al.* 2011; Larousse *et al.* 2014; Larousse and Galiana 2017). These studies suggest that biofilm formation could be prevalent in many disease situations, including blasts, mildews, rots, rusts and wilts caused by fungi with a global significance. Further work demonstrates that certain plant pathogenic fungi, including *A. niger* and *Sclerotinia sclerotiorum*, form biofilms that can be targeted bymolecules that act as antifungal antibiofilm agents (Abdel-Aziz, Emam and Elsherbiny 2019; Harding *et al.* 2019). Therefore, there is relevance in studying the contribution of biofilms in pathogenicity and how we leverage this information to treat biofilm-related plant diseases.

FUNGAL BIOFILMS IN BENEFICIAL INTERKINGDOM INTERACTIONS

Plant-associated microbial communities are located on or inside the leaves, stems and roots. These communities are chiefly known to participate in the uptake of soil nutrients, promotion of stress tolerance and resistance to pests and pathogens as well as growth hormone production, typically in exchange for photosynthesis-derived carbon (Schlaeppi and Bulgarelli 2015; van der Heijden 2016). Microbes associated with plants achieve most of their roles via a collective effort established through microbe–microbe interactions. How plant health is beneficially modulated by fungal biofilms remains poorly defined, although

fungi can form beneficial associations with bacteria and plants as part of a single or mixed species biofilm. Notable examples include mycorrhizae with diverse species from the phyla Mucoromycota, Ascomycota and Basidiomycota (Plassard, Becquer and Garcia 2019). These fungi can derive functional heterogeneity in biofilms as subterranean hyphal networks connecting plants. However, it is not fully understood to what purview does biofilm formation influences how such networks bring water and nutrients to the roots.

It has been suggested that the underground hyphal networks form part of the biofilm maturation phase or part of the 'detachment' phase (Harding *et al.* 2017), especially because most microbes in nature exist as biofilms, including on plant roots (Fig. 1A). In the case of ectomycorrhizae, symbiosis with plants is initiated by the development of extra-radical hyphae that develop into underground mycelia networks and intraradical hyphae, which act as a point of contact between fungal and plant cortical cells (Plassard, Becquer and Garcia 2019). The extra-radical hyphae emerge from mycorrhizal sheaths developed outside the plant root surface, and extends into intercellular spaces between the plant cells in the cortical area of the root, forming a structure known as the Hartig net (Fig. 1A). The development of the sheath likely follows the steps initiating fungal biofilm development, and it is in-between layers of the sheath that EPS is deposited (Harding *et al.* 2017).

Underground hyphal networks formed by ectomycorrhizae play an important role in transmitting and sharing growth and defense signals (e.g. water, nutrients and minerals) between plants by linking the roots of different species (Fig. 1A). Plants connected through these mycorrhizal networks can spread toxins, allelochemicals or other defense cues through the network, and this may discourage 'uninvited guests' such as pathogenic and parasitic invaders (Gorzelak *et al.* 2015). The shared network signals may also be important for widely separated plants to protect themselves (e.g. by thickening their cell walls, priming or boosting the immune response and/or secreting insect repellents) by 'eavesdropping' on signals from other partner plants already under pathogen or pest attacks (Berendsen, Pieterse and Bakker 2012; Philippot *et al.* 2013). From these notions, it is reasonable to argue that mycorrhizae act as conduits for signalling molecules facilitating plant–plant dialogue (Fig. 1A). In other words, fungal biofilms are not only a source of these complex networks, but may also act as a strategy for enhancing the networks' resilience to ecological stress.

According to recent studies, existing fungal biofilms could act as a source of filamentous hyphal networks that provide a platform for adhesion and movement of soil bacteria beneficial to the plant (Crognale *et al.* 2019; Hao *et al.* 2020). Such associations are displayed by many fungal species, including *Laccaria bicolor*, *A. niger*, *Agaricus bisporus* and *Rhizoglomus irregular*, and act as drivers of nutrient fluxes between the soil and plants (Taktek *et al.* 2017; Miquel Guennoc *et al.* 2018; Kjeldgaard *et al.* 2019; Hao *et al.* 2020).

Because fungal biofilms can act in symbiotic relationships with plants (Tian *et al.* 2020), they could boost agricultural productivity (Klein, Siegwolf and Korner 2016). Fungi such as *Penicillium*, *R. irregular* and *L. bicolor* can physically interact with diverse soil bacteria (e.g. *Bradyrhizobium elkanii*, *Rhizobium miluonense*, *Burkholderia anthina*, *B. phenazinium* and *Pseudomonas fluorescens*) (Bianciotto *et al.* 2001; Jayasinghearachchi and Seneviratne 2004; Taktek *et al.* 2017; Noirot-Gros *et al.* 2018). These interactions often promote the secretion

of enzymes and compounds required for plants to fix nitrogen, liberate certain key nutrients needed by the plant or display plant growth-promoting properties (Table 1). Numerous reports of bacterial associations with fungal species (e.g. species of *Aspergillus*, *Pleurotus* and *Penicillium*) include those that promote the uptake of phosphorus by the plants (Hao *et al.* 2020). The absorption of phosphorus takes place in the extra-radical mycelium and is largely facilitated by the high-affinity fungal inorganic phosphate transporter PT2, where phosphorus is slowly translocated off the Hartig net to the plant cortical cells of the roots (Plassard, Becquer and Garcia 2019). Therefore, bacterial-fungal biofilm associations can agriculturally improve nutrient bioavailability in plants (Kaminsky *et al.* 2019; Tian *et al.* 2020).

FUNGAL BIOFILMS, SECONDARY METABOLITES AND MEMBRANE VESICLES

Fungi can influence plant health through production and secretion of secondary metabolites. Yet, the link between biofilms with secondary metabolite production is less explored in fungal biofilms, with most of the knowledge emanating from studies of medically important fungi. Opportunistic pathogens of mammalian species such as *Candida albicans* have been reported to elicit the release of arachidonic acid from host cell membranes. This is a polyunsaturated fatty acid and a primary linoleic acid product often converted to secondary metabolites including prostaglandin E2 (PGE2). PGE2 is widely studied in mammals and pathogenic fungal species (Pohl and Kock 2014). Upon release from arachidonic, PGE2 acts as a potent oxygenated metabolite (also known as eicosanoid) that exerts pleiotropic effects over many bodily functions in mammals including inflammation or immunity. Interestingly, exposure of fungi to PGE2 promotes dimorphism, biofilm formation, and ultimately host colonization (Alem and Douglas 2004, 2005; Krause, Geginat and Tammer 2015; Wang *et al.* 2017; de Carvalho and Caramujo 2018). Several reports have shown that *C. albicans* biofilms produce PGE2 at concentrations greater than that of planktonic cells (Alem and Douglas 2004, 2005; Ells *et al.* 2011; Fourie *et al.* 2017).

It is possible that filamentous fungal pathogens can colonize host tissues by deploying the production of secondary metabolites that tightly control or coordinate interspecies and interkingdom communication within a biofilm (Pohl and Kock 2014). Although this is yet to be verified for filamentous plant fungal pathogens, it has been demonstrated in the filamentous human fungal pathogen *A. fumigatus* (Zheng *et al.* 2015). The study found that mycotoxins released by cells in biofilms can regulate oxidative stress and act as interspecies signalling molecules in mixed species biofilms between *A. fumigatus* and bacteria. An earlier study used functional genomic profiling to show that a secondary metabolite gene cluster associated with the production of gliotoxin, a sulfur-containing mycotoxin with immunosuppressive properties, was highly induced in biofilm cultures (Bruns *et al.* 2010). The enhanced production of this toxin in *A. fumigatus* biofilm cultures, relative to culture supernatants, has been suggested to explain why the fungus is able to evade the immune system and cause infections in humans (Bruns *et al.* 2010). Taken together, these studies support the findings reported in eicosanoid studies in human pathogenic yeasts (Alem and Douglas 2004, 2005; Ells *et al.* 2011; Fourie *et al.* 2017), and may apply to fungal biofilms of plants.

Numerous studies suggest that membrane vesicles may be involved in toxin production and virulence by fungal biofilms. These include studies that show the role of these vesicles in

delivering microbial toxins from the pathogen to the target cell, including in enterotoxigenic *Escherichia coli* and *Bacillus anthracis* (Kesty *et al.* 2004; Rivera *et al.* 2010). Membrane vesicles are naturally formed by cells from all three domains of life (i.e., archaea, bacteria, and eukarya) during endocytosis (e.g. endosomes, multivesicular bodies and intraluminal vesicles) and exocytosis (e.g. microvesicles and exosomes). These vesicles are responsible for storing and transporting a trove of molecular cargos and other key materials within the plasma membrane and between cells. This effectively allows them to regulate cell–cell and interkingdom communication through bidirectional transfer of materials they carry (Woith, Fuhrmann and Melzig 2019). Notably, membrane vesicles have been discovered in the biofilms of several yeast fungal species (Brown *et al.* 2015 and references therein; Leone *et al.* 2018; Zarnowski *et al.* 2018; Dawson *et al.* 2020). These vesicles were concentrated in biofilms than in planktonic cells, and highly associated with the EPS (e.g. Zarnowski *et al.* 2018). Presumably, the prevalence of membrane vesicles in biofilms may represent a strategy in which biofilms produce high secondary metabolite (e.g. mycotoxins) levels than planktonic cells in fungi. It should be noted that the discovery of membrane vesicles in fungal biofilms has been made in non-pathogenic fungi, including the dimorphic yeast *Pichia fermentans* (Leone *et al.* 2018). This suggests that virulence is not the only function of membrane vesicles in biofilms, but nevertheless an important one.

ADAPTATION, FITNESS AND GENETICS OF FUNGAL BIOFILMS

Myriad aspects define adaption and fitness held by fungi in the environment and these include (*inter alia*) taking shelter in a biofilm EPS matrix, the deployment of gene exchange mechanisms and articulating a transcriptional profile that is unique to that of planktonic cells. Many of these mechanisms have been described in the biofilms of several fungal species (Nobile *et al* 2012; Gravelat *et al.* 2013; Lee *et al.* 2016), and are often ascribed to the relentless adaptability of human pathogens (Fanning and Mitchell 2012), yet remain elusive in plant fungal pathogens.

EPS provides the encased microbial cells with several rewards, including protection from wide-ranging environmental conditions (e.g. UV radiations, rapid changes in pH, nutrient limitations, antimicrobials agents etc.) and allows the exchange of beneficial genes, metabolites and nutrients (Zarnowski *et al.* 2014; Costa, Raaijmakers and Kuramae 2018). EPS keeps the biofilm cells together since it is made up of several important components, including exopolysaccharides and nucleic acids (e.g. extracellular DNA and RNA), proteins and lipids. Taken together, these components constitute what has recently been called the 'matrixome' (Karygianni *et al.* 2020). As a result, EPS has come to be seen as the hallmark for structural support and protection of cells within a biofilm (without which, the cell mass is not a full-fledged biofilm) that effectively enables these cells to tap into shared community resources. Therefore, a detailed analysis of microbial 'lifestyle in a gel matrix' will lead to an in-depth understanding of complex communities of multiple interacting species. Given these views, we explain life in the matrix in terms of two scenarios: first, EPS affords the microbes a recalcitrant phenotype, and second, the arrangements of the polysaccharides in the EPS determine the nature of the 'barrier'.

In the first scenario, EPS allows remodeling of the microbial biomass to be phenotypically distinguishable from the planktonic cells through the maintenance of the structural integrity of the biofilm. EPS thus subscribes microbial cells to a crowded

community to such an extent that biofilms, when matured, can be visualized by an unaided eye (Fig. 1A, plate assay). In the second scenario, exopolysaccharides in the EPS interact physically, so as to not only provide mechanical stability to the biofilm but also modulate antimicrobial sequestration, immune evasion (by masking of fungal cells within the biofilm from recognition by the host immunity cells) and virulence (Costa, Raaijmakers and Kuramae 2018). The arrangement of different polysaccharides and their physical interaction enables the matrix to act as a physical barrier to antimicrobial infiltration or biotic and abiotic stressors. In filamentous fungi including *A. fumigatus*, partial deacetylation of the exopolysaccharide galactosaminogalactan (GAG, composed of α-1,4-linked galactose and *N*-acetylgalactosamine) mediates attachment to the host and masks fungal cells from host immune attack (Gravelat *et al.* 2013; Lee *et al.* 2016). The GAG gene cluster includes a glucose 4-epimerase gene (or *uge3*) that is required for GAG biosynthesis; this process appears to be highly conserved in fungi and analogous to the bacterial biofilm glycosyltransferase systems (Gravelat *et al.* 2013; Lee *et al.* 2016). It is therefore possible that a similar system contributes similarly to fungal cell attachment to plant tissues, biofilm formation and pathogenicity.

Further contributing to adaptability of fungal biofilms to the environment are a range of genetic exchange mechanisms. The acquisition of genetic material can contribute to the adaptability of fungal biofilms and the fungi themselves. The species boundaries of these organisms were recently considered as 'semipermeable' (Steenkamp *et al.* 2018). Permeability of species boundaries can influence pathogenic and virulent phenotypes, including the ability of fungi to develop better biofilms. Indeed, biofilms appear to be a 'hotbed' for genetic exchange achievable through horizontal gene transfer (HGT) (Abe, Nomura and Suzuki 2020). This process has been implicated in the development of numerous disease phenotypes in plants (Ma *et al.* 2010; Fitzpatrick 2012; Soanes and Richards 2014; Qiu *et al.* 2016; Feurtey and Stukenbrock 2018). Biofilms may thus be an important vehicle for HGT as the microbial cells are kept in close proximity, which facilitates the spread of virulence genes between different species in a biofilm. Extracellular DNA in the EPS of *C. albicans* and *A. fumigatus* has been found to increase biofilm biomass (Martins *et al.* 2010; Shopova *et al.* 2013; Rajendran *et al.* 2014). In *C. albicans*, however, extracellular DNA is mostly non-coding (Zarnowski *et al.* 2014). Furthermore, close inspection of *C. albicans* biofilm gene regulation has revealed interaction of a regulatory network with relatively 'young' genes, some of which suggested to have been acquired through HGT (Nobile *et al.* 2012). However, additional studies are needed to unpack how HGT-mediated genetic exchange can influence environmental adaptation through biofilms formed by plant fungal pathogens. Biofilm-mediated genetic exchange in *C. albicans* appears to be dominated by mating (Fanning and Mitchell 2012). For example, biofilms promote efficient mating by providing protection to the underlying mating competent cells and by stabilizing the pheromone gradients between the competent mating cell partners (Daniels *et al.* 2006; Ene and Bennett 2014). Biofilms are also reliant on these gradients and pheromone signaling networks for structural thickening (Daniels *et al.* 2006). This likely ensures the possible obscured genetic exchange functions and could be conserved or mostly utilized by plant fungi that form 'sexual biofilms', i.e. biofilms induced by sexual pheromones (Perry, Hernday and Nobile 2020).

With the help of transcription profiling, unveiling the differences between planktonic and biofilm cells has provided detailed insights into environmental responses of these growth forms. For instance, expression of genes involved in protein synthesis, multi-drug resistance, sterol biosynthesis, attachment, primary metabolism, and cell wall biogenesis is often increased during biofilm growth compared with planktonic growth (Fanning and Mitchell 2012). Many of these processes have been reported to play a role in biofilm fitness. The difference in terms of transcriptional regulation between planktonic cells and biofilms also provides key lifestyle features of how the two growth states adapt to their surroundings. This has been demonstrated in *C. albicans*. In this human yeast fungal pathogen, an intricately large biofilm regulatory network, controlled by nine core transcriptional regulators (TRs), coordinates *in vitro* and *in vivo* biofilm development (Perry *et al.* 2020). This TR network controls ∼1000 target genes, some of which are additional TRs and might largely explain why *C. albicans* biofilms respond flexibly to environmental stimuli such as stress or antifungal treatment (Nobile *et al.* 2012). In *A. fumigatus* and other filamentous fungi, such a comprehensive network is yet to be defined. In the case of *A. fumigatus*, a TR (SomA) that corresponds to the Flo8/Som1 regulator described in other fungi operates downstream of the cyclic AMP/protein kinase A pathway to regulate biofilm formation (Lin *et al.* 2015). SomA, in partnership with other TRs important to biofilm formation, enables the biofilms of this fungus to manipulate the environment more effectively than its planktonic cells (Lin *et al.* 2015).

PERSPECTIVES ON FUNGAL BIOFILMS AND CLIMATE CHANGE

Climate effects are naturally or anthropogenically driven, and such effects touch nearly all life on earth, including plants and microbes (Fig. 1B). They can also significantly reshape biological interactions, which might permit microbes to mount a planktonic- and/or community-level response. Microbes are pertinent to climate change according to a recent consensus statement dealing with climate–microbe interaction (Cavicchioli *et al.* 2019). Similarly, microbial biofilms are important role players in soil and plant ecosystems (Ahmad and Husain 2017). Therefore, climate–biofilm effects will surely impose unpredictable consequences for the health of plants (Fig. 1B). The effects of climate changes in increasing the frequency and severity of plant diseases by pathogens has been well documented (for reviews, see Compant, Van Der Heijden and Sessitsch 2010; Bidartondo *et al.* 2018; Velásquez, Castroverde and He 2018; and Chanda *et al.* 2020). However, there is little focus on the effect of climate change on biofilms in this regard and most current studies seemingly focus on biofilms in aquatic systems (Villanueva *et al.* 2011; Smith *et al.* 2016; Mohit *et al.* 2017; Kent, Garcia and Martiny 2018). These studies seem to support a strong correlation between climate change and increased microbial biofilm formation. For example, biofilm formation by *Roseobacter* isolates increases in response to lower ocean oxygen solubility and high ocean temperatures, which is due to genomic variations influencing phenotypic responses (Kent, Garcia and Martiny 2018). Nonetheless, empirical evidence to support the impact of changing climate on biofilms of plant-associated fungi is still lacking, and this could be due to several reasons. For instance, it might be challenging to separate the short- from long-term climatic impacts (e.g. extensive drying, temperature variations and wetness) on the plant host and associated fungi. One way to address this is to study impacts of beneficial plant-associated fungi or negative impacts of pathogenic fungi on short- and mid-term large temperature, moisture and dryness variations on plants in controlled and field environments.

Biomass, composition and diversity of microbial communities play a critical role in plant ecological functioning. However, the impacts of climate change on beneficial plant–microbe interactions appear to be overlooked, although there are emerging themes from which we can conceptualize and comprehend the outcomes of such impacts as mediated by biofilms (e.g. Compant, Van Der Heijden and Sessitsch 2010; Classen *et al.* 2015; Cavicchioli *et al.* 2019; Crowther *et al.* 2019). Potential consequences that climate change has on biofilms could include an increase in pathogenic interactions with plants (Fig. 1B). Hypothetically speaking, climate change can cause biofilm structural upheavals and subsequent inadvertent resuscitation of 'persister' cells. Indeed, the resurgence of persister cells, which are often metabolically latent within the biofilm, has been linked to multidrug resistance in pathogenic bacteria and fungi (LaFleur, Kumamoto and Lewis 2006; Lewis 2008; Martins *et al.* 2018; Gollan *et al.* 2019). Equivalent persistent fungal cells could thus be tolerant to antifungal agrichemicals, and render fungicide disease intervention strategies ineffective. Of note, mycelial growth may preclude the need for fungi to develop a 'formal biofilm' simply by virtue of the structure of the mycelia (e.g. branching and septate thread-like hyphae). Under asexual reproduction, the hyphae can intermittently or regularly release stress tolerant spores that, due to exposure to different dispersal mechanisms, are capable of traveling over short and long distances during night and day conditions, respectively. However, this phenomena could lead to the settling of these asexual spores in environmental conditions that are conducive for formation of resilient biofilms.

Alternatively, climate change could negatively impact plants (Timmusk *et al*. 2014, 2019; Wang *et al.* 2019), in that the physiology and environmental adaptability of beneficial microorganisms may be altered during changes in climatic conditions (Pennisi and Cornwall 2020). In the Pinaceae forests in North America, for example, fungal diversity of mycorrhizae is predicted to drastically decline due to climate change (Steidinger *et al.* 2020); undoubtedly, this can cause ecologically poor plant performance and negatively impact forest ecosystems. By extension, these climatic effects may impact on the services provided by beneficial microbes if such services are dependent on biofilm formation. Climate changes can impact enormously on how beneficial microbes cooperate with plant immunity and potentially bestow pathogenic advantages by enabling otherwise benign fungi to cause disease. The ability for harmless species to acquire virulent traits has previously been demonstrated (Miskinyte *et al.* 2013; Pérez, Kumamoto and Johnson 2013; Proença, Barral and Gordo 2017).

CHALLENGES AND FUTURE DIRECTION

Fungal microbes often exist in single or multispecies biofilms colonizing anthropogenically altered or pristine natural habitats. Therefore, the ecological flora may depend as much, if not more, on biofilms. In essence, the ecological flora may depend as much, if not more, on biofilms. Research behind the contribution of fungi to plant ecological roles is, however, primarily focused on free-living cells, and for most filamentous fungal species, biofilms have escaped notice. Although the limited plant fungal biofilm research provides some explanations underlining the biofilm model, it also reveals 'unknowns' with questions still outnumbering answers for the modern-day plant biologist (Table 2). Among is a narrow understanding of *in planta* and virulence functions of biofilms, and how they could be altered by climate change. These are potentially exciting new areas of research in the field of plant pathology and ecology.

Table 2 Key knowledge gaps that limit current progress in the field of plant-associated fungal biofilms and proposals for the future.

Fungal biofilms and virulence: Biofilms for several pathogenic fungi of plants have been described, but there is currently a narrow understanding about how these biofilms contribute to plant infections. In only a few fungal species (e.g. *Botrytis cinerea* and some species of *Fusarium*) have *in vitro* biofilm morphologies and properties been compared with those produced *in vivo* (Harding *et al.* 2010). This knowledge gap can be addressed by developing robust *in planta* assays to test putatively virulent *in vitro* biofilm phenotypes of fungi. Such studies should preferably also be complemented with microscopic analyses of samples to provide insights into if and how biofilm-like structures contribute to virulence and disease of plants.
Beneficial fungal biofilms: Work on beneficial fungal biofilms remains highly lacking even though it is a common knowledge that beneficial fungi greatly influence plant communication and resilience to dynamic conditions below and above ground. Therefore, it is hoped that future biofilm studies will incorporate the different beneficial fungal biofilms formed under different environmental conditions (e.g. drought or other biotic factors) and define how these influence plant–microbe interactions and plant environmental responses.
The link between fungal biofilms, mycotoxin production and climate change: To date, the plant science community can only speculate on the interaction of climate change with secondary metabolism in fungal biofilms. Furthermore, fungal biofilms produce secondary metabolites, including mycotoxins, in higher concentrations, potentially impacting negatively on food and human health. An exciting frontier for the plant science community is to catalog the biofilm-forming capacity of mycotoxigenic fungi under conditions resembling changing climate. Complementary studies can then compare the level of mycotoxins in biofilms and planktonic cells. To the best of our knowledge, only one study has looked at biofilm formation in a plant mycotoxigenic fungus (i.e. *F. verticillioides*), establishing that the mycelia of this fungus are well organized in a biofilm-like fashion (Miguel *et al.* 2015). Perhaps the findings of this study will enable plant pathologists to begin to correlate fungal biofilm development with mycotoxin production.
Membrane vesicles in plant-associated fungal biofilms: There is increasing data supporting the role of membrane vesicles in fungal virulence, both in human and plant fungal pathogens. Furthermore, studies in pathogenic bacteria suggest these vesicles may play a role in fungal mycotoxin production. However, currently no study has analyzed the role of vesicles in plant-associated fungal biofilms. Work on the discovery of membrane vesicles involved in pathogenic and non-pathogenic yeast biofilms (Leone *et al.* 2018; Zarnowski *et al.* 2018) can be used by plant pathologists as a framework for studying membrane vesicles in plant-associated fungal biofilms.
Gene regulatory network underpinning plant-associated fungal biofilm formation and maintenance: There is very limited data on gene regulatory networks governing the development of fungal biofilms associated with plants, with much of our current understanding drawn from in-depth analyses of bacteria and yeasts;). Fungal 'omics' tools can be used to map gene regulatory networks and to provide key information on the spatial and temporal patterns of gene expression during the development of fungal biofilms where they occur in association with plants.

Moreover, we lack the scientific insight into interaction of fungal biofilms and climate change and how the pair will influence secondary metabolite production. This is relevant given the prediction that climate change will directly affect pre- and post-harvest mycotoxin contamination of most plant crops and foods derived from these crops (Paterson and Lima 2010; Magan, Medina and Aldred 2011; Medina, Rodríguez and Magan 2015). Therefore, biofilm fungal mycotoxin production and its association with climate change are likely to be of future importance for sustainable crop production and food supply. Our understanding of the gene regulatory network that governs biofilm development in plant-associated fungi is also far from comprehensive. Unpacking this regulatory network is essential to prioritize candidate genes that will represent targets for the development of antibiofilm agents to treat biofilm-related diseases in plants. Therefore, gene regulotry network analyses of plant-associated fungal biofilms will inform evidence-based development of antibiofilm agents for agricultural use, and offer unique opportunities for disease control.

Generally speaking, as plant and microbial ecologists, we need to proceed thoughtfully and in alignment with medical mycologists to formulate robust strategies to analyze how fungi grow, survive, adapt, and exploit hosts and resources through biofilms. As we think about how best to proceed, it is valuable to take leaf from biofilm research pertaining to fungi of medical importance such as *A. fumigatus*, *C. albicans*, *Cryptococcus neoformans* and *Exophiala dermatitidis*, *inter alia*, that are responsible for many chronic human diseases (e.g. Kaur and Singh 2014; Aslanyan *et al.* 2017; Kirchhoff *et al.* 2017; Lohse *et al.* 2018). Likewise, microbial ecologists need to increase the scope of knowledge by integrating research on biofilms' influence on the ecological response of fungi, and how this can be directed to improving plant productivity. This research is highly relevant to plant environmental responses and the relationship with the microbial 'mob response', as well as how to strategically combat or exploit this response for the benefit of plants and their ecosystems.

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