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Harnessing exogenous membrane vesicles for studying *Fusarium circinatum* and its biofilm communities

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ABSTRACT

Extracellular vesicles (EVs) are tiny messengers that convey bioactive molecules from donor to recipient cells, leading to changes in their physiology and function. We investigated the role of EVs in shaping growth and the biofilm biology of the tree pathogen *Fusarium circinatum* and its interaction with the susceptible host, *Pinus patula*. Vesicles were collected from fungal planktonic and biofilm cultures and from pine seedling needles and roots. The physical properties of these vesicles were analysed using nanoparticle tracking analysis and transmission electron microscopy, which revealed a diverse range of sizes and shapes, respectively. Furthermore, uptake of vesicles by conidia was conducted. The results demonstrated that *F. circinatum* EVs significantly but variably affected spore viability during the early phase (2–4 h) although they enhanced fungal biofilm integrity. In contrast, *P. patula* EVs greatly inhibited hyphal formation and biofilm biomass, but failed to inhibit matrix production in the fungal biofilm. Our results therefore show that conidial germination is essential for late fungal development including hyphal and biofilm formation while matrix production is a counter measure against harsh environmental conditions including the effects of plant-derived EVs.

1. Introduction

Many laboratories are increasingly investigating the role of extracellular vesicles (EVs) as transporters of bioactive cargo to recipient cells and their influence on the recipients' cellular behaviour. When eukaryotic EVs bud inwardly or outwardly from the parent cell (respectively forming exosomes or microvesicles), they engulf payloads that such as proteins and nucleic acids (DNAs, miRNAs, siRNAs, and lncRNAs). This cargo is then delivered to the recipient cell through membrane fusion [1] and the inward budding of the membrane (e.g., clathrin-mediated endocytosis) [2,3]. Because of this, EVs can orchestrate coordinated responses among cells, organs, and even entire organisms [1,3–6]. The role of EVs also extends to biofilms where they are crucial for cellular communication [7–14], likely by mediating synergistic and antagonistic interactions among inhabitants [15].

All microbes can grow attached to biotic and abiotic surfaces, forming complex communities embedded in self-produced slimy extracellular matrix (ECM) [16,17]. As with EVs, the varied biological traits associated with biofilms have garnered significant research interest from the scientific community [18]. This is particularly true for the yeast and bacterial pathogens of humans and animal, where biofilm formation is often integral to infection and disease development [19–22]. Although this growth form has been investigated in certain bacterial pathogens of plants [23], relatively little is known about biofilm formation in the filamentous fungal pathogens of plants [18,24]. The ECM is a biofilm's major structural constituent and is made up largely of extracellular polymeric substances comprising of organic polymers such as carbohydrates, extracellular DNA, lipids, and proteins [16]. Because the ECM also contain EVs, it is essentially involved in various complex and intricate processes within the biofilm ecosystem [16]. In addition to accounting for much of the characteristic 3D architecture of a biofilm, ECM is thus also responsible for a biofilm's emergent properties, which include physical and social interactions, chemical heterogeneity, and greater tolerance to chemical and environmental stresses [25].

Here we explored the role of EVs and biofilm formation in the interaction of the filamentous fungus *Fusarium circinatum* (Ascomycota,

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Nectriaceae) with its *Pinus* (Plantae, Pinaceae) host. This fungus is the causal agent of Pine Pitch Canker, which annually causes substantial economic losses wherever pine species are cultivated commercially [26]. In production nurseries, it causes devastating root and root collar disease of seedlings, which often hampers the establishment of new plantations [27–30]. In established plantations or natural stands, the pathogen causes large resinous cankers on tree trunks and branches, leading to branch die-back, stunted growth and overall yield reductions [31]. Although some nurseries utilize antifungal treatments for controlling *F. circinatum*-associated seedling disease, the pathogen is mostly controlled using standard nursery and silvicultural practices, with the most efficient strategy involving deployment of resistant planting stock [31,32].

In addition to growing as free-living mycelium (i.e., planktonic growth form/mycelium free of the ECM), *F. circinatum* can also grow as biofilms in which ECM-embedded mycelia form compact mat-like colonies [33]. This is similar to reports from other notable *Fusarium* species, where biofilm formation appears to provide the pathogen protection against environmental stresses and the plant host's defence responses [34]. Although the role of biofilm formation during the pine-*F. circinatum* interaction is currently unknown, biofilms of the pathogen are more resistant to environmental stresses [33]. In this fungus, enhanced biofilm formation and increased ECM production during exposure to fungicide or elevated treatments [33] might thus also point to an adaptation for dealing with pine's defence response. Here we further explored this hypothesis by bringing pine and fungus-derived EVs into consideration.

Our aim was therefore to examine how ECM production and biofilm formation by *F. circinatum* influences its responses to EVs produced by itself and by pine. Overall, our findings highlighted an EV-mediated complex interaction by both the planktonic and biofilm states of the fungus with the plant host. Understanding the involvement of EVs in *F. circinatum* communication biology will contribute a foundational step for probing deeper into the impact of vesicles on the regulation of interactions among tree pathogens, as well as their interactions with host organisms.

2. Materials and methods

2.1. Isolate and growth conditions

We sourced isolate FSP34 of *F. circinatum* [35] from the *Fusarium* collection of the Forestry and Agricultural Biotechnology Institute (FABI; University of Pretoria, South Africa). For routine culturing, the fungus was incubated on potato dextrose agar (PDA; 10 g/L; Merck Group) in darkness at 25 °C for 7 days. For obtaining conidia, a 7-day--old culture was flooded with 2 mL of 0.2 M phosphate-buffered saline (PBS) consisting of 10 mM NaH_2PO_4, 10 mM Na_2HPO_4, and 150 mM NaCl at pH 7.2. Following collection with a sterile pipette, the Countess 3 Automated Cell Counter (ThermoFisher Scientific) was used to quantify the concentration of the spore suspension.

3. Isolation and quantification of EVs from fungal and plant tissue

Fungal EVs were isolated from 72-h planktonic and biofilm cultures, prepared in 200 mL potato dextrose broth (PDB; 10 g/L; Merck Group). This involved sequential centrifugation steps (4000 *g* for 15 min and 15,000 *g* for 30 min at 4 °C) for pelleting fungal tissue, after which the supernatant was passed through a 0.45 μm membrane filter (Merck Millipore) to eliminate any remaining cells or debris. By making use of the Amicon Ultra-4 Centrifugal system (100-kDa pore size, Millipore), the supernatant was concentrated to approximately 20 mL. The concentrated supernatant was then subjected to ultracentrifugation (100,000×*g* for 1 h at 4 °C) using Beckman Coulter equipment. The supernatant was discarded, and the EV-enriched pellet was washed twice with PBS at 100,000×*g* for 1 h at 4 °C. Finally, the EV pellet was stored in PBS at 4 °C until further use.

Isolation of EVs from the roots and needles of *P. patula* seedlings were performed as previously described [36]. Briefly, a total of two healthy seedlings per isolation were used, whereby entire leaves and roots were rinsed and weighed, and distal zones of needles and roots from the same plant were collected. After removing excess water, the plant material was ground using a pestle and mortar in the presence of vesicle isolation buffer containing 5.9 g/L NaCl, 0.002 g/L $CaCl_2$, and 4.3 g/L 2-(N-Morpholino)ethanesulfonic acid hydrate (MES hydrate). This homogenate was filtered by passing it through a cheesecloth, and then centrifuged for 20 min at 700×*g* at 4 °C to separate the extracts. Concentrated EVs obtained using the Amicon Ultra-4 Centrifugal system (10-kDa pore size) were then subjected to size-exclusion chromatography (SEC) as described previously [36,37]. This involved application of a mixture containing EVs labelled with a cell membrane-specific dye (FM4-64; Thermo Fisher Scientific) to a Sepharose CL-2B column. Fractions with fluorescence were generated and those above 3.0 RFU were pooled as "EV signal" after measurement.

4. Analyses of the general properties of vesicles

The general features of the extracted EVs were studied using electron microscopy and nanoparticle tracking analysis (NTA). Purified EVs were spotted onto carbon-coated grids for adsorption for 5 min, negatively stained with 1 % (w/v) uranyl acetate for 3 min and subjected to transmission electron microscopy (TEM) using the JEM-2100F Field Emission Electron Microscope (JOEL Ltd., Tokyo, Japan). Scanning electron microscopy (SEM) was carried out as previously described (Ratsoma et al., 2023b) for biofilms in order to observe EV-like structures on the surface of the cells. Briefly, biofilms formed on glass slides were rinsed with PBS and treated with a 2.5 % glutaraldehyde/formaldehyde solution, followed by fixation with 1 % osmium tetroxide Subsequent dehydration steps using graded ethanol concentrations (30 %, 50 %, 70 %, 90 %, and absolute ethanol) and a 50:50 mixture of hexamethyldisilazane (HMDS) were performed. The dried samples were mounted on aluminum stubs, carbon-coated (Quorum Q150T ES sputter coater), and observed using a JEOL JSM 6490LV scanning electron microscope (GenTech Scientific Inc., Arcade, NY, USA). For NTA, 5 mL of an EV suspension was vortexed for 1 min, and diluted with PBS to a total of 10 mL, of which 1.5 mL was immediately analysed with the NanoSight NS500 (Malvern Panalytical, UK) in triplicate (each run = 30 s video). PBS was treated as a blank. The videos were captured and analysed using the NanoSight NTA software (NTA 3.3 Dev Build 3.3.104, Malvern Panalytical, UK). The camera sensitivity and detection threshold were optimized per video, and the temperature was set at 22 °C. We further determined the concentrations of EVs derived from planktonic cells (p^{kc}dEVs), biofilms (bdEVs), and pine seedling needles (ndEVs) and roots (rdEVs) based on protein content using the QuantiPro™ BCA Assay Kit (Merck) following manufacturer's instructions.

5. Evaluating the effects of EVs on conidial germination and biofilm formation

The analyses of the effects of EV on conidial viability and germination, as well as biofilm formation were conducted using previously published methodologies ([38]; Honorato et al., 2021; [39]). To induce spore (conidia) germination, the experiments were conducted using ¼ strength potato dextrose broth (PDB), a standard approach to promote fungal germination under reduced nutrient conditions. In one experiment, 15 μg/ml EVs derived from planktonic cells (p^{kc}dEVs), biofilms (bdEVs), and pine tissues (pdEVs) were co-incubated with conidia to a final concentration of 5 x 10^5 spores/mL at room temperature. The mixture was incubated for 16 h under shaking conditions (100 rpm, Shake-O-Mat, Labotec South Africa). Following this, cultures treated and not treated with EVs were examined using an automated cell counter

(Countess 3 FL, Thermo Fisher Scientific) that incorporates Trypan Blue staining to selectively colour dead cells blue, distinguishing them from viable ones. We also observed the cultures between 2 and 24 h following shaking using a haemocytometer to determine the number of germ tubes emerging from viable conidia. In the second experiment, all types of EVs isolated were co-incubated with conidia as before, but incubated statically to allow fungal spores to settle at the bottom of the plate in order to achieve biofilm formation. After this, the biomass and ECM of biofilms were analysed as described above, at 72 h and 7 days. Biofilms formed without EV treatments were treated as a positive control, while a negative control included PDB with EVs only and EVs that were heat-inactivated in PDB at 90 °C for 15 min.

6. Biofilm biomass and matrix quantification

Biofilm and planktonic cultures were grown as described previously (Ratsoma et al., 2023b) using either 96-well polystyrene plates (Greiner bio-one, South Africa) or 500 mL pyrex Erlenmeyer flasks (Merck, South Africa). In case of the 96-well plates, conidia were suspended to a final concentration of 2 x 10^5 spores/mL in 400 μL PDB, followed by incubation at room temperature for 72 h and 7days under stationary conditions to allow biofilm formation. Planktonic cell cultures were grown in the same way in these plates, except that they were incubated on a shaker (100 rpm). In case of the Erlenmeyer flasks, we inoculated 200 mL of PDB with conidia to a final concentration of 1 x 10^6 spores/mL, followed by incubation at room temperature for 72 h on an orbital shaker at 100 rpm for the planktonic cultures or statically for the biofilm cultures.

Biomass and ECM analysis of planktonic and biofilm (72 and 7 days) cultures of FSP34 in 96-well polystyrene plates were conducted as before [33]. Briefly, biofilms were rinsed with PBS to remove non-adherent cells, while planktonic cells were collected by centrifugation (10000 *g* for 10 min) and rinsed the same way as biofilms. Cells from cultures were then fixed with methanol and air-dried, followed by respectively crystal violet and fuchsin staining [33]. The former allowed visualization of fungal cells alongside certain components within the biofilm matrix, while the latter allowed for visualization and distribution of polysaccharides in the matrix. We then used a spectrophotometer (SpectraMax paradigm, Multimode detection platform) to quantify biomass (590 nm) and ECM production (530 nm).

7. Morphological analyses of fungal cells exposed to vesicles

In order to determine the morphological effects in culture of EVs on fungal cells, a conidial suspension (5 x 10^5 spores/mL) was pre-treated with 5 μg/mL p^{kc}dEVs and bdEVs, as well as 15 μg/mL ndEVs and rdEVs for 24 h. The pre-treated spores were then allowed to grow for 7 days at room temperature on triplicate PDA plates (Merck, Modderfontein GP, South Africa; 4 g/L Potato Infusion, 20 g/L D (+)-Glucose), and 15 g/L agar). Following this, mycelial growth was quantified from by measuring the diameter of colonies after 3, 7, and 14 days. In addition, a loopful of cells taken from the periphery of a 7-day-old and 14-day-old culture and analysed using a light microscope and a scanning microscope.

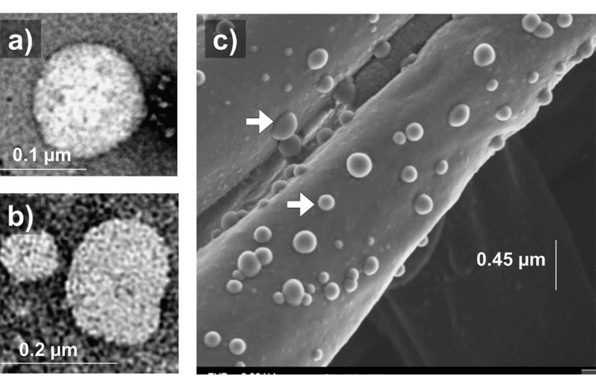
8. Reproducibility and statistical analyses

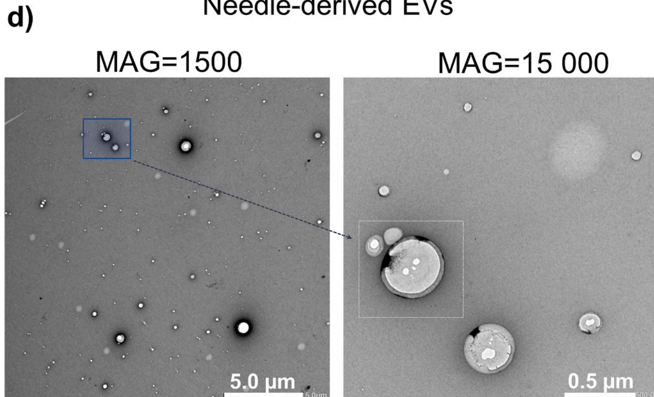
All experimental data, unless specified otherwise, were subjected to one-way ANOVA or non-parametric Student's t-test for comparison. Statistical analyses were performed using GraphPad statistical software (GraphPad 5 Software, San Diego, CA, USA). The level of significance was determined through the representation of mean ± Standard Deviation (SD) and defined using probability thresholds: *, $P \leq 0.05$; **, $P \leq 0.002$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$. In most cases, each experiment was conducted with three technical replicates and five biological replicates, unless stated otherwise. Additionally, all experiments included both positive and negative controls to assess the validity and reliability of the experimental results.

9. Results

9.1. *Pine and Fusarium circinatum-derived EVs have diverse sizes and shapes*

We employed NTA and TEM analysis to investigate the physical properties of EVs derived from *F. circinatum* planktonic and biofilm cultures, as well as from *P. patula* roots and needles. The EVs from all these sources exhibited a range of sizes and various shapes, including spherical, rosette, and cup-shaped (Fig. 1a–d). NTA measurements





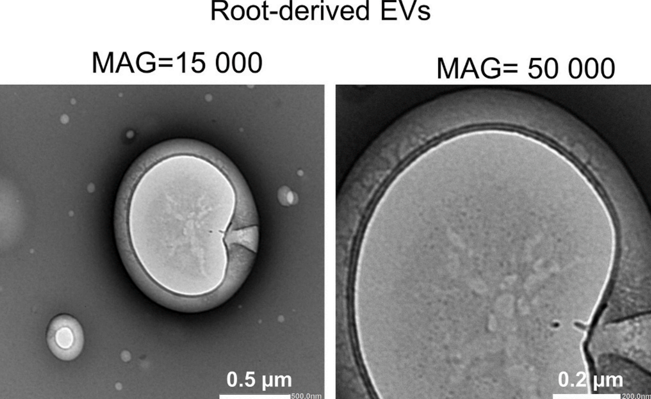


Fig. 1. Physical properties of extracellular vesicles (EVs) visualized using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Vesicles were purified from Fusarium circinatum planktonic cells **(a)** and biofilms **(b, c - whit arrows)** as well as from Pinus patula needles and roots **(d).** Images are shown at varying magnifications, as indicated by 'MAG.

indicated that *F. circinatum* EVs ranged in size from 100 to *ca*. 400 nm (Fig. 2a), while *P. patula* EVs ranged in size from 5 to *ca*. 300 nm (Fig. 2b). Furthermore, SEM analysis of the *F. circinatum* biofilms showed the presence of EV-like structures on the surface of hyphal cells, which appeared to be budding from the plasma membrane (Fig. 1c). The EVs of *P. patula* displayed a distinct outer layer with low density and an inner compartment with high density (Fig. 1d).

9.2. Pine-derived EVs inhibit conidial germination

We investigated the influence of pine-derived EVs on the germination of *F. circinatum* conidia. This was done by first examining the effect of these EVs (pdEVs) on spore viability using Trypan blue, which stains dead cells blue, distinguishing them from live cells (unstained). We found that exposure to ndEVs and rdEVs led to a reduction in spore viability, primarily noticeable after 2 h of incubation, with the former having a more pronounced effect relative to the latter (Fig. 3a–b). Additionally, these EVs also inhibited germ tube formation to varying degrees depending on time of exposure and EV concentration. In the case of ndEVs, the inhibition of conidial germination was more pronounced at higher EV concentrations at the earlier (4 h, 8 h) time points (Fig. 3c), while rdEVs exerted their inhibitory effects at all concentrations and time points after 4 h (Fig. 3d). This time and dose-dependent inhibition suggests that the pine-derived EVs may gradually impair germ tube development, pointing towards a role in regulating the early stages of fungal growth. Intriguingly, at a lower concentration (5 μg/mL), ndEVs appeared to promote conidial germination (Fig. 3c; $P \leq 0.002$). Taken together, our results indicate that *P. patula* EVs have dynamic effects on *F. circinatum* spore viability and germ tube formation, with the potential to impact the early stages of fungal growth and development.

9.3. Pine-derived EVs inhibit mycelial growth of Fusarium circinatum

Fungal cultures established from conidial suspensions that were first co-incubated with pine-derived EVs grew significantly slower on PDA than those grown from untreated conidia (Fig. 4a). This inhibitory effect was evident for both ndEVs and rdEVs after 14 days of growth on PDA, suggesting that these vesicles have effects on fungal growth lasting for at least two weeks. Radial growth was also measured in order to quantify the effects of vesicles on hyphal growth on agar plates. The results confirmed what we observed in terms of hyphal growth by *F. circinatum* colonies of cells exposed to vesicles (Fig. 4b). Further corroborating this data was the highly reduced hyphal density in cells taken from a culture grown for up to 14-days following exposure to EVs (Fig. 4c–d), suggesting that the observed effects on colony morphology had a cellular origin. Taken together, these results support the idea that pine-derived EVs inhibit mycelial growth of *F. circinatum*.

9.4. Pine-derived EVs negatively impact biofilm biomass in Fusarium circinatum

A 24 h exposure of fungal spores to pine-derived EVs also significantly influenced biofilm formation in *F. circinatum* (Fig. 5). When conidial suspensions that were first co-incubated with pine-derived EVs were grown statically in PDB, we observed a significant inhibitory effect on biofilm biomass production, with rdEVs showing a more potent impact across all concentrations (Fig. 5a–b), consistent with inhibition of spore germination (Fig. 3d). Taken together, this data suggests that pine-derived EVs, especially rdEVs, can reduce the size of a biofilm.

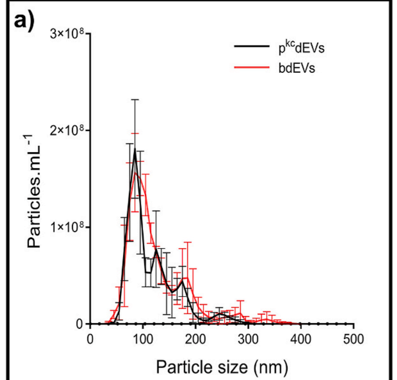
9.5. Pine-derived EVs failed to inhibit matrix production in Fusarium circinatum biofilms

ECM production was inhibited only by the rdEV treatment in the 72-h-old biofilm, however, neither ndEVs nor rdEVs appeared to impair ECM production in the older biofilm (Fig. 5c–d). Furthermore, in the older biofilms, pre-treatment with ndEVs and rdEVs both resulted in higher ECM-to-biomass ratios (Fig. 5d–f). These results collectively suggest that pine-derived EVs have no discernible impact on matrix production in biofilms, although they may possess the ability to modulate the ECM-to-biomass ratio. Therefore, pine-derived EVs may be unable to inhibit an already established biofilm since it is usually characterized by increased matrix production. This was confirmed when preformed biofilms were exposed to EVs, where they maintained relatively the same level of biomass and matrix production as the controls (Fig. S1).

9.6. Fungal EVs promote conidial germination and biofilm formation in Fusarium circinatum

We investigated the influence of *F. circinatum*-derived EVs on the germination of its conidia over a 24-h period. At the earlier time points, both p^{kc}dEVs (2 and 4 h) and bdEVs (4 h) appeared to negatively impact conidial viability (Fig. 6a). At later time points (16 and 24 h), significantly more viable cells were observed in the EV-exposed cultures. Notably, bdEVs exhibited a more pronounced effect ($P \leq 0.001$) on cell viability compared to p^{kc}dEVs ($P \leq 0.002$) during these time intervals. This suggests that the impact of bdEVs on cell viability may be stronger and longer-lasting than that of p^{kc}dEVs. However, unlike pdEVs, neither of the EV treatments significantly affected conidial germination (Fig. 6b).

Interestingly, pre-exposure to *F. circinatum*-derived EVs enhanced biofilm formation in this fungus (Fig. 7). Although the pre-treatment with p^{kc}dEVs and bdEVs had mixed effects on biofilm biomass, the impact was marginally significant ($P > 0.05$) for p^{kc}dEVs and highly



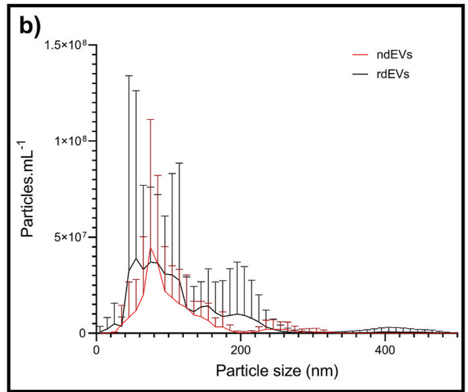


Fig. 2. Physical properties of extracellular vesicles (EVs) analysed using nanoparticle tracking analysis (NTA), with particle sizes measured in nanometres (nm). **(a)** Vesicles purified from Fusarium circinatum planktonic cells (pkcdEVs) and biofilms (bdEVs). **(b)** Vesicles purified from Pinus patula seedling needles (ndEVs) and roots (rdEVs).

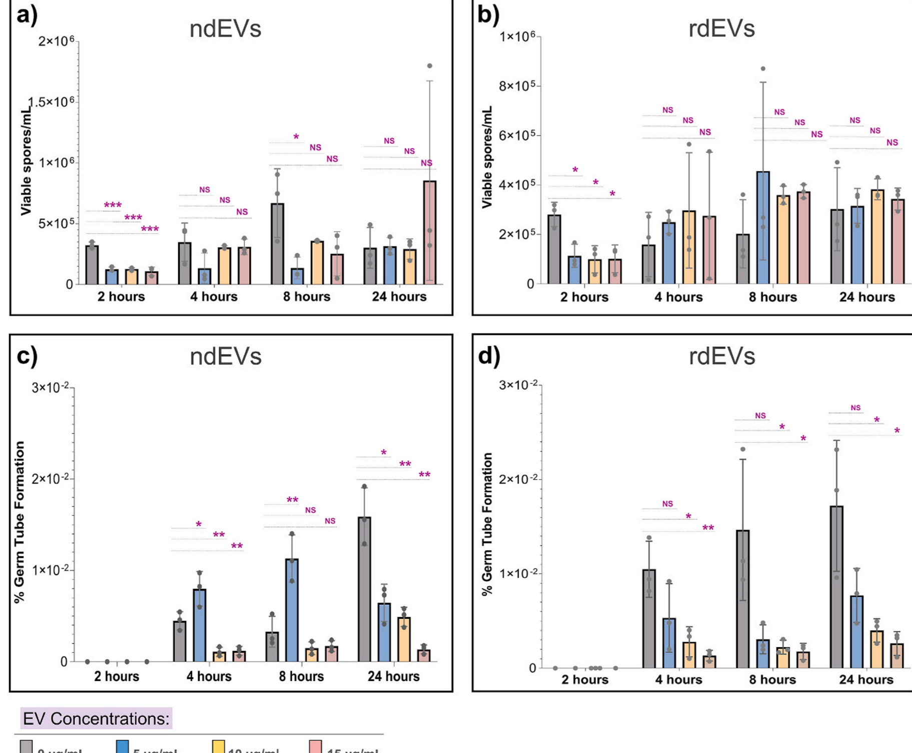


Fig. 3. Effect of extracellular vesicles (EVs) on Fusarium circinatum hyphal growth in ¼ strength potato dextrose broth. **(a)** and **(b)** show the impact of needle-derived EVs and root-derived EVs on cell viability, respectively. **(c)** and **(d)** illustrate the effects on spore germination, expressed as the percentage (%) of germ tube formation. Probability thresholds: *, P≤0.005 **, P≤0.002; ***, P≤0.001 ****, P≤0.0001 NS, not significant.

significant (P ≤ 0.002) for bdEVs (Fig. 7a). Additionally, we found that pre-treatment with both EV types, but especially bdEVs, significantly promoted ECM production (Fig. 7b). In terms of the abundance and distribution of matrix components relative to cellular biomass, we also observed a stimulatory effect of *F. circinatum*-derived EVs; for both with p^{kc}dEVs and bdEVs, significantly higher ECM-to-biomass ratios compared to the control groups were observed (Fig. 7c–d).

10. Discussion

Germ tube formation demonstrates the potential for fungi to form intricate hyphal clusters (mycelia), which account for much of the fungal biofilm biomass [18,20,24,33,40,41]. Furthermore, in dimorphic fungi such as *C. albicans*, the transition to the hyphal form is one of the key requirements for the establishment of both infections and a fungal biofilm, with both spores (or yeast cells) and hyphal cells often found wrapped in the matrix of a fungal biofilm ([42]; Honorato et al., 2021). Therefore, targeting spore germination could serve as a crucial strategy in effectively combating fungal infections.

Several studies have reported the potential inhibitory effects of plant-derived EVs (pdEVs) against spore germination in several fungal pathogens [39,43,44]. For instance, rdEVs of tomato (*Solanum lycopersicum* L.) plants were found to inhibit spore germination in *Botrytis cinerea*, *F. oxysporum*, and *Alternaria alternata* [44]. Similar findings were reported earlier using vesicles derived from a sun flower plant [39]. In line with these studies, we found that *P. patula* EVs isolated from seedling needles and roots not only inhibited cell viability and germ tube formation in *F. circinatum*, with rdEVs seemingly showing more inhibition than ndEVs. We also demonstrated that the inhibition of spore viability and germination results in impaired colony growth on nutrient agar and pigmentation, radial growth, and significant impairments in hyphal growth.

Since previous studies only investigated the impact of exogenously applied vesicles on spore germination and not on biofilm formation [39,44], our study attempted to address this gap in knowledge by assessing how the host-derived EVs interact with and influence surface-associated complex community structures such as biofilms. Indeed, during biofilm formation in the presence of pdEVs, we found that rdEVs of *P. pautula* inhibited biomass of *F. circinatum* biofilms, one of the most important biofilm traits previously demonstrated to be crucial in response to abiotic factors [33]. This data also suggests that spore germination may be a key step in biofilm initiation in *F. circinatum*. In *C. albicans*, for instance, the initiation of biofilm formation involves germ tube and hyphal formation [45]. Furthermore, many genes involved in hyphal growth also hold great significance in biofilm formation [46]. However, it is important to mention that *F. circinatum* is different from *C. albicans* due to the absence of a yeast phase. This means that the *F. circinatum* spores serve as reproductive propagules, and if they fail to germinate, the fungus is practically dead. This contrasts with *C. albicans*, which can still thrive in its yeast state. Overall, our results on uptake of pdEVs by *F. circinatum* spores suggest that pdEVs have the potential to be harnessed as an effective biocontrol agent against *F. circinatum* infections.

We have also discovered that pdEVs had no impact on matrix production in *F. circinatum* biofilms. The matrix is a network of extracellular polymeric substances produced by fungal cells within the biofilm and has several key functions including providing structural support and protection [16]. In *C. albicans*, EVs majorly influence fungal matrix production, dispersal, and antifungal resistance ([38]; Honorato et al., 2021; [12–14]). For these reasons, we were interested to know if vesicles of *F. circinatum* pathogen may be involved in countering the effects of pdEVs. We argued that pathogen-derived vesicles may play a crucial role in enhancing the structural integrity and protective properties of the biofilm, as previously described in *C. albicans*. To achieve this goal, we looked at whether planktonic cell-derived EVs (p^{kc}dEVs) and biofilm-derived EVs (bdEVs) can enhance matrix production in the biofilms of this fungus. Indeed, our results supported this notion,

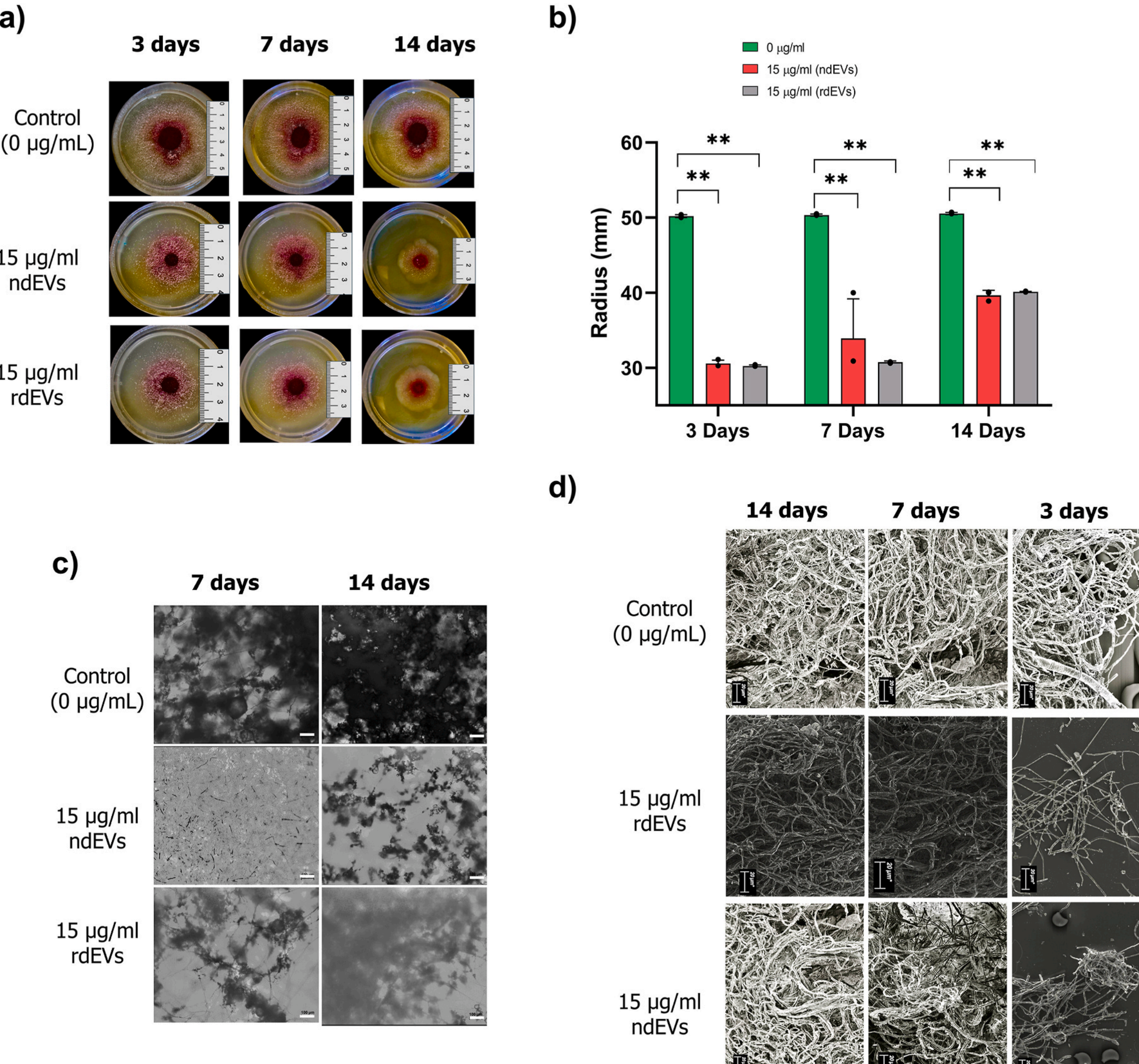


Fig. 4. Phenotypic effects of extracellular vesicles (EVs) on Fusarium circinatum during incubation on potato dextrose agar. **(a)** Radial growth of spores after 24-h exposure to needle-derived EVs (ndEVs) and root-derived EVs (rdEVs). **(b)** Quantification of radial growth by measuring colony diameter (cm) as shown in panel **(a)**. Probability thresholds: *, P≤0.005 **, P≤0.002 ***, P≤0.001 ****, P≤0.0001 **(c)** Cells from a radially growing culture were analysed using a light microscope at 10x magnification (Scale bar = 10 μm) and **(d)** a scanning microscope (Scale bar = 20 μm).

showing that, although both p^{kc}dEVs and bdEVs showed no significant negative or positive impact on early growth, they nonetheless promoted matrix production when applied to *F. circinatum* biofilms. This suggests that EVs from *F. circinatum* biofilms are potentially involved in antifungal resistance, and according to a recent study, biofilms are possibly involved *F. circinatum* resistance to azole antifungals [33].

The fact that pdEVs fail to inhibit matrix production in *F. circinatum* may have serious implications for field isolates. In a recent study exploring the possibility of biofilm growth on wheat, it was noted that fungal biofilms on plant surfaces are probably harmless (epiphytic) [47]. The rationale behind this is that these biofilms experience exposure to various physical factors, such as UV light and fungicides used to combat infectious fungi, which may prompt the biofilms to prioritize survival over pathogenesis. Similarly, enteric pathogens like *Salmonella enterica* and *Escherichia coli* can form persistent biofilms on plant surfaces [48], which may give rise to numerous outbreaks associated with fresh produce. The ability of these human and animal pathogens to establish biofilm communities on plant surfaces may enable them to evade host surveillance systems such as vesicles released by the plant as they are covered in a self-produced matrix. Furthermore, as we demonstrated in this study and previously [33], heightened matrix production could sustain pathogenic biofilms on plant surfaces. It is also important to consider that under specific favourable conditions, which may facilitate biofilm dispersal and allow the matrix-encased cells to break free from the biofilm structure [49], fungi growing within the biofilm on plant surfaces could potentially act as a more effective inoculum for invasion and in the process, alter plant immunity through the biofilm matrix.

P. patula is known to be highly susceptible to *F. circinatum* infections compared to other pines within the same genus [27,29,30]. Therefore, the formation of biofilms is likely to enhance the pathogen's persistence and ability to colonize and cause disease in the susceptible host [47]. Our results are thus intriguing as the EVs from this pine species failed to inhibit matrix production in the biofilm of this fungus. This may point towards a nuanced interaction between the host and the pathogen; while the inhibition of biomass by EVs could signify the host's defense mechanism targeting the pathogen's growth and proliferation within the biofilm, the failure to curtail matrix production might reflect the pathogen's ability to sustain its protective matrix despite the host's countermeasures, potentially serving as a survival strategy. Indeed, we observed that EVs isolated from both planktonic cells and biofilms

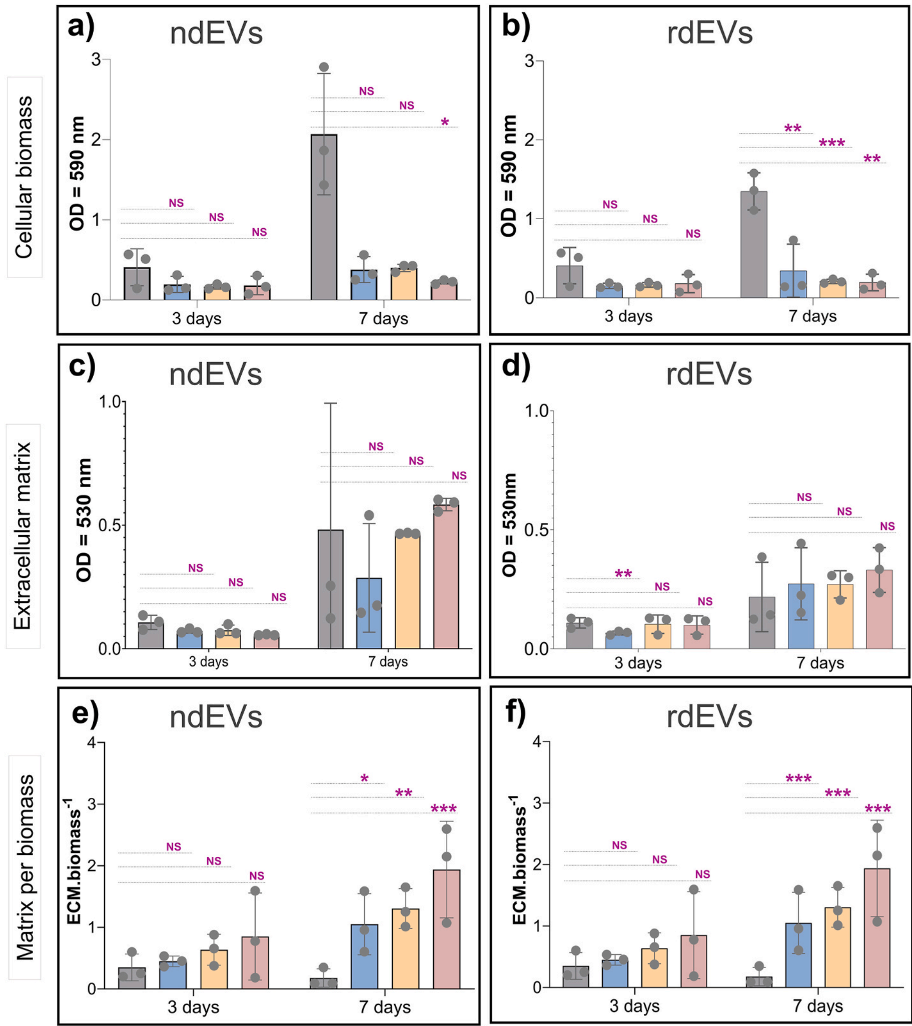


Fig. 5. The impact of plant-derived extracellular vesicles (pEVs) on biofilm biomass (OD = 590 nm), extracellular matrix (ECM) production (OD = 530 nm), and ECM per cellular biomass (ECM.biomass[1]). Effects induced by EVs derived from the needles **(a–c)** the roots **(d–f)**. Probability thresholds: *, P≤0.005 **, P≤0.002 ***, P≤0.001 ****, P≤0.0001 NS, not significant.

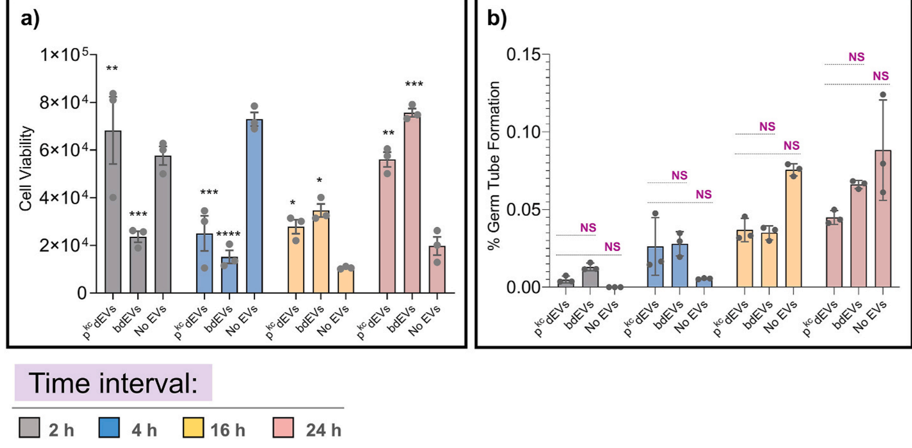
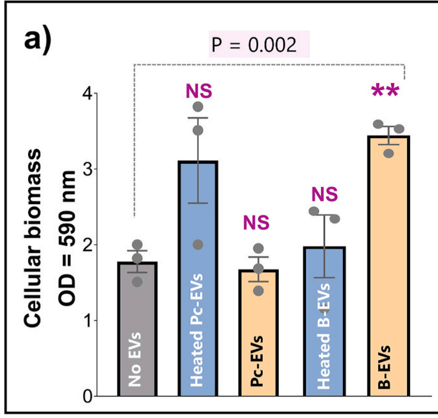
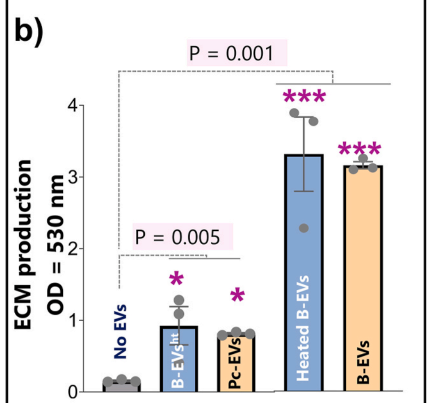
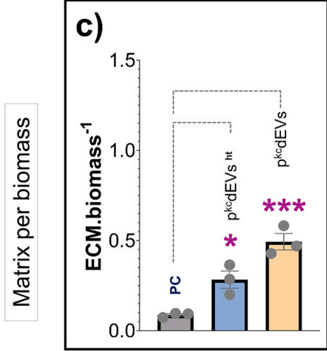


Fig. 6. Effects of extracellular vesicles (EVs) on Fusarium circinatum hyphal growth during incubation in 1¼ strength Potato Dextrose Broth. Effects of planktonic cell-derived EVs (pkcdEVs) and biofilm-derived EVs (bdEVs) on cell viability **(a)** and spore germination **(b)**, expressed as % germ tube formation. Probability thresholds: *, P≤0.005 **, P≤0.002 ***, P≤0.001; ****, P≤0.0001 NS, not significant.

promoted matrix production, which may partly explain why pdEVs were unable to reduce matrix production in *F. circinatum* biofilms. As EVs are known to play an important role in defence against pathogens and often in part mirror the biology of their parent cells [12–14,50–52], our data suggest that *P. patula* may be prone to infections associated with the biofilm, given that infectious propagules may take cover under the







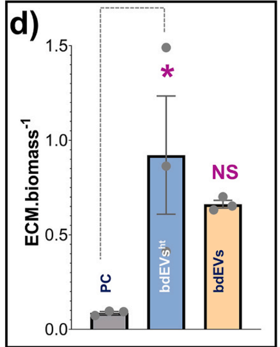


Fig. 7. Impact of extracellular vesicles (EVs) (5 μg/mL) on biofilm characteristics. **(c)** EV-mediated effects induced by planktonic cell-derived EVs (pkcdEVs). **(b)** EV-mediated effects induced by biofilm-derived EVs (bdEVs). **(c–d)** The impact of vesicles determined as the ratio of extracellular matrix (ECM) to cellular biomass (ECM.biomass-[1]). PC, positive control (No EVs added); pkcdEVsht, heat-treated pkcdEVs; bdEVht, heat-treated bdEVs. Probability thresholds: *, P≤0.005 **, P≤0.002 *** 1 P≤0.001 ****, P≤0.0001 NS, not significant.

matrix during a period when conditions are unfavourable. In addition, the biofilm-associated vesicles may carry compounds or enzymes that may later mobilize the biofilm-embedded cells [49,53], perhaps to seed a new biofilm, or to even penetrate the same surface hosting the matrix-embedded biofilm community.

Furthermore, the findings from pathogen-derived EVs, clearly demonstrates a distinct divergence between p^{kc}dEVs and bdEVs in terms of their impact on matrix production. This distinction is a direct reflection, albeit partial, of the unique characteristics of their respective parent cells i.e., planktonic cells and biofilms. Specifically, bdEVs demonstrated a significantly higher capacity to enhance matrix production compared to p^{kc}dEVs, which was also evident when considering the ratios of these characteristics. Therefore, there is a clear divergent response exhibited by EVs derived from planktonic cells and biofilms that may reflect the difference between their parent cells in *F. circinatum*, consistent with other studies [14]. Recently (Ratsoma et al., 2023b), we provided evidence of the superior heat-resistance attributes of *F. circinatum* biofilms compared to planktonic cells, among other attributes (13). This further strengthens the observed differentiation in EV-mediated effects exhibited by these distinct cell types. The demonstrated heat-resistance superiority of *F. circinatum* biofilms suggests the significance of biofilms in this fungal species, and likewise, that of their corresponding EVs. These results also suggest that bdEVs maybe more involved in shaping the biofilm architecture, an idea that is quite logical since the biofilm environment significantly modifies the metabolic profiles of its constituent cells [15,16,54]. Consequently, EVs released from these biofilm cells are expected to elicit distinct effects compared to cells situated outside the biofilm environment, which is what we observed. Therefore, our findings enhance current understanding of the specific functions and behaviours of EVs in *F. circinatum*, shedding some light on the intricate dynamics within biofilm communities of plant pathogens.

Taken together, our findings are crucial as they shed light on how a tree pathogen may exploit biofilms as a survival strategy and they could inform the development of targeted interventions to control such infections in *P. patula* and other susceptible species.

11. Conclusions

Typically, interactions entailing plant hosts and microbial colonizers are predominantly investigated from the perspective of microbial cells obtained from nutrient agar or sometimes from shaken flasks, both of which do not fully support biofilm formation. As a result, our understanding of how plants interact with microbial cells enveloped in biofilm-produced matrices remains highly limited. As we showed in this study, the application of EVs holds immense promise in unravelling the intricate interplay between plant hosts and complex community structures such as biofilms. For instance, pathogen-derived vesicles *of F. circinatum* promoting its matrix production could signify the pathogen's strategy to fortify the structural integrity of the biofilm, potentially aiding in its resilience and protection against external stresses. Enhancing biomass production, on the other hand, increased biofilm formation and energy utilization, potentially enabling the pathogen to divert resources towards other activities such as immune evasion or host exploitation. As a result, the pathogen's matrix is not affected by the EV-mediated response of the host. This underscores the intricate arms race between the pathogen's persistence and the host's defense strategies. Our results therefore highlight the need for further investigation to decipher the underlying molecular mechanisms governing these interactions, which could potentially offer insights into developing novel strategies for disease control and management. For this reason, the

findings provide a foundation to highlight the importance of a biofilm lifestyle in contributing to infections caused by filamentous plant fungal pathogens. This is crucial considering that this lifestyle maybe a preferred mode of living for microbes in their natural environments [17,55,56]. Additionally, our study opens up exciting possibilities for utilizing EVs as tools for studying fungal biology and plant-fungal interactions in non-model systems such as pine, which differ significantly from model systems like the thale cress and tobacco in terms of their growth habit, life cycle, and ecological context.

CRediT authorship contribution statement

Thabiso E. Motaung: Writing – review & editing, Writing – original draft, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Francinah M. Ratsoma:** Writing – review & editing, Methodology, Investigation, Data curation. **Sithembile Kunene:** Writing – review & editing, Methodology, Investigation, Data curation. **Quentin C. Santana:** Writing – review & editing, Supervision. **Emma T. Steenkamp:** Writing – review & editing, Supervision, Resources, Project administration. **Brenda D. Wingfield:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2025.107368.

Data availability

No data was used for the research described in the article.

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