# Biofilms and extracellular vesicles of *Fusarium verticillioides* and their implications for virulence

**by**

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## Declaration of Originality

I, Chizné Peremore declare that the dissertation, which I hereby submit for the degree Master of Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

**C.V. Peremore**

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## Preface

Extracellular vesicles (EVs) can internalize and transmit payloads (e.g., proteins, nucleic acids, and secondary metabolites) from a source to a recipient cell. In fungi, EVs influence cellular and kingdom-level communications and are associated with a variety of virulence factors, including biofilms, an architecturally complex community often essential for microbes to survive harsh environments. Like all other cell types in fungi, biofilms produce EVs, however, these are not identical to their planktonic (free-living) counterparts. Nothing is known about the biofilm and EV properties of *Fusarium verticillioides,* an important maize pathogen, as well as other filamentous fungi.

The first chapter of this dissertation focuses on the importance of EVs, including their participation in plant-microbe interactions, with a particular emphasis on the involvement of nucleic acids, proteins, and secondary metabolites. The significance of fungi-derived EVs during plant-pathogen interactions in crops such as maize has yet to be thoroughly investigated. Furthermore, while the involvement of fungal EVs in biofilm formation has been described in a small number of human fungal pathogens, it is not well understood for plant pathogenic fungi. Understanding how microorganisms, such as fungi, use EVs to cause plant diseases may lead to innovative therapeutics, such as using EVs as immune system boosters, disease biomarkers, and priming agents against abiotic and biotic stresses.

Chapter 2 focuses on biofilms, the aim of which was to characterize biofilm formation in *F. verticillioides* using *in vitro* assays. What precisely structurally resemble biofilms were observed and successfully characterized using techniques such as microscopy (Confocal Laser Scanning Microscopy (CLSM) and Scanning Electron Microscopy (SEM)) and colorimetric assays. These were performed to quantify metabolic activity, overall biomass and EPS of these so-called biofilms. To further strengthen the case that the structures formed by *F. verticillioides* under biofilm-inducing conditions indeed represents a biofilm, the extracellular polymeric substances (EPS) was extracted and quantified, and then, from it, extracted and analysed extracellular DNA (eDNA) and the effect DNase has on biofilm formation. Among other cell-free markers found to be associated with the EPS of a biofilm, such as extracellular proteins, RNA, and lipids, the eDNA is the most studied from the EPS of fungal and bacterial biofilms and has been reported to play many roles including in maintaining biofilm structural integrity

and resistance to antimicrobial compounds. This research examines the function of eDNA in a filamentous plant fungal pathogen for the first time, and it paves the way for future research on this fascinating facet of fungal biology.

Chapter 3 focuses on EVs that provide a method for the controlled transport of heterogeneous cargo from donor to recipient cells. The aim of chapter 3 was to isolate and characterize EVs derived from and compare their biophysical properties as they pertain to their donor cells i.e., planktonic and biofilm cells. During this study, *F. verticillioides* vesicles were isolated from biofilms and planktonic cells and characterized following the International Minimal Information for Studies of Extracellular Vesicles guidelines, which included transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA), and EV uptake studies.

The research for this project was conducted at the Forestry and Agricultural Biotechnology Institute (FABI), and the Department of Biochemistry, Genetics, and Microbiology (BGM) at the University of Pretoria. This study was performed under the supervision of Drs Thabiso Motaung and Quentin Santana, and Professor Emma Steenkamp. The dissertation is divided into three distinct sections, with certain content and references repeated. The first chapter is prepared as a literature review study based on existing literature, and the second and third chapters are written in the manner necessary for submission to scientific publications.

# Chapter 1:

# Literature review

## 1. Introduction

Extracellular vesicles (EVs) important function as mediators of signals from source to recipient cells throughout all kingdoms of life (Gill et al., 2019). EVs fulfil their functions as a consequence of encapsulated internalized payloads (e.g., proteins, nucleic acids, lipids and secondary metabolites) and payloads that are carried on their membrane surfaces (e.g., transmembrane proteins and lipid rafts) (Bielska et al., 2018; Kalra et al., 2012). These cargos are involved in both localized and distant cell-to-cell communication, but also in inter-organismal and inter-kingdom communication, such as in the case of pathogens interacting with their hosts (Bielska et al., 2018; Kalra et al., 2012). Oftentimes, EVs internalize their cargos from producer cells, which largely explains why EV properties and functions overlap with those of their parent cells (Bleackley et al., 2019; Cai et al., 2018; Costa et al., 2021; Regente et al., 2017).

According to the International Society for Extracellular Vesicles (ISEV), the term "extracellular vesicle" represents a generic phrase for particles spontaneously released from the cell that is bounded by a lipid bilayer and cannot multiply, i.e., they do not include functioning nuclei (Théry et al., 2018). EVs are grouped into distinct categories (i.e., exosomes, apoptotic bodies, and ectosomes) based on their size and mode of release (Mathieu et al., 2019). The phrase "extracellular vesicle" is the recommended general word and in this review, the term EV will predominately be used (Théry et al., 2018).

Exosome-type EVs have been identified in both pathogenic and non-pathogenic microbes, as well as in plants in recent decades and have essentially led to redefining the area of plant-pathogen interactions (Bleackley et al., 2019, 2020; Cai et al., 2018; Gill et al., 2019). However, in comparison to mammalian and bacterial vesicles, research on fungal EVs is presently in its infancy, with most studies focusing on pathogenic yeasts that infect humans (Rodrigues et al., 2015; Rybak and Robatzek, 2019). However, *in vitro* functional studies of vesicles support a variety of fungal EV functions in host–pathogen interactions, including several reports showing that EVs play a role in virulence factor production such as biofilm formation (Wang et al., 2015).

Given the above as background, this literature review discusses what EVs are, and role of EVs in plant-microbe interaction whereby the focus specifically is on the involvement of nucleic acids, proteins, and secondary metabolites. Uncovering the role

of EVs in microbe-host interaction may provide new insights into how microbes, especially fungi, exploit non-canonical pathways to produce plant illnesses, understanding of which will lead to novel therapies, such as employing EVs as immune system boosters, disease biomarkers, and priming agents against abiotic and biotic stressors.

## 2. Current understanding of EVs

EVs are membrane-bound structures that participate in cell-to-cell material transfer (Rutter and Innes, 2017). They are implicated in a diversity of functions in fungi, including molecular cell wall transport (Rodrigues et al., 2007), drug resistance (Zarnowski et al., 2018), virulence factor delivery (Ikeda et al., 2018; Rodrigues et al., 2007) and export of RNAs (Cai et al., 2018; da Silva et al., 2016). In humans, EVs are sub-classified depending on size and manner of biogenesis but it is uncertain if the same system holds in fungi. This is despite the isolation of fungal EVs of various sizes that correlate to EVs typically defined in other systems.

Among the various EV types (e.g., ectosomes and apoptotic bodies – size range: 0.1-2 μm), exosomes (~30-150 nm) appear to be the most studied, especially in mammalian systems (Doyle and Wang, 2019; Pariset et al., 2017; Zaborowski et al., 2015). Exosomes are the smallest vesicles that are produced during the exocytosis of multivesicular bodies (MVBs) containing intraluminal vesicles (ILVs) (Yáñez-Mó et al., 2015). On the other hand, MVBs are produced during endocytosis, and once made, they can merge with the receiving cells' lysosomes or plasma membrane to release ILVs, at which point they are referred to as exosomes as they are released during exocytosis (see Figure 1A) (Doyle and Wang, 2019; Yáñez-Mó et al., 2015; Zaborowski et al., 2015).

Larger vesicles such as ectosomes are formed by budding or pinching off from the plasma membrane, thereby promptly releasing their contents into the extracellular space (Figure 1A) (Cocucci and Meldolesi, 2015; Doyle and Wang, 2019). Ectosomes have similar roles to exosomes and share similar markers (Doyle and Wang, 2019). However, they differ from exosomes not only in terms of size but also in terms of transmembrane proteins and other markers of EV identification (Doyle and Wang, 2019). In exosomes, endocytosed transmembrane proteins are transported to early endosomes, while in ectosomes outward membrane budding is promoted by

transmembrane proteins clustered in discrete membrane domains (Cocucci and Meldolesi, 2015). Tetraspanins, small transmembrane proteins that are critical for trapping both membrane and luminal proteins, are abundant in exosome membranes. In addition, the membrane of ectosomes contains many proteins, including receptors, glycoproteins, and metalloproteinases (Meldolesi, 2018).

EVs transport bioactive compounds as transmembrane proteins and/or as internalized cargos from the parent cell, either to cells nearby or cells distant from the parent cell (Figure 1B). The absorption and transfer of EV-associated molecules by recipient cells happen via evolutionary conserved processes such as membrane fusion and phagocytosis, clathrin-mediated endocytosis (Gurung et al., 2021; Mulcahy et al., 2014; Rodrigues et al., 2015). Knowing how EV-associated cargos are taken up by recipients is critical for their usage as potential therapeutic agents.

## 3. EVs in plant-microbe interaction and fungal virulence

Over the last few decades, the understanding of plant-microbe interactions has improved substantially, but how EVs are involved in the process remained largely unclear. This is attributed to significant technological difficulties, such as inadequate EV identification, as well as the presumption that EVs cannot be released and picked up by plant cells due to their cell walls (Rybak and Robatzek, 2019). While the function of EVs in cell-to-cell contact has been well-studied in a variety of human pathogens, researchers have only recently started to focus on the role of EVs in controlling the interaction between plants and microbes (He, et al., 2021; Regente et al., 2017; Zhou et al., 2022a). This has led to the realization that EVs are integral to inter-microbial and host-microbe interactions, which frequently also involve the formation of virulence factors such as biofilms, as well as surface binding and host cell immunomodulation (Rybak and Robatzek, 2019; Zhou et al., 2022b).

The study of membrane vesicles, called outer membrane vesicles (OMVs) since they are derived from the outer membrane of Gram-negative bacteria, has contributed significantly to the understanding of EVs at the host-pathogen interface (Pathirana and Kaparakis-Liaskos, 2016). For instance, a study by An et al., (2006) on the powdery mildew pathogenic fungus shows that in barley leaves, multivesicular structures contribute in a cell wall-associated defence response. Another study by Bahar et al. (2016), shows that plants can detect and respond to OMV-associated molecules

generated by bacteria by activating their immune systems. Another study by Ionescu et al. (2014) showed that OMVs are involved in the migratory abilities and pathogenicity of *Xylella fastidiosa*. However, surprisingly, little is known about EVs produced by plant-interacting microbes, despite the prevalence of EVs in cultured phytopathogens and plant samples contaminated with microbes. Based on the previous work on mammalian models, EVs produced by plant pathogens would play a key role in intracellular communication between cells to maximize the pathogen's possibility for successful infection. EVs are also expected to be involved in intracellular communication between the pathogen and the plant host by eliciting prototypic pattern-triggered immune responses by delivering small RNAs for suppressing host immune response genes (Bleackley et al., 2019; Rybak and Robatzek, 2019). In response to infection, plants produce and load EVs, which serve as a protective mechanism, transporting silencing small RNAs (sRNAs) into pathogens by silencing target genes during infection (He et al., 2021; Cai et al., 2018).

## 4. Key breakthroughs regarding fungal EV research

In 2007, a landmark study found that RNAs (messenger RNAs and microRNAs) were predominantly carried by EVs of mouse and human cells, providing one of the first important indications that cargo associated with EVs could have physiological effects on target recipient cells (Valadi et al., 2007). Recent studies, including one by Cai et al. (2018), have since shed light on the role of EVs in facilitating cargo exchange between plants and their pathogens, specifically RNAs. Key developments in fungal EV research mostly emerged from studies on species such as *Cryptococcus neoformans, Candida albicans*, *Pichia fermentans* and *Fusarium oxysporum* (Figure 2).

Fungal EVs were first reported in 2007 from *C. neoformans* in a study that explored how a virulence-associated polysaccharide reaches the extracellular environment (Rodrigues et al., 2007). A year later, the same research group demonstrated the role of EVs in transporting pathogenesis-related to the effector cells of its human host (Rodrigues et al., 2008). A few years later the first report of filamentous fungal EVs by *Alternaria infectoria* was reported (Silvia et al., 2014). In 2015, da Silva et al. (2015) reported that RNA is exported by fungal EVs (da Silva et al., 2015). Two years later, research showed that in *P. fermentans* EVs have a role in the development of biofilms

(Leone et al., 2018). In 2018, EVs were found at the plant-fungal interface and may have resulted from multivesicular bodies-plasma membrane fusion (Cai et al., 2018). In addition, in 2018, *C. albicans* biofilm EVs and endosomal sorting complex required for transport (ESCRT) machinery (Zarnowski et al., 2018), as well as Cryptococcus gattii EVs and its part in virulence transmission (Zarnowski et al., 2018), were investigated (Bielska et al., 2018). In 2020, phytotoxicity in *F. oxysporum f. sp. vasinfectum* and the role of EVs were studied (Bleackley et al., 2020), and in *C. neoformans* virulence which is mediated by EVs was also interrogated (Hai et al., 2020). Taken together, these pioneering studies have led to the exploration of the EV-mediated relationships between plants and microbes (Motaung and Steenkamp, 2021).

## 5. Diverse cargos are carried by EVs

EVs have been shown to function in a range of signalling pathways (Yáñez-Mó et al., 2015), including those that govern the outcome of interactions between plants and their microbial pathogens. Notable examples include calcium signalling and mitogen-activated protein kinase (MAPK) activation (Yáñez-Mó et al., 2015). Due to the variety of payloads carried by EVs, these structures have a wide range of biological effects. Some of these bioactive compounds are briefly addressed in the sections that follow.

### 5.1. Nucleic acids

Many studies have now confirmed that EVs can disseminate nucleic acids (Bielska et al., 2018; Kalra et al., 2012). These structures are known to mediate long-distance transport of both coding and noncoding RNAs in mammals (Valadi et al., 2007). RNAs that are protected within the lumen of an EV are transferred to distant cells, where they continue to function after delivery (Ratajczak et al., 2006; Valadi et al., 2007). However, the RNA most often found in EVs are noncoding small RNAs (sRNAs), which range in size from 21 to 24 nucleotides (nt). These RNAs are important signalling molecules and involved in a wide range of processes, including plant growth, reproduction, and defence (Samad et al., 2017).

Based on their synthesis and mechanism of action, sRNAs are classified into two types: microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Song et al., 2019; Zhu et al., 2019), and both types have been recovered from EV preparations (Song et al., 2019; Zhu et al., 2019). It is also possible that EV-associated RNAs function as

alarm signals or as a way of communicating the metabolic condition to neighbours in order to enable group behaviours (Tsatsaronis et al., 2018). The protozoan pathogen *Trypanosoma cruzi*, for example, was discovered to bundle tRNA fragments in intracellular vesicles that were then released into the surrounding media and transmitted to other parasites and host cells (Garcia-Silva et al., 2020). This suggests that EV-associated RNAs from various pathogenic and symbiotic species may not only be transported to host cells, but can also impact gene expression by sequence-specific targeting of host gene transcripts (Tsatsaronis et al., 2018).

DNA is also known to be associated with EVs, although most studies did not explore the role of this nucleic acid in detail, thereby leaving a gap in the knowledge regarding the role of EV-associated DNAs in the biology of organisms (Elzanowska et al., 2021). Nevertheless, the distribution of genomic and mitochondrial DNA varies among EV subpopulations (Malkin and Bratman, 2020). In human cancer and immune diseases, for example, EV-associated DNA is involved in pathological intercellular communication (Elzanowska et al, 2021). Accordingly, EV-associated DNA can be used in liquid biopsy applications for disease detection and management because it is a type of cell-free DNA (Malkin and Bratman, 2020). Additionally, EVs extracted from planktonic cultures of *Streptococcus mutans* were shown to include DNA (Liao et al., 2014). In this microbe, EV-associated DNAs are thought to functions as a structural component of biofilms (Bose et al., 2020). In fact, EVs and their DNA cargo play a role in biofilm formation, as well as bacterial colonization. There have been no reported investigations of EV-associated DNAs from fungal species, although similar outcomes may be seen when investigated.

## 5.2. Proteins

The protein contents of EVs are cell and disease-type specific, conferring unique characteristics to the EVs that influence their biological functions (Rosa-Fernandes et al., 2017). Proteomic technologies, western blotting, and fluorescent-activated cell sorting are used to catalogue the protein components of EVs obtained from various cell types and biofluids. The majority of EVs cargo proteins identified to date are essential for cellular, metabolic, and biosynthetic processes (Bleackley et al., 2019). Table 1 below summarizes a few of the protein/cargo of interest known specifically to be associated with fungal EVs, and these will be discussed briefly in this section.

Common proteins discovered in EVs from fungal cells of many species include those involved in carbohydrate/protein metabolism, stress response, transport, oxidation/reduction, translation, and signalling, while others are unique to certain fungal species (reviewed in de Toledo et al., 2018). Examples of proteins normally isolated from fungal EVs include proteases, heat-shock proteins, fungal cell wall proteins, nuclear proteins, mitochondrial proteins and polyketides synthases (Bleackley et al., 2020; Garcia-Ceron et al., 2021; Hill and Solomon, 2020; Rodrigues et al., 2008; Silva et al., 2014).

Polyketide synthases have been identified to play an important role in the biosynthesis of toxins in the fungal pathogens of maize (*Cochliobolus heterostrophus*), wheat (*Fusarium graminearum*), and banana *(Pseudocercospora fijiensis*) (Garcia-Ceron et al., 2021; Noar et al., 2019; Yang et al., 1996). Enniatin synthase, also detected in EVs of *F. oxysporum f. sp. vasinfectum*, are involved in the production of cyclic mycotoxins responsible for cytotoxicity and anticancer properties (Garcia-Ceron et al., 2021; Liuzzi et al., 2016). EVs of *F. oxysporum f. sp. vasinfectum* can also internalize tyrosinase, which is involved in the synthesis of pigments such as melanin (Eisenman and Casadevall, 2012), a pigment thought to increase the virulence of fungal plant pathogens (Garcia-Ceron et al., 2021). Several proteases, a family of proteins that contribute to the pathogenesis of various fungi, are also packaged into EVs (Bleackley et al., 2020; Olivieri et al., 2002). Furthermore, many proteins with unclear functions have been found to be associated with EVs in, e.g *F. oxysporum f. sp. vasinfectum* and *Penicillium digitatum* (Bleackley et al., 2020). A subset of the undescribed proteins, as well as proteins that have been inaccurately annotated based on sequence homology, are likely to have a function in pathogenesis but this hypothesis needs to be tested further.

In addition, other proteins associated with EVs include guanosine triphosphate(GTP) binding carbohydrate metabolism, peptidases, GTPase activity, the unfolded protein binding, and ribosome subunit proteins (Bleackley et al., 2020; Garcia-Ceron et al., 2021). Basic cellular metabolic and biosynthetic processes were mostly identified by these cargo proteins. This broadly mirrors the annotated functions of EV proteins in other fungi, especially considering the recent findings that fungal EVs are consistently enriched in proteins involved in protein and carbohydrate metabolism, as well as cell

wall maintenance and remodelling (Bleackley et al., 2020; Garcia-Ceron et al., 2021; Hill and Solomon).

Knowledge about EV-associated proteins have also aided research into marker proteins that can be used to investigate the purity of EVs. For example, during the preparation of EVs for further study, crude extracts are passed through magnetic beads that have been coated with antibodies against common EV surface proteins (Carnino et al., 2019). Although this method offers high selectivity, some category of EVs elute in solution and cannot be isolated in the absence of surface protein markers selected for separation(Carnino et al., 2019). It is thought that such lack of protein markers and adequate isolation procedures have impeded research into EVs from filamentous fungi. The mammalian marker proteins (e.g., integrins, selectins, and CD40) are either absent from fungi, or their homologs are not present in EVs (Dawson et al., 2020; Pinedo et al., 2021).

Heat shock proteins (Hsp70) is among the few proteins identified in most of the previously reported proteomic data sets for many fungal species such *as C. albicans* and *C. neoformans* (Bleackley et al., 2020; Rodrigues et al., 2008; Hill and Solomon, 2021). It is uncertain if Hsp70-like proteins which are present in EVs and soluble supernatant is due to sample preparation which is contaminated or if the protein is released through other mechanisms. Due to the ambiguity, it is unlikely that Hsp70 can be used as marker in fungi until the presence of this co-occurrence is investigated (Hill and Solomon, 2020). Potential EV marker proteins from the human fungal pathogen *C. albicans* have recently been described and include Sur7 identified in *C. neoformans* and *Z. tritici* (Hill and Solomon, 2020; Rizzo et al., 2020). However, more research is needed to establish if Sur7 is loaded into EVs in planta and whether it is present in other species.

## 5.3. Secondary metabolites

Secondary metabolites are low-molecular-weight organic products that are often synthesized after growth of an organism has stopped (Keller et al., 2005). Secondary metabolites are formed by fungi, bacteria, or plants and do not specifically play a role in the normal growth, development, or reproduction of these organisms (Nigam et al., 2014). Instead, they usually mediate ecological interactions, which may result in a selective advantage for the organism (Wang et al., 2020). Upon contact, certain

secondary metabolites, depending on the concentration, may be harmful to humans and animals (Forbey et al., 2009). In fact, some secondary metabolites are highly toxic, and notable examples include fumonisin and ricin produced by the plants *F. verticilliodes* and *Ricinus communis* (Alexander et al., 2009; Miller, 2001). Numerous fungal examples of toxic secondary metabolites are also known (reviewed by Keller et al., 2005), and many of these are associated with severe medical conditions, while others can serve as virulence factors during pathogenic encounters with plants and animals (Macheleidt et al., 2016).

Secondary metabolite investigations on the nanovesicles (nanovesicles are similar to mammalian exosomes) produced by various plant species showed that lipophilic molecules, in particular, were vesicle-associated (Table 2). For example, Woith et al. (2021) discovered that curcuminoids and chlorophylls were abundant in *Nicotiana tabacum* vesicles. Their observations on secondary metabolites suggested that they are not actively packed into EVs but are enriched in the membrane of the EVs when sufficiently lipophilic. These findings supported the notion that plant EVs contain secondary metabolites, that thus can be delivered over short and long distances.

Research on fungal secondary metabolites have also shown that EVs are important for their secretion and transport (Table 2). For example, Chanda et al. (2009) demonstrated that *Aspergillus parasiticus* employs EVs to perform a range of functions, including the biosynthesis of aflatoxin, one of the most active naturally occurring carcinogens (Coppock and Christian, 2007). The role of EVs in the biosynthesis and export of aflatoxins was verified by inhibiting vesicle-vacuole fusion via disruption of the gene vb1 (encoding a protein in the small GTPase superfamily) and/or treatment with the compound Sortin 3. The vb1 gene which encodes a small GTPase which is known to regulate vesicle fusion in *Aspergillus nidulans*. While Sortin 3 blocks Vps16 (a protein in the class C tethering complex that permits intracellular molecules to be separated into various organelles) function. This seminal study demonstrated how the late steps in aflatoxin synthesis and storage that take place primarily in EVs and vacuoles (Chanda et al., 2009), and how these give rise to specialized trafficking vesicles known as Aflatoxisomes (Chanda et al., 2010). As the synthesis of aflatoxins begins, vesicle-vacuole fusion is stimulated by the global regulator Velvet, resulting in the build-up of aflatoxisomes containing most of the enzymes needed for aflatoxin synthesis (Chanda et al., 2010; Linz et al., 2012). As

these enzymes must either enter the vesicles via a shuttle mechanism or be present on their surface (Chanda et al., 2010).

Research on *P. digitatum* EVs revealed that tryptoquialanine, a major secondary metabolite and an important metabolite in the pathogenic process of this pathogen, is exported extracellularly by EVs (Costa et al., 2021). Investigations on this fungus showed that its EVs are enriched for mycotoxins such as fungisporin and indole alkaloids (tryptoquialanines), although they lacked the intermediates of the biosynthetic pathway of tryptoquialanine. The findings suggest that *P. digitatum* EVs and tryptoquialanine A have a novel phytopathogenic function, and that somehow this alkaloid is transported in EVs during plant infection.

During a study involving *F. oxysporum f. sp. vasinfectum*, a serious pathogen of the cotton plant, EVs from the pathogen were found to have a distinct purple colour (Bleackley et al. 2020). This was considered to be caused by a naphthoquinone dye packed into the EVs. A proteomic analysis of *F. oxysporum f. sp. vasinfectum* EVs showed that these vesicles are abundant in proteins involved in polyketide synthesis. Only a few studies have been conducted to investigate the involvement of EVs in the transfer of secondary metabolites from fungi to the host and environment.

## 6. Biofilms, secondary metabolite and EV relationships

EVs have also been shown to be formed by biofilm cells of some bacteria (e.g., *Helicobacter pylori, Pseudomonas aeruginosa*) and fungi (e.g., *C. albicans*), and have frequently been linked to harmful consequences of these pathogens (Leone et al., 2018; Wang et al., 2015; Zarnowski et al., 2018). A proteomic analysis revealed that planktonic and biofilm EVs contain a significant amount of unique cargo, including the induced during hyphae development protein 1 (involved in hyphal growth and virulence), survival factor 1 (for cell survival pathways), cell surface Cu-only superoxide dismutase 5 (escape innate immune surveillance), and survival factor 1 (survival in response to oxidative stress) (Zarnowski et al., 2018). These proteins are exclusively present in biofilms, and their rarity may help the biofilm with virulence and survival tactics. This may explain why biofilms act differently from planktonic cells. Furthermore, numerous proteins shared by vesicles from both origins were shown to be 10-to 100-fold more prevalent in fungal biofilm EVs compared to planktonic cells (Zarnowski et al., 2018).

The link between biofilm and vesicle has been extensively investigated, although the intricacies remain elusive. Studies conducted on bacterial vesicles determined that the release of OMVs by cells in response to various stress events increased the hydrophobicity of the cell surface, resulting in greater biofilm formation (Wang et al., 2015). It was shown that *H. pylori* have a great ability to produce biofilms and that its OMVs play an essential role in the production of the extracellular matrix of biofilms. In addition, Subra et al. (2010) investigated whether exosomes contained lipid-related proteins and bioactive lipids. The exosomes included all of the phospholipases (A2, C, and D), as well as associated proteins such as aldolase A and Hsp 70. The phospholipase D (PLD) / phosphatidate phosphatase 1 (PAP1) pathway was also present. Exosomes enriched in prostaglandin$E_2$ assist in tumour immune evasion and promote tumour development (Record et al., 2014).

The composition of biofilm EVs lead to two potential functions in biofilm extracellular matrix synthesis (Wang et al., 2015; Zarnowski et al., 2018). Firstly, vesicle composition is highly comparable to matrix composition in terms of protein and carbohydrate content, indicating that vesicles might be a key source of matrix material. Second, vesicle cargo may have a catalytic role in the creation of matrix macromolecules. A subset of ESCRT (endosomal sorting complexes required for transport machinery) subunits, which is a key pathway in a variety of cellular activities, including the formation of multivesicular bodies (MVBs) as well as improved matrix biogenesis and function by participating in the formation of biofilm EVs (Zarnowski et al., 2018).

Only a few studies have been conducted on mycotoxins and how biofilms influence their production. Because of their inherent microbial biomass, biofilms can emit more mycotoxins than planktonic cells (Bruns et al., 2010). For instance, studies show that as opposed to planktonic cells, biofilms contain more biomass, correlating with more compounds of importance, such as mycotoxins and other secondary metabolites (Henriques et al., 2016; Ramage et al., 2011). The above-mentioned findings offer a new aspect to the knowledge of EV function as they suggest that EVs play a role in the sharing of community resources, namely biofilm matrix material as it is with bacteria. Isolating and characterizing EVs from planktonic and biofilm populations may thus give quantitative and qualitative differences between the two, as well as clues about potential activities for EVs within biofilms.

## 7. *Fusarium verticillioides*

Numerous fungal species are pathogenic to plants, and one such pathogen is *F. verticillioides* (Blacutt et al., 2018). *F. verticillioides*, formally known as, is widely spread around the world and is particularly affiliated with maize (*Zea mays L.)*, where it can cause stalk rot and cob rot, resulting in severe yield losses and grain quality reductions (Blacutt et al., 2018; Gulya et al., 1980). The fungus was first identified in maize in Italy in 1877 (Yilmaz et al., 2021). *F. verticillioides* belongs to *the Fusarium fujikuroi* species complex (FFSC) in African clade A (Yilmaz et al., 2021), which is well-known for containing a large number of well-reported plant pathogens and mycotoxin producers (Kvas et al. 2009). *F. verticillioides* is a filamentous ascomycete fungus (Ma et al., 2013) and initially develops as white mycelia and develops purple pigments within the mycelia as it ages (Leslie and Summerell, 2006).

This fungus is frequently a concern for maize farmers in Africa, especially in Sub-Saharan Africa and by creating mycotoxins, which can concentrate in food and feed and damage humans and animals (Braun and Wink, 2018 and references therein). The fungus is most commonly found as an endophyte within hosts like maize (Blacutt et al., 2018). The endophytic pathogenic state is very temporary, with the fungus switching between a hemibiotrophic pathogen and a symptomless biotrophic condition depending on the plant's biotic and abiotic environment (Bacon and Hinton, 1996; Bacon et al., 2008). The mechanistic understanding of the molecular basis of *F. verticillioides* infections with regards to EVs and biofilms will be essential to developing reliable biocontrol to curb the spread of infections in maize plantations and post-harvest products and ultimately mitigate risks to the end-user.

### 7.1. Plant-fungus interactions and their importance

*F. verticillioides* infect mainly maize species but can also infect other plant species such as barley, wheat and soybeans (Castellá et al.,1999). As a result, it is not a host-specific pathogen, which suggests that the fungus' life cycle may also be influenced by a change in the host (Blacutt et al., 2018). *F. verticillioides* has an intimate host association, meaning it has constant evolutionary pressure on both the pathogen and host (Blacutt et al., 2018). It responds to the evolving features that come with the host and has necrotrophic and endophytic aspects that are associated with its host as mentioned (Blacutt et al., 2018).

In South Africa, maize (*Zea mays L.)* is an important crop and amounts to an average to be around 9.2 million tonnes per year, of which 8 million tonnes are used as food and fodder in the country (Ncube et al., 2011). Commercial producers in South Africa adopt loss-reduction measures because maize quality and yield are important in South Africa since 600 000 households rely on subsistence farming (Ncube et al., 2011). However, maize grown in subsistence farming systems is frequently impacted by both pre-and post-harvest damage, with fungal infection being one of the most serious issues. *F. verticillioides* can account for up to 95% of all *Fusarium* strains obtained from African maize fields (Ncube et al., 2011).

*F. verticillioides* may infect maize in a number of ways, the most common being airborne conidia infecting the silks and eventually the kernels. Another method is for systemic infection to begin with mycelia or conidia located in the seed or on the surface (Oren et al., 2003). The fungus grows inside immature plants before spreading to the stalk and infecting the cob and kernels (Oren et al., 2003). The fungus often develops as the kernels near physiological maturity and continues to proliferate until the conclusion of the growing season. According to electron microscopic examinations, the fungus is located at the tip cap of both symptomatic and asymptomatic maize kernels (Bacon et al., 1992; Leslie and Summerell, 2006).

Traditional plant-microbe interactions rely on the release of compounds and enzymes by microorganisms; for instance, the *F. verticillioides* fungalysin destroys class IV chitinases used in host defence in maize (Naumann et al. 2011). The orthologous wheat antimicrobial peptides, fungalysin, found in maize can block this new metalloprotease (Slavokhotova et al. 2014). Cross-kingdom signalling molecules appear to be crucial in influencing the consequences of the *F. verticillioides*, in addition to secreted effectors and their inhibitors and maize interaction (Blacutt et al., 2018).

Despite not having been thoroughly examined in *F. verticillioides*, some research has suggested that this pathogen may form a biofilm. For instance, a study on *F. verticillioides* was conducted by Miguel et al. (2015) on mycelial morphology and how fungicide affects it. They saw fine fibrils which allow for hyphae to attach and extracellular material around the hyphae, resembling biofilms. Fludioxonil and metalaxyl-M both had an impact on the development of these communities that resembled biofilms, suggesting that *F. verticillioides* is actually the organism that forms

biofilms. There has not been any research done to determine if these biofilms are important in the infection process of *F. verticillioides* with maize. However, when considered collectively, the research on biofilms in plants raises the real likelihood that fungal biofilms related to plants have an infectious potential that requires immediate attention.

## 7.2. EVs and biofilms

Little is known about EVs in *F. verticillioides* and the role of these EVs and their potential cargo is poorly understood. Mycotoxins may be present in the cargo, especially since mycotoxins such as aflatoxins have been shown to be synthesized in EVs (Bruns et al., 2010). Aflatoxins are furanocoumarins formed from polyketides that are principally produced by the filamentous fungus *A. parasiticus* and *Aspergillus flavus* (Bennett and Klich, 2003). Furthermore, Menke et al. (2013) suggested that type B trichothecenes are formed cellular vesicles known as "toxisomes," and found proof that the HMG-CoA reductase of the isoprenoid pathway localizes to toxisomes when toxins are created. In addition to the above-mentioned studies, Garcia-Ceron et al. (2021) discovered biosynthetic enzymes in EVs such as polyketide synthase indicating that EVs may carry phytotoxic secondary metabolites. Although the cellular processes involved in fumonisin synthesis have been reported in *Fusarium* and with fumonisin also being formed by a polyketide (Huffman et al., 2010), it is hypothesized that fumonisins could be synthesized and transported in EVs. As a result, it is hypothesized that EVs play a significant role in *F. verticillioides* toxin-mediated infection, and the same assumption can be extended to other mycotoxigenic fungal species.

As mentioned, EVs have also been shown to be formed by biofilm cells of some bacteria and fungi. This brings us to the importance of studying biofilms including the fact that once understood, they will offer new methods to study plant pathogens such as members of the genus *Fusarium*. Plant pathogen fungicide resistance is a big issue, and this results in significant treatment failures. There are many reasons why this could happen, but one of them could be the lack of understanding of how biofilm formation in fungi contributes to infections. For example, in *Candida*, one of the major human fungal pathogens, the biofilm protects the pathogenic yeast from antifungal drugs and the hosts immune system (Seneviratne et al., 2008). Left unnoticed and

untreated, the biofilms can cause superficial and systemic infection in patients and makes it difficult to treat (Cavalheiro and Teixeira, 2018; Villa et al., 2017). Biofilms generated by filamentous fungal pathogens, such as filamentous yeasts, have been significantly associated with clinical illnesses. For example, *F. solani* biofilms have a role in the etiology of fungal keratitis, a corneal infection of the eye (Mukherjee et al., 2012). *Botrytis, Didymella, Fusarium*, and *Verticillium* isolates have been found to produce biofilms in plants, which may contribute to the diseases caused by these fungi (Harding et al., 2010, 2017).

Biofilms also develop more biomass than planktonic cells (Henriques et al, 2016; Ramage et al., 2011), and hence more compounds of interest such as mycotoxins, enzymes, and other important agents. According to Bruns et al. (2010), mycotoxin-related genes were increased when *A. fumigatus* was developing as biofilm cultures, which may shield the fungus from the immune system while the host is in the disease state. Therefore, it is hypothesized that *F. verticillioides* biofilms have a high concentration of fumonisin, some of which are contained in EVs, as compared to free-living (planktonic) cells, and that EVs contribute to the extracellular release of fumonisin.

### 7.3. Secondary metabolites of *Fusarium verticillioides*

The ability of the fungus to generate secondary metabolites, especially mycotoxins, is important as they negatively influence animal and human health (Venkatesh and Keller et al., 2019). Mycotoxins are defined as "small organic compounds generated by a filamentous fungus that elicit a hazardous reaction when administered in low concentration through a natural route to higher vertebrates and other animals" (Bennett,1987). Mycotoxins have been demonstrated to improve fungus pathogenicity, virulence, and/or aggressiveness. Fumonisins are certainly the most significant toxins generated by *F. verticillioides,* and certain strains may produce these mycotoxins at extremely elevated levels (Miller, 2001). In addition, *F. verticillioides* may also generate fusaric acid and its derivatives, as well as trace levels of beauvericin (Sreenivasa, 2017; Leslie et al., 2004). Fusaric acid is widely found in grains, has significant phytotoxicity and can impair maize seedling growth (de Oliveira, 2009).

Fumonisins are dangerous to both animal and human health. Fumonisins are generated within the mycelia of this filamentous fungal pathogen, which is one of the major elements that comprise a biofilm (Miller, 2001). Mycotoxins are not essential for the growth and development of *F. verticillioides*, however, they do aid in the assault on host plants by having a range of phytotoxic effects on germinating seeds, young seedlings, and isolated plant tissues (Marasas ,1995). There are at least 15 Fumonisins compounds although B1 and B2 are the most toxic (Mannaa and Kim, 2017). Fumonisin B1 is mostly found on maize kernels and B2 corn silage. Additionally, mycotoxigenic fungi, along with their mycotoxins, pose a danger to global food security. Fumonisins cause infections in maize and left undetected, will lead to oesophageal and liver cancer in humans due to immune suppression and nephrotoxicity (Nazareth et al., 2019). In addition, these fungal toxins can contaminate feed products and cause pulmonary edema syndrome (PES) in pigs, leukoencephalomalacia (LEM) in horses and liver cancer in rats (Marasas ,1995). FB1 is hepatotoxic to horses, pigs, rats, and vervet monkeys and cancer initiator and promotor in rat liver.

Mycotoxins also affect the quality of the pre-harvest during storage (Bhat et al., 2010). Garcia-Diaz et al. (2020) determined the moment when contamination occurred and if it was related to mycotoxin appearance. The study found that fumonisins are present at pre-harvest but did not exceed legal limits, which means fumonisin are able to colonize at earlier stages of the cycle. The maize kernel stage is important for FB1 production. Shim et al., (2003) performed a study on FB1 production and found that growth of *F. verticillioides* is the same in the germ tube and degermed kernel but fumonisin production is five times higher in the degermed kernel, likely due to the fact that the degermed kernels became acidic after a certain period (Shim et al., 2003). Therefore, these conditions should be kept in mind while designing experiments to develop mycotoxin-related fungal biofilms.

## 8. Conclusion and future prospects

The area of fungal EV research is still in its infancy, which explains why there are so many unknowns such as how these structures might influence disease progression. The intricacy of fungal EVs is tremendous, yet their ability to influence fungi-host cell interactions implies that they have considerable promise in the development of novel

strategies to fight fungal infections. Dissecting the physiological and pathogenic activities of fungal EVs will help us better understand their potential use in vaccine models

Despite significant efforts to understand the role of EVs in plant-fungal interaction, there are still many gaps in the knowledge of these vesicles as the field is relatively new to plant sciences. For instance, it is not known for sure if all microbes that interact with plants produce EVs. It is known that the existence of multiple EV internalized cargos that can be delivered to influence plant behaviour, but the extent to which these influence the donor and recipient cell functions and behaviour, including plant's immune response, is unknown. Understanding these mechanisms will contribute to the current understanding of molecular plant-pathogen interactions. Information on EV cargos, secondary metabolite production by fungal phytopathogens and their impact on maize, and how to use EVs as an alternative strategy to combat plant infections, such as using host-derived EV phytochemicals for control of toxigenic fungi and mycotoxins.

The functions of EVs and biofilms during the infection process are also poorly understood for many fungal pathogens including *F. verticillioides*. Therefore, characterization of EVs and biofilms from *F. verticillioides*, including applying them exogenously to understand their effects more deeply on fungal (planktonic) cells and maize early growth stages (e.g., seeds and seedlings) will enhance a mechanistic understanding of fungal infection processes.

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## 10. Figures

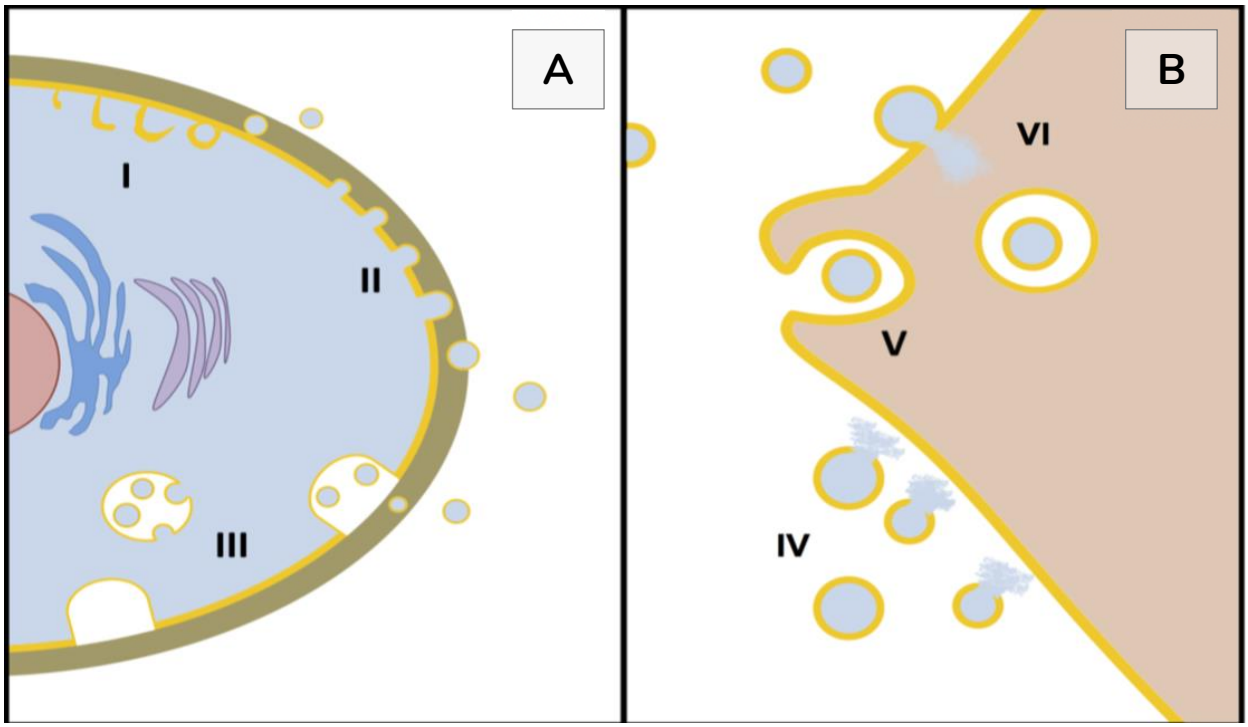


**Figure 1:** An overview of the practical features of fungal EVs. A: The biogenesis of fungal EVs is depicted by (I) plasma membrane remodelling (II) Development of ectosomes is caused by membrane budding. (III) Development of multivesicular bodies (MVBs), accompanied by fusion with the plasma membrane for extracellular release of exosomes. B: Internalization of fungal EVs is possible (V) or fusion with host cell plasma membrane and as result, vesicular cargo is likely to be released intracellularly (VI). Picture adopted from Rodrigues et al (2015).

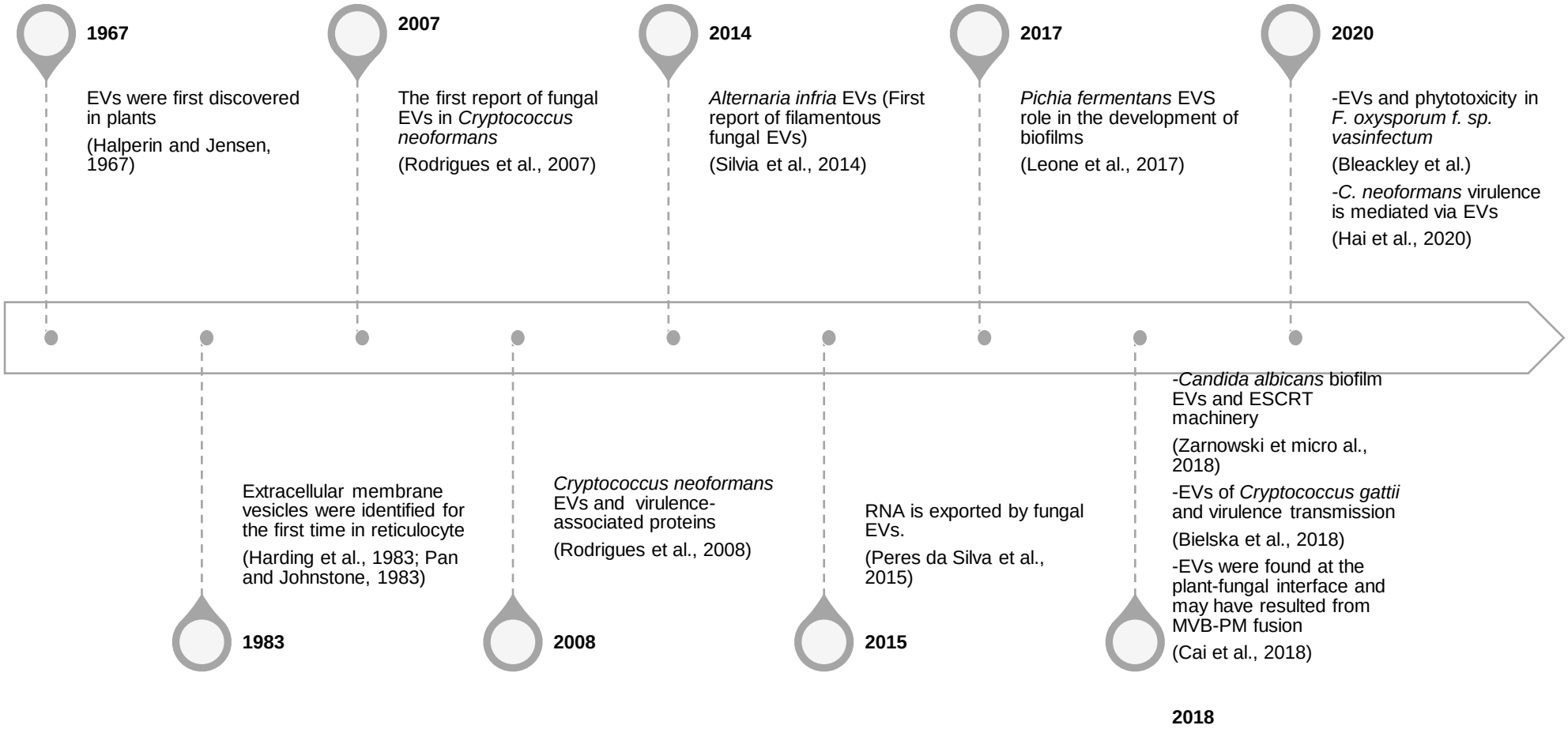


**Figure 2**: Timeline of key developments in EV research

**Table 1:** Overview of the types of protein cargo internalized and released by EVs during cell-cell communication studies in fungi, and their effect on recipient cells/tissues.

| **Fungal species (Source cell)** | **Type of cargo** | **Role of EVs** | **Recipient cell/tissue** | **Effect on recipient** | **Reference** |
|---|---|---|---|---|---|
| *Fusarium oxysporum f. sp. vasinfectum* | Polyketide synthase; Proteases; Naphthoquinone pigments; ribosomal proteins; HSP70 like proteins; unfolded proteins; and proteins involved in glucose metabolism, GTP binding and GTP hydrolysis | A plausible role in the infection process such as toxin production | Cotton plant cells | Discolouration of cotyledon or leaf | Bleackley et al., 2020; Garcia-Ceron et al., 2021 |
| *Penicillium digitatum* | Tryptoquialanine A; Polyketide synthases | Possibly a site of pigment biosynthesis and secondary metabolite transfer | Citrus fruit cells | Colour alteration of seeds and tissue damage | Costa et al., 2021 |
| *Sporothrix brasiliensis* | Proteins related to metabolism, transport, signalling, DNA repair and stress response; Virulence associated proteins; cytokines and serine/threonine protein kinases | Transport of fungus-produced molecules involved in virulence and immune system regulation | Human dendritic cells | nr | Ikeda et al., 2018 |

| | | | | | |
|---|---|---|---|---|---|
| *Saccharomyces cerevisiae* | Glucan synthase; Chitin synthase | Regulation of cell wall dynamics | ns. | ns | Zhao et al., 2019 |
| *Cryptococcus neoformans* | Glucuronoxylomannan; Chaperone proteins; ribosomal proteins | Contain major virulence factor | Macrophage cells | nr | Rodrigues et al., 2007,2008 |
| *Aspergillus fumigatus* | RNA/carbohydrate/protein binding proteins, proteins with structural activities; hydrolases; transferases; peptidases; oxidoreductases | Transport of fungus-produced proteins involved in growth and development | ns | ns | Souza et al., 2019 |
| *Aspergillus parasiticus* | Polyketide synthase | Aflatoxin biosynthesis and export | ns | ns | Chanda et al., 2009 |

nr = not reported; ns = not studied

**Table 2:** Overview of the types of Secondary metabolite internalized and released by EVs during cell-cell communication studies in fungi, plant and bacteria, and their effect on recipient cells/tissues.

| Source cell | EV type | Metabolite | Effect on target cell/tissue | Reference |
|---|---|---|---|---|
| Fungal: *Penicillium digitatum* | nr | Tryptoquialanines | Colour alteration and tissue damage | Costa et al., 2021 |
| Plant: *Nicotiana tabacum* and *Aconitum napellus L.* | Nanovesicles | Curcuminoids Chlorophylls | nr | Woith et al., 2021 |
| Plant: Ginger | Nanovesicles | Shogaol | Prevent the onset of liver-related disorders | Zhuang et al., 2015 |
| Fungal: *Aspergillus parasiticus* | nr | Aflatoxin | Vesicles converts sterigmatocystin to aflatoxin B1 | Chanda et al., 2009 |
| Fungal: *Fusarium oxysporum f. sp. vasinfectum* | nr | Polyketides | Phytotoxic effect to leaves | Bleackley et al., 2020 |
| Bacterial: *Myxococcus xanthus* | OMV | DK xanthene; cittilin A, myxovirescin A, myxochelins and myxalamids | Yellow pigmented OMVs<br>Assist in killing of *M. xanthus* microbial prey | Evans et al., 2012;<br>Schwechheimer and Kuehn, 2015 |

nr = not reported

# Chapter 2:

# Characterization of *Fusarium verticillioides* biofilms

## Abstract

Nearly all microbes, including fungal pathogens, form biofilms, which are structured communities of microbial aggregates enclosed in self-produced extracellular polymeric substances (EPS) and attached to a surface. Studying plant-associated fungal biofilms can enhance understanding of fungal biology and knowledge of the links between fungal diseases and plants. However, only a few plant-associated fungi are reported to form biofilms. This study aimed to examine the ability of a mycotoxigenic fungus of maize, *Fusarium verticillioides*, to form biofilms under laboratory conditions. During our investigation, *F. verticillioides* stationary phase cultures incubated in liquid media developed a biofilm-like pellicle with a hyphal assemblage that appears in the form of a cloudy and thin slime material. Under the microscope, the biofilms exhibited a highly heterogeneous architecture made of dense, entangled, and compact hyphae, which were accompanied by a quantifiable EPS and extracellular DNA (eDNA). The biofilm was also found to respond to different abiotic conditions including pH and temperature, suggesting their relevance in a field setting. We further demonstrate the biofilm structural maintenance role of eDNA through treatment with DNase, which was only marginally effective during late biofilm stages, suggesting that it forms complex interactions with the EPS during biofilm maturation. Based on these results, we propose that *F. verticillioides* forms a 'true' biofilm that may act as a potential virulence factor.

# 1. Introduction

After rice and wheat, maize (*Zea mays*) is the third most abundant grain crop, feeding millions of people, especially in Sub-Saharan Africa (FAO. 2021). However, its development and yield are threatened by *Fusarium verticillioides*, which systemically colonizes leaves, stems, roots, and kernels. The fungus can therefore induce serious damage that often manifests in *Fusarium* ear and stalk rot (Gai et al., 2018; Oren et al., 2003). These diseases have food safety and security implications due to mycotoxin contamination that is associated with them. Primarily, *F. verticillioides* secretes the mycotoxin fumonisin $B_1$, which contaminates symptomatic and asymptomatic maize kernels and stored grains (Munkvold and Desjardins, 1997). The toxicity of this compound is due to the inhibition of ceramide synthase and subsequent toxic intracellular accumulation of sphingosine and other sphingoid bases (Marin et al., 2013), ultimately imposing detrimental health effects on consumer populations (Wild and Gong, 2010). Indeed, studies from around the globe, including some from Africa, Asia, and Latin America, paint a disconcerting picture of how the prevalence of mycotoxins leads to a variety of human pathologies, including oesophageal and liver cancer in adults who consumed contaminated maize (Wild and Gong, 2010).

The mechanisms by which *F. verticillioides* invades maize have been outlined (Gai et al., 2018; Oren et al., 2003), providing important clues on the circumstances leading to infection symptoms and the precise anatomical locations of the maize plant that would probably harbour mycotoxins. Undoubtedly, the production of mycotoxins in *F. verticillioides* has been heavily emphasized over virulence factor production in numerous papers on mycotoxigenic fungi. The current study posits the development of biofilms as a virulence factor is somehow closely related to the accumulation of mycotoxins in fungi. Tell-tale is the biofilm 3D structure and biomass, both of which covered in extracellular polymeric substances (EPS). This structural design gives rise to emergent characteristics that are only seen in the biofilm mode of microbial life, such as surface adhesion-cohesion, spatial organization, physical and social interactions, chemical heterogeneity, and greater tolerance to antimicrobials (Karygianni et al., 2021). In the case of mycotoxigenic fungi, the biofilm EPS may exert a substantial effect on mycotoxin production; it may permit mycotoxins to stably accumulate and persist for longer periods of time due to the fact that that it glues the

cells together, in the process creating pockets and channels through which mycotoxins can be concentrated and distributed within a biofilm, respectively. *Aspergillus fumigatus* biofilms, for instance, augment the production of gliotoxin, a sulphur-containing mycotoxin with immunosuppressive properties (Bruns et al., 2010). An interesting area of research will be determining to what extent the components of a biofilm, including the EPS and its associated cell-free components such as the extracellular DNA (eDNA), influence mycotoxin production. Therefore, research on biofilms will provide a fresh perspective on mycotoxin synthesis in economically important fungi.

As biofilms present a cross-sectoral challenge, affecting a wide range of sectors including healthcare, built environment, food and agriculture (Cámara et al., 2022), our lack of understanding of how filamentous fungal biofilms originate, and how they adapt to their microenvironments once developed, will restrict our capacity to identify and counteract their detrimental impacts. This is apparent in the clinical setting, where most clinical infections associated with medical instruments, including indwelling devices (e.g., catheters, pacemakers, dentures, orthopedic prostheses, and heart valves) colonized by biofilms are difficult to treat due to antifungal resistance of these cell community structures (Bryers, 2008; Donlan, 2001; Lindsay and von Holy, 2006). Marine biofilms, on the other hand, which are created by both microorganisms and macroorganisms (such as algae), play a significant role in the environmental effects of biological fouling, also known as marine biofouling, which is the accumulation of undesirable biological matter on artificial submerged surfaces. Ships and underwater surfaces (such as undersea cables and acoustic instruments) are examples of colonized surfaces, and their treatment is difficult due to concurrent and intolerable environmental impacts on non-target species (Bixler and Bushan, 2012; Callow and Callow, 2011). The same type of challenges might apply to agriculture, where some of the essential and most used tools and machinery are contaminated with harmful fungal biofilms that are challenging to remove. When employed across many fields, farming equipment colonized by biofilms has the potential to contaminate unaffected fields with biofilm-derived propagules that may have acquired novel traits within a biofilm, including resistance to fungicides.

Somewhat formal descriptions of biofilms in plant fungal pathogens have started to emerge (Motaung et al., 2020), with the latest being provided for *F. graminearum* (Shay et al., 2022). However, to date, we have no knowledge pertinent to the role of biofilm formation in many disease-causing plant fungi, including key biofilm components such as eDNA and EPS. The current study sought to see if the morphologies of surface-associated development by *F. verticillioides* might be included in current biofilm descriptions. Our research will contribute to a better understanding of how an important maize fungal pathogen coordinates survival by forming a robust community structure.

## 2. Materials and methods

### 2.1. Fungal strains and maintenance

Strains of *F. verticillioides* were obtained from the Culture collection in the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria, following isolation from maize samples in taken from fields in the Eastern Cape province of South Africa. Two strains (CMWF 1196, and CMWF 1197) were screened for their ability to form biofilms in this study. The strains were allowed to grow for two weeks at room temperature by plating on ¼ strength PDA (Potato Dextrose Agar; 10 g of PDA powder and 12 g of Difco agar in 1 L distilled $H_2O$). They were then used to develop a biofilm in liquid media as described below.

### 2.2. Screening for biofilm formation

Rapid screening of biofilm formation was performed for all fungal isolates by cutting a block of agar (5 mm x 5 mm) from the sporulating culture and inoculating it into 15 ml of three different growth media. These were: ¼ Potato Dextrose Broth (PDB, Potato Dextrose Broth; 6 g of PDB powder (Difco) in 1 L distilled $H_2O$), Roswell Park Memorial Institute-1640 broth (RPMI) and Sabouraud Dextrose Broth (SDB or Sabouraud liquid medium) in 50 ml falcon tubes. Varied media are used to determine if biofilms can form under different nutritional circumstances, as well as which medium is optimal for biofilm formation. After mixing, the inoculate solution was poured into petri dishes (100 mm × 15 mm) and incubated at 25 and 30 °C for 24-72 hrs. They were visually analysed every 24 hrs for biofilm formation which is a hyphal assemblage that appears

in the form of a cloudy and thin slime material. Photos were taken with an Epson Perfection V700 Photo scanner.

Biofilm formation for the remaining experiments was conducted using a cell counting method. To do this, the inoculum for counting cells was prepared by adding 2 ml of 1x Phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO4$, and 1.8 mM $KH_2PO_4$) onto sporulating culture of *F. verticillioides* growing on ¼ PDA for seven days. The plate was swirled to allow spores to be released into the PBS buffer, and then counted using a haemocytometer placed under a Zeiss Axioskop 2 plus Light Microscope. A volume of 40 µL of fungal cell culture (i.e., PBS spore suspension) was diluted by adding 40 µL of 0.4% Trypan Blue solution (Sigma-Aldrich) into an Eppendorf tube, in order to distinguish between dead and living cells. The concentration of the harvested conidia was adjusted to 1 x $10^5$ conidia/ml in ¼ PDB. Of this 200 µL and left to form a biofilm in 48 wells plates (Corning® Costar® TC-Treated Multiple Well Plates from Sigma-Aldrich) or chamber slides (Lab- Tek® Chamber SlideTM System, 8 Well Permanox® Slide) and incubated for seven days at 25 °C. Once biofilms had matured, the culture was then analysed using different microscopy techniques as explained later.

## 2.3. Analyses of biofilm-like structures

To better describe the biofilms formed by *F. verticillioides*, colony and cell morphology were first analysed on plates containing liquid media (PDB), where the former was examined visually every 24 hrs for 7 days. Following the visual screening, the spores were cultured in ¼ PDB under shaking (180 rpm) (Shake-O-Mat, LABOTEC) and stationary conditions for 72 hrs in chamber slides, harvested and then analysed under a light microscope (Zeiss Axioskop 2 plus Light Microscope (LM)). A scanning electron microscope (SEM) was used to study the ultrastructure of biofilms formed as previously described, in chamber slides or 48 well plates with glass coverslips (LASEC SA). Sample preparation for SEM was performed according to Harding et al. (2010). Samples were examined with Zeiss Ultra PLUS FEG SEM Confocal Laser Scanning Microscopy (CLSM). This was used to analyse of *F. verticillioides* biofilms by forming biofilms in chamber slides at different time points (24 hrs and 7 days) and staining them for 30 mins in a dark room with 100 µl of FUN-1, which fluorescent viability probe

for fungi (Chandra et al., 2001). Biofilms were then visualized using Zeiss LM 880 CLSM (Carl Zeiss Microscopy, Munich, Germany) with excitation wavelength at 488nm and emission at 650nm.

## 2.4. Analyses of biofilm-derived cells

This study considered whether biofilm-derived cells are different from planktonic cells in *F. verticillioides.* To do this, biofilms of *F. verticillioides* were formed as previously mentioned. After 7 days of incubation, cells were extracted from the biofilm by scraping and briefly agitated (30 s) to loosen the cells from the EPS matrix. This suspension was filtered through Mira cloth (Sigma-Aldrich) to separate the cells from the EPS material. In parallel, *F. verticillioides* planktonic cells were scraped from 7-day old sporulating cultures on ¼ PDA and added to PBS. The cells were then counted and adjusted to the desired concentration (1 x $10^5$ conidia/ml). A volume of 10 µl was aliquoted onto ¼ PDA and incubated at 25 °C for 7 days. The agar plates were then examined to study differences in growth and colony morphology, with three sections of each biofilm-derived and planktonic cells examined with Zeiss Ultra PLUS FEG SEM (Carl Zeiss Microscopy, Munich, Germany).

## 2.5. Quantification of biomass, EPS and metabolic activity

Biofilm biomass was determined in relation to the production of EPS and metabolic activity. To measure the biofilm biomass, crystal violet was used as it is known to be a good indicator of the amount of cellular biomass (Cruz et al., 2018). Biomass was then quantified according to the method described by Mello et al. (2016). Biofilms in 96 well plates (Corning® Costar® TC- Treated Multiple Well Plates (Sigma-Aldrich)) were fixed for 15 min in 200 µl of 99% methanol. The supernatant was then removed, and the biofilm in microtiter plates was air-dried for 5 min prior to adding 200 µl of 0.3% crystal violet solution (stock solution diluted in PBS; Sigma-Aldrich) to each well. The stained biofilms in microtiter plates were then incubated for 20 min at room temperature prior to being rinsed twice with PBS to remove excess dye. The biomass in each well was then decolourized with 200 µl of 99% ethanol for 5 min. A volume of 100 µl of this solution was then transferred to a new 96-well plate and the absorbance was measured at 590 nm using a microplate reader (SpectraMax ® Paradigm® Multi-Mode Detection Platform).

Non-fixed biofilms were stained for 5 min at room temperature with 20 µl of 0.1% safranin (stock solution diluted in PBS:Sigma-Aldrich). After that, the stained biofilms were rinsed with PBS till the supernatants became transparent. With 200 µl of 99% ethanol, the extracellular matrix was decolorized. A volume with 100 µl of the supernatant was transferred to a fresh 96-well plate, and the absorbance at 530 nm was measured using a microplate reader as previously reported. To measure the metabolic activity of the biofilm, biofilms were formed in 96 well plates as before and incubated at 25 °C. Once biofilms had been formed, their metabolic activity was then quantified using a colorimetric assay, XTT (sodium 3´-[1- (phenylaminocarbonyl)- 3,4-tetrazolium]-bis (4- methoxy6-nitro) benzene sulfonic acid hydrate) (Sigma-Aldrich), according to the manufacturer's recommended specifications. The activity of fungal mitochondrial dehydrogenase converts XTT tetrazolium salt to XTT formazan resulting in a colour change that can be measured using the microplate reader as previously described, with the absorbance from each well measured at 492 nm.

## 2.6. The impact of abiotic conditions on biofilm growth

The impact of abiotic conditions on biofilm production by *F. verticillioides* was assessed at different pH values (2, 3, 4, 5, 6, 7, 8) and temperatures (10, 15, 20, 25 and 35 °C) in ¼ PDB. The pH was adjusted using HCl and NaOH. To conduct these experiments, standardized spore suspensions were inoculated as previously described and biofilms were allowed to develop under the aforementioned abiotic conditions for 7 days. Quantification of biomass, EPS, and metabolic activity was then performed using the microplate reader as previously described. Following this, EPS and biomass were determined, with EPS expressed per biomass (i.e., EPS/Biomass) and metabolic activity percentages calculated to compare the response of biofilms under the different physical factors.

## 2.7. Effect of DNase treatment on biofilm formation

The eDNA release was measured using a microplate fluorescence assay using a DNA binding dye (SYBR® Green I), as previously described by Rajendran et al. (2014). First, EPS was extracted following a method described by Rajendran et al. (2013). For this study, biofilms were cultivated in ¼ PDB for 7 days at 25 °C before being scraped from the plates using sterile cell scrapers and rinsed with PBS. The EPS was extracted

from the disaggregated biofilm using 0.2 M EDTA. Following this the samples were centrifuged at 10,000 x g for 30 min, the EDTA supernatant was collected and filtered through a 0.45 m syringe filter (Millipore). SYBR® Green I (Invitrogen), whose binding results in fluorescence that is directly proportional to DNA content, was applied at a 1:4 ratio to biofilm supernatants in a black well microtiter plate (Costar3603; Corning). The levels of eDNA were then quantified using a fluorescence plate reader (SpectraMax ® Paradigm® Multi-Mode Detection Platform) at 485 and 525 nm, respectively. The concentration of eDNA in the sample was quantified using the DNA standard curve as previously described by Leggate et al. (2006).

The role of eDNA in *F. verticillioides* biofilm formation was investigated by depletion of eDNA within the biofilm using the hydrolytic enzyme (Rajendran et al., 2013), DNase I from bovine pancreas (Sigma-Aldrich). The DNase I was prepared in 0.15 M NaCl supplemented with 5 mM of $MgCl_2$. To assess the effect of DNase I on biofilm formation, biofilms were formed in ¼ PDB as described above and were incubated with DNase I at the concentration of 0.25, 1, and 2 mg/ml, and incubated at 25 °C for 72 hrs and 7 days. Untreated controls were included for direct comparison. After each treatment, the biofilms were washed in PBS and their metabolic activity, biomass, and EPS were quantified as mentioned prior.

### 2.8. Statistical analysis

All experiments were performed in duplicate, in two independent experimental sets. The data were expressed as mean ± standard deviation (SD). The results were evaluated using the GraphPad Prism 9 computer program. A *p*-value of 0.05 or below was deemed statistically significant in all analyses (ns= $p > 0.05$; *= $p \leq 0.05$; **= $p \leq 0.01$; *** =$p \leq 0.001$; ****= $p \leq 0.0001$).

## 3. Results

### 3.1. Colony morphology of *in vitro* biofilm-like structures

The ability of *F. verticillioides* to form biofilm-like structures was observed in both strains examined in this study (Figure 1). Then, using visual inspection of liquid cultures, colonies emerging from a biofilm culture were observed in PDB, SDB and

RMPI, incubated at 25 °C from 24 hrs to 7 days (data not shown). The biofilm is normally distinguished from planktonic cells by their dense, highly hydrated clusters of cells enmeshed in a gelatinous matrix (Coraça-Huber et al., 2020; Hurlow and Bowler, 2009, Metcalf and Bowler et al., 2013). Indeed, *F. verticillioides* biofilm-like colonies displayed a dense, thin, and cloudy material (Figure 1). Based on these morphological traits, the biofilm-like formations will be referred to as simply biofilms from this point on.

### 3.2. *Fusarium verticillioides* biofilm development

Since, morphologically, both the strains appeared to be forming similar biofilms, only one CMW 1196 was then selected and utilized for subsequent studies. It was anticipated that a biofilm would form most effectively under stationary conditions and assumed that the shear stress from shaking would prevent the formation of the EPS matrix, a distinguishing feature of microbial biofilms. Therefore, the cells from *F. verticillioides* CMW 1196 were cultured under both shaking and stationary conditions. Following this, it was found that planktonic cells incubated under shaking conditions did not typically clump together when observed under a light microscope (Figure 2A), but those incubated under stationary conditions developed a community of cells resembling a biofilm (Figure 2B). When these cells were analysed under SEM, little to no EPS formation in cells incubated under shaking conditions was observed (Figure 2C). However, cells that were incubated without shaking formed a visible EPS (Figure 2D), which was not surprising to us given that EPS have been observed often after growth under non-shaking conditions in a variety of biofilm investigations (Cavalheiro and Teixeira, 2018).

### 3.3. *Fusarium verticillioides* biofilms and impact on cells therein

The development of a biofilm in *F. verticillioides* may influence the metabolic status of cells and by extension, phenotype (Ramage et al., 2012). Confirming this were that the spores at the dispersion stage (Figure 3D) appeared to be morphologically distinct from the normal microconidia spores initially used as inoculum to initiate a biofilm (indicated in Figure 3A). Usually, microconidia of *F. verticillioides* are club- or elliptical-shaped or pointed at both ends (Figure 3A). However, the biofilm-derived spores appeared to be more globose/lemon-shaped and slightly larger than typical conidial

cells (Figure 3D). Therefore, biofilm-derived cells, as indicated in Figure 3D, may influence phenotypic diversity in *F. verticillioides*. For instance, when these cells were harvested from a biofilm and plated on ¼ strength PDA, they displayed a colony morphology that is different from cells not derived from a biofilm i.e., they formed a colony smaller (Figure 3D) than that of their planktonic counterpart (Figure 3C). This implies that the physiological response of cells contained within a biofilm is greatly influenced by the biofilm ecosystem. However, the morphology of cells derived from a biofilm had no apparent differences when compared to the morphology of normal cells (Supplementary Figure 2), suggesting that the differences between these cells might largely be in their response to environmental signals, as in the case with observations in Figure 3, as opposed to their morphology.

### 3.4 EPS/Biomass and metabolic activity as indicators of biofilm response

The complexity of the biofilm is for the most part brought on by the release of EPS (Ramage et al., 2011). This means that the biofilm may possess the ability to affect the physiology of the cells within it by virtue of holding them in place, thus maintaining the biofilm's 3D structure while also optimising the exchange of nutrients and genetic material. As previously indicated, the results showed that unlike cells cultured under shaking conditions (Figure 2A, C), the generation of a visible EPS occurs concurrently with the establishment of a mature biofilm (Figure 2B, D). Since establishing that *F. verticillioides* biofilms produce EPS, in this study, we were also interested in how much of this material was being produced during biofilm formation and to what extent metabolically active cells contributed to the total biofilm biomass. For this reason, colorimetric assays were applied, namely crystal violet and XTT, to analyse the biomass and EPS by the absorption of safranin, and to assess the metabolic activity (cell viability) of the biofilm, respectively. In the case of the XTT reduction assay, the production of soluble coloured formazan salts by sessile cells is a direct reflection of cellular metabolic activity. According to the results, an increase in cell mass and EPS production (Figure 4A) seems to be accompanied by an increase in metabolic activity of the cells inside a biofilm (Figure 4B); EPS/Biomass increased significantly from 13% (3 days) to 45% (7 days) (*p*-value=0,002), suggesting the more the biofilm matures the more EPS is produced.

## 3.5 Biofilm formation in response to abiotic factors

Having shown that *F. verticillioides* asexual cells could develop into a biofilm, this study then investigated how the biofilm will react to different environmental conditions, namely different temperature and pH conditions. As shown in Figure 5A, EPS/Biomass was highest at pH 5 at around 65%, suggesting that *F. verticillioides* may prefer pH 5 for biofilm formation. The media that was used in the initial experiments (¼ PDB) has a pH of around 5, and it was in this medium that all the stages of a biofilm were observed (Figure 2). The EPS and metabolic activity were essentially the same at acidic pH levels (i.e., 2, 3, and 4, below the optimal pH of 5), whereas, at pH levels higher than the optimal (i.e., pH 6, 7, and 8, higher than pH 5), the EPS was produced at lower levels than at pH 5 and essentially stayed at these levels. From the optimal pH (pH 5) point of view, *F. verticillioides* biofilms seem to produce significantly more EPS/Biomass than at pH 6 and pH 8 ($p$ value ≤ 0.05). However, this biofilm had a significantly lower metabolic activity at a range of pHs from 2-8 (2, 3, 4, 6, 7, and 8) ($p$ value ≤ 0.001). This suggests that, although pH 5 permits better biofilm formation, the biofilm response to pH is versatile, spanning a range of pH conditions, which could influence the adaptability of this pathogen to a range of field conditions.

Figure 5C and D depict the influence of temperature on the production of *F. verticillioides* biofilms. The best temperature tested for metabolic activity is 25 °C, and the fungus displayed similar metabolic activities and formed robust biofilms at 20 and 25 °C with no significant differences. The fungus also formed biofilms at 10, 15 and 35 °C, but these were not as robust as 20 and 25 °C and had lower metabolic activity. Different temperatures do not seem to significantly affect the EPS/Biomass percentage except for 10 °C where the biomass was beyond detection.

## 3.6 The structural maintenance role of extracellular DNA

The biofilm was treated with DNase I to show how eDNA maintains the structure of the biofilm. The DNAse caused the collapse of biofilm formation during early stages of development i.e., at 72 hrs (Figure 6A). Also, the structural integrity of the biofilm was revealed to be significantly impacted by the addition of DNase I in a concentration-dependent manner. Unfortunately, the EPS/Biomass percentages could not be determined during the early biofilm maturation phase (72 hrs) as the biofilm was too

weak to perform the relevant assays. In comparison to the biofilm growth control at 7 days, the application of 0.25 and 1 mg/ml, DNase I slightly reduced EPS/Biomass by 17% ($p$>0.05) while 2 mg/ml significantly reduced it by 40% ($p$=0.0095) (Figure 6B). Interestingly, although DNase I had an impact on the EPS/Biomass, the metabolic activity of the biofilm was not drastically affected (Data not shown), which could show that eDNA has more impact on the structure of *F. verticillioides* biofilm than on cellular activity, as has been demonstrated in other fungi

## 4. Discussion

Members of the genus *Fusarium* cause economically important and hard-to-control diseases including cankers, crown rot, head blight, scabs, and wilts. Although many of these diseases likely have strong links with the biofilm formation (Harding et al., 2010; Harding and Daniels, 2017; Motaung et al., 2020) given that microbes predominantly exist in a state of a biofilm in their natural environments, biofilm formation has been formally described in just a few *Fusarium* species including *F. oxysporum* f. sp. *cucumerinum* and *F. graminearum* (Li et al., 2014; Shay et al., 2022). Therefore, for many fungal pathogens of plants, including those belonging to *Fusarium*, it is unclear how biofilms form, let alone how they impact infections and disease outcomes.

Seven years ago, Miguel and his colleagues saw what looked like *F. verticillioides* biofilms, in which the mycelium was structured in an extracellular material around the hyphae (Miguel et al., 2015). These researchers also discovered a flocculent substance over the cells or small fibrils of hyphae connecting to one another, like a biofilm. To the best of my knowledge, this is the only time that evidence of a biofilm-like structure for *F. verticillioides* has been reported *in vitro*. There is no formal description of biofilms by this fungal pathogen. In the current study, we addressed this knowledge deficit by describing how *F. verticillioides* forms biofilms under *in vitro* conditions.

Information that describes biofilm colony morphologies in stationary liquid cultures is generally lacking in fungal plant pathogen biofilm studies. The filamentous fungus *F. graminearum* has recently been described as being able to form biofilm colonies at the air/liquid interface that are growing as pellicle–floating masses of cells that cling to

each other and move as a unit (Shay et al., 2022). In Figure 1 of this study, it is shown that floating masses for *F. verticillioides* can be distinguished from free-living (planktonic) cells by forming colonies displaying a dense, thin, and cloudy material. The results of this study are consistent with several other studies also reporting similar features in plant and animal tissues, as well as surgically implanted equipment such as catheters and pacemakers colonized by fungal and bacterial biofilms (Coraça-Huber et al., 2020; Hurlow and Bowler, 2009; Santos et al., 2011; Trautner and Darouiche, 2004).

In the current study, it was also observed that biofilms in *F. verticillioides* also developed most efficiently under stationary conditions, while shear stress from shaking conditions prevented proper biofilm formation. Cells incubated in the stationary conditions without shaking had hyphal cells tangled with EPS that appeared to behave like a matrix binding the hyphae together, and cells cultured under agitated conditions seldomly clumped together or not at all. These findings are similar to those of Hawser et al. (1998), who demonstrated that only a few cells are visible on the surface of cultures when they are shaking, in contrast to stationary conditions, whereby cultures are characterized by dense networks of hyphae. This might be due to the severe shaking influencing cell architecture, matrix deposition, and biofilm formation (Soll and Daniel, 2016). In *C. albicans*, shaking at a speed of 60 rpm prevents biofilm growth, with biofilms exposed to shear stress being thinner than those exposed to non-shaking conditions (Cavalheiro and Teixeira, 2018). Hawker et al. (1998) also found that lower speeds result in the production of biofilms with no extracellular matrix but only hyphae, while shaking at higher-speed results in a biofilm which consists of a few cells on the surface. In this study, it was observed that some EPS material is present in planktonic cultures (Figure 2C); however, this was not as abundant as in biofilm cells (Figure 2D), suggesting that under conditions causing agitation of the fungal cells, the cells struggle to produce the EPS matrix.

In light of the above, the findings presented here align with the biofilm concept previously proposed (Córdova-Alcántara et al 2019; Harding et al. 2009, 2010; Motaung et al., 2020). The biofilms of *F. verticillioides* seem to develop through spore adhesion, microcolony formation, maturation, and dispersion. Although many fungal and bacterial species have recorded comparable developmental stages for biofilm

formation, filamentous fungal biofilm formation seems to differ from strain to strain (Li et al., 2014; Mowat et al., 2007; Ramage et al., 2011,2012). In contrast to unicellular life forms such as yeast and bacteria, most fungi contain many planktonic forms that can disperse and continue the cycle (e.g., sporangia asexual spores, sexual spores, and hyphal fragments), and these dispersive forms most usually float in air rather than water (Harding et al., 2009). In this study, the dispersal phase of biofilms leads to a substantial number of free-living cells in the form of conidia, but these cells appeared to be morphologically distinct from the normal microconidia spores initially used as inoculum to initiate a biofilm (Supplementary Figure 1). This suggests that the dispersed biofilm cells differ from normal microconidia. Similar findings were reported in a study on *Bacillus cereus* where the cells in the biofilm have different cell-surface characteristics than their planktonic counterparts. For example, the structure of a polysaccharide linked to peptidoglycan in *B. cereus* has been discovered to change during biofilm development (Majed et al., 2016). In addition, according to Boles et al. (2004), the short-term development of *P. aeruginosa* in biofilms causes considerable genetic diversity in the resident bacteria. They also discovered that genetic diversity forms bacterial subpopulations in biofilms with specialized functions and that functional diversity improves the biofilm community's capacity to endure physiological stress. Equivalent diversity has not been shown in fungal biofilms and should be investigated further.

The biofilm detachment phase is important in disease development, and there has been growing evidence that specific techniques of dispersion can result in releasing biofilm cells that are more pathogenic than their planktonic counterpart (Beitelshees et al. 2018). The dispersed biofilm-derived conidia may be critical to the pathogen’s invasive nature during fungal infections. In other studies, biofilm-derived cells have frequently been shown to be resistant to antifungals and stress (Guilhen et al., 2017). Therefore, *F. verticillioides* biofilm-derived conidia need to be further investigated for agriculturally relevant traits including heightened antifungal resistance against fungicides currently employed in agriculture, which could make the disease caused by this fungus challenging to treat.

Traditional methods for probing biofilm biomass, extracellular matrix, and metabolic were used in this study. In *A. fumigatus* biofilm have been previously linked with

metabolism, biomass, and hyphal growth (Mowat et al. 2007). It was also shown that metabolic activity and biomass increased during the first 24 hours of biofilm formation and subsequently reaching a plateau of development (Mowat et al. 2007). Mello et al. (2016) focused on the assessment of biofilm formation by *Scedosporium* species and reported similar outcomes in some species in terms of biomass, metabolic activity, and EPS production. The production of the matrix has a high energetic cost, which may be evolutionary justified given the matrix's structural and physicochemical significance in the growth and operation of the biofilm, without which the beneficial emergent properties of biofilms would not be possible (Flemming et al., 2016; Saville et al., 2011). This goes to show that the formation of biofilms is closely linked to the formation of the matrix, the bulk of which is extracellular material (Li et al., 2014).

As biofilms grow, metabolic activity and EPS production also rise, suggesting that they may become more resistant to abiotic stress (Motaung et al., 2020). In the current study, it was found that *F. verticillioides* developed biofilms under a range of pH and temperature conditions, and a similar trend was previously reported (Li et al., 2014). These results show that *F. verticillioides* biofilms are produced under different conditions and have an optimum pH of 5 and an optimum temperature of 25 °C. Under field conditions it has been reported that the optimum temperature for spore development is 27 °C a temperature at which biofilm development could occur. Evaluation of biofilm formation under different pH conditions showed that biofilms were much more robust in somewhat acidic (pH 4-5) conditions than in acidic (pH 2, 3), neutral (pH 7) or alkaline (pH 8) environments. These findings are consistent with other reports (Cornet and Gaillardin, 2014) since the pH range for fungal development is fairly broad, ranging from pH 3.0 to more than pH 8.0, with the optimum at pH 5.0 assuming nutritional needs are met. The capacity to develop a biofilm under varying physical conditions may provide the fungus with survival benefits in inhospitable environments.

In the current study, it was demonstrated that DNase I has an impact on the biofilm stability. In a similar study using *A. fumigatus*, DNase I was effective at all concentrations (0.25, 1, 4 mg/ml) (Rajendran et al., 2013, 2014), but the maximal effect was observed with 4 mg/ml of DNase I ($p$ value < 0.001) which is similar to the observations in this study. The discovery that eDNA contributes significantly to the

biofilm EPS in both bacteria and fungi suggests that this may be a conserved and possibly active microbial biofilm process. Unlike other components of the biofilm EPS matrix, the eDNA has by far attracted the most attention and is considered a useful tool to study the biology of biofilms (Ibáñez de Aldecoa et al., 2017). In addition, many studies have uncovered that eDNA plays many important roles in bacteria, such as in biofilm structural maintenance, assisted by the action of DNA binding proteins (Buzzo et al., 2021; Kavanaugh et al., 2019; Whitchurch et al., 2002), antimicrobial resistance (Okshevsky and Meyer, 2015; Rajendran et al., 2013), and acting as a reservoir for the interexchange of genes through natural transformation (Merod and Wuertz, 2014). Furthermore, eDNA assumes more unusual roles, including acting as a source of energy and nutrients (e.g., carbon, nitrogen and phosphorus) (Ibáñez de Aldecoa et al., 2017; Pinchuk et al., 2008), and forming higher-order conformations (e.g., G-quadruplex DNA) that further strengthen the biofilm through extracellular EPS-eDNA networks (Seviour et al., 2021). Given these roles, eDNA is an attractive target of antimicrobial drugs to manage biofilm-related infections. However, there are only a few studies exploring the existence and function of eDNA in filamentous fungi, with no studies conducted in plant pathogenic fungi.

## 5. Conclusions

The study showed that the maize fungal pathogen, *F. verticillioides*, can develop a biofilm under laboratory conditions. Even though the description described here of fungal biofilms may have limitations when the natural environment is considered, at the very least *F. verticillioides* biofilm development seems to follow a typical model that entails attachment, colonization, development (including EPS synthesis), maturity, and dissociation from EPS, with the last phase producing conidia to restart the biofilm cycle. However, in the natural environment, plant stem and leaf surfaces can be sparsely or densely colonized by diverse fungal biofilms and are likely more complex than conditions used in this study. Laboratory-grown biofilms are a simple surface-covering, frequently exhibiting confluent and compact uniformity that is consistent with the original definition of biofilms. Documenting biofilm formation in the natural environment by analysing heavily infected maize plants and a population of field strains, as opposed to a few ones, is needed to better understand what is happening in field conditions. By better understanding the complexity of plant-

associated fungal biofilms and their phenotypic traits, it would be possible to develop novel antifungal drug targets and treatment alternatives to decrease the prevalence of fungal infections. This study thus established a baseline with regards to *F. verticillioides* biofilms, showing its intricate structure and response to the environment. The findings presented further showed that eDNA degradation causes biofilms to collapse, suggesting it is a candidate for antifungal development. These findings represent the first investigation on eDNA analysis in a plant fungal pathogen. Taken together, *F. verticillioides* ability to form biofilms may give it an ecological edge in its battle to keep its place as a commensal and pathogen of maize. The biofilm might enable this fungus to evade host immunity, withstand antifungal treatment and competition from other microbes. The current study, together with earlier studies, therefore, will deepen the understanding of the relationship between disease outcomes and biotic interactions in *F. verticillioides*.

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## 7. Figures

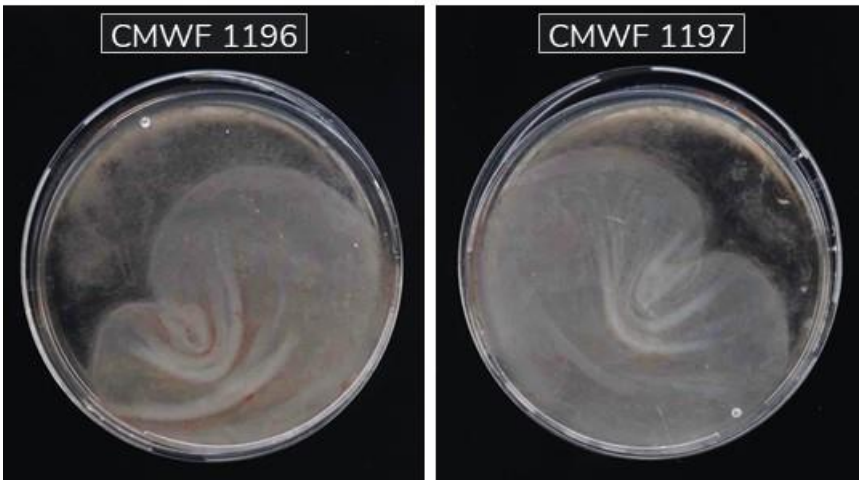


**Figure 1:** *Fusarium verticillioides* strains that formed biofilm-like cultures in Petri-dishes containing ¼ strength Potato Dextrose Broth. The strains were left to grow, without shaking for 7 days at 25 °C.

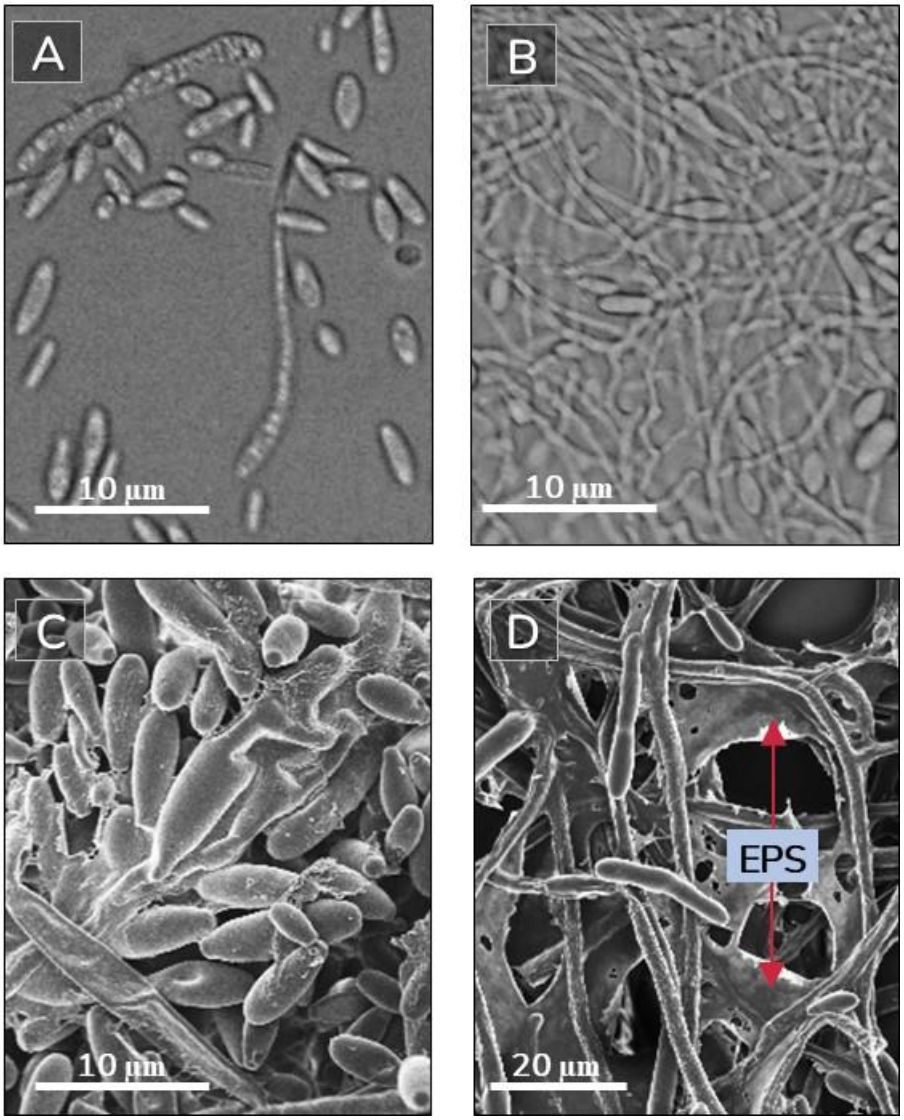


**Figure 2**: *Fusarium verticillioides* cultured for 7 days under shaking conditions (**A, C**), remained in the planktonic (free-living) state, and without shaking **(B, D),** formed biofilms with observable extracellular polymeric substances (EPS, indicated by red arrows in **D**).

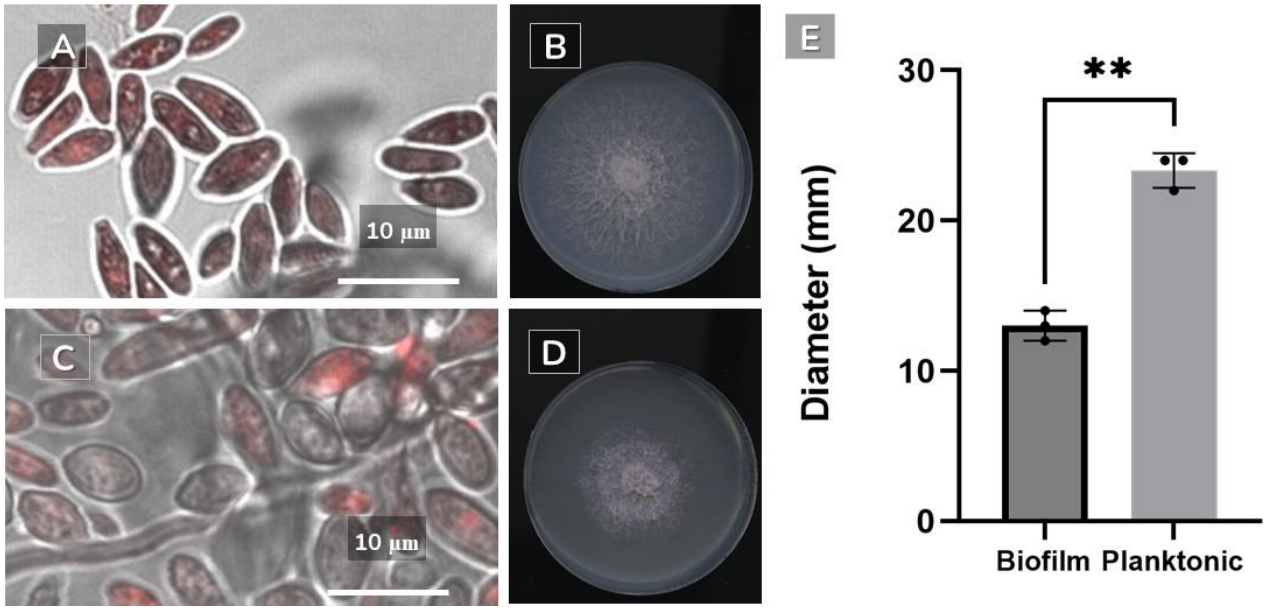


**Figure 3:** Biofilm-derived and planktonic cultured *Fusarium verticillioides* in chamber slides for **(A)** 24 hrs and **(C)** 7 days**.** The cells were stained with FUN-1 to determine biofilm developments. *F. verticillioides* cells from shaking **(B)** and non-shaking **(D)** cultures established colonies on ¼ PDA. Colonies derived from biofilm cells of asexual cells that were formed were significantly smaller (**=$p \leq 0.01$) than those derived from planktonic cells **(E).** This is despite the fact that the respective cells were plated at the same concentrations and incubated under the same conditions. Each dot on the bar graphs represents an independent biological replicate. ns= $p > 0.05$; *= $p \leq 0.05$; **= $p \leq 0.01$; *** =$p \leq 0.001$; ****= $p \leq 0.0001$

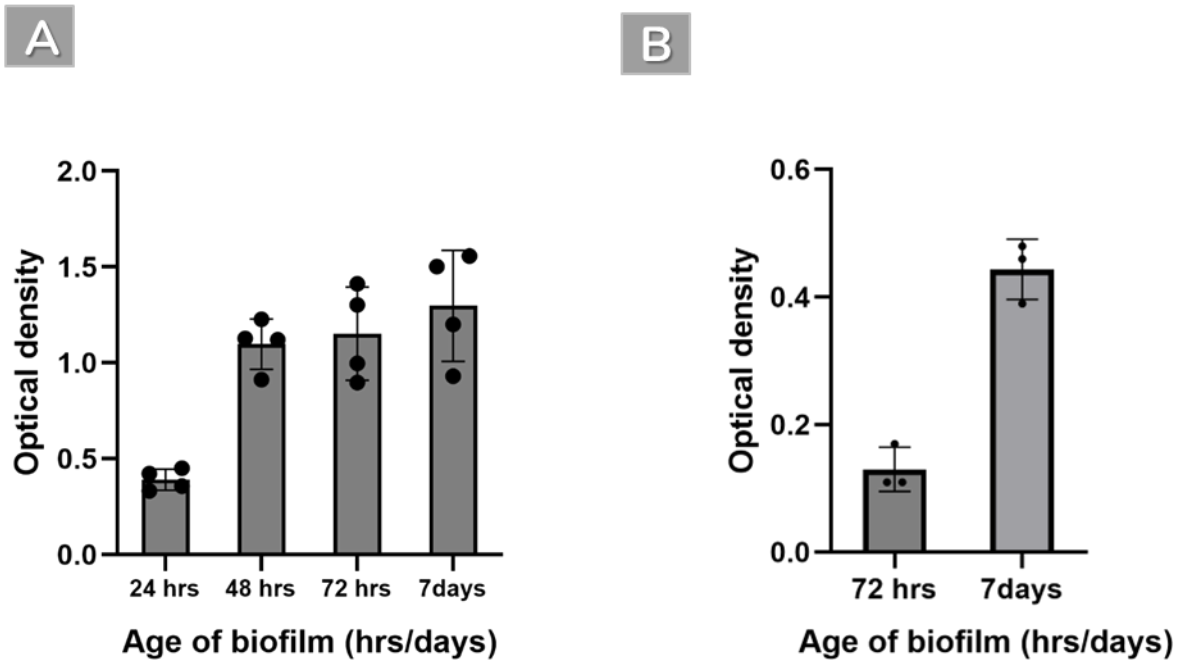


**Figure 4**: *Fusarium verticillioides* biofilm formation was assessed based on **(A)** metabolic activity, evaluated by XTT reduction assay and **(B)** biomass and extracellular polymeric substances (expressed as EPS/Biomass) evaluated using crystal violet ($OD_{590nm}$) and safranin ($OD_{530nm}$), respectively ($p$-value=0.0013). An increase in cell mass and EPS production is observed. Each dot on the bar graphs represents an independent biological replicate. ns= $p > 0.05$; *= $p \leq 0.05$; **= $p \leq 0.01$; *** =$p \leq 0.001$; ****= $p \leq 0.0001$

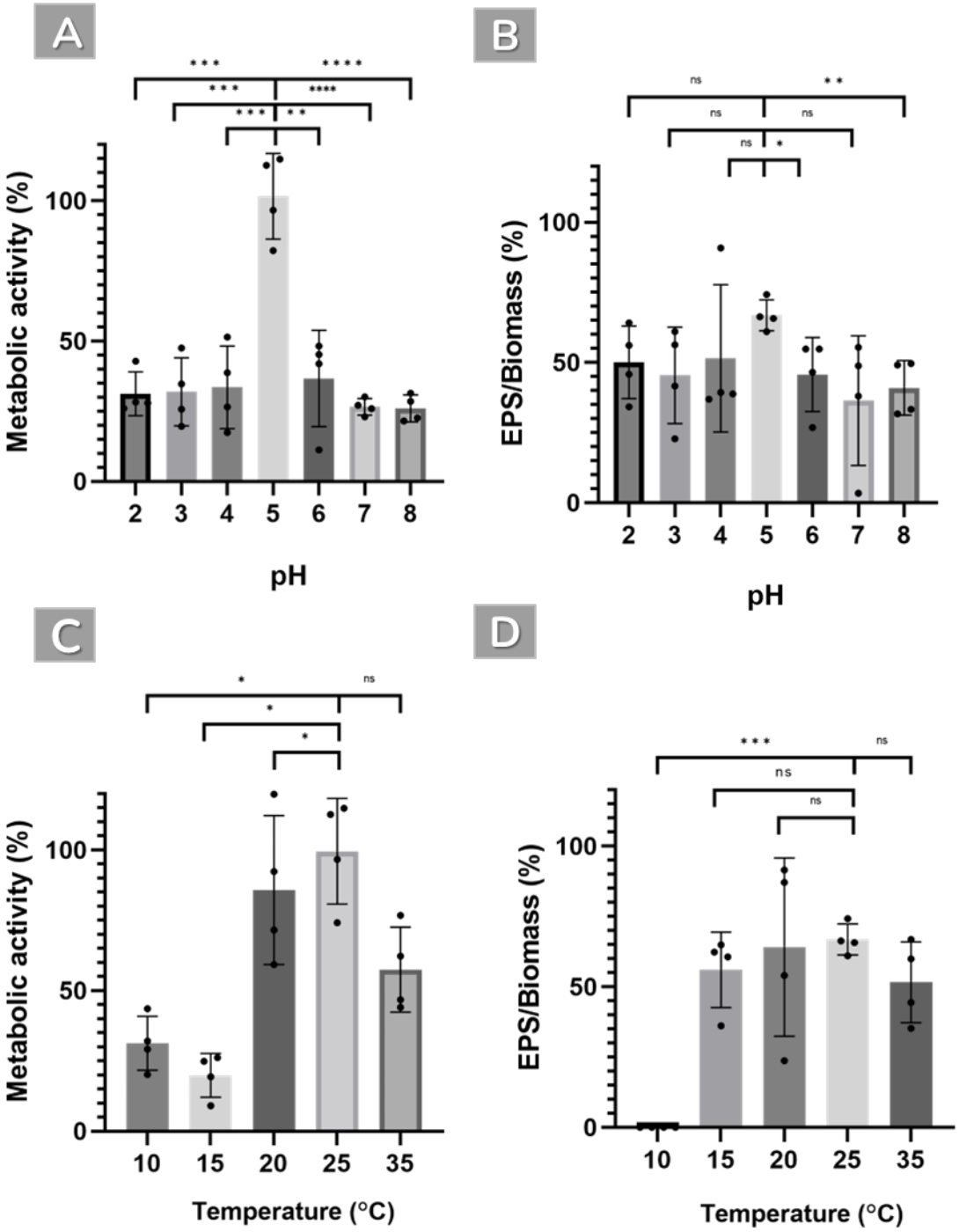


**Figure 5**: *Fusarium verticillioides* biofilm formation assessed at various pH and temperature conditions by measuring **(A, C)** metabolic activity (XTT reduction assay ($OD_{475nm}$; *p* value<0.05) (expressed as metabolic activity percentage)), and **(B, D)** biomass and extracellular polymeric substances (expressed as EPS/Biomass percentage), evaluated using crystal violet ($OD_{590nm}$) and safranin ($OD_{530nm}$), respectively (*p* value<0.05). This data shows that pH 5 permits better biofilm formation, and the biofilm response to pH is versatile, spanning a range of pH conditions. The

optimum temperatures evaluated for metabolic activity were 20 and 25°C. It also generated biofilms at 10, 15, and 35°C, but these were less robust and had lower metabolic activity. Different temperatures do not appear to have a significant impact on the EPS/Biomass %, except for 10°C, when the biomass could not be quantified. Each dot on the bar graphs represents an independent biological replicate. ns= $p > 0.05$; *= $p \leq 0.05$; **= $p \leq 0.01$; *** =$p \leq 0.001$; ****= $p \leq 0.0001$

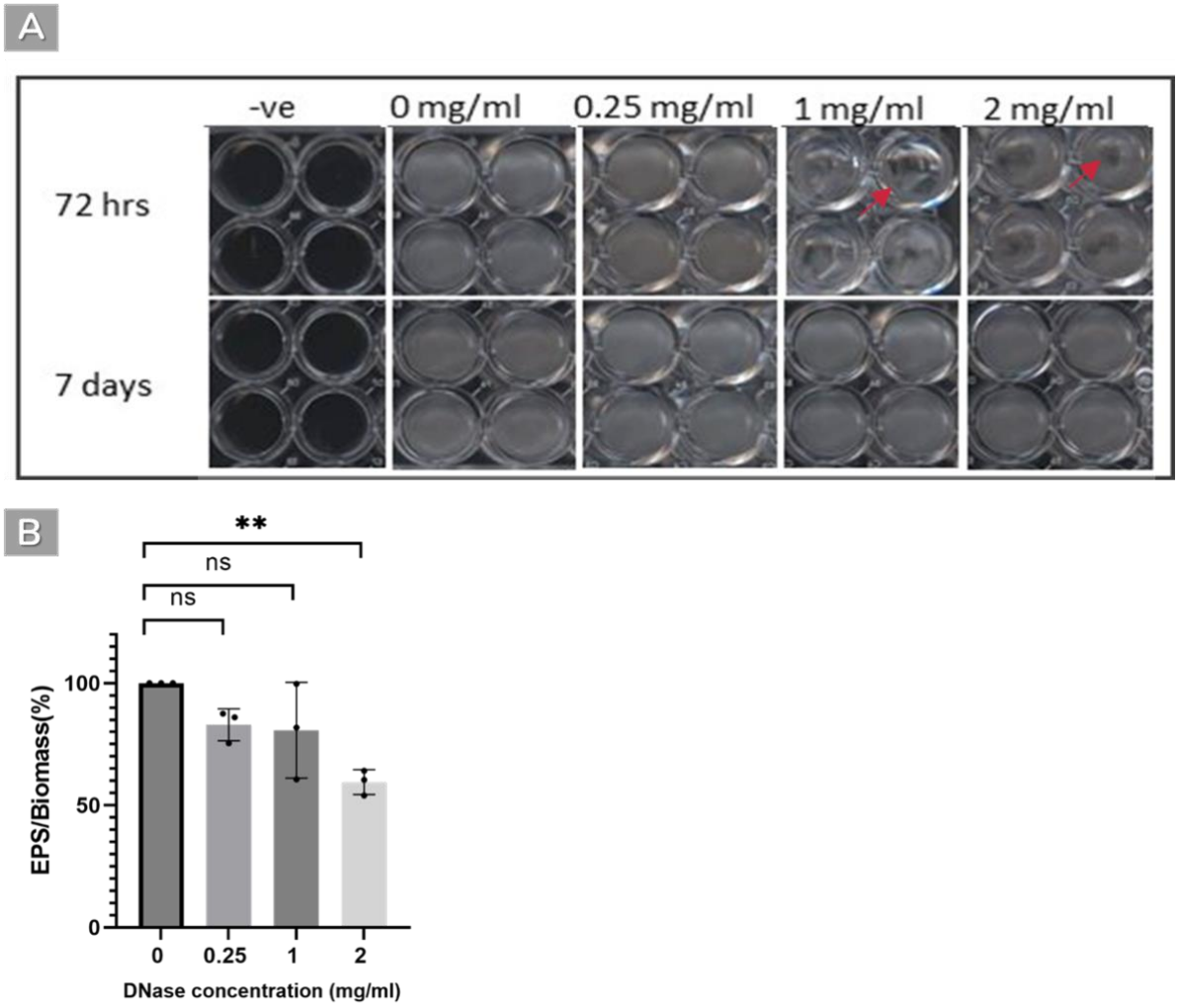


**Figure 6**: *Fusarium verticillioides* biofilm response to DNase treatment. **(A)** The response of a 72hr-old biofilm to DNase at different concentrations (0.25, 1 and 2 mg/ml). **(B)** The response of biofilms measured in biomass and extracellular polymeric substances (expressed as EPS/Biomass percentage). This data shows that DNase I caused the collapse of biofilm formation at the early stages of growth, i.e., at 72 hrs, and the structural integrity of the biofilm was found to be strongly influenced by DNase I in a concentration-dependent manner. Each dot on the bar graphs represents an independent biological replicate. ns= $p > 0.05$; *= $p \leq 0.05$; **= $p \leq 0.01$; *** =$p \leq 0.001$; ****= $p \leq 0.0001$

## 8. Supplementary figures

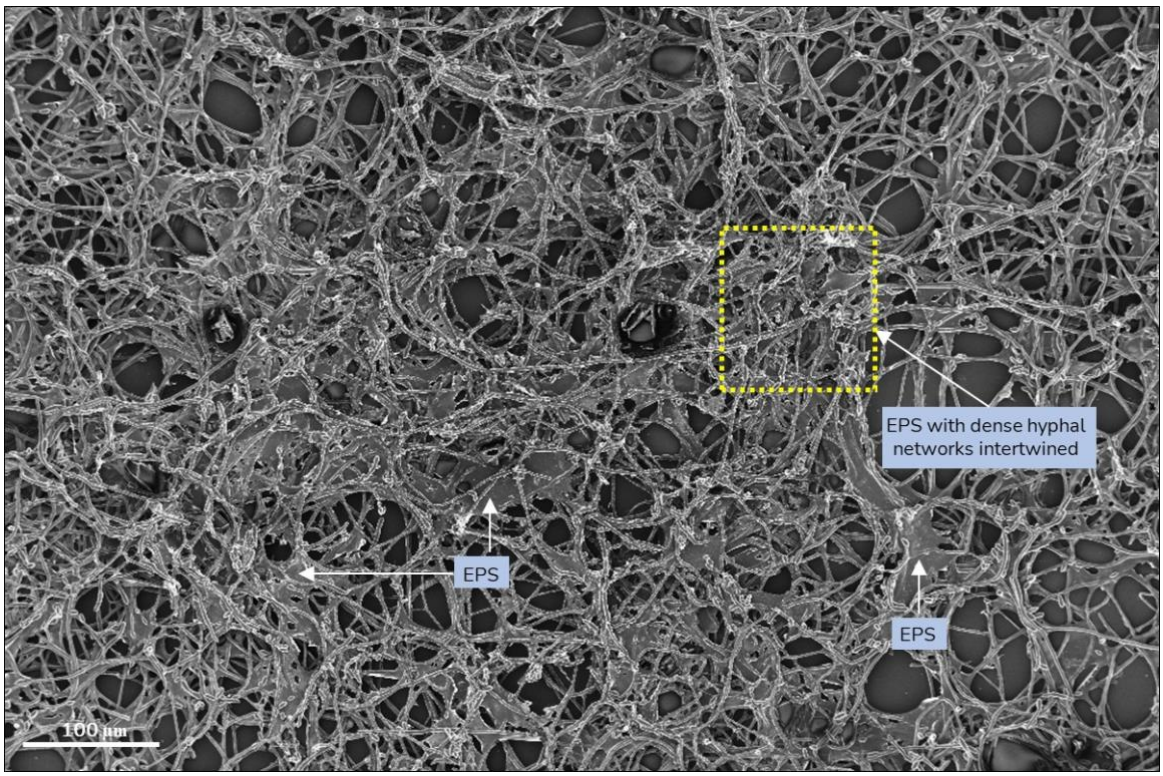


**Figure S1**: *Fusarium verticillioides* biofilms cultured for 7 days at 25 °C under stationary conditions. The extracellular polymeric substances (EPS, indicated by white arrows) appear to fill the spaces between hyphal cells, forming a highly organized network that strengthens a biofilm.

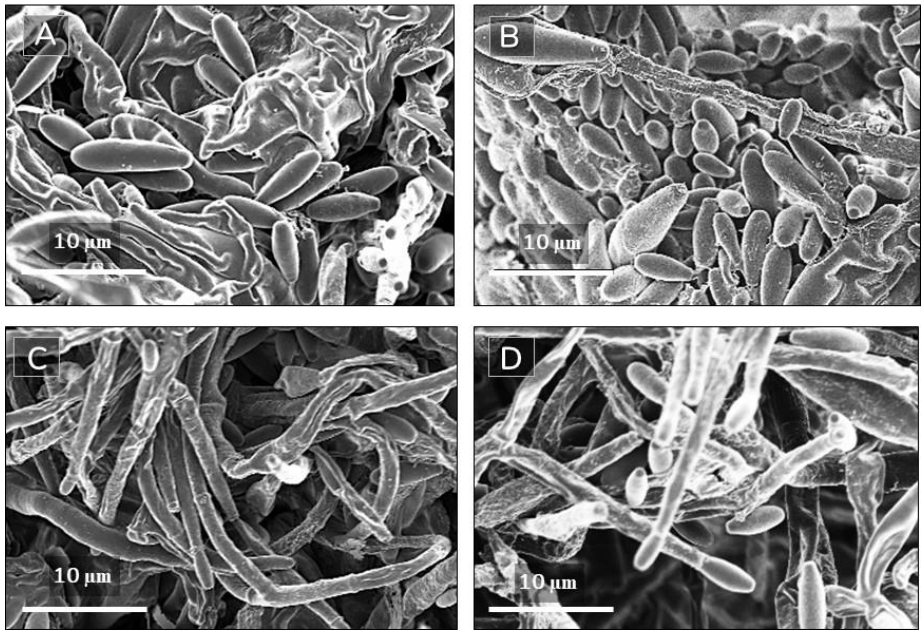


**Figure S2:** *Fusarium verticillioides* planktonic cells (**A:** Conidia; **C:** Hyphae) and biofilm-derived cells (**B:** Conidia; **D:** Hyphae). The cells were plated at the same concentration ($1x10^5$ cells/ml) on ¼ PDA and incubated at 25 °C.

# Chapter 3:

# Characterization of extracellular vesicles from *Fusarium verticillioides* planktonic and biofilms cells

## Abstract

Extracellular vesicles (EVs) are small lipid bilayers important in cell-to-cell communication. EVs have lately been linked to host-pathogen interactions, although fungal EV research has mostly centred around yeasts that cause human infections. In addition, EVs have, in the last five years, been associated with biofilm functions. *Fusarium verticillioides* is a serious pathogen of maize and threatens food security through mycotoxin (Fumonisin) contamination of maize-related products. For this purpose, the aim of this chapter was to characterized fungal EVs isolated from *F. verticillioides* biofilm and planktonic cells and compared their properties. The results revealed, among other things, that the size distribution and shape of these EVs are comparable to EVs obtained from other fungal pathogens. Both planktonic and biofilm EVs had the usual cup-like form of EVs, and the sample had an equal amount of multi-lobed, rosette-shaped particles. Furthermore, the average quantity and size of EVs recovered from *F. verticillioides* planktonic cells were 1,5x $10^9$ particles/ml, with a mean diameter of 189.33 +/- 14.1 nm. The average quantity and size of EVs extracted from *F. verticillioides* biofilm cells were 7,13x $10^8$ particles/ml, with a mean diameter of 185.95 +/- 12,9 nm. In addition, uptake analysis shows that spores can uptake EVs and when stained with FM4-64 led to a distinct labelling pattern compared to control samples. This is the first in vitro study of *F. verticillioides* EVs, and it serves as a foundation for future research to investigate their role in the infection cycle of the maize pathogen and, more broadly, to enhance the understanding of EV production in filamentous phytopathogens.

## 1. Introduction

The conventional secretion pathway is the best-documented transport mechanism in eukaryotes that takes proteins from the ER to the Golgi apparatus, then to the trans-Golgi network, and finally to the plasma membrane (Viotti, 2016). On the other hand, extracellular vesicles (EVs) are tiny lipid bilayers that provide non-conventional transportation of biologically active molecules that have been released from the cells. Since they contain bioactive compounds (e.g., nucleic acids, proteins, polysaccharides, lipids, and secondary metabolites), EVs possess the capacity to influence many aspects of cell biology, and they are produced by organisms from all kingdoms of life (Théry et al., 2018; Yáñez-Mó et al., 2015).

Not only do cargos enclosed in EVs make them a useful communication system, but cells also produce distinct types of EVs, including exosomes, ectosomes, and apoptotic bodies, which have been classified based on their biogenesis and release mechanisms (Théry et al., 2018; Yáñez-Mó et al., 2015). Exosomes are generated when endosomes mature into multivesicular bodies or late endosomes. Once assembled, these endosomes fuse with the plasma membrane and emit vesicles they contain in their luminal space, called intraluminal vesicles (ILVs), into the extracellular space. When ILVs are released, they are then referred to as exosomes, ranging in diameter from species to species, typically around 40 to 120 nm in size (Cocucci and Meldolesi 2015; El Andaloussi et al. 2013). The other type of EVs is called ectosomes, microvesicles or, simply called shedding vesicles as they are shed directly from the plasma membrane. These range in size from 100 nm to 1 μm (Heijnen et al. 1999), while dying cells produce vesicular apoptotic bodies that are typically 50 nm–2 μm (Théry et al. 2001).

EVs can carry cargo either on the vesicles membrane (e.g., transmembrane proteins and lipid rafts) or the cargo can be internalized (e.g., proteins, nucleic acids, lipids and secondary metabolites) (Bielska et al., 2018; Kalra et al., 2012). Unlike in humans where EVs have been classified into sub-classes depending on size and manner of biogenesis, it is uncertain if the same holds true in fungi, despite the isolation of EVs of various sizes that correlate to ectosomes and exosomes (Rodrigues et al., 2013). EV biology in filamentous fungi is poorly understood and has only been analysed in a few species, including *Fusarium oxysporum f. sp. vasinfectum* (Bleackley et al., 2020;

Garcia-Ceron et al., 2021), *Alternaria infectoria* (Silva et al., 2014), *Rhizopus delemar* (Liu et al., 2018) and *Penicillium digitatum* (Costa et al., 2021). In the fungal pathogens studied so far, EVs have been found to promote fungal virulence, and have even been referred to as 'fungal virulence bags' (Hill and Solomon, 2020). For instance, Zhao et al. (2019) discovered that yeast EVs may play a function in the remodelling of the cell wall implying that they are a crucial role in immune system regulation during infection (Bleackley et al., 2020; Ikeda et al., 2018). Proteins involved in polyketide production, cell wall remodelling, proteases, and possible effectors were also found that in fungal EVs by Bleackley et al. (2020). However while most EV-associated proteins have been well characterized, many proteins remain uncharacterized or incorrectly annotated.

Biofilms are defined as surface-associated communities with a high cell density and an extracellular polymeric matrix (Hall-Stoodley et al., 2004). This microbially generated matrix typically protects cells against antimicrobial treatment, external stresses and enables microorganisms to survive in hostile environments. In the medical environment, biofilm development and antimicrobial drug resistance lead to disease recurrence (Baldrich et al., 2019). Interestingly, EVs have been shown to play a crucial role in the biology of microbial biofilms, such as biofilms formed by some bacteria (e.g., *Helicobacter pylori, Pseudomonas aeruginosa*), whereby they have often been associated with pathogenic outcomes of these microbes (Leone et al., 2018; Wang et al., 2015).

In recent years, Zarnowski et al. (2018, 2021, 2022) began to unpack the role of EVs in the biofilm biology of *Candida albicans*. For instance, they have revealed that biofilm EVs enhance extracellular matrix development and related resistance to antifungal drugs. Their findings in 2018 demonstrated that *C. albicans* biofilms produce a distinct EV population and cargo. Specific cargos of these biofilm-associated EVs have been identified and include surface Cu-only superoxide dismutase 5 (escape innate immune surveillance), survival factor 1 (for cell survival pathways), and survival factor 1 (survival in response to oxidative stress) (Zarnowski et al., 2018). Multiple EV cargo proteins' roles in biofilm matrix building, drug resistance, fungal cell adhesion, and dissemination were then reported in 2021 (Zarnowski et al., 2021). The authors achieved this by producing 63 homozygous deletion mutants, which were identified in the ESCRT cargo clustering study, and by analysing their biofilm properties. These

findings support earlier work, demonstrating that EVs enable community coordination during biofilm production in *C. albicans* (Zarnowski et al., 2021). Most recently they identified the composition and function of biofilm-associated vesicles in five *Candida* species (Zarnowski et al., 2022). Here the similarities in particle sizes and release throughout the biofilm lifecycle were found, where proteomes varied greatly between species, and a subset of 36 similar proteins was enriched for orthologs of *C. albicans* biofilm mediators. Furthermore, vesicles were found to impact the formation of beneficial monomicrobial and mixed microbial biofilm populations. Indeed, the role of EVs in aiding microbial community functions is beginning to emerge, not only for biofilms (Motaung et al., 2020) but also for microbiota (Motaung and Steenkamp, 2021).

The filamentous fungal pathogen, *F. verticillioides*, causes diseases that negatively impacts the health of maize, either through its invasive cellular states (e.g., mycelia and biofilms) or secretion of bioactive molecules such as degradative enzymes (e.g., amylases, cellulases, proteases, and pectinases) and secondary metabolites (e.g., fumonisin mycotoxins). This fungus is often problematic to maize producers in Africa, and Sub-Saharan Africa (Braun and Wink, 2018 and references therein) by releasing toxins that can accumulate in food and nutrition and can negatively affect humans and animals. It is commonly associated with maize as an asymptomatic endophyte (Bacon and Hinton, 1996; Bacon et al., 2008). As was demonstrated previously (Chapter 2 of this dissertation), *F. verticillioides* is capable of forming biofilms. Whether EVs are implicated in the fungus pathogenic lifestyle is not known.

Only a few studies have begun to unpack the role of EVs in ferrying secondary metabolites and other virulence molecules from fungi to the host and surroundings. To fill a gap in our understanding of how EVs contribute to fungal disease in plants, the aim of this study was to isolate and characterize EVs from the maize pathogen *F. verticillioides* in both biofilm and planktonic cells. This was done in accordance with the Minimal information for studies of extracellular vesicles (MISEV) criteria (Théry et al., 2018).

## 2. Materials and methods

### 2.1. Strains and growth conditions

The *F. verticillioides* strain CMWF 1196 was donated by the Culture collection in the Forestry and Industrial Biotechnology Institute (FABI), University of Pretoria. The fungus was maintained by plating on ¼ strength PDA (Potato Dextrose Agar; 10 g of PDA powder and 12 g of Difco agar in 1L distilled $H_2O$) and incubated for 2 weeks at room temperature.

### 2.2. Biofilm formation and isolation of extracellular vesicles

For EV isolation, *F. verticillioides* conidia were cultured in ¼ PDB inoculated with 1 x $10^5$ conidia/ml at 25 °C for 72 hrs in a shaking incubator at the speed of 120 rpm. In parallel, *F. verticillioides* spores were cultured in ¼ PDB inoculated with 1 x $10^5$ conidia/ml at 25°C for 72 hrs under stationary conditions to allow them to form biofilms. Ultracentrifugation was then used to isolate and concentrate EVs from the fungal cultures following previously published methods (Bleackley et al., 2020; Rodrigues et al., 2007). Briefly, through sterile Mira cloth, the mycelium was filtered and removed from the culture medium. The resulting filtrate was then centrifuged at 4500 x *g* for 20 min at 4 °C, followed by 15,000 x *g* for 45 min at 4°C to remove spores and cell debris. The resulting supernatants were then filtered through 0.45 µm pore syringe filters (Corning® syringe filters from Sigma-Aldrich) and concentrated using the Amicon® 30 kDa Pro Purification System (Sigma-Aldrich). Following this, the concentrated sample was ultracentrifuged at 100,000 x *g* for 1 hr at 4 °C using a swing bucket rotor. The resulting pellets were washed once with Phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO4$, and 1.8 mM $KH_2PO_4$.) and ultracentrifuged at 100,000 x *g* for 1 hr at 4 °C. The pellet was resuspended in PBS and stored at -80 °C for downstream analyses.

### 2.3. Transmission Electron Microscopy (TEM)

A Transmission Electron Microscope (TEM) is typically used to identify EVs from non-EV particles since it provides resolution at the nanometre level (Théry et al., 2018). Furthermore, TEM enables single-particle analysis and is utilized to measure numerous EVs with more statistical power than typical single-EV techniques of EVs.

For these reasons, crude EV samples were visualized by TEM by negative staining. The samples were deposited on carbon-coated copper/palladium grids for 2 min then washed twice with ultrapure water drops for 10 sec. The grids with samples were negatively stained with a drop of 1% (w/v) uranyl acetate by floating the grids for 1 min on the stain and later examined with a TEM (JEM. 2100F, JEOL Tokyo, Japan) Microscope, followed by image capture.

## 2.4. Nanoparticle Tracking Analysis (NTA)

Extracellular vesicles were isolated in the same method as previously described, and the particle size and quantity of pure EVs were measured using a Nanosight NS300 (NTA NS500, Malvern, UK) equipped with a 405 nm (blue) laser. Samples (DNA-0.1 g/ml) were diluted in sterile Phosphate-buffered saline in a ratio of 1:1000 before injection using a syringe pump at a 25 AU flow rate. Three biological duplicates, with 1 min video recordings taken for analysis. All samples were tested in triplicate, and the data were analyzed with NTA 3.3 Dev Build 3.3.104 and the auto-analysis parameters.

## 2.5. Extracellular vesicles labelling and uptake

Cells have the ability to absorb EVs produced by the biofilm (Schooling and Terry, 2006). If such is the case, our hypothesis was that planktonic cells that are not part of a biofilm might also absorb EVs derived from biofilms, possibly conveying important genes and other key compounds. In order to explore this, a freshly prepared EV sample was tagged with FM4-64 dye (Molecular Probes, ThermoFisher Scientific, Argentina), with a 1:5 dilution of the probe based on the manufacturer's procedure. Isolated vesicles in 40 µl of PBS were gently mixed with FM4-64 (1 g/ml) and incubated on ice for 1 hr. PBS was added to make a volume of 3 ml and access dye was removed via ultracentrifuged at 100 000 *g*. The resulting pellets were washed once with PBS and ultracentrifuged at 100 000 and then resuspended in 20 µl PBS. An aliquot of 5 µl of *F. verticillioides* spores (~10 000 cells) was incubated with 2 µl of FM4-64-labelled biofilm-derived EVs directly on glass slides and after 3-5 min of incubation at room temperature. The results were then viewed under a confocal laser scanning microscope. As controls, *F. verticillioides* spores were incubated in PBS without FM4-64 as well incubating the spores with FM4-64 final concentration 5 g/ml) without EVs. Images were captured using an excitation wavelength of 488nm and

detected at a range of 650-750nm using the Zeiss LM 880 CLSM (Carl ZeissMicroscopy, Munich, Germany).

### 2.6. Effect of extracellular vesicles on fungal spores

To see if EVs had any morphological impact on fungal cells after uptake of biofilm-derived EVs, a typical qualitative test for the inhibition of fungal spore germination was done on microscopic slides using standard methods (Regente et al., 2017). In a final volume of 20 µl, the incubation mixture comprised of 1500 spores, 4% sucrose, and the biofilm-derived EVs sample. After 16 hours of incubation at 25 °C, the slides were examined at 100 x magnification using light microscopy (Zeiss Axioskop 2 plus Light Microscope). There were further controls that involved substituting the EVs with the exact same volume of PBS.

### 2.7. Statistical analysis

All experiments were performed in triplicate. The data were expressed as mean ± standard deviation (SD). The results were evaluated using the GraphPad Prism 9 computer program. A *p* value of 0.05 or below was deemed statistically significant in all analyses.

## 3. Results

### 3.1. *Fusarium verticillioides* biofilm and planktonic cells produce extracellular vesicles

In this study EVs were isolated from *F. verticillioides* broth cultures using the differential ultracentrifugation technique derived from Bleackley et al. (2020) and Rodrigues et al. (2007). While both TEM and NTA provided data about the size of EVs, NTA also provides information relating to the concentration of EVs. In addition, both these techniques showed that *F. verticillioides* release EV particles that resemble fungal and mammalian EVs. For instance, according to TEM analysis of EVs from planktonic (Figure 1A-B) and biofilm (Figure 1C-D) cells, both types of cells release EVs in the manner described for other fungi (Bleackley et al., 2020). The EVs of both planktonic and biofilm displayed the typical cup-like shape of EVs as described by other authors (Thery et al., 2001). In addition to the cup-shaped particles, the sample included an equal number of multi-lobed, rosette-shaped particles. Moreover, the

average number and size of EVs isolated from *F. verticillioides* planktonic cells were 1,5x $10^9$ particles/ml, mean diameter of 189.33 +/- 14.1 nm. The average number and size of EVs isolated from *F. verticillioides* biofilm cells were 7,13x $10^8$ particles/ml, mean diameter of 185.95 +/- 12,9 nm. The vesicles of planktonic and biofilm cells are similar in size but have a significant variation in concentration ($p$-value < 0.05). Biofilm cells had a concentration much lower than planktonic cells, and the reasons for this are currently unknown.

## 3.2. Uptake of biofilm-derived extracellular vesicles by *Fusarium verticillioides* fungal spores

To see if biofilm-derived EVs might be internalized by *F. verticillioides* planktonic cells, the vesicles were tagged with the probe FM 4-64 before being rinsed and ultracentrifuged to remove any unbound probe. FM 4-64 is an amphiphilic styryl dye that embeds into the membranes of EVs. The tagged EVs were then incubated with *F. verticillioides* conidia and examined to see if the dye was transmitted from the biofilm extracellular vesicles to the fungal planktonic cells. *F. verticillioides* conidia appeared red in the presence of labelled EVs, although not glowing red in the presence of PBS (control) (Figure 3A), indicating that the EVs were internalized (Figure 3B). Since the fluorescence was identified within the cells just 3-5 min after incubation, this uptake was considered to have taken place quite rapidly. On the other hand, the control direct treatment of the spores with FM4-64 led to a distinct labelling pattern (Figure 3C). In this instance, both the cell inside and the cell surface displayed fluorescence. While with the sample the spores have taken up the EVs and colocalized in the cell due to EVs being taken up by the fungal spores (Figure 3B). While with the control fluorescence it is on the surface and within the whole cell.

A conventional qualitative spore germination test was conducted to see if EVs produced from biofilms had any impact on the morphology of fungal cells. Before being examined under a microscope, spores were grown for 16 to 24 hours with EVs generated from biofilms. When spores were incubated with PBS as a control, they grew into many, straight-extending hyphae (Figure 4A-C). In contrast, no morphological alterations were found in spores incubated with EV from biofilms (Figure 4D-F), which could suggest that biofilm EVs do not control the growth of planktonic cells.

## 4. Discussion

During this study, EVs from *F. verticillioides* were successfully isolated using ultracentrifugation. To characterize the EVs, a checklist was first developed according to the MISEV (minimal information for studies of EVs) which included EV purification/enrichment, quantification and identity (size and morphology), and cellular uptake (Théry et al., 2018). Although the characterization is currently partial, it was found that EVs recovered from *F. verticillioides* displayed the typical cup-like appearance, with no apparent morphologic differences observed between EVs of planktonic and biofilm cells. The typical morphology that was observed has been reported in many other fungal species such as *F. oxysporum f. sp. vasinfectum* (Bleackley et al., 2020), *Alternaria infectoria* (Silva et al., 2014) and *Cryptococcus gattii* (Bielska et al., 2018) to mention a few. The rosette-shaped particles were previously identified in isolation of *S. cerevisiae* EVs and *F. oxysporum f. sp. vasinfectum* (Bleackley et al., 2020; Giardina et al., 2014). Since the current study characterized the EVs for the first time in the plant pathogen *F. verticillioides*, this work lays down the groundwork for future investigations of *F. verticillioides* EVs and their involvement in the invasion of maize.

The vesicles of planktonic and biofilm cells were similar in size and no significant differences were detected. NTA confirmed that the EV population had a similar size distribution to that reported for *F. oxysporum f. sp. vasinfectum, Zymoseptoria tritici C. albicans and Candida neoformans,* both of which employed NTA for particle size analyses (Bielska and May 2019; Bleackley et al., 2020; Hill and Solomon, 2020). Larger vesicles (diameter larger than 450 nm) have been found in *C. albicans, C. neoformans,* and *Malassezia sympodialis* (Bielska and May, 2019), and were also discovered in this research, but in extremely low quantities. The EVs of *F. verticillioides* have a significant variation between planktonic and biofilm cells in terms of concentration which could affect how the microbe interacts with its host.

The cargo ferried by the biofilm and planktonic-derived EVs of *F. verticillioides* is still unknown. However, The difference in some biofilm EV cargos relative to planktonic EV cargos comes unsurprising since EVs tend to mirror features of their donor cells, regardless to varying extents (Mathieu et al., 2019; Théry et al., 2018; Yáñez-Mó et al., 2015). Zarnowski et al., (2018, 2022) findings show that EVs generated by biofilms

differ from those produced by planktonic cells. According to their proteomic analysis, planktonic and biofilm EVs both carry a sizable amount of unique cargo, with the biofilm cells having 34% of the unique cargo. Furthermore, it was discovered that the abundance of several proteins in vesicles from both origins was 10- to 100-fold higher in biofilm EVs (Zarnowski et al., 2018). Their research also suggests that biofilm EVs may secrete cargo that directly affects matrix structure and catalytic activities involved in the synthesis of matrix polysaccharides. To increase biofilm drug resistance and biofilm matrix formation, biofilm EVs transport valuable cargo (Vitse and Devreese, 2020; Zarnowski et al., 2018, 2022). By introducing wild-type biofilm vesicles into a vesicle-deficient ESCRT mutant, Zarnowski et al. (2018) demonstrated that vesicles are directly involved in matrix formation, which should correct matrix synthesis and matrix-related abnormalities. Unexpectedly, adding wild-type vesicles to drug-sensitive ESCRT mutants significantly improved drug resistance. The results show that biofilm EVs transport beneficial cargo to increase biofilm drug resistance.

Schooling and Beveridge (2006) researched *Pseudomonas aeruginosa, Escherichia coli, Shewanella oneidensis,* and *Azotobacter sp.* vesicles from biofilm and planktonic cells. The study revealed that biofilm vesicles assemble inside the extracellular matrix, in contrast to planktonic bacterial vesicles, which disperse from donor cells once liberated. They also showed that during processes like infection and ecological succession, biofilms might successfully serve as "safe home" producers, vesicle depositories, and export depots. Their results further demonstrate that biofilm vesicles had higher proteolytic activity than planktonic cell vesicles, which is a crucial point to consider when taking into account the potential roles that vesicles may play in the infection process, nutrient release, and possibly surface modification. Before anti-microbial action affects the cells, biofilm vesicles may act as "decoys or sponges" to reduce it. Last but not least, the researchers show that biofilm vesicles seem to be independent, "extracellular extensions of the cell," which extend the typical confines of the cell (Schooling and Beveridge, 2006). Together, the results of these investigations make a compelling case that EVs derived from biofilms reflect the biology of biofilms and their potential influence on the biology of fungal pathogens. When uptake of EVs originating from biofilms was performed by *F. verticillioides* cells for this analysis It is important to highlight that, due to time constraints, only the germination of asexual cells was addressed in these investigations; additional

research is necessary, including an examination of the production of virulence factors by cells that have ingested EVs produced from the biofilm.

## 5. Conclusion

A better understanding will be gained of molecular plant-pathogen interactions and how these affect maize's response to internal and extrinsic stimuli by characterizing EVs from *F. verticillioides* and other agriculturally significant fungal pathogens. While EVs from human pathogenic fungi have been strongly associated with virulence, it is unclear how EVs play a role in the interactions between plant pathogens and their hosts. The EVs recovered from both planktonic and biofilm *F. verticillioides* cells displayed the typical appearance of EVs from other species, according to TEM. Both planktonic and biofilm EVs had the characteristic cup-like and also contained an equal amount of multi-lobed, rosette-shaped particles. NTA shows that the sizes between planktonic and biofilm EVs were not significantly different. In terms of concentration, there was a significant difference in biofilm and planktonic cell-derived EVs (P-value < 0.05) with biofilms secreting a lot fewer EVs, which could affect how the microbe interacts with its host.

Although membrane vesicles as cellular messengers are described in a small number of human fungal diseases. There are currently very few studies reporting on fungal plant pathogen EVs, and even those that do exist are limited since they do not contain EVs produced from biofilms. The current studies in plant-fungal pathogens show that fungal vesicles can deliver their bioactive cargos, including several virulence factors (polyketides or mycotoxins) from the releasing fungal pathogen to a plant host. They have often been associated with a complex array of outcomes on recipient cells including subverting host immunity genes and inducing other immunomodulatory effects such as phytotoxicity. These biofilm-derived EVs may significantly contribute to pathogenesis, pathogenicity, and ultimately the outcomes of disease. In addition, the relevance of fungi-derived EVs during plant-pathogen interactions in crops such as maize has yet to be thoroughly investigated. This is even though plant pathogenic EVs from both planktonic and biofilm cells could be important in the secretion and distribution of fungal virulence factors including secondary metabolites such as mycotoxins.

## 6. Future work

It is important to explore the potential activities of *F. verticillioides* EVs through a proteomics approach. The proteomes of EVs from planktonic cells and biofilms of this fungal pathogen are presently being uncovered in our laboratories as a result, which will provide us with new insights into its biology. The plan is also to perform infection studies on maize plants to determine whether EVs produced by *F. verticillioides* have any impact on maize plant tissue. This is particularly important because previous studies reported EV-induced phytotoxicity in plants (Bleackley et al., 2020), indicating that EVs are the cause of the reaction. Enzyme assays should be carried out for biofilm-derived EVs to further demonstrate the pathogenicity potential of these EVs, just as they were for biofilm-derived cells in Chapter 2 of this dissertation. This is significant because extracellular enzymes are required by a fungus not only for digesting but also frequently for activities that are harmful to the plant host. As a result, future studies like the ones already stated will enable us to comprehend the contents of the vesicles and the impact that they have, providing us with a better understanding of the biology of the EVs produced by *F. verticillioides*.

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## 8. Figures

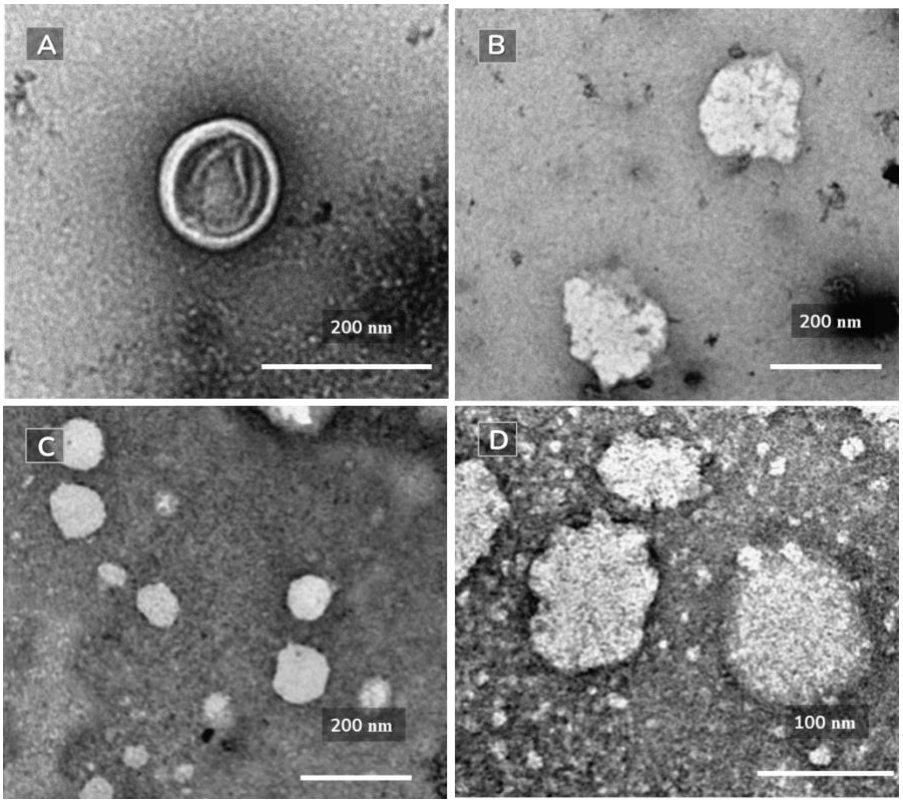


**Figure 1:** *Fusarium verticillioides* planktonic and biofilm cells secrete extracellular vesicles (EVs). TEM characterization of EVs from *Fusarium verticillioides* TEM images of EVs from planktonic **(A-B)** and biofilms **(C-D).**

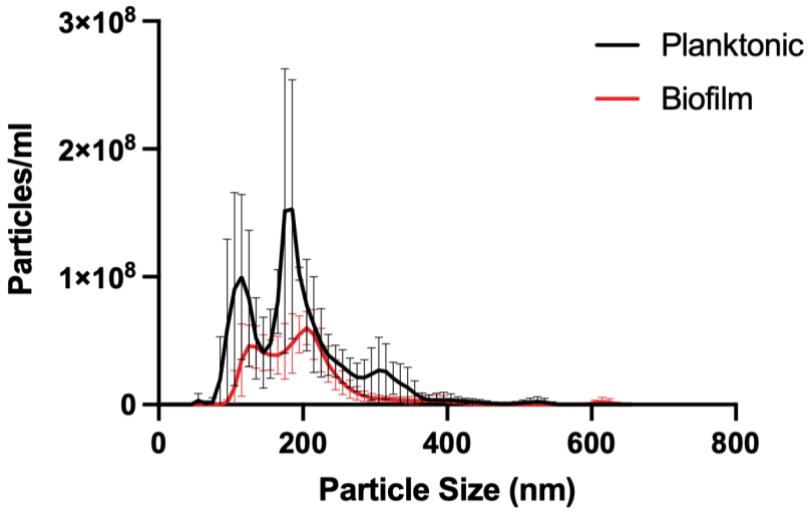


**Figure 2:** Characterization of the size and concentration of EVs isolated from *Fusarium verticillioides* growing as both biofilms and planktonic cells. Nanoparticle tracking analysis was used to assess the quantity and size distribution of the isolated EVs. The average number and size of EVs isolated from *F. verticillioides* planktonic cells were 1,5x $10^9$ of culture, mean diameter 189.33 +/- 14.1 nm. The average number and size of EVs isolated from *F. verticillioides* biofilm cells were 7,13x $10^8$ of culture, mean diameter 185.95 +/- 12,9 nm. Planktonic and biofilm cell vesicles are identical in size but differ significantly in concentration (*p*-value 0.05).

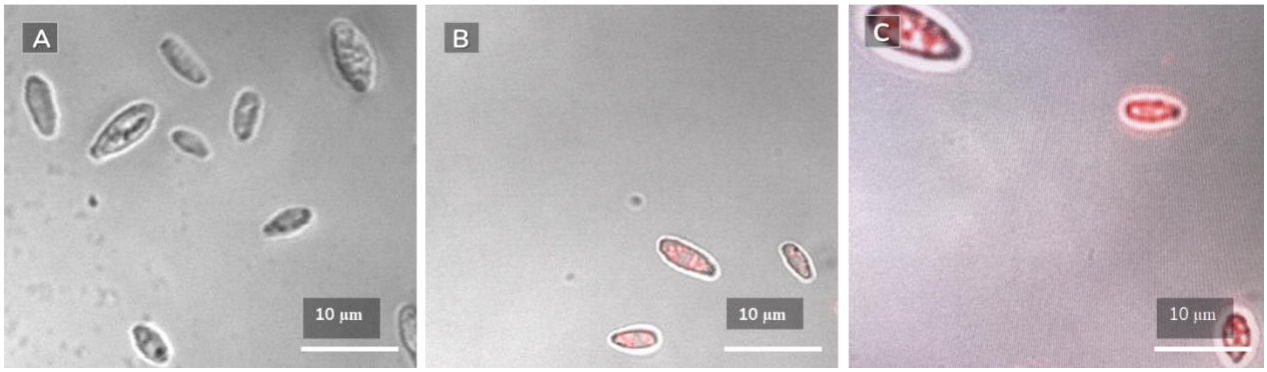


**Figure 3**: Uptake of biofilm extracellular vesicles (EVs) by *Fusarium verticillioides* spores. Control spores in PBS show negative red autofluorescence**(A)**. Spores incubated for 5 min with FM4–64-labelled EV **(B)**. Spores treated with FM4–64 **(C)**. *F. verticillioides* conidia became red in the presence of tagged EVs but not in the presence of PBS (control), suggesting that the EVs had been absorbed. Direct treatment of the spores with FM4-64 on the other hand resulted in a different labelling pattern.

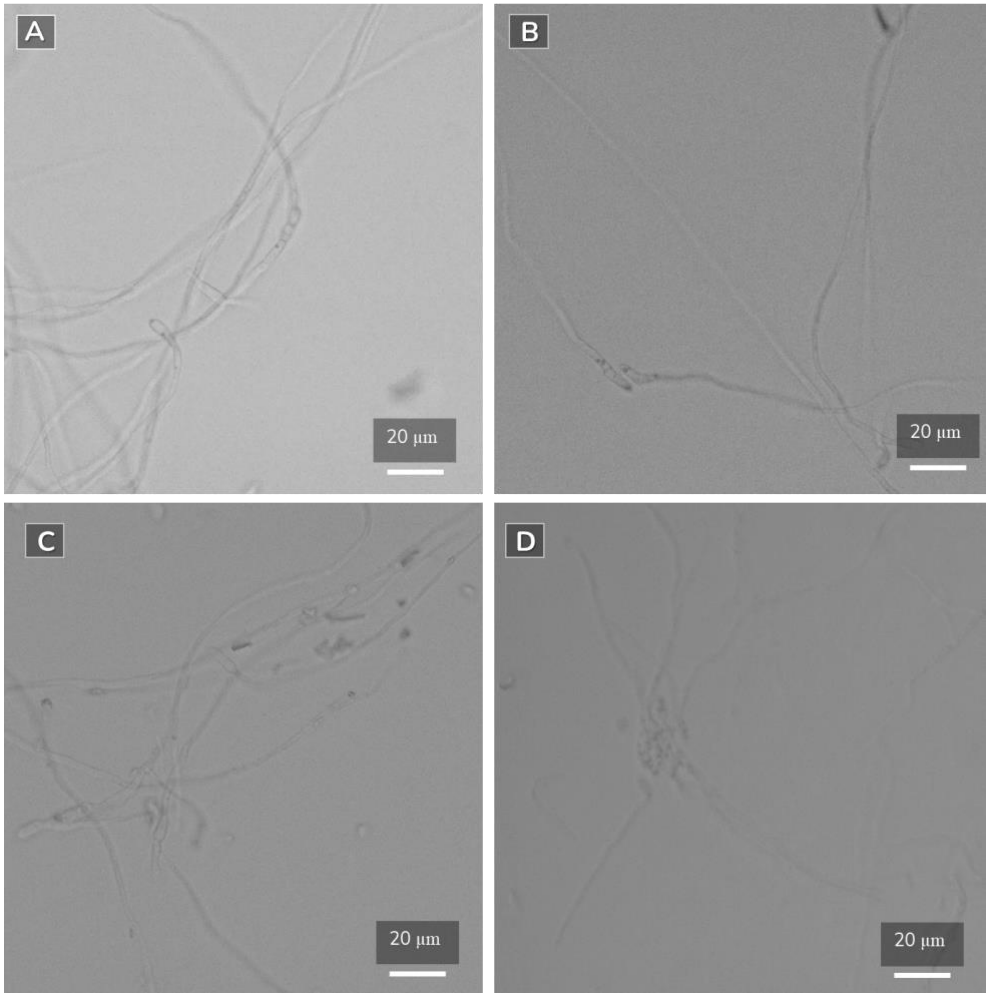


**Figure 4**: Extracellular vesicles (EVs) have no impact on *Fusarium verticillioides* germination of spores or mycelial growth. *Fusarium verticillioides* spores were incubated for 16 h with EVs **(A-B)** (1.5 µg of proteins) or PBS **(C-D).**

## Summary

Extracellular vesicles (EVs) have substantial functional consequences in fungi because they internalize bioactive molecules (e.g., proteins, nucleic acids, and secondary metabolites) from their source cells. Internalized contents can be released into the extracellular environment, where they can either persist for long periods of time because they are protected by the vesicle membrane from potentially harmful agents (e.g., degradative enzymes), ensuring their potency, or they can be readily absorbed by recipient cells, causing a variety of physiological effects. In fungi such as *Candida albicans*, EVs enhance virulence factor production such as biofilms, a matrixed and architecturally complex microbial community typically attached to a surface and embedded in a gelatinous matrix composed of extracellular polymeric substances (EPS). As EVs are also produced by biofilms, which are a key virulence determinant in nearly all microbial pathogens, a thorough understanding of their role in biofilm formation is key. During this study, it was first thoroughly characterized biofilm formation in the important disease-causing agent in the maize crop, *Fusarium verticillioides*. Then, EVs were recovered and characterized from free-living (planktonic) cells and biofilms of *Fusarium verticillioides*, offering fresh information on how key signals (e.g., nutrients, enzymes, genes, RNA, DNA, metabolites, and so on) are exchanged to contribute to biofilm development and structural integrity. Given that both EV and biofilm biology in phytopathogens are poorly understood, these findings will greatly contribute to this knowledge gap and serve as a reference point for further research into biofilms as a poorly represented virulence determinant in fungal phytopathogens.