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The role of *Pectobacterium brasiliense* 1692 outer membrane vesicles on members of soft rot *Enterobacteriaceae* and *Phytophthora parasitica*

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Declaration

I, Sedibane Kothibe Precious, declare that the dissertation, which I hereby submit for the degree Master of Science in Microbiology 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.

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Research outputs

Conferences

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Summary

Outer membrane vesicles (OMVs) are spherical nanoparticles formed from bulging and the subsequent fission of the outer membrane of Gram-negative bacteria. OMVs play a role in the secretion and transport of molecules in bacteria. They can transfer molecules from the inside to the outside of the cell, and as a result, they are regarded as a type zero secretion system. Furthermore, OMVs enclose multiple molecules such as enzymes (e.g., hydrolases), nucleic acids (e.g., DNAs and RNAs), and virulence factors. As a result, bacteria utilise OMVs in various microbial interactions, including competition. Potato (*Solanum tuberosum*) is one of the stable crop plants worldwide and has been under the threat of microbial infections. *Pectobacterium brasiliense* 1692 (*Pbr*1692), a soft rot bacterium, has resulted in a massive loss in the potato production industry and affected food security. Previously, it was reported that *Pbr*1692 cells could outcompete potato co-infectors using various mechanisms, namely, the secretion of antibiotic carbapenem, bacteriocins, and the type six secretion system.

In this study, we investigated the role of *Pbr*1692 OMVs in microbial interactions, especially in competition. To achieve this, OMVs were isolated from *Pbr*1692 and confirmed through transmission electron microscopy and SDS-page. As anticipated, *Pbr*1692 OMVs exhibited antimicrobial activity against *Dickeya dadantii*. However, our data indicated that OMVs do not play a role in competition against *Pectobacterium atrosepticum*, *P. carotovorum* and *P. parmentieri*.

Multiple studies have shown that bacterial OMVs participate in both intra and inter-kingdom interactions. Therefore, we investigated the role of *Pbr*1692 OMVs on plant pathogens from other kingdoms, specifically *Phytophthora parasitica*. *Phytophthora parasitica* is an oomycetes plant pathogen notorious for root and stem rot in economically important crops such as tobacco, potato, tomato, and peppers, as well as citrus plants. Since both *P. parasitica* and *Pbr*1692 are potato pathogens, we hypothesised that there is a likelihood that *Pbr*1692 could interact with *Phytophthora parasitica*. Therefore, we investigated the type of interaction that might occur between these two pathogens. From our analysis, it was evident that *Pbr*1692 cells do not inhibit the growth of *P. parasitica* INRA 310. Since OMVs reflect the biology of their parental cells, we were motivated to investigate whether *Pbr*1692 OMVs do not exhibit inhibition on *P. parasitica*. Despite the fact that *P. parasitica* INRA 310 zoospores were able to internalise *Pbr*1692 OMVs, they germinated in the same way as the untreated zoospores, implying that *Pbr*1692 OMVs do not inhibit *P. parasitica* INRA 310. Taken together, these

results suggested that there might be an interaction between the two pathogens through the OMVs, but not competition. To shed some light on this, we conducted a phenotypic microarray analysis where *Pbr*1692 OMVs were shown to potentially assist *P. parasitica* INRA 310 zoospores in utilising some of the carbon sources tested. These preliminary results showed that zoospores co-inoculated with *Pbr*1692 OMVs showed improved growth in some chemicals, and these results were subsequently validated. Therefore, we could conclude that *Pbr*1692 OMVs participate in intra and inter-kingdom interactions. This study assists in understanding OMVs as a mechanism of interaction between plant pathogens. Also, it paves a way to target this mechanism to combat plant diseases caused by *Pbr*1692 or to look into *Pbr*1692 OMVs as antimicrobial agents in microbial communities.

List of Abbreviations

°C	Degrees Celsius
CDT	Cytolethal distending toxin
CFU	Colony forming units
CMA	Corn meal media
dH_2O	Distilled water
ddH_2O	Double distilled water
DNA	Deoxyribonucleic acid
ETEC	Enterotoxigenic *E. coli*
FF-IF	Filamentous fungi inoculating fluid
GN	Gram-Negative
GP	Gram-Positive
Hrs	Hours
LB	Luria Bertani
LCMS	Liquid chromatography-mass spectrometry
mg	Milligram
Mins	Minutes
ml	Millilitre
MV	Membrane vesicles
OD	Optical density
OM	Outer Membrane
OMVs	Outer membrane vesicles
OU	Omnilog units
Pbr	*Pectobacterium brasiliense*

PBS	Phosphate Buffered Saline
PCWDEs	Plant cell wall degrading enzymes
PM	Phenotypic microarray
PQS	*Pseudomonas* quinolone signal
PVDF	Polyvinylidene difluoride
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscope
TEM	Transmission electron microscope
T6SS	Type VI secretion system

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Chapter one

1.1 Introduction

Research in microbial interactions allows us to understand the complex dynamics of microbes and paves the way in the biotechnology and medicine industry. Microbial communities involve the three kingdoms of life, each with its own set of interactions that can either benefit or damage the surrounding microbial partners (Braga *et al.*, 2016). Microorganisms adopt various mechanisms to navigate relationships within microbial communities. These relationships can be antagonistic (competition) or cooperative. These include the secretion of effector proteins (among other molecules) and antimicrobial peptides that can assist in competitive interactions (Shyntum *et al.*, 2019). With regard to cooperative interactions that benefit members of within communities, enzymes that may degrade harmful chemicals or secretion of iron chelators that sequester iron from the environment have been demonstrated (Schink, 2002, Tshikantwa *et al.*, 2018).

One particular mechanism used in microbial interactions is represented by the outer membrane vesicles (OMVs), also called the type zero secretion system or T0SS (Guerrero-Mandujano *et al.*, 2017), among other TXSSs (X stands for any number from 1-10). OMVs are blebs of the outer membrane of Gram-negative (GN) bacteria (Schwechheimer & Kuehn, 2015). The composition of the OMVs reflects that of the bacterial outer membrane (OM) (Beveridge, 1999, Kuehn & Kesty, 2005, Kulp & Kuehn, 2010). The OMVs are composed of lipopolysaccharides, glycerol phospholipids, and outer membrane proteins (OMPs) (Ellis & Kuehn, 2010). OMVs harbour other molecules such as cytoplasmic proteins and periplasmic proteins (**Fig 1.1a**) (Whitchurch *et al.*, 2002, Kuehn & Kesty, 2005). They also contain nucleic acid and virulence factors which may be crucial for the survival of GN bacteria (Gankema *et al.*, 1980, Renelli *et al.*, 2004).

Various studies have shown that OMVs play essential roles in bacterial activities/interactions, such as pathogenesis through biofilm formation and also through the transport of virulence factors (Gankema *et al.*, 1980, Kadurugamuwa & Beveridge, 1995, Kulp & Kuehn, 2010, Jan, 2017, Wang *et al.*, 2020). OMVs also form part of the bacterial defence system through the secretion of toxins, enzymes, and antibiotic inhibitors (Li *et al.*, 1998, Evans *et al.*, 2012, Kim *et al.*, 2018, Wang *et al.*, 2020). One of the novel characteristics of the OMVs is their ability to transfer genes between bacteria and modulate the metabolic activities of each other (Yaron *et al.*, 2000, Kim *et al.*, 2018). Besides being used for self-benefits, it has been suggested that

the production of OMVs may also benefit bacterial communities, for example, by promoting antibiotic resistance (Allan & Beveridge, 2003).

Pectobacterium brasiliense 1692 (*Pbr*1692) uses its secretion systems to secrete antimicrobial compounds such as carbapenem and bacteriocins for competitive advantage (Shyntum *et al.*, 2019). Notably, *Pbr*1692 is the causal agent of potato blackleg and soft rot worldwide (Pérombelon, 2002, Merwe *et al.*, 2010, Ashmawy *et al.*, 2015). Other microorganisms, including *Enterobacteriaceae* bacteria, interact with *Pbr*1692 in potato tubers, potato rhizosphere, and phyllosphere, suggesting that they may compete, cooperate, or act in synergy for the available nutrients (Toth *et al.*, 2011, Shyntum *et al.*, 2019).

This chapter reviews the roles of OMVs in microbial interactions and how they may participate in *Pbr*1692 interactions with other micro-organisms.

1.2 *Pbr*1692 in microbial interactions

Soft rot *Enterobacteriaceae* (SRE) are bacterial pathogens that cause plant diseases worldwide, resulting in significant economic losses (Pérombelon, 2002, Toth *et al.*, 2011). The SREs are composed of the genera *Dickeya* and *Pectobacterium* (Pérombelon, 2002, Duarte *et al.*, 2004). The broad host range bacteria: *Dickeya* and *Pectobacterium* species, cause stem wilt, tuber soft rot, and blackleg diseases. The SREs are characterised by the production of plant cell wall degrading enzymes (PCWDEs) that allow bacteria to infiltrate and macerate plant tissues (Charkowski *et al.*, 2011, Czajkowski *et al.*, 2011). Potato blackleg caused by *Pectobacterium* and *Dickeya* species appears slimy and wet with black rot lesions that spread from the rotting mother tuber up the stems. Furthermore, the yellowing, wilting, and desiccation of the stems and leaves can also be observed due to potato blackleg (Czajkowski *et al.*, 2011). On the other hand, potato tuber soft rot is characterised by small cream-to-tan, water-soaked spots on the surface of the potato, which progresses to a mushy and slimy appearance with an unpleasant odour, especially under moist conditions (Rosenzweig *et al.*, 2016).

Microbes are involved in constant inter and intraspecies interactions that create, maintain, and sustain microbial communities in the ecology or host environment. Through these interactions, microbes have evolved and adapted various mechanisms to survive constant environmental changes such as nutritional limitations, competition, cooperation, and parasitism/predation (Peleg *et al.*, 2010). As such, they secrete and exchange chemicals and signals to respond accordingly to maintain their survival (Caruana & Walper, 2020). *Pbr*1692 ecological niche

includes interacting with various bacteria on their hosts, including potato plants (Shyntum *et al.*, 2019). Furthermore, it was reported that the microbial composition of both diseased and healthy potato tubers is constituted mainly by the gammaproteobacteria. Some of the bacteria from the family *Enterobacteriaceae* found in the potatoes included *Erwinia*, *Citrobacter*, *Pectobacterium*, *Enterobacter*, *Pantoea*, *Serratia,* and *Klebsiella* species (Shyntum *et al.*, 2019).

Within microbial communities, some species can produce secondary metabolites, such as siderophores which sequester iron and exhibit antibiotic activity, suggesting that they are involved in both competitive and cooperative microbial interactions (Johnstone & Nolan, 2015). Furthermore, bacteria also use signalling systems such as quorum sensing to allow the cells to communicate and respond to environmental conditions (Braga *et al.*, 2016). In competitive interactions, bacteria may produce antimicrobial compounds that may inhibit co-infectors. For example, *Pbr*1692 employs various mechanisms, including the T6SS, in the interbacterial competition of phytopathogens and commensals. In this regard, the bacteria use the T6SS to inject toxins into its competitors, thus providing a competitive advantage to the attacking strain (Bernal *et al.*, 2018). In addition, the T6SS secretes bactericidal effectors, which can inhibit the growth of the targeted bacteria (Ma *et al.*, 2014, Bernal *et al.*, 2018).

Pectobacterium spp. produce bacteriocins which are extracellular toxins. These include carotovoricin, pectocin M1 and M2, and carocin (Roh *et al.*, 2010, Grinter *et al.*, 2012). These bacteriocins demonstrate antimicrobial activity against bacteria of the same species, particularly carotovoricin, which degrades the phospholipids making up the cell walls of bacteria (Roh *et al.*, 2010). Bacteria such as *Serratia*, *Dickeya,* and *Pectobacterium* spp. can produce antibiotics like carbapenem (McGowan *et al.*, 2005, Monson *et al.*, 2018). Carbapenem is a β-lactam antibiotic that targets the cell wall of both GN and Gram-positive (GP) bacteria, and compared to bacteriocins, carbapenem has a broad-spectrum activity (Papp-Wallace *et al.*, 2011, Shyntum *et al.*, 2019). However, in some cases, bacteria display antimicrobial resistance against compounds produced by their competitors as a survival mechanism (Meletis, 2016).

Previous studies have shown that *Pbr*1692 interacts with various bacteria on potato plants, including the bacteria from their *Enterobacteriaceae* and endophytes such as *Serratia* spp. (Shyntum *et al.*, 2019). Could the *Pbr*1692 microbial interactions involve other kingdoms? *Phytophthora parasitica* is an aggressive oomycete plant pathogen commonly reported as the

causal agent of tobacco black shank (Gallup *et al.*, 2006). Even so, *P. parasitica* has also been reported to cause pink rot in potato tubers (Taylor *et al.*, 2008, Taylor *et al.*, 2014). Furthermore, *P. parasitica* produces flagellate single-celled zoospores, which are the infectious agents of the pathogen (Larousse & Galiana, 2017). *Pbr*1692 and *P. parasitica* are both potato pathogens, thus occupying the same ecological niche and possibly competing or helping with obtaining nutrient sources.

Understanding the mechanisms microorganisms use for survival in their communities will improve our understanding of microbial pathogenesis and provide insights into developing new control methods.

1.3 Outer membrane vesicles

Membrane vesicles are one of the mechanisms used in microbial interactions to promote survival. Membrane vesicles are bi-layered spherical particles found in the three kingdoms of life; however, they are described as outer membrane vesicles (OMVs) in GN bacteria because of the membrane structure of the GN bacteria (Ellis & Kuehn, 2010, Brown *et al.*, 2015). OMVs were first identified from *Escherichia coli* cultures grown under lysine-limiting conditions (Bishop & Work, 1965). It was later reported that OMVs are formed from the bulging and the subsequent fission of the OM (Ellis & Kuehn, 2010).

OMVs are formed at all stages of bacterial growth and under different environmental conditions (Ellis & Kuehn, 2010). Furthermore, they can also be produced *in vivo* and *in vitro*; however, the production of OMVs is mainly associated with the bacterial stress response (Ellis & Kuehn, 2010). Reports have shown that the production of OMVs has predominantly been triggered by stress on the membrane envelope (MacDonald & Kuehn, 2012), with stressors ranging from iron reduction, fluctuating temperatures, and oxidative stress (Klimentová & Stulík, 2015). Therefore, this indicates that vesiculation may be one of the critical processes for the survival of bacteria in unfavourable environments.

The evolution of OMVs is suggested to increase the delivery efficiency of bacteria substances (Li *et al.*, 1998). Therefore, this has made OMVs perfect particles to promote interactions within bacterial communities and between bacteria and their hosts. The characteristic ability of OMVs to take part in these interactions has favoured the survival of the bacteria. OMV-mediated bacterial interactions can result in pathogenesis, antibiotic resistance, nutrient

acquisition, cooperation and killing competition (Renelli *et al.*, 2004, Lee *et al.*, 2016, Guerrero-Mandujano *et al.*, 2017).

1.3.1 OMVs biogenesis and the delivery mechanisms

OMVs are classified based on their biogenesis and biological functioning (Gill *et al.*, 2019); however, there are limited studies on each category. There are three proposed potential mechanisms of OMVs biogenesis. The first mechanism suggests that the production of OMVs may result from the bending and blebbing of the bacterial OM due to turgor pressure build-up and stress (**Fig 1.1b**), caused by the accumulation of peptidoglycan fragments and misfolded proteins in the periplasm causing the OM to bulge out (Zhou *et al.*, 1998, McBroom & Kuehn, 2007, Macdonald & Kuehn, 2013). In addition, the increase in the number of OMVs produced as the OM blebs reduce the turgor pressure (Jan, 2017). In the second mechanism, vesiculation is caused by raising the region of the relaxed cell wall-cell membrane interactions due to the turnover differences between the cell wall and cell membrane (Berleman & Auer, 2013). The third mechanism involves ionic repulsion and cell membrane destabilisation, resulting in curvature of the OM, which contributes to the OMV release (Berleman & Auer, 2013, Jan, 2017). This was observed in *Pseudomonas aeruginosa*, where OMVs were released through the interaction between the *Pseudomonas* quinolone signal (PQS) (**Fig 1.1c**) and lipopolysaccharides and the sequestered divalent cations (Berleman & Auer, 2013, Jan, 2017).

Interestingly, pathogenic bacteria appear to produce more OMVs than non-pathogenic bacteria. This has been reported in enterotoxigenic *E. coli* (ETEC), which produce more vesicles than in non-pathogenic *E. coli* (Horstman & Kuehn, 2002). Furthermore, it has been discovered that comparing leukotoxic and non-leukotoxic *Actinobacillus actinomycetemcomitans*, the pathogenic strain produces more than 25-fold the number of vesicles implying that OMVs play a role in the pathogenesis in these species (Lai *et al.*, 1981).

McBroom *et al.* (2006) showed that there is an OMV overproduction by an *E. coli* mutant (induced envelope stress) compared to the wild-type strain. Studies have reported that *E. coli* and *P. aeruginosa* showed increased OMV production when treated with ethanol and exposed to oxygen saturation, respectively (Sabra *et al.*, 2003, McBroom & Kuehn, 2007, Macdonald & Kuehn, 2013). Antimicrobial treatments have been shown to affect vesiculation. Dutta *et al.* (2004) reported that treating *Shigella dysenteriae* with mitomycin C results in high production of OMVs. However, there was low vesiculation when *S. dysenteriae* was treated with other antibiotics (Dutta *et al.*, 2004), suggesting that OMV production may vary depending on the

antimicrobial agent used and the specific target on that bacterium. In addition, Chan *et al.* (2017) reported that an *E. coli* strain naturally sensitive to gentamycin produced more OMVs when exposed to the drug. Despite reports that OMVs are primarily generated in response to stress, it should be noted that OMV production is a natural process that occurs at all stages of bacterial development (Li *et al.*, 1998, McBroom *et al.*, 2006, Brameyer *et al.*, 2018).

For the OMVs to effectively deliver their cargo, they should be able to interact with and have entry into other micro-organisms. Although the process by which OMVs enter the cells is not yet fully understood, some fluorescence studies have shown the possibility of OMVs using endocytic entry, such as clathrin-mediated as the method of entry and delivery of molecules in the OMVs (Pérez-Cruz *et al.*, 2016). Besides clathrin-mediated endocytosis, some bacterial cells use other entries such as micropinocytosis, caveolin-mediated endocytosis, lipid raft-mediated endocytosis, and membrane fusion (Bomberger *et al.*, 2009, Furuta *et al.*, 2009, Parker *et al.*, 2010, Jin *et al.*, 2011, Jäger *et al.*, 2015, Yang *et al.*, 2020, Zingl *et al.*, 2021).

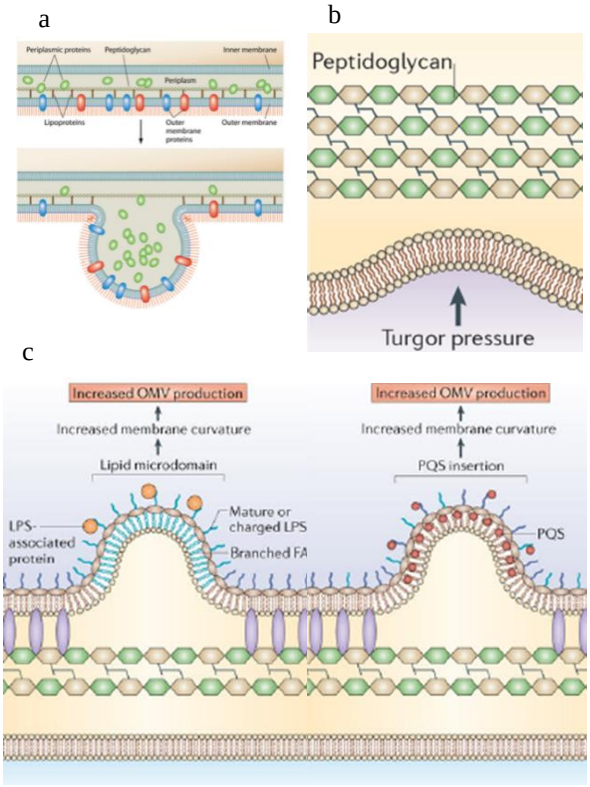

Figure 1. 1: The illustration of OMV cargo and biogenesis. **a**. OMVs contain molecules that form part of the outer membrane. **b**. OMVs are formed from turgor pressure on the periplasm. **c**. When the outer membrane is enriched with molecules such as PQS of *P. aeruginosa* or LPS, it causes the curvature of the outer membrane (Ellis & Kuehn, 2010, Brown et al., 2015, Schwechheimer & Kuehn, 2015).

1.3.2 OMVs in bacterial defence

Bacteria tend to utilise various mechanisms to defend themselves against harmful agents as well as predators. OMVs are regarded as one of the examples that can be used as antimicrobial agents. Intercellular exchanges such as cell-cell signalling have shown that bacteria can produce OMVs that kill their co-infector to minimise and eradicate competition (Ellis & Kuehn, 2010). OMVs can also neutralise and impair the host's defence system, thus helping bacterial communities survive and manifest infections (Ellis & Kuehn, 2010). For example, OMVs produced by *Helicobacter pylori* carry an antioxidant enzyme, KatA, which counteracts the oxidative radicals produced by the host through catalytic hydrolysis activity or the oxidation of methionine residues of katA (Lekmeechai *et al.*, 2018).

Through proteomic studies, it was discovered that some OMVs carry cell degradation enzymes, and the bacteria that produce OMVs with such functions induce a less susceptible mode, allowing the OMVs only to recognise and destroy their target (Kadurugamuwa & Beveridge, 1996, Kadurugamuwa *et al.*, 1998). Bacteria also produce OMVs through the adhesion of phage during a phage treatment before phage DNA infection (Kulp & Kuehn, 2010). Interestingly, OMVs produced by *E. coli* can adsorb T4 bacteriophage and reduce the ability of the phage for infection (Manning & Kuehn, 2011).

Li *et al.* (1998) discovered that OMVs could lyse a spectrum of GN and GP bacteria targeting the peptidoglycan chemotype and this study further demonstrated that *P. aeruginosa* PAO1 OMVs showed the most lysis activity, which could be due to the hydrolases that are enclosed in the OMVs (Li *et al.*, 1998). There are also other enzymes used by bacteria to target the peptidoglycan, such as *Lysobacter* sp. OMVs secrete endopeptidase L5, a bacteriolytic enzyme that can kill competitors by targeting the peptidoglycan (Vasilyeva *et al.*, 2008, Jan, 2017). The mechanisms through which OMVs execute their function are not entirely understood. Still, for GN bacteria, it is suspected that OMVs bind by fusing on the OM and releasing peptidoglycan hydrolase into the periplasmic space, attacking the peptidoglycan of the bacteria of interest (Kadurugamuwa *et al.*, 1998). While there is no definitive explanation of how OMVs lyse GP bacteria, it is suggested that the OMVs bind to the cell wall and release enzymes that break it (Brown *et al.*, 2015, Nagakubo *et al.*, 2020). Vesicles are commonly known to contain cell wall modifying or degrading enzymes such as PCWDEs and endopeptidase L5 from *Lysobacter* sp. (Vasilyeva *et al.*, 2008, Katsir & Bahar, 2017).

Pseudomonas aeruginosa OMVs enclose quinolones and murein hydrolases with antibacterial activity against other bacteria (Tashiro *et al.*, 2013). During natural development, the bacterium also produces membrane vesicles containing periplasmic components such as hydrolytic enzymes (e.g., protease, phospholipase C, and peptidoglycan hydrolase). Under nutrient-limiting conditions, *P. aeruginosa* OMVs attack the neighbouring bacteria, lyse them, and provide the parent strain with nutrients to feed on (Tashiro *et al.*, 2013) and this ability can be beneficial in microbial communities. Therefore, hydrolytic enzymes and other bioactive molecules associated with bacterial vesicles may be broadly used by bacteria as mechanisms employed to obtain resources vital from the environment. The PQS molecule found in *P. aeruginosa* OMVs required for iron acquisition can also inhibit the growth of other bacteria or stimulate virulence genes (Toyofuku *et al.*, 2009, Tashiro *et al.*, 2013). To this end, it is possible for a cell to select what to secrete thus, determining the contents of these OMVs.

1.3.3 OMVS-mediated defence through antibiotic inhibitors and antibiotic resistance

Some OMVs package β-lactamases that act against β-lactam antibiotics, promote antimicrobial resistance, and allow the survival of the bacteria (Kim *et al.*, 2018) (**Fig 1.2a**). This suggests that the β-lactamase OMVs can confer resistance to bacteria carrying β-lactam molecules or protect other existing bacteria from that antibiotic treatment (Ellis & Kuehn, 2010). For example, *A. baumannii* shelters carbapenem-susceptible bacteria through the production of carbapenemase such as Oxa-58 found in OMVs (Liao *et al.*, 2015). Similarly, *Moraxella catarrhalis* protects amoxicillin-susceptible *M. catarrhalis*, *Haemophilus influenzae*, and *Streptococcus pneumoniae* from amoxicillin (Schaar *et al.*, 2011). Besides the use of antibiotic inhibitors, OMVs may transfer resistance to other bacteria, as seen when gentamycin-treated *P. aeruginosa* released gentamycin OMVs which were able to pass through a permeable barrier of *Burkholderia cepacia* and induced resistance to gentamycin treatment (Allan & Beveridge, 2003). As such, bacteria use OMVs as potential mechanisms to induce antimicrobial resistance, which has been a great challenge in medicine.

Antibiotic treatment is regarded as a stressor in the bacterial community, and as such, bacteria tend to defend themselves when they encounter them. OMVs produced by *M. catarrhalis* can inhibit the bactericidal effects and the lysis of the peptide antibiotic polymyxin B (Roszkowiak *et al.*, 2006), also illustrated in **Fig 1.2b**. To our interest, hypervesiculating *E. coli* mutants survived polymyxin B and colistin treatment compared to the wild-type (Manning & Kuehn,

2011) because OMVs mimic the outside environment and act as a decoy for the defence and survival (Kulp & Kuehn, 2010).

Although there is less to no information on OMVs in interkingdom interactions, bacterial OMVs have been reported to decrease the susceptibility of pathogenic yeast to a combination of antifungals (Roszkowiak *et al.*, 2006), which is an indication that OMVs play a role during inter-kingdom interactions. Furthermore, various studies have reported that bacterial OMVs may carry and transport antifungal agents (Meers *et al.*, 2018, Yue *et al.*, 2021). According to Wang *et al.* (2020), OMVs of *Burkholderia thailandensis* can inhibit the growth of drug-sensitive and drug-resistant bacteria and fungi such as *A. baumannii*, *Staphylococcus aureus*, *Candida albicans*, and *Cryptococcus neoformans.* They further reported that the growth could be inhibited by the antimicrobial compounds peptidoglycan hydrolases, 4-hydroxy-3-methyl-2-(-2-non-enyl)-quinoline (HMNQ), and long-chain rhamnolipid contained in *B. thailandensis* OMVs.

A myxobacterium, *Cystobacter velatus* (strain *cbv34*), showed inhibitory activity over *E. coli*, and the liquid chromatography-mass spectrometry (LCMS) showed the presence of cystobactamids in the OMVs (Schulz *et al.*, 2018). Cystobactamids are bacterial isomerase inhibitors (Baumann *et al.*, 2014).

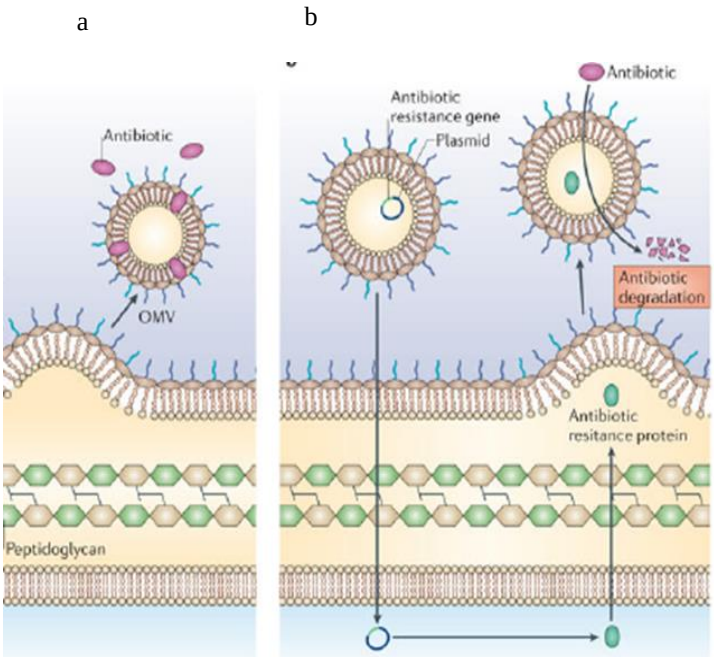

Figure 1. 2: Illustration of the OMVS transporting antibiotics (**a**) or antibiotic resistance genes (**b**) and the degradation of the antibiotics (Schwechheimer & Kuehn, 2015).

1.3.4 OMVs pathogenesis and virulence

OMVs transport and deliver toxins and virulence factors that help bacteria to invade the host cells and modulate their immune response (**Fig 1.3**). Furthermore, the toxins and virulence factors can also hijack the host's nutrient-acquiring machinery (Jan, 2017). Firstly, when bacteria interact with their hosts, they release OMVs with adhesin molecules, and this has been reported in *Helicobacter pylori* which produced OMVs with VacA adhesin (Parker *et al.*, 2010). Adhesins are very important during the pathogenesis and colonisation of host tissues as they mediate coaggregation (Kulp & Kuehn, 2010). OMVs from Enterotoxigenic *E. coli* reportedly release more heat-labile enterotoxin (LT) (Gankema *et al.*, 1980). On the other hand, *P. aeruginosa* OMVs are associated with virulence factors such as alkaline phosphatase, hemolytic phospholipase C, and Cif, delivered to their host through the N-wasp channel, which delivers the OMVs in the cytoplasm (Bomberger *et al.*, 2009). In mammalian studies, *Aggregatibacter actinomycetemcomitans* and *Campylobacter jejuni* OMVs are enriched with

toxic proteins such as cytolethal distending toxin (CDT), which are toxins that cause mammalian DNA damage (Lindmark *et al.*, 2009, Rompikuntal *et al.*, 2012).

1.3.5 OMVs as public goods

Most of the studies have focused on OMVs as a negative system, but could these OMVs serve as public goods in microbial communities? Allan & Beveridge (2003) have shown that *P. aeruginosa* OMVs can transfer OMVs to *B. cepacia*, thus promoting gentamycin resistance. Therefore, this ability may prove beneficial within microbial communities during antibiotic treatment. OMVs can provide nutrients in various ways. OMVs-associated enzymes have been reported to break down substrates and secure nutrients that can be used within bacterial communities (Elhenawy *et al.*, 2014). Besides the transfer of molecules, OMVs that are used in nutrient-limiting environments to eliminate competitors may also increase nutrient availability to the surviving bacteria. Biller *et al.* (2014) reported that Prochlorococcus vesicles could support the growth of heterotrophic bacteria through the carbon influx systems. OMVs from *Mycobacterium tuberculosis* can carry high amounts of an iron-chelating molecule, mycobactin, which forms iron-scavenging OMVs (Prados-Rosales *et al.*, 2014). Once released into the environment, these mycobactin-OMVs can now be accessed by neighbour bacteria as a community resource, thereby contributing to the social life of that community (West *et al.*, 2007). OMVs can also be used by bacterial hosts, for example, *Pseudomonas syringae* pv. tomato T1 OMVs carry superoxide dismutase, an antioxidative enzyme used in cased of high production of reactive oxygen species (ROS) when plants are exposed to excessive stress.

1.4 Outer membrane vesicles in *Pectobacterium*

The production of OMVs in *Pectobacterium* was first reported in 1992, with the characterisation reported between 2020 and 2021 (Fukuoka, 1992, Piotrowska *et al.*, 2020, Jonca *et al.*, 2021, Maphosa & Moleleki, 2021). To date, the characterisation of OMVs was reported in *Pectobacterium zantedeschiae*, *P. ordoriferum*, *P. versatile*, *P. brasiliense* 1692 *(Pbr*1692), and *P. betavasculorum* (Piotrowska *et al.*, 2020, Jonca *et al.*, 2021, Maphosa & Moleleki, 2021).

Proteomics studies revealed that *Pectobacterium* OMVs harbour Cdi effectors and Cdi toxins responsible for contact independent inhibition, which is suspected of playing a role in the competition (Jonca *et al.*, 2021, Maphosa & Moleleki, 2021). Amongst the identified proteins were PCWDEs which are widely responsible for the maceration of potato tubers, and this further shows that OMVs may play a role in *Pectobacterium* pathogenesis (Jonca *et al.*, 2021,

Maphosa & Moleleki, 2021). In addition, *Pectobacterium* OMVs are suspected of mediating antibiotic resistance through deflecting bacteriophages and carrying β-lactamases (Jonca *et al.*, 2021, Maphosa & Moleleki, 2021). The characteristics of the OMVs produced by *Pectobacterium* further show that OMVs play a role in microbial communities.

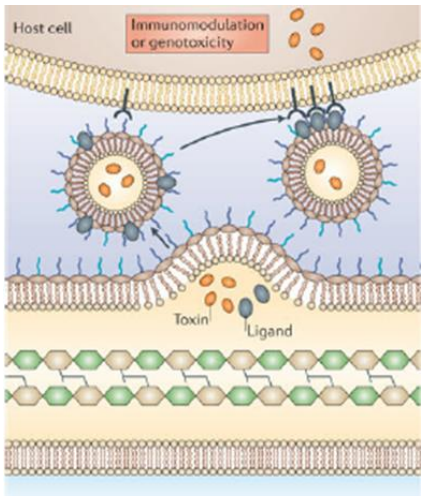

Figure 1. 3: OMVs package, transport, and deliver toxins to the bacteria of interest (Schwechheimer & Kuehn, 2015).

1.5 Understanding the isolation of OMVs

The critical aspect of the study of OMVs is obtaining OMVs from bacterial cells. The isolation of OMVs includes the removal of intact bacterial cells through centrifugation, followed by removing the residual cells through a vacuum pump filtration system and high-speed centrifugation (Klimentová & Stulík, 2015). It should be noted that the sizes of OMVs vary amongst bacterial species. Therefore, the membrane pore size corresponds to the bacterial cells (Klimentová & Stulík, 2015).

Several studies have used the precipitation method or the ultrafiltration system to concentrate the OMVs and subsequently collected the OMVs via ultracentrifugation (Chutkan *et al.*, 2013). Some researchers have opted to use crude OMVs for their studies with just polyvinylidene difluoride (PVDF) membrane filtration after ultracentrifugation (Ko *et al.*, 2016, Deo *et al.*, 2018). In contrast, some proceed to various methods that allow the removal of possible contaminants (Kwon *et al.*, 2009, Wang *et al.*, 2020). The multiple methods include multiple washes to purify the pellet or sucrose gradient ultracentrifugation and Size Exclusion

Chromatography (SEC) with Sepharose CL-2B column (Schulz *et al.*, 2018). However, it should be noted that the use of crude OMVs and what is considered purer OMVs showed similar results.

One of the challenges in OMVs isolation and purification is low concentration, and unfortunately, the concentration of OMVs could be affected at every step of the isolation (Chutkan *et al.*, 2013). Therefore, gentle preparation and improvisations are required to obtain a higher OMV concentration, and this includes taking into account the amount of the resuspension buffer that should correlate with the size or the visibility of a pellet (Chutkan *et al.*, 2013). Notably, the concentration of OMVs affects antimicrobial activity (Li *et al.*, 1998).

Based on the literature review, we designed a study to investigate the role of OMVs from *Pbr*1692 in microbial communities. The study's objectives were to establish the interaction between *Pbr*1692 and *P. parasitica* INRA 310 through antagonism assays and to investigate the effect of *Pbr*1692 OMVs on SREs and *P. parasitica*. The last objective was to utilise phenotypic microarray (PM) technology to examine the role of *Pbr*1692 OMVs on the metabolism of *P. parasitica* INRA 310. This study will significantly contribute to the understanding of OMVs in microbial communities and possible ways they may be employed in agriculture for the survival and control of crops and microorganisms, respectively.

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Chapter two

2.1 Methods and materials

2.1.1 Strain growth conditions

Pectobacterium brasiliense 1692 (Charkowski, 2018), *Dickeya dadantii* LMG 25911$^{T(Type\ strain)}$ (Samson *et al.*, 2005), *P. carotovorum* (FABI) and *P. parmentieri* ATCC 43316^{T} (Khayi *et al.*, 2016) cultures were incubated at 30 °C, while *P. atrosepticum* ATCC 33260^{T} (Panda *et al.*, 2015) was incubated at 28 °C. All bacterial cells were shaken at 150 rpm (incubation time varies as per experiment). The bacterial cultures were grown in Luria Bertani (LB) media, where necessary, supplemented with 15 µg/ml gentamicin (Sigma-Aldrich, South Africa). *P. parasitica* isolates (FABI) were grown in V8 agar, and cornmeal meal media (CMA) incubated at 25 °C.

2.1.2 Electrocompetent cells and transformations

Following the protocol described by Shyntum *et al.* (2019), *Dickeya dadantii*, *P. carotovorum,* and *P. parmentieri* were grown overnight at 30 °C, while *P. atrosepticum* was grown at 28 °C, shaking at 150 rpm for 16 hrs. One millilitre (1 ml) of each culture was separately inoculated in 100 ml LB broth and incubated at either 30 °C or 28 °C, shaking at 150 rpm for 2-3 hrs until an OD600 = 0.4 was reached. Subsequently, 50 ml of each culture was transferred into 50 ml centrifuge tubes, pre-cooled on ice, and centrifuged for 8 mins at 6000 rpm and 4 °C. The pellets were resuspended in dH_2O (50 ml, 40 ml, and 30 ml) and centrifuged for 8 mins at 6000 rpm for each volume. After the 30 ml dH_2O wash, the pellets were resuspended in 10% (v/v) glycerol, centrifuged at 6000 rpm for 8 mins at 4 °C, and resuspended in 2 ml 10% glycerol and stored at -80 °C before use. Plasmid pMP7605 conferring gentamicin resistance was added in 20 µl bacterial cells chilled in cuvettes. The cuvettes were then placed on the electroporation machine, and the gentamicin plasmid was transformed into bacterial cells through electrical shock. A volume of 200 µl LB broth was aliquoted into the transformants, resuspended, and transferred to an Eppendorf tube with 600 µl LB and the cells were recovered by incubating at either 30 °C or 28 °C, shaking at 150 rpm for 2 hrs. Each bacteria suspension was separately plated on gentamicin-supplemented agar plates for subsequent experiments.

2.1.3 Isolation of OMVs

*Pbr*1692 OMVs were isolated as described by Maphosa & Moleleki (2021), *Pbr*1692 cultures were grown overnight for 12 hrs until an OD_{600} = 0.8 - 0.9 was reached. The overnight cultures were centrifuged at 5000 rpm for 30 mins at 4 °C to pellet the cells. Using the vacuum filtration system, the supernatant was filtered through a stericup 0.22 µl membrane (Merck). The Amicon

centrifugal filter units (10-50 kDa, Merck) were used to concentrate the supernatant at 2500 rpm for 25 mins. Subsequently, the supernatants were centrifuged using an ultracentrifuge at 36 000 rpm at 4 °C for 5 hrs. The pellet obtained was resuspended in Phosphate buffered saline (PBS buffer) and washed in PBS buffer by centrifuging for 1 hour at 36 000 rpm. The pellet was resuspended in a PBS buffer. The protein concentration that makes up the OMVs was approximated using Bradford assay (Bio-Rad) and nanodrop spectrophotometer, and it ranged between 6 mg/ml to 7 mg/ml, which are the concentrations used in the forthcoming experiments. The samples were stored at -20 °C and -80 °C.

2.1.4 Transmission electron microscopy

Negative staining: To confirm and visualise the isolated OMVs, negative staining was performed according to Wang *et al.* (2020). OMVs were placed on a copper grid for 3 mins and stained with 1% uranyl acetate for 20 mins until they dried out. The samples were visualised through FEGTEM Joel 2100 microscope.

Thin section: As described by Maphosa & Moleleki (2021), *Pbr*1692 cells were cultivated overnight for 12 hrs. The cells were pelleted at 6000 rpm for 5 mins. The pelleted cells were washed with 0.075 M phosphate buffer and fixed with 2.5% glutaraldehyde at room temperature for 3 mins. Subsequently, the pellet was rinsed three times with phosphate buffer. The cells were post-fixed with 1% Osmium tetroxide (OsO_4) for 30 mins and pelleted. The pelleted cells were dehydrated through a series of ethanol (30%, 50%, 70%, 90% all v/v, and 3x100%); thereafter, the cells were left in 100% ethanol for dehydration for 30 mins. A mixture of 50:50 epoxy resin and 100% ethanol were added to the dehydrated cells for 1 hour and pelleted. The cells were then left in an epoxy resin mixture for 1 hour before being embedded in epoxy resin for another 1 hour, then incubated in an oven for 24 hrs for polymerisation. The cells were trimmed and sectioned, then mounted on the copper grid stained with 1% v/v uranyl acetate.

2.1.5 The effect of *Pbr*1692 OMVs on soft rot *Enterobacteriaceae*

The effect of *Pbr*1692 OMVs was screened through co-inoculations assays, following the protocol described by Shyntum *et al.* (2019). The targeted bacterial cells, namely *D. dadantii*, *P. atrosepticum*, *P. carotovorum*, and *P. parmentieri* transformed with gentamicin plasmid pPM7605 were grown at 30 °C or 28 °C, shaking at 150 rpm for 16 hrs, subsequently standardised to an OD_{600} = 0.1, and individually mixed with *Pbr*1692 OMVs (7 mg/ml) to a ratio of 1vol:1vol. The mixed suspensions were inoculated at the centre of the LB agar plates

and left to air dry. The bacterial plates were grown at 30 °C or 28 °C for approximately 12 hrs, and the colony was scrapped and resuspended in ddH_2O; serial dilutions were performed and plated on gentamicin-supplemented agar plates. The bacterial plates were incubated for 12-14 hrs, colonies were counted, and the results were presented as CFU/ml.

2.1.6 The effect of *Pbr*1692 cells on *Phytophthora parasitica* INRA 310

2.1.6.1 Antagonism assays

To investigate whether *Pbr*1692 cells interacted with *P. parasitica* INRA 310, *in vitro* competition assays were employed through the bacteria-oomycete dual culture method, according to Chen *et al.* (2018). *P. parasitica* INRA 310 was grown for 7 days on V8 agar media, and a mycelium plug was obtained from the edges of the growing culture using a 4 mm cork borer. This was then placed on a 9 cm cornmeal agar plate. *Pbr*1692 cells were standardised (OD_{600} = 0.5) and inoculated 2 cm from the mycelium plug. Double-distilled water was used as the negative control. The experiments were all performed in triplicates, repeated three independent times, and incubated and monitored at 25 °C for 5 days. The radius of the mycelium growth was then measured from the centre 5 days following incubation.

2.1.6.2 *Pbr*1692 cells on *Phytophthora parasitica* INRA 310 zoospore germination and motility

Germination of the zoospores was performed on micro slides (Lasec®) using the protocol described by Regente *et al.* (2017) with modifications. Briefly, *P. parasitica* INRA 310 was grown on V8 agar media for 7 days. Thereafter, 10% (w/v) soil water was prepared, poured on the *P. parasitica* mycelial plates and cultures incubated for 48 hrs under light to induce sporangia. To induce *P. parasitica* zoospores, soil water was poured out, replaced with 4 °C cold water, and incubated for 1 to 3 hrs at room temperature. *Pbr*1692 cells were grown overnight. The micro slide was inoculated with 4% (w/v) glucose and approximately 1500 zoospores, 4% (w/v) glucose, and *Pbr*1692 cells (OD_{600} = 0.5) adjusted to 20 µl and covered with coverslips. The micro slides were placed in a tightly sealed container and incubated at 25 °C for 16 hrs. Double-distilled water was used as a negative control. The results were evaluated using Zeiss brightfield microscopy.

Scanning electron microscopy of *Pbr*1692 cells

Following the protocol described by Maphosa & Moleleki (2021) with slight changes, *Pbr*1692 cells grown for 12-13 hrs were incubated with approximately 70 000 zoospores of *P. parasitica* INRA 310 and 4% (w/v) glucose in a total volume of 1.7 ml and incubated for 16

hrs with ddH_2O as the negative control. The samples were pelleted at 4000 rpm for 4 mins and washed with 0.75 M phosphate wash buffer for 15 mins on a rotor. Samples were fixed with 2.5% glutaraldehyde for 1 hour and hydrated with a graded series of ethanol (30%, 50%, 70%, 90%, and 3x 100%) for 15 mins per hydration cycle. After that, the samples were left in the third 100% ethanol hydration for 30 mins. The samples were pelleted and embedded in a hexamethyldisilazane (HDMS): ethanol mixture for 1 hour. Subsequently, the samples were added to fresh HDMS and left to dry. The samples were mounted on aluminium stubs, coated with carbon, and visualised using Zeiss Scanning Electron Microscope (FE-SEM) at 2kv. The experiment was conducted in triplicates.

The effect of *Pbr*1692 cells on *Phytophthora parasitica* zoospore motility

Phytophthora parasitica INRA 310 zoospores were induced from the 7 days mycelium plates that were soaked with soil water and then poured out and replaced with cold water. The *Pbr*1692 cells were grown overnight and standardised (OD_{600} = 0.5), and *P. parasitica* INRA 310 zoospores were mixed at a 1 vol: 1vol ratio and incubated for 3 hrs recording the motility every 30 mins using a mobile device and ddH_2O as the negative control. This experiment was repeated three independent times.

2.1.7 The effect of *Pbr*1692 OMVs on *Phytophthora parasitica* mycelium

To investigate the antimicrobial activity of *Pbr*1692 OMVs on *P. parasitica* mycelium, the targeted assay was performed according to Meers *et al.* (2018) with slight modifications. A 4 mm mycelium plug was excised from the edges of the 7-day *P. parasitica* mycelium culture and inoculated at the centre of a 9 mm CMA plate, then incubated for 3 days at 25 °C. The sterilised filter papers were inoculated with the OMVs at two different concentrations of 3 mg/ml and 6 mg/ml. *Pbr*1692 cells (OD_{600} = 0.5) were grown overnight, and a negative control PBS buffer was prepared, left to air dry, and placed at the edges of the 3-day-old mycelium plate. The experiment was incubated for 2 more days at 25 °C, conducted in triplicates, and repeated three times.

2.1.8 *Pbr*1692 OMVs on *Phytophthora parasitica* INRA 310 zoospore germination and motility

Germination of the zoospores was performed on micro slides using a protocol described by Regente *et al.* (2017). Briefly, *P. parasitica* INRA 310 was grown on V8 agar media for 7 days. Thereafter, 10% (w/v) soil water was prepared and poured on the *P. parasitica* INRA 310 mycelial cultures, then incubated for 48 hrs under light to induce sporangia. To induce *P.*

parasitica INRA 310 zoospores, soil water was poured out and replaced with 4 °C cold water; thereafter, incubated for 1 to 3 hrs at room temperature. An aliquot of 10 µl of *P. parasitica* INRA 310 zoospores (~1500 zoospores), 4% (w/v) glucose, and *Pbr*1692 OMVs (6 mg/ml) to the final volume of 20 µl were added on micro slides. The slides were placed in a tightly sealed container and incubated at 25 °C, with PBS buffer serving as a negative control. Zeiss brightfield microscope was used to evaluate the morphology and germination of the zoospores.

Scanning electron microscopy of *Pbr*1692 OMVs

Four hundred microliters (400 µl) of *Pbr*1692 OMVs or PBS buffer were incubated with 900 µl *P. parasitica* INRA 310 zoospores (~70 000 zoospores) and 4% (w/v) glucose to a total volume of 1.7 ml for 16 hrs. The samples were pelleted at 4000 rpm for 4 mins and washed with 0.75 M phosphate wash buffer for 15 mins on a rotator. The sample was fixed with 2.5% glutaraldehyde for 1 hour and hydrated with a graded series of ethanol (30%, 50%, 70%, 90% all v/v, and 3x 100%) for 15 mins per hydration cycle. Notably, at the third 100% ethanol hydration, the sample was incubated for 30 mins. The sample was pelleted and embedded in a hexamethyldisilazane (HDMS): ethanol mixture for 1 hour. The experiment was conducted in triplicates and repeated three times. The samples were added to a fresh HDMS solution and left to dry. Subsequently, they were mounted on aluminium stubs, coated with carbon, and visualised using Zeiss Scanning Electron Microscope (FE-SEM) at 2kv.

The effect of *Pbr*1692 OMVs on *Phytophthora parasitica* motility

Phytophthora parasitica zoospores were induced from mycelium that was grown for 7 days at 25 °C and soaked with soil water for 48 hrs. The soil water was poured out and replaced with ice-cold water containing zoospores. The *Pbr*1692 OMVs or PBS buffer as the negative control and *P. parasitica* INRA 310 zoospores were mixed at a 1:1 ratio and incubated at 25 °C for 3 hrs recording the motility on the brightfield microscope every 30 mins using a mobile device. This experiment was repeated three independent times.

2.1.9 Staining of OMVs and their uptake by *Phytophthora parasitica* zoospores

Fresh *Pbr*1692 OMVs were labelled with Biotracker™ 640 C2 (FM4-64) Synaptic dye as described by Regente *et al.* (2017). Briefly, an aliquot of 60 µl of the 6 mg/ml OMVs previously resuspended in PBS buffer isolation was gently mixed with FM4-64 to the final concentration of 1 µg/ml. The sample was incubated on ice for 60 mins and later diluted in 4 ml PBS buffer. The diluted OMV sample was ultracentrifuged at 36 000 rpm for 1 hour at 4 °C to wash off the dye, and the pellet was resuspended in 60 µl PBS buffer kept at -20 or -80 °C.

The germination test of *P. parasitica* INRA 310 zoospores was performed directly on the micro slide according to Regente *et al.* (2017), with slight modifications. Approximately 2500 *P. parasitica* INRA 310 zoospores were incubated with 2.5 µl labelled OMVs (~6 mg/ml) and 4% (w/v) glucose directly on the slide and placed in a tightly sealed container for 16 hrs at 25 °C PBS buffer as the negative control. The experiment was conducted in duplicates, repeated three independent times, and the images were acquired using Zeiss confocal and fluorescent microscopes.

2.1.10 *Phytophthora parasitica* viability tests

The viability of germinated hypha was investigated using viability assays adapted from Regente *et al.* (2017). About 1500 zoospores were incubated with *Pbr*1692 OMVs and 4% (w/v) glucose on a micro slide and incubated in a tight sealed container for 16 hrs at 25 °C. After the incubation, Evans blue stain (0.05% w/v) was added to the micro slides and left to dry at room temperature. The viability of the zoospores was evaluated on the Zeiss brightfield microscope. Following a similar protocol, membrane permeability assays were performed on the micro slide. Propidium iodide (Millipore Corp) (50 µg/ml) was added on micro slides for 1 hour, and viability was investigated on Zeiss fluorescent microscope using a filter of 530-585 nm excitation and 615 nm emission. Phosphate-buffered saline (PBS) was used as the negative control, and the experiment was repeated three independent times.

2.1.11 Phenotypic Microarray

The *P. parasitica* INRA 310 mycelium was grown on CMA plates for seven days at 25 °C. Soil water was prepared and poured on the CMA plates of the mycelium and incubated for approximately 48 hrs to induce sporangia. Subsequently, the soil water was discarded and replaced with ice-cold distilled water to induce the *P. parasitica* INRA 310 zoospores and incubated for 3 hrs at room temperature. The zoospores were harvested by gently rubbing the sterile cotton swab on the mycelium surface, and the haemocytometer counting chamber was used to count the zoospores. Following Biolog protocol (**Appendix A**), approximately $30x10^7$ zoospores were inoculated in the filamentous fungi inoculating fluid (FF-IF) (Biolog, Inc) with the Biolog Redox Mix E dye (Biolog, Inc) and transferred to the 96 wells of PM1 and PM21D (Biolog, Inc) microplates for carbon sources and chemical sensitivity, respectively. Yeast nitrogen base and glucose were used as additives to supplement FF-IF used for the PM21D microplate. The plates were incubated in the Omnilog incubating system for 48 hrs, recording

the results every 15 mins. This experiment was conducted once, in duplicates for PM1 and a single plate for PM21.

2.1.12 *Phytophthora parasitica* carbon source utilisation in the presence of *Pbr*1692 OMVs

To confirm the carbon sources that were utilised by *P. parasitica* INRA 310 zoospores when treated with *Pbr*1692 OMVs, *P. parasitica* INRA 310 mycelium was used. *P. parasitica* INRA 310 was grown on V8 media for 7 days; thereafter, 4 mm mycelium plugs were taken from the mycelium and inoculated at the centre of the 65 mm CMA plates supplemented with 0.05% (v/v) formic acid and *Pbr*1692 OMVs for the test, or PBS buffer as the negative control. The plates were incubated at 25 °C for 7 days, and the mycelium diameter was measured. This experiment was performed in triplicates.

2.1.13 *Phytophthora parasitica* zoospore germination and viability in carbon source in the presence of *Pbr*1692 OMVs

Germination of the zoospores was performed on micro slides similar to the protocol described by Regente *et al.* (2017), with slight changes. *Phytophthora parasitica* was grown on V8 agar media for 7 days, and 10% (w/v) soil water was prepared and poured on the *P. parasitica* INRA 310 mycelial plates and incubated for 48 hrs under light to induce sporangia. To induce *P. parasitica* INRA 310 zoospores, soil water was poured out and replaced with 4 °C cold water; thereafter, incubated for 1 to 3 hrs at room temperature. An aliquot of 10 µl of *P. parasitica* INRA 310 zoospores (~1500 zoospores), 0.05 (v/v) formic acid, and *Pbr*1692 OMVs (6 mg/ml), all to the final volume of 20 µl were added on micro slides. The micro slides were placed in a tightly sealed container and incubated at 25 °C for 16 hrs, and PBS buffer was used as the negative control. The germination of the *P. parasitica* INRA 310 zoospores was evaluated using a brightfield microscope. To further test the viability of the germinated zoospores, the slides were stained with propidium iodide (50 µg/ml). Zeiss Fluorescence and brightfield microscopes were used to evaluate the viability of the germinated zoospores. The experiment was performed in triplicates.

2.2 Appendices

2.2.1 Appendix A

Table 2. 1: Procedure for inoculation into phenotypic microarrays.

Preparation of glucose stock solution			
Ingredients	Concentration (mM)	Grams/100 ml	Concentration factor
D-glucose	3200	57.664	32x
Preparation of PM21 additive solution			
Yeast nitrogen base	-	8.04	12x
PM inoculating fluids from stock solutions			
PM stock solution		PM1 (ml)	PM21(ml)
FF-IF		20.00	60.00
Redox dye		2.00	12.00
D-glucose (32x)		-	4.50
PM additive (12x)		-	12.00
Zoospores		0.05	0.1
*Pbr*1692 OMVs		0.1	0.2
Sterile water		1.85	54.60
Total		24.00	144.00

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Wang Y, Hoffmann J, Chou CW, Höner Zu Bentrup K, Fuselier J, Bitoun J, Wimley W & Morici L (2020) *Burkholderia thailandensis* outer membrane vesicles exert antimicrobial activity against drug-resistant and competitor microbial species. *Journal of Microbiology* **58**: 550-567.

The physiological effects of *Pectobacterium brasiliense* 1692 outer membrane vesicles on soft rot *Enterobacteriaceae* and *Phytophthora parasitica*

Chapter three

3.1 Results

3.1.1 Production of OMVs by *Pectobacterium brasiliense* 1692

Outer membrane vesicles are reported to take part in the secretion of virulence factors and molecules involved in cellular communication, biofilm formation, and pathogenesis (Yonezawa *et al.*, 2009). Therefore, this study explored the possibility that *Pbr*1692 uses OMVs to secrete and transport molecules used in microbial communities. Towards this end, OMVs were isolated and purified from the stationary phase of the *Pbr*1692 culture. Their size and morphology were screened through TEM analysis. The thin-section electron micrograph revealed that *Pbr*1692 produces OMVs derived from the outer membrane (**Fig 3.1a)**, as previously reported (Maphosa and Moleleki, 2020). Based on the TEM analysis conducted in this study, the OMVs produced by *Pbr*1692 were shown to vary in size from approximately 35 to 100 nm in diameter, and they mostly showed a spherical appearance enclosed in a single membrane (**Fig 3.1b**). After ultracentrifugation, the pellet was resuspended in PBS buffer and filtered with PVDF 0.22 μm to filter and trap contaminants. The TEM micrographs confirmed the purity of the OMVs through the absence of flagella material. The purified OMVs were further plated on LB agar and incubated for 24 hrs to determine whether there were no residual bacterial cells. There was no bacterial growth (results not shown) on the agar plate, indicating no residual cells that contaminated the OMVs sample. The SDS page was performed to confirm the isolated OMVs through the protein patterns of the membrane of OMVs. The three independent isolation of OMVs showed a consistency in the band pattern of the proteins that form part of the outer membrane of the OMVs, demonstrating the reproducibility of the isolated OMVs. The proteins have sizes between 25 kDa and 100 kDa (**Fig 3.1c**).

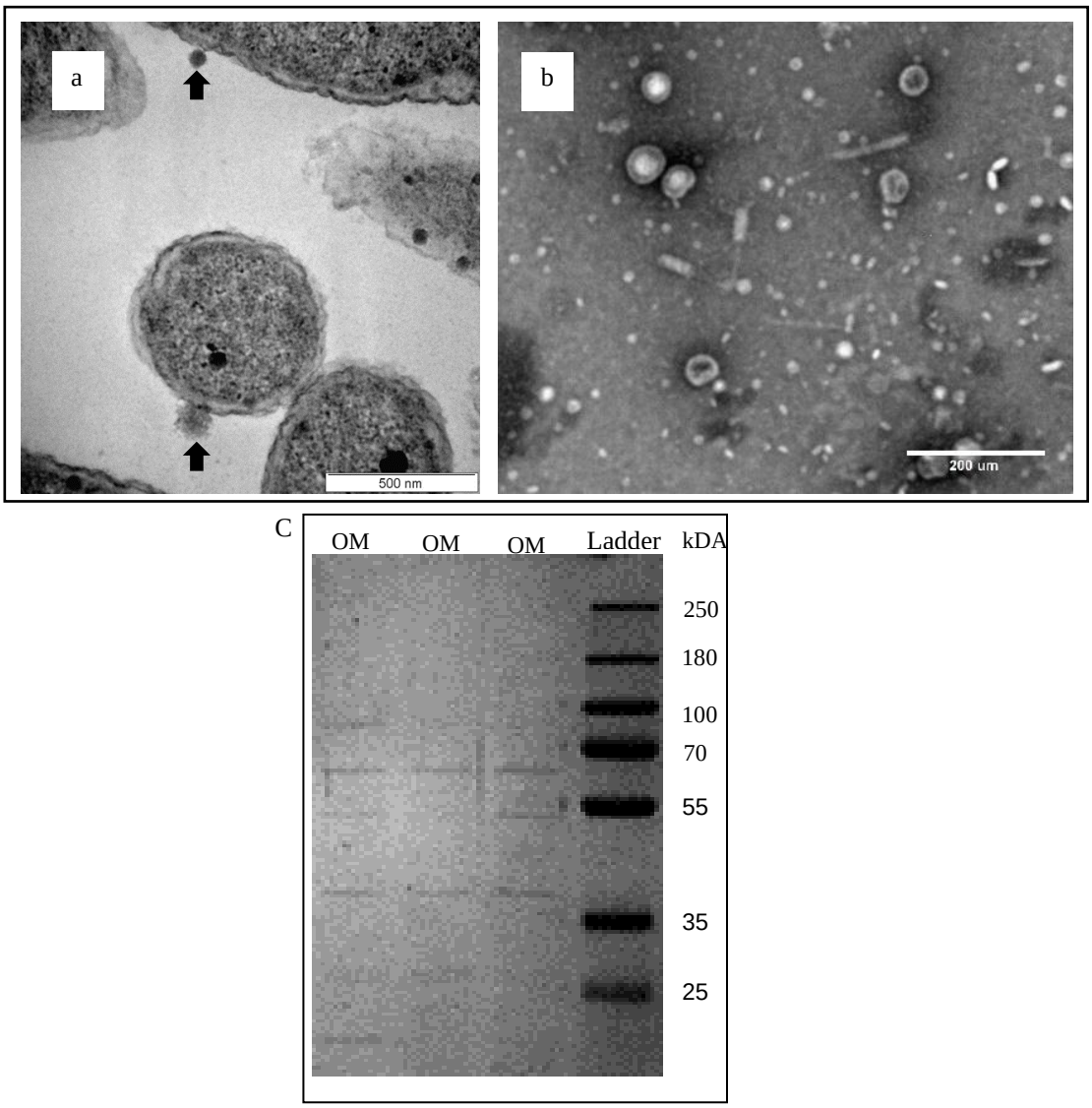


Figure 3. 1: Isolation of OMVs from *Pbr*1692 at the stationary phase. (**a**) Thin section micrographs depicting OMVs bulging from the *Pbr*1692 cell membrane. **(b**) Transmission electron micrographs of OMVs isolated from *Pbr*1692 negatively stained with 1% uranyl acetate. (**c**) SDS page gel of three independent OMV isolations confirming the reproducibility of the isolated OMVs.

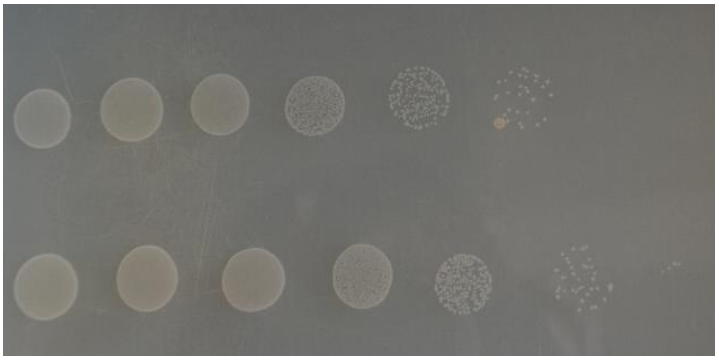
3.1.2 The effect of *Pbr*1692 OMVs on soft rot *Enterobacteriaceae*

Microbes are involved in constant inter and intraspecies interactions that maintain microbial communities, including competition and cooperation (Peleg et al., 2010). *Pbr*1692 shares a niche with various bacteria and other microbes within the host potato plants (Shyntum *et al.*, 2019, Motyka-Pomagruk *et al.*, 2021); these microorganisms likely compete for available nutrients to survive. Previously, it was reported that *Pbr*1692 outcompetes multiple SREs, including *D. dadantii*, *P. atrosepticum*, and *P. carotovorum* (Marquez-Villavicencio *et al.*, 2011, Shyntum *et al.*, 2019). To investigate whether *Pbr*1692 OMVs play a role in these interactions, we screened for any effects, whether negative or positive, that OMVs could have on selected SREs. Shyntum *et al.* (2019) showed that *Pbr*1692 cells did not inhibit *P. . parmentieri*. However, we included it as part of the study to investigate whether *Pbr*1692 OMVs (instead of cells) could affect the bacterium. We used a co-culture method, where *Pbr*1692 OMVs of 7 mg/ml were co-inoculated with the gentamicin-transformed SREs. Subsequently, serial dilutions were conducted, and the bacterial cells were plated on gentamicin-supplemented LB agar. The statistical analysis showed a significant reduction in CFU/ml of *D. dadantii* when co-cultured with *Pbr*1692 OMVs ($P < 0.05$), suggesting that an antimicrobial effect was enacted (**Fig 3.2**). In contrast, there was no inhibition for *P. atrosepticum*, *P. carotovorum*, and *P. parmentieri* (**Fig 3.2 b**), suggesting that *Pbr*1692 OMVs have antimicrobial activity against *D. dadantii* only. This is consistent with proteomic analysis from Maphosa & Moleleki (2021), showing that *Pbr*1692 OMVs may have proteins associated with inhibition.

a

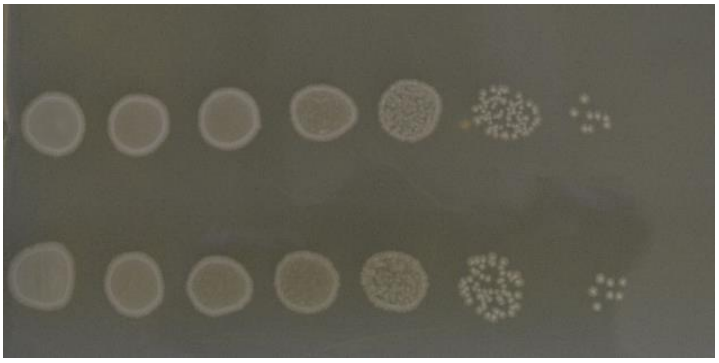
D. dadantii + *Pbr*1692 OMVs

D. dadantii + *PBS*



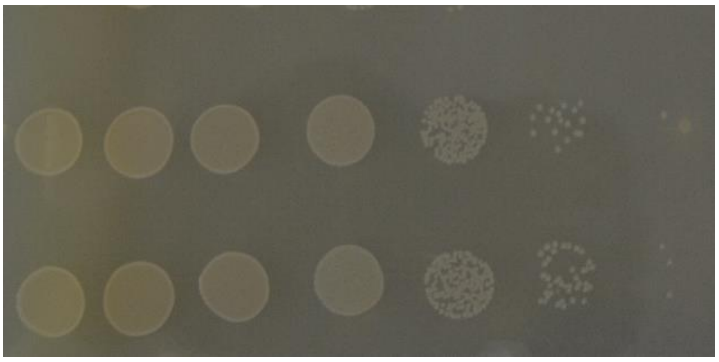
P. atrosepticum + *Pbr*1692 OMVs

P. atrosepticum + PBS



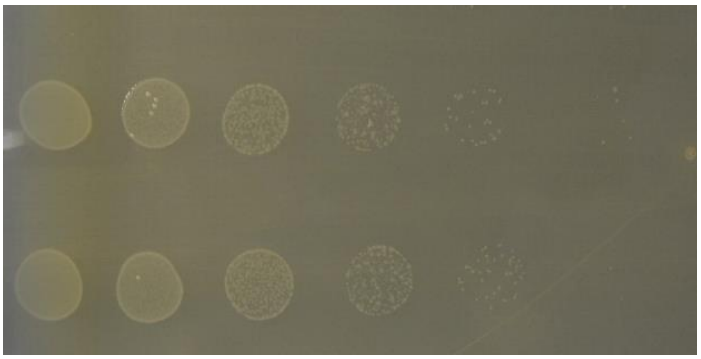
P. carotovorum + *Pbr1692* OMVs

P. carotovorum + PBS



P. parmentieri + *Pbr1692* OMVs

P. parmentieri + *PBS*



b

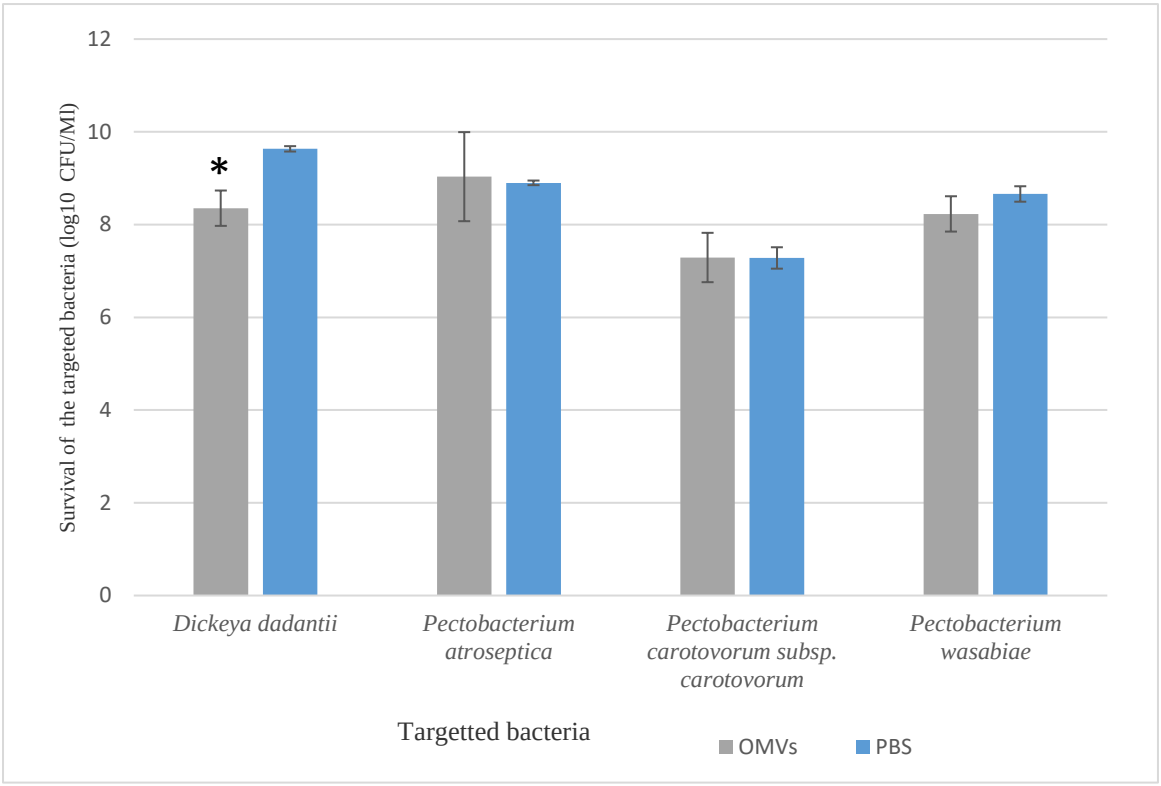


Figure 3. 2: The effect of *Pbr*1692 OMVs on SREs. (**a**) Gentamicin-resistant targetted bacteria *D. dadantii*, *P. atrosepticum*, *P. carotovorum*, and *P. parmentieri* were treated with *Pbr*1692 OMVs (**b**) The quantification of the targetted and treated bacteria presented as log10 CFU/ml. The asterisk shows a statistical significance where $P < 0.05$ compared to the PBS buffer negative control using the t-test.

3.1.3 *Pbr*1692 cells do not inhibit the growth of *P. parasitica* INRA 310

In figure 2.2., we observed that *Pbr*1692 OMVs inhibited the growth of *D. dadantii*, which means that *Pbr*1692 OMVs may harbour antimicrobial compounds. *Pbr*1692 and *P. parasitica* INRA 310 are soilborne pathogens that both infect potato tubers and may interact in the fields (Grisham *et al.*, 1983, van der Merwe *et al.*, 2010, Taylor *et al.*, 2012, Taylor *et al.*, 2014). Therefore, we next explored the possibility that *Pbr*1692 OMVs could have antimicrobial activity against oomycetes that infect potatoes. Hence, to investigate whether there is competition between *Pbr*1692 and *P. parasitica* INRA 310, we performed an *in vitro* dual culture assay where *P. parasitica* INRA 310 mycelium plugs were placed 2 cm away from *Pbr*1692 inoculation. Both *P. parasitica* INRA 310 and *Pbr*1692 could grow with no inhibition observed (**Fig 3.3a**). Furthermore, we measured the *P. parasitica* INRA 310 mycelial growth radius from the point of inoculation to detect and quantify possible inhibition. We found that the mean radius of the *P. parasitica* INRA 310 mycelium co-cultured with *Pbr*1692 was 3.5 cm compared to the mean radius of the mycelium of the negative control, which was 3.45 cm (**Fig 3.3b**). Hence, using the *P*-value where $P > 0.05$, concluding that there is no significant difference in the mycelium radius between the dual cultured of *Pbr*1692 and *P. parasitica* INRA 310 compared to the negative control.

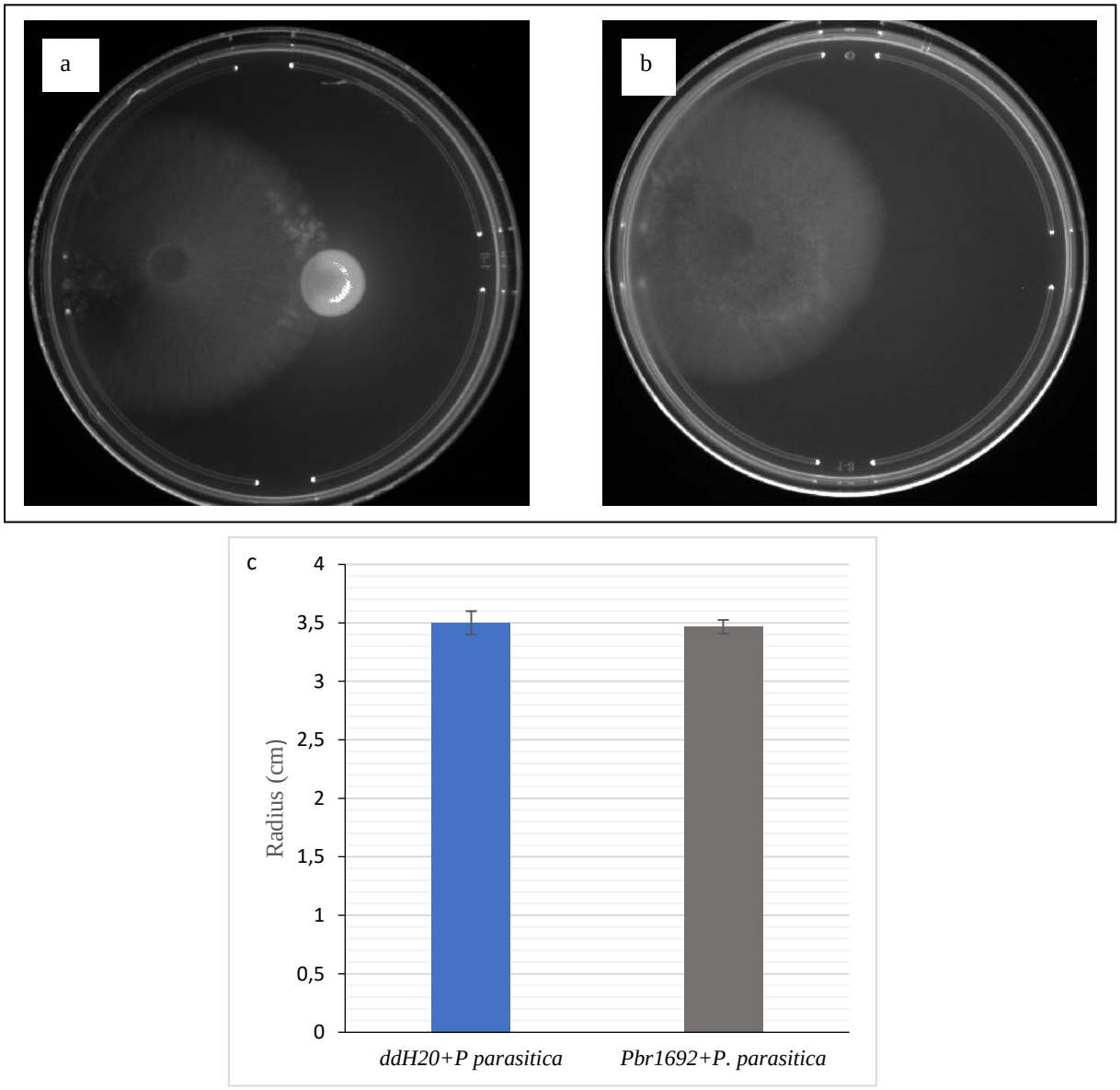


Figure 3. 3: Antagonism assay of (**a**) *Pbr*1692 cultured with *P. parasitica* INRA 310 mycelia and (**b**) ddH2O as the negative control on CMA. (**c**) Comparison of the length of the radius of the mycelium growth from the point of inoculation for both the test and the negative control. The error bars represent the standard deviations from the data, and asterisks denote a statistical significance at $P < 0.05$, where the absence of an asterisk shows insignificance.

Since we did not observe any inhibition of *P. parasitica* INRA 310 mycelia *by Pbr*1692 cells, we tested whether *Pbr*1692 cells can inhibit *P. parasitica* zoospores. A zoospore germination test was performed to investigate the effect of *Pbr*1692 on *P. parasitica* INRA 310 zoospores germination. The zoospores were incubated overnight with *Pbr*1692 cells and glucose and submitted for microscopic observations. **Fig 3.4a** shows the germination of *P. parasitica* INRA 310 zoospores treated with *Pbr*1692 cells, similar to the negative control where germination was observed (**Fig 3.4b**).

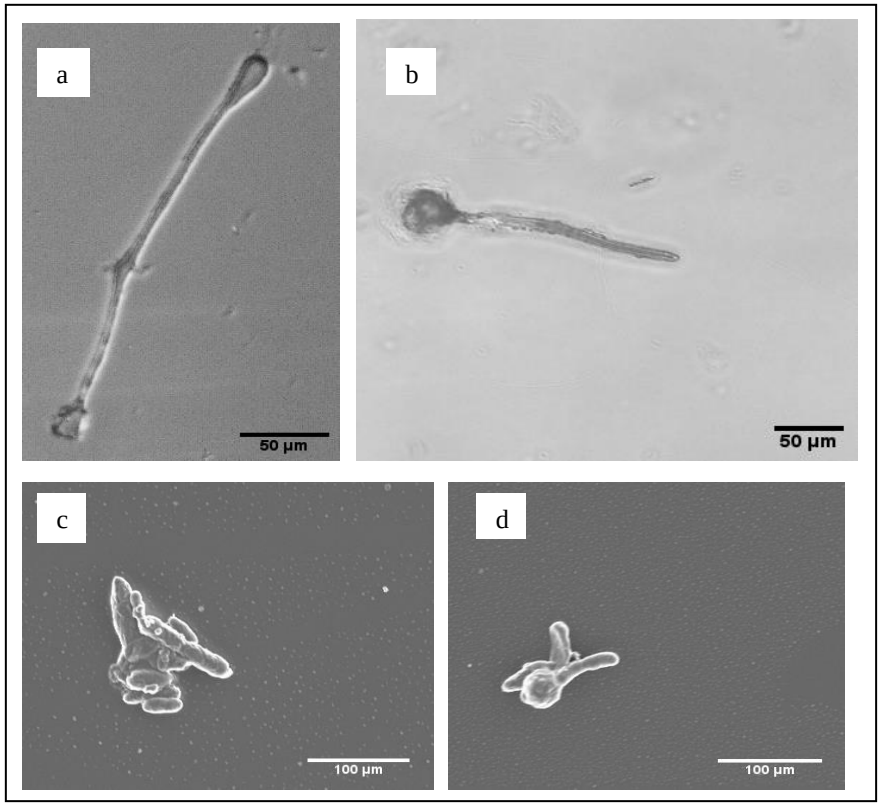


Figure 3. 4: Zoospore germination test. *Phytophthora parasitica* INRA 310 zoospore with *Pbr*1692 cells (**a**, **c**) and water is the negative control (**b**, **d**) image obtained using a brightfield microscope 40x objective (**a**, **b**) and a scanning electron microscope (**c**, **d**).

A scanning electron microscope was employed to evaluate if there are morphological effects that *Pbr*1692 cells could have on the zoospores. The SEM micrographs show that *Pbr*1692 cell-treated *P. parasitica* INRA 310 zoospores were able to germinate similarly to the negative control (**Fig 3.4c, d**), and no morphological deformities were observed. The zoospores were motile for the first two hrs, maintaining the same pace as the negative control in ddH_2O; however, after 2 hrs 30 mins, the zoospores were treated with *Pbr*1692 cells slowed down as shown on the links (**Sup Fig 3.13a**). Based on the results, it was concluded that *Pbr*1692 cells have no antimicrobial activity against *P. parasitica* INRA 310 zoospores. However, these cells may affect the motility of the zoospores.

3.1.4 *Pbr*1692 OMVs do not inhibit the growth of *Phytophthora parasitica*

We previously demonstrated that *Pbr*1692 cells do not exhibit inhibition against *P. parasitica* INRA *310* (**Fig 3.3**). As vesicles often reflect the biology of their donor cells, we, therefore, argued that *Pbr*1692 OMVs might also lack antimicrobial activity against *P. parasitica* isolates. We employed a mycelium targetted assay to test this hypothesis where a mycelium plug was grown on a CMA plate for three days. The sterile filter papers were inoculated with the variables and incubated for two additional days, and the results were observed. Both the two independent batches of *Pbr*1692 OMVs at 3 mg/ml and 5 mg/ml did not inhibit the mycelial growth of all the *P. parasitica* isolates, and these results were similar to that of the negative control PBS buffer (**Fig 3.5**).

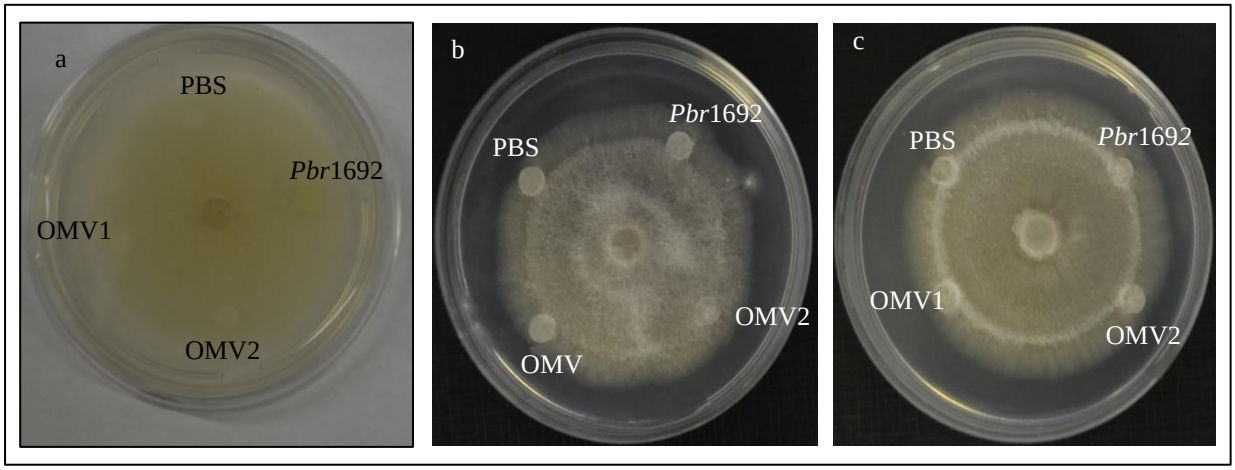


Figure 3. 5: Mycelium targetted assay investigating the non-inhibitory activity of OMVs on the isolates of *P. parasitica* mycelium at two different concentrations, inoculated with *Pbr*1692 cells and PBS buffer as the negative control. (**a**) *P. parasitica* INRA 310, (**b**) *P. parasitica* 149, and (**c**) *P. parasitica* 329.

We also tested the non-inhibitory effects of *Pbr*1962 OMVs on *P. parasitica* INRA 310 zoospore germination, given that zoospores play a huge role in disease initiation and development (Meng *et al.*, 2014). To this end, we first determined whether zoospores could internalise exogenously applied OMVs as a function of *trans*-kingdom communication. OMVs were labelled with a lipophilic dye, FM4-64, which selectively stains the vacuole membrane of the OMVs red and shows red fluorescence for OMVs uptake. Subsequently incubating the zoospores with glucose and the labelled OMVs. The germinated zoospores showed red fluorescence during confocal microscopy, suggesting that *P. parasitica* INRA 310 zoospore can internalise the FM4-64 stained *Pbr*1692 OMVs (**Fig 3.6a, b**). In contrast, the negative control showed no fluorescence (**Fig 3.6c**), and the brightfield micrograph of the negative control, as indicated in **Fig 3.6d,** showed no fluorescence. Therefore, *P. parasitica* INRA 310 zoospores can take up *Pbr*1692 OMVs, and germination was also recorded. This result suggests that, although there is OMV-mediated *trans*-kingdom interaction between the pair, it bears no visible inhibitory effect on the recipient cells of *P. parasitica*, and whatever effect is present, it cannot be detected by this assay.

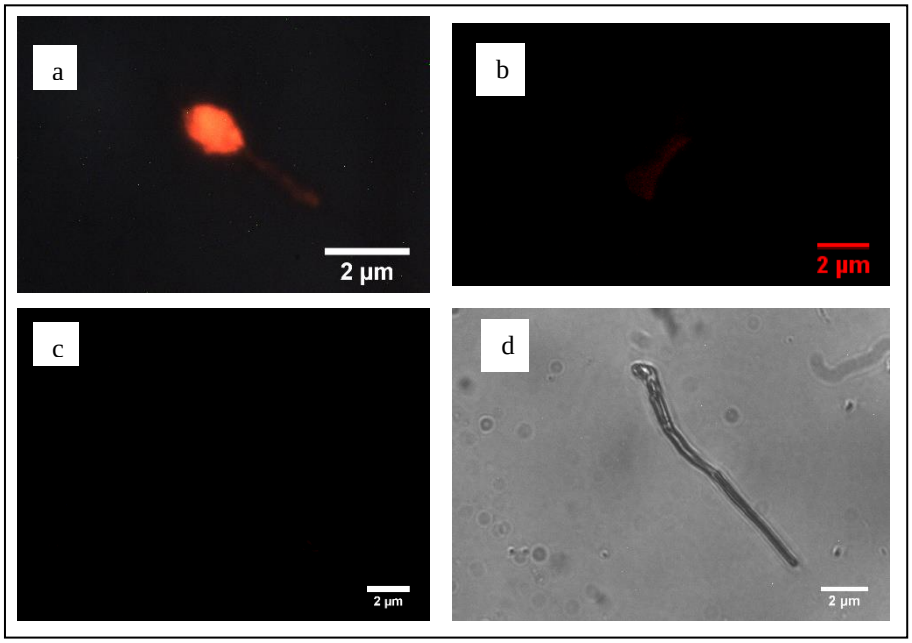


Figure 3. 6: OMVs uptake test. (**a**, **b**) FM4-64 labelled OMVs with *P. parasitica* INRA 310 zoospores. (**c**, **d**) the negative control with PBS buffer. (**a**, **c**) micrographs obtained with a fluorescent microscope, (**b**) a confocal microscope image of the *Pbr*1692 OMV uptake by *P. parasitica* zoospores (**d**) the micrographs were generated with a brightfield microscope using a 40x objective.

Next, the zoospores were incubated overnight with OMVs and glucose and then submitted for microscopic observations. **Fig 3.7a** shows the germination of *P. parasitica* INRA 310 zoospores in the treatment of OMVs, similar to the negative control in PBS buffer (**Fig 3.7b**). To evaluate the morphological effect of *Pbr*1692 OMVs on the zoospores, we used SEM, which showed that *P. parasitica* INRA 310 zoospores could germinate with no deformities in both *Pbr*1692 cells and the negative control (**Fig 3.7c, d**). Furthermore, the zoospores treated with OMVs maintained the same pace of motility as the negative control in the PBS buffer (**Sup Fig 3.13b**).

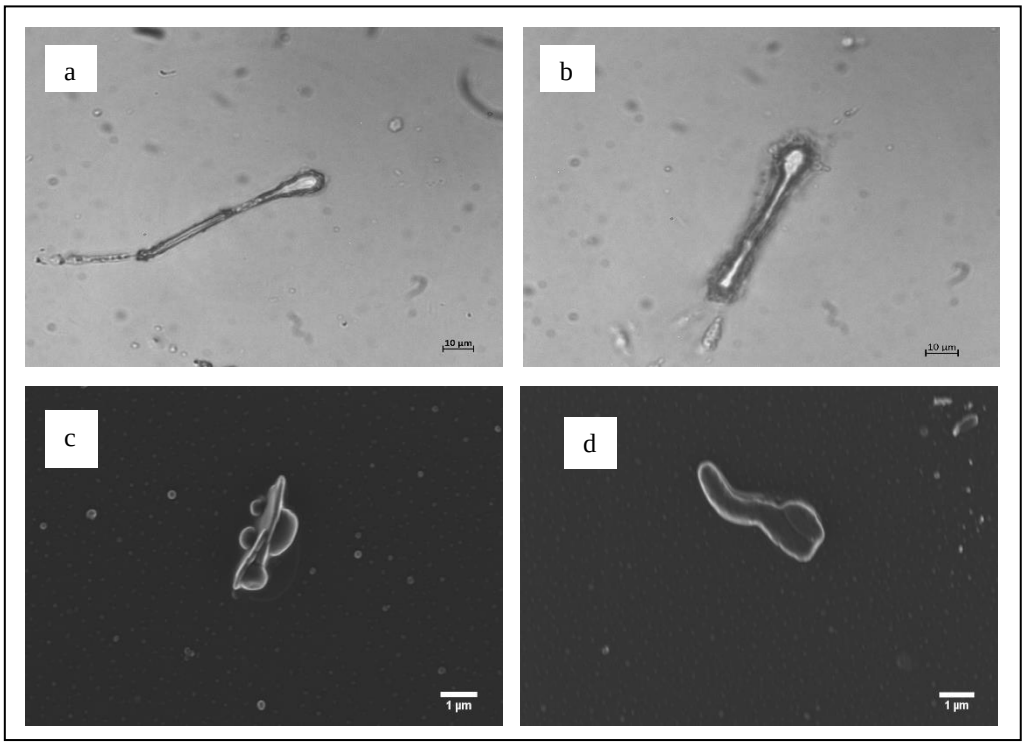


Figure 3. 7: Zoospores germination test. *P. parasitica* INRA 310 zoospores treated with OMVs (**a**, **c**) and the negative control with PBS buffer (**c**, **d**). The micrographs were generated using a brightfield microscope 40x objective (**a**, **b**) and a scanning electron microscope (**c**, **d**).

To further ascertain the non-inhibitory effects of *Pbr1962* OMVs on the growth of *P. parasitica* INRA 310, an inhibition test was performed to investigate whether the germinated hyphae were viable. *P. parasitica* INRA 310 zoospores with glucose and OMVs were incubated overnight. Subsequently, the germinated zoospores were stained with Evans blue, which targets membrane damage and stains the cytoplasm and the nucleus blue. The hypha did not stain blue (**Fig 3.8a**), suggesting no membrane damage to the zoospores treated with OMVs, similar to the negative control (**Fig 3.8d**). Another approach was employed to assess the membrane permeabilisation of the zoospores when treated with *Pbr*1692 OMVs. In this case, the

zoospores with a compromised membrane are expected to take up the red propidium iodide. The zoospores treated with OMVs and the negative control with PBS buffer did not exhibit any fluorescence (**Fig 3.8b, c**), confirming that the *P. parasitica* germinated zoospores were viable when treated with *Pbr*1692 OMVs. These results show that *Pbr*169*2* OMVs do not cause *P. parasitica* cell death, even though they can be internalised showing *trans*-kingdom communication (**Fig 3.6**).

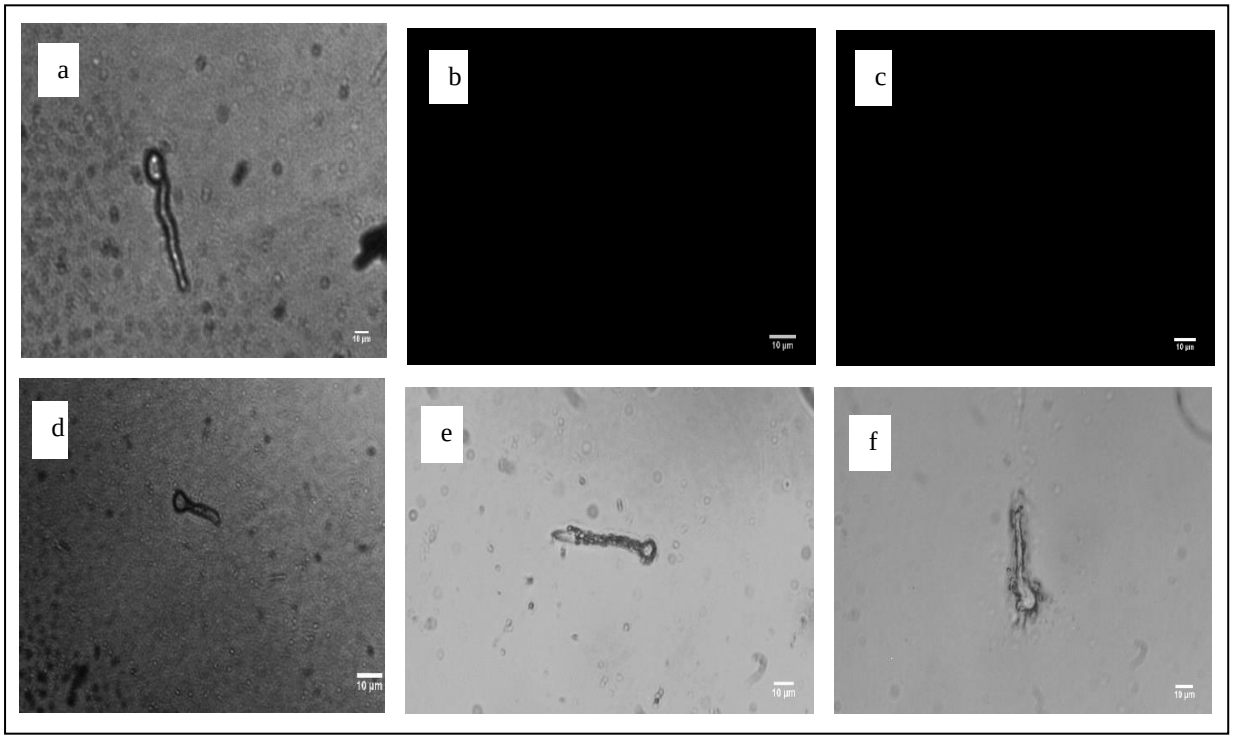


Figure 3. 8: Zoospore viability test. *P. parasitica* INRA 310 zoospores were incubated for 16 hrs with OMVs (**a**, **b**, **c**) or PBS buffer as the negative control (**d**, **e**, **f**) were stained with Evan's blue (**a**, **d**) or propidium iodide (**b**, **c**, **e**, **f**). The micrographs were obtained with a fluorescent (**c**, **d**) or brightfield microscope 20x objective (**a**, **d**, **e**, **f**).

3.1.5 Phenotypic Microarray preliminary results

3.1.5.1 *Pectobacterium brasiliense* 1692 OMVs assist *Phytophthora parasitica* INRA 310 zoospores in utilising some carbon sources.

As previously reported (**Fig 3.6)**, *P. parasitica* INRA 310 zoospores could internalise *Pbr*1692 OMVs, suggesting interkingdom interaction between *P. parasitica* INRA 310 and *Pbr*1692. We then questioned what effect *Pbr*1692 OMVs had on *P. parasitica* INRA 310 if they did not suppress its growth, given that uptake of these OMVs resulted in non-inhibitory effects (**Fig 3.5**-**3.8**). Several studies have shown that bacterial OMVs are involved in interspecies and intraspecies interactions (Wang *et al.*, 2020) through potential antimicrobial compounds contained in their lumen. It is, therefore, likely that OMVs can also release compounds that mediate other forms of interactions, thus serving as 'public goods' for the coexistence of various bacterial species in the same habitat (Elhenawy *et al.*, 2014). For instance, OMVs can help bacterial communities by disseminating enzymes, at a cost to the producer, that break down extracellular material into nutrients, recruiting iron, functioning as bacteriophage or antibiotic decoys, and transferring beneficial DNA material between the OMV donor and bystander cells (Schwechheimer & Kuehn, 2015). Therefore, it is possible that, instead of inhibiting growth, the *Pbr*1962 OMVs could benefit *P. parasitica* INRA 310. As we could not detect any growth-inhibiting effects with several assays that we performed between *Pbr*1962 OMVs and *P. parasitica* INRA 310, we argued that any effects resulting from this interaction could be detected through a more sensitive assay. For this reason, we turned our attention to the metabolic activity of the zoospores measured using an OmniLog phenotypic microarray platform provided by Biolog.

During this study, we investigated the ability of zoospores treated with *Pbr*1692 OMVs (referred to as treated zoospores) to utilise various carbon sources provided by Biolog. These were compared to the negative control zoospores that were not treated with *Pbr*1692 OMVs (referred to as untreated zoospores). We separately added the treated and untreated zoospores into the inoculating fluid, aliquoted them into the 96 well microplates containing carbon sources at unknown concentrations, as per the manufacturer's recommended instructions, and incubated the microplates for 48 hrs. Results were then recorded every 15 mins using an Omnilog plate reader.

After 48 hrs of incubation, Biolog software was employed to construct a parametric graph that compares the carbon utilisation and the growth of the treated and untreated zoospores, of which

the green and red represent treated and untreated zoospores, respectively, and yellow is the overlap of the treated and untreated zoospores. The results showed that treated and untreated zoospores utilised 85 similar carbon sources out of 95 wells. Based on the Biolog system, these carbon sources belong to the carbohydrates, carboxylic, amino acids, and alcoholic groups (**Fig 3.9**). Only three carbon sources were utilised by neither treated nor untreated zoospores; interestingly, seven carbon sources, namely, D-glucosamic acid, formic acid, thymidine, acetoacetic acid, N-acetyl-D-mannosamine, m-Hydroxyphenylacetic acid, and glucoromide were used by treated zoospores only (**Fig 3.9b**). These carbon sources belong to the carbohydrates, carboxylic, and amide groups. The results suggest that OMVs may aid *P. parasitica* INRA 310 zoospore in assimilating specific carbon sources. Based on the KEGG database, the carbon sources that were utilised and showed growth of treated zoospores only were indicated to be involved in various metabolic pathways, including the metabolism in diverse environments, biosynthesis of secondary metabolites, and metabolism of amino acids (**Table 3.1**). In addition, some of the carbon sources participate in pyruvate metabolism and the two-component systems.

Of the 85 carbon sources that were utilised by both treated and untreated zoospores, some carbon sources showed enhanced growth in the treated zoospores compared to the untreated. Therefore, to determine the carbon sources that showed improved carbon utilisation, we calculated the omnilog unit difference between the treated and the untreated zoospores of each corresponding well using the cut-off value of 10 Ou. About 31 carbon sources showed improved growth in the treated zoospores, showing a cut-off of 10 Ou and above (**Table 3.2**). Furthermore, using the KEGG database, the carbon sources were predicted to be involved in various metabolic pathways that may assist in the metabolic reactions in *P. parasitica* INRA 310 zoospores indicated in **Table 3.2**. From the results, the *P. parasitica* INRA 310 zoospores treated with *Pbr*1692 OMVs could utilise specific carbon sources that the untreated zoospores were unable to utilise, and the treated zoospores further showed enhanced growth in some carbon sources compared to the untreated zoospores.

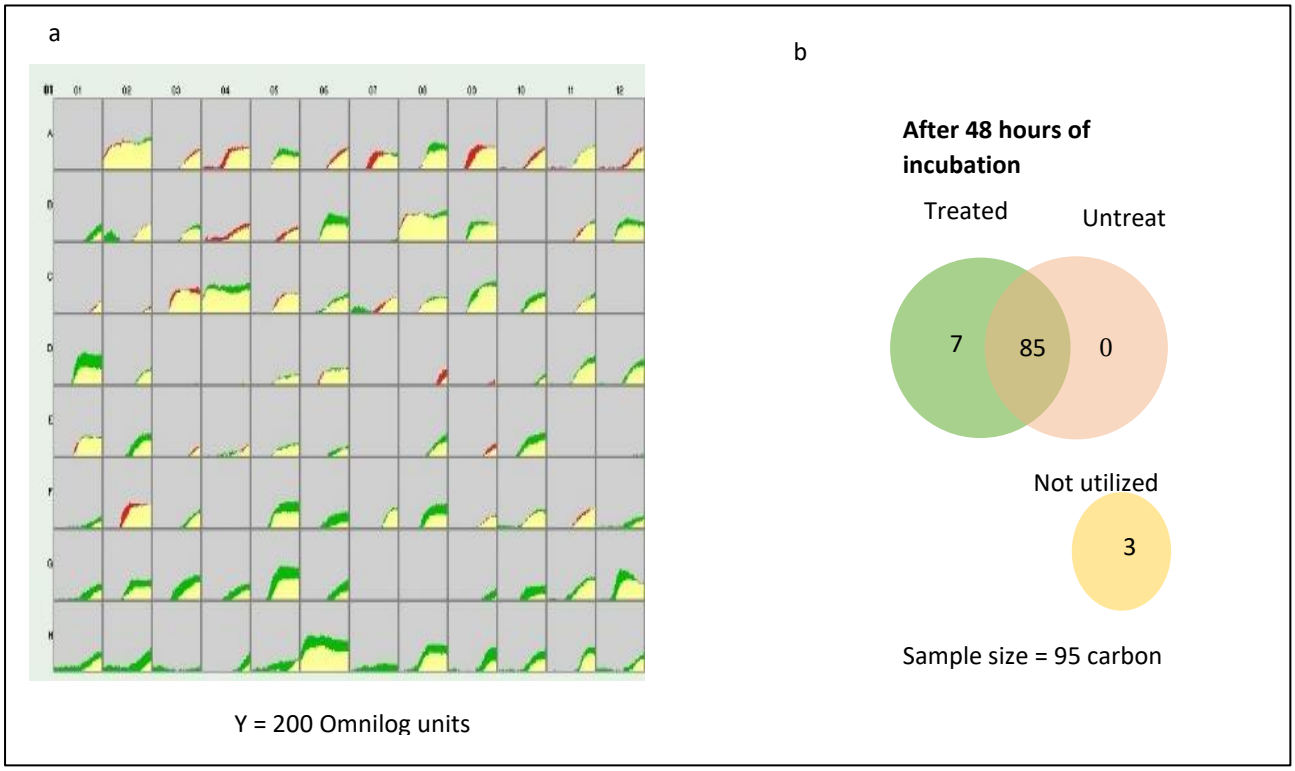


Figure 3. 9: PM comparing the carbon utilisation ability of *P. parasitica* INRA 310 zoospores treated with *Pbr*1692 OMVs to those not treated with *Pbr*1692 OMVs for 48 hrs incubation. The colours green and red show the utilisation of the carbon sources by treated and untreated zoospores, respectively, and yellow is the overlap of both treated and untreated zoospores (**a**). The Venn diagram shows the carbon sources utilised by either treated or untreated *P. parasitica* INRA 310 zoospores, treated and untreated (**b**).

Table 3. 1: The metabolic pathways of the seven carbon sources that *P. parasitica* INRA 310 zoospores treated with *Pbr*1692 OMVs utilised using the KEGG database.

Carbon sources	**Pathways in which the carbon sources engage in**
Thymidine	Metabolic pathways
D-Glucosaminic acid	Microbial metabolism in diverse environments
Formic acid	Pyruvate metabolism, microbial metabolism in diverse environments, and carbon metabolism
Aceto-acetic acid	Microbial metabolism in diverse environments, and two-component system
N-acetyl-D-mannose	Amino sugar and nucleotide sugar metabolism
Glucuronamide	Ascorbate and aldarate metabolism, amino sugar and nucleotide sugar metabolism, Inositol phosphate metabolism, biosynthesis of cofactors, and nucleotide sugars
m-Hydroxyphenyl acetic acid	Microbial metabolism in diverse environments, and tyrosine metabolism

Table 3. 2: The list of carbon sources showed the most growth and metabolism of zoospores treated with *Pbr*1692 OMVs compared to the untreated by calculating the difference between the treated and untreated zoospores using the cut-off of 10 Ou and the predicted metabolic pathways they participate in using KEGG database.

Carbon source	**Cut-off value (OM)**	**Pathways involved**
Succinic acid	10	Citrate cycle (TCA cycle), oxidative phosphorylation, alanine, aspartate, glutamate metabolism, pyruvate metabolism, glucagon signalling pathway, biosynthesis of secondary metabolites, microbial activity metabolism in diverse environments, and carbon metabolism.
D-Sorbitol	10	Metabolism of fructose, mannose, galactose metabolism, ABC transporters, and phosphotransferase system (PTS).
Gluconic acid	17	Pentose phosphate pathway, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, and carbon metabolism.
L-Glutamic acid	10	ABC transporter, two-component system, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, Neomycin, kanamycin, and gentamicin biosynthesis.
D-Ribose	18	Pentose phosphate pathway and ABC transporters
Maltose	10	Starch and sucrose metabolism, biosynthesis of secondary metabolites, ABC transporters, and phosphotransferase system
L-Asparagine	28	Biosynthesis of secondary metabolites and amino acids, protein digestion and

		absorption, mineral absorption, alanine, aspartate, and glutamate metabolism
Sucrose	10	Starch and sucrose metabolism, biosynthesis of secondary metabolites, ABC transporters, and phosphotransferase system (PTS)
Uridine	11	ABC transporters
m-tartaric acid	14	Glyoxylate and dicarboxylate metabolism
Maltotriose	16	ABC transporters, carbohydrate digestion and absorption, glyoxylate and dicarboxylate metabolism
Gly-Asp	12	Glycine and aspartate metabolism, alanine, aspartate and glutamate metabolism, glycine, serine, and threonine metabolism
Fumaric acid	19	Citrate cycle (TCA cycle), oxidative phosphorylation, alanine, aspartate, and glutamate metabolism, nicotinate and nicotinamide metabolism, metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, carbon metabolism, two-component system, and glucagon signalling pathway
Bromosuccinic acid	19	Citric cycle
Mucic acid	20	Ascorbate and aldarate metabolism
Inosine	40	Purine metabolism, nucleotide metabolism, and ABC transporters
Gly-Glu	17	Amino acid metabolism and glyoxylate metabolism
Tricarboxylic acid	17	Citric cycle (TCA cycle)
L-Serine	21	Biosynthesis of various other secondary metabolites, microbial metabolism in diverse environments, carbon metabolism, ABC transporter, mineral absorption, glycine,

		serine and threonine metabolism, cysteine, and methionine metabolism
L-Threonine	14	Glycolysis / Gluconeogenesis and citrate cycle (TCA cycle)
L-Alanine	24	Alanine, aspartate and glutamate metabolism, cysteine and methionine metabolism, ABC transporter, biosynthesis of various other secondary metabolites and amino acids, metabolic pathways, microbial metabolism in diverse environments, and carbon metabolism
Methylpuruvate	16	Glycolysis / Gluconeogenesis, citrate cycle (TCA cycle), phosphotransferase, metabolic pathways, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, and carbon metabolism
L-Malic acid	12	Biosynthesis of secondary metabolites, microbial metabolism in diverse environments, carbon metabolism, two-component system, citrate cycle (TCA cycle), pyruvate, glyoxylate, and dicarboxylate metabolism
Gly-Pro	15	Glycine, serine, threonine, and proline metabolism
D-Hydroxyphenyl acetic acid	30	Tyrosine metabolism, phenylalanine metabolism, and microbial metabolism in diverse environments
Tyramine	11	Tyrosine metabolism, and microbial metabolism in diverse environments
L-Lyxose	28	Pentose and glucuronate interconversions
Pyruvic acid	15	Glycolysis / Gluconeogenesis, citrate cycle (TCA cycle), pentose phosphate pathway, alanine, aspartate and glutamate metabolism,

		glycine, serine and threonine metabolism, phosphotransferase, biosynthesis of secondary metabolites, microbial metabolism in diverse environments, and carbon metabolism
L-Galactonic acid-g-Lactose	26	Ascorbate and aldarate metabolism, metabolic pathways, biosynthesis of secondary metabolites and cofactors
D-Galacturonic acid	14	Pectin degradation and
Phenylethylamine	10	Metabolism of amino acids

3.1.5.2 *Pbr*1692 OMVs affect the chemical sensitivity of *Phytophthora parasitica* INRA 310

A PM21 microplate was used to investigate the effect of *Pbr*1692 OMVs on the chemical sensitivity of *P. parasitica* INRA 310 zoospores. The PM21 microplates contain various chemicals such as antimicrobials, efflux pump inhibitors, detergents, chelators, chaotropic agents, biofilm inhibitors, and phosphodiesterase inhibitors. Four consecutive wells on the PM21 microplate contain the same chemical at different unknown concentrations. The parametric graph shows the quantification of the metabolism of zoospores in the `presence of different chemicals (**Fig 3.10**), where increased sensitivity to the chemical will decrease the metabolic rate of the zoospores, and a decrease in sensitivity will allow the metabolism and growth of the zoospores.

The experiment had no replicates, and because of that, wells with consistent increase or decrease in the omnilog units across the four wells of the same chemical were selected to analyse the effect of the OMVs on zoospores' metabolism. The chemicals nystatin, sodium dichromate, and D-cycloserine showed increased metabolic growth with increased chemical concentration for both the treated and the untreated zoospores, indicated by an increase in the steepness of the yellow graph (**Table 3.3)**. However, the treated zoospores showed the most metabolism in the early hours of incubation in D-cycloserine and at the highest concentration of sodium dichromate. In contrast, the untreated zoospores showed increased metabolic activity in nystatin compared to the treated. The thiourea, nickel chlorate, sodium selenite, and 1-Hydroxypyridine-2-thione wells showed a decrease in the metabolism and growth of both the treated and untreated zoospores (**Table 3.3**). However, increased metabolic activity was observed for the treated zoospore during the early hours of incubation in 1-Hydroxypyridine-2-thione nickel chlorate, sodium selenite, and thiourea.

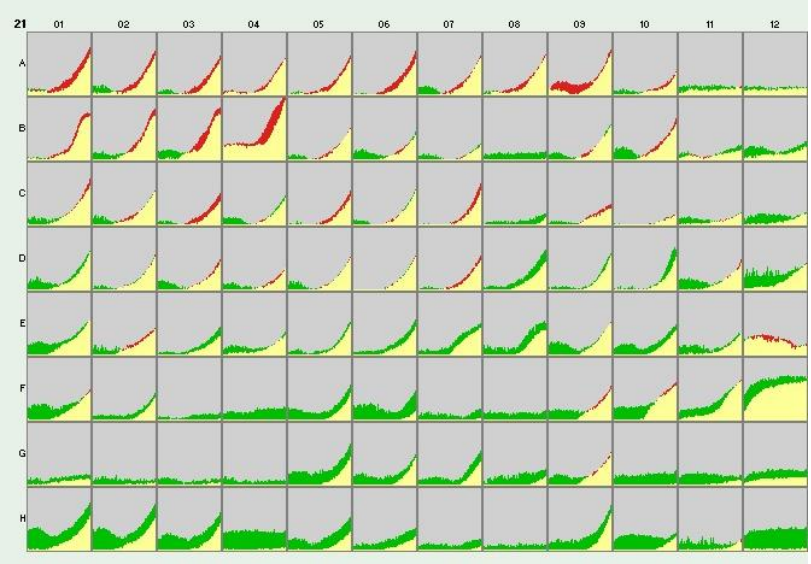


Figure 3. 10: A parametric graph compares the chemical sensitivity of *P. parasitica* INRA 310 zoospores treated with *Pbr*1692 OMVs to zoospores that are untreated at 48 hrs. The concentration of the chemicals increases from left to right. Green and red show the utilisation of the carbon sources by the treated and untreated zoospores, respectively, and yellow is the overlap of both the treated and untreated zoospores.

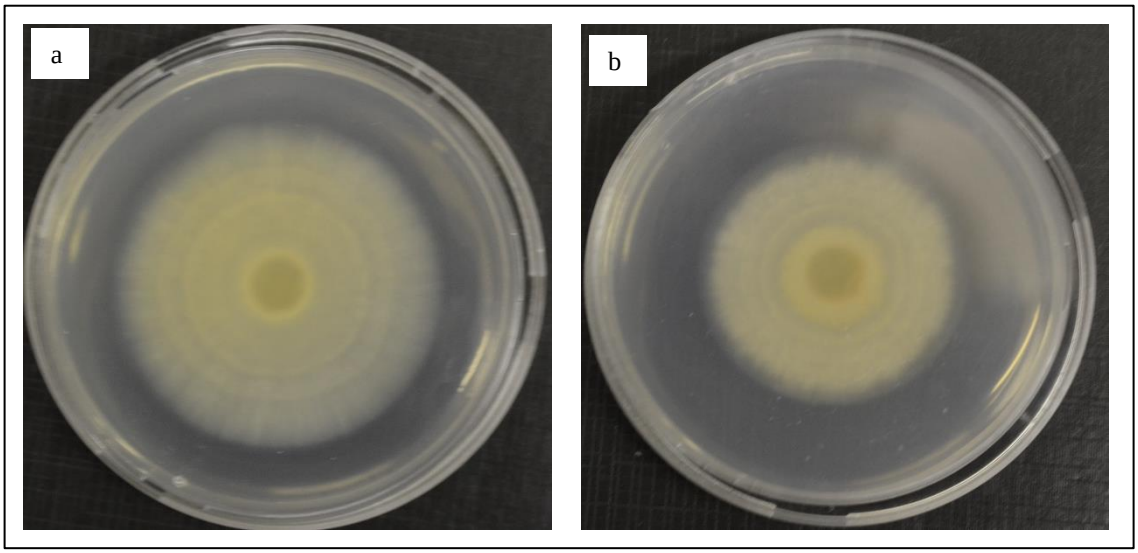
Table 3. 3: Chemicals that elicited a decrease or increase in metabolism and growth of the *P. parasitica* INRA 310 zoospores treated with *Pbr*1692 OMVs with an increase in the concentration of the chemical.

Wells	**Chemical**	**Function**	**Metabolism: Based on the increase in the concentration of the chemicals**	
			Treated and untreated zoospores	The one performing better
B1-B4	Nystatin	Increase permeability	Increase	Untreated
D1-D4	1-Hydroxypyridine-2-thione	Antifungal/inhibit biofilm formation	Decrease	Treated in the early hours of incubation
D9-D12	Sodium dichromate	Toxin anion	Increase	Treated at the highest concentration
F9-F12	D-cycloserine	Targets cell wall	Increase	Treated at the early hours of incubation
G1-G4	Sodium selenite	Toxin cation	Decrease	Treated
G5-G8	Nickel chlorite	Toxin cation	Decrease	Treated in the early hours of incubation
H5-H8	Thiourea	Chaotropic agent	Decrease	Treated

3.1.6 Validation of the phenotypic microarray results

3.1.6.1 *Pbr*1692 OMVs assist *Phytophthora parasitica* INRA 310 with the utilisation of formic acid

The PM results showed that *Pbr*1692 OMVs might assist *P. parasitica* INRA 310 zoospores in the utilisation of certain carbon sources. For instance, *P. parasitica* INRA 310 zoospores could not completely utilise D-glucosamic acid, formic acid, thymidine, acetoacetic acid, *N*-acetyl-D-mannosamine, *m*-Hydroxyphenylacetic acid, and glucoromide. However, when treated with OMVs, utilisation was recorded. Formic acid was selected to confirm the PM results using *P. parasitica* INRA 310 mycelium and zoospore germination and viability. Depending on the concentration, formic acid has been shown to induce growth inhibition and cell death in various fungi (Clevström *et al.*, 1989, Lastauskienė *et al.*, 2014), for this reason, it was prioritised for testing. *P. parasitica* INRA 310 mycelium was grown on CMA supplemented with formic acid (0.05%) and *Pbr*1692 OMVs. The results showed that in the presence of *Pbr*1692 OMVs, *P. parasitica* INRA 310 mycelium grows significantly well in formic acid compared to the negative control (**Fig 3.11**). The statistical analysis showed a significant ($p < 0.05$) mycelium growth (measured diameter in cm) in the presence of *Pbr*1692 OMVs compared to the negative control (**Fig 3.11c**). These results suggest that in the media supplemented with formic acid, *Pbr*1692 OMVs enhance *P. parasitica* INRA 310 mycelium growth.



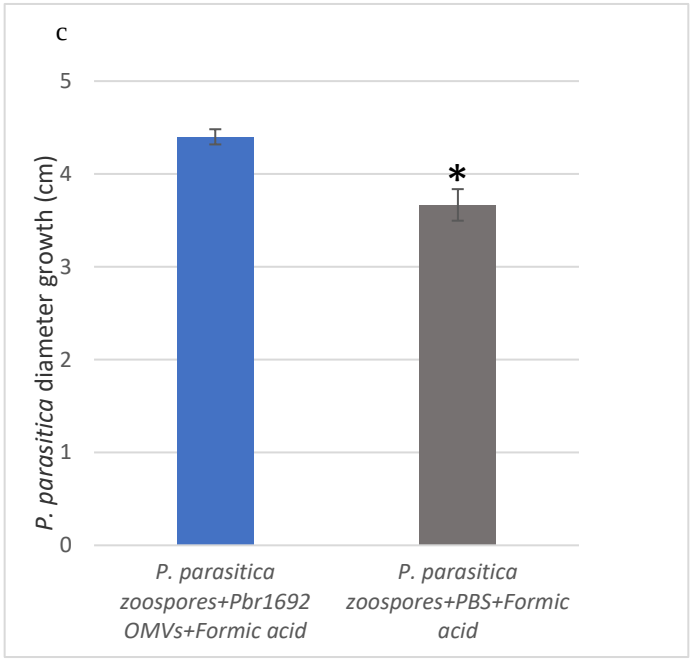


Figure 3. 11: The effect of *Pbr*1692 OMVs on *P. parasitica* INRA 310 carbon utilisation. (**a**) *P. parasitica* INRA 310 mycelium grown on CMA supplemented with formic acid and *Pbr*1692. (**b**) Mycelium grown on CMA supplemented with formic acid and PBS buffer as the negative control. (**c**)The error bars represent the standard deviations from the data, and the asterisk shows a statistical significance where $P < 0.05$ compared to the PBS buffer negative control using the t-test.

3.1.6.2 *Phytophthora parasitica* INRA 310 zoospores germination and viability in formic acid carbon source in the presence of *Pbr*1692 OMVs

To test whether *P. parasitica* INRA 310 zoospores treated with *Pbr*1692 OMVs would germinate in a formic acid carbon source, we inoculated the zoospores with formic acid and *Pbr*1692 OMVs followed by incubation for 16 hrs. Our results revealed that the zoospores were able to germinate. However, there was an accumulation of particles around the hyphae (**Fig 3.12 a, b**). Therefore, we performed a viability test by staining the micro slides with propidium iodide. Results showed that the hyphae treated with *Pbr*1692 OMVs did not exhibit fluorescence (**Fig 3.12 c**), while the negative control showed fluorescence (**Fig 3.12d**), suggesting that the germinated zoospores were viable. **Figure 3.12 e, f** represents the bright field micrographs of **Fig 3.12 c, d,** respectively, which are the specimens of the fluorescence micrographs. These results show that *P. parasitica* INRA 310 zoospores could germinate in both the test and the negative control. However, only the germinated zoospores are viable in *Pbr*1692 OMVs compared to the negative control PBS buffer, indicating that *Pbr*1692 OMVs may assist *P. parasitica* INRA 310 zoospores in formic acid utilisation.

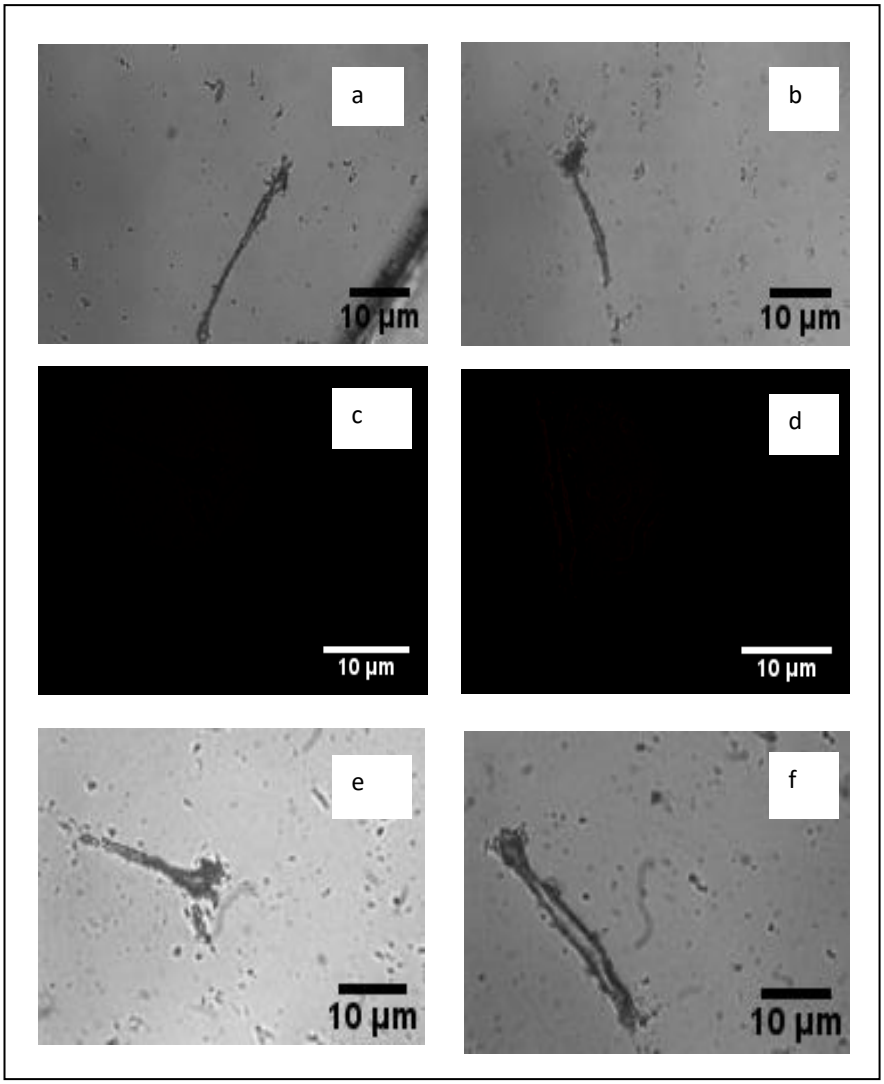


Figure 3. 12: Zoospore germination and viability test in formic acid. *P. parasitica* INRA 310 zoospores incubated with *Pbr*1692 OMVs (**a**) and the negative control with PBS buffer (**b**). Zoospores and *Pbr*1692 OMVs (**c**) and the negative control with PBS buffer (**d**), both stained with propidium iodide. (**e**,**f**) brightfield micrographs of (**c**,**d**) showing the specimen. The micrographs were obtained with brightfield and fluorescence microscopes, all at 40x objective.

3.2 Discussion and conclusions

The role of *Pbr*1692 OMVs in soft rot *Enterobacteriaceae* competitions

Outer membrane vesicles (OMVs) harbour proteins, such as hydrolases and chitinases that have shown antimicrobial activity against microorganisms, and this suggests that OMVs may play an essential role in the competition and survival of the parental bacteria (Kadurugamuwa & Beveridge, 1996, Meers *et al.*, 2018, Wang *et al.*, 2020, Maphosa & Moleleki, 2021). *Pbr*1692 is an important potato pathogen that relies on different bacterial secretion systems for access to nutrients and pathogenesis (Pérombelon, 2002, Shyntum *et al.*, 2019). *Pbr*1692 causes blackleg and soft rot of potatoes in the field; however, it can also be transmitted during storage. SREs including *Pbr*1692, *P. atrosepticum*, *P. carotovorum*, *Dickeya* spp., and *P. parmentieri* were isolated from the same diseased potato tubers in South Africa (Shyntum *et al.*, 2019), showing that SREs co-infect of potato tubers promoting multiple interactions amongst the SREs (Shyntum *et al.*, 2019, Motyka-Pomagruk *et al.*, 2021). Furthermore, Shyntum *et al.* (2019) showed that *Pbr*1692 secretes bacteriocins and antibiotics such as carbapenem and employs the T6SS to inhibit and outcompete micro-organisms in their niche. Another mechanism reported to take part in microbial interactions is OMVs, through the secretion and transport of crucial proteins that may aid the survival of bacteria (Kulp & Kuehn, 2010).

Pectobacterium spp., including *Pbr*1692, were reported and confirmed to produce OMVs through microscopic studies, nanoparticle tracking analysis and proteomic studies (Piotrowska *et al.*, 2020, Jonca *et al.*, 2021, Maphosa & Moleleki, 2021). Therefore, this study investigated the interactive physiological effects, whether antimicrobial or beneficial, of *Pbr*1692 cells and OMVs against some SREs, namely, *D. dadantii*, *P. atrosepticum*, *P. carotovorum*, *P. parmentieri*, and the oomycetes plant-pathogen *P. parasitica* INRA 310, and this is because *Pbr*1692 interacts these pathogens on potato and has been reported to also outcompete some of them (Marquez-Villavicencio *et al.*, 2011, Shyntum *et al.*, 2019).

We looked into OMVs as a possible mechanism *Pbr*1692 utilised in the competition (**Fig 3.2**). The results showed that *Pbr*1692 OMVs have antimicrobial activity against *D. dadantii*, which was also supported by Maphosa & Moleleki (2021). Furthermore, *Pbr*1692 OMVs proteomic analysis revealed that OMVs harbour proteins such as hydrolases, Cdi toxins, lyases, and peptidoglycan murein, which are suspected to be involved in microbial interactions and in inhibiting bacteria (Maphosa & Moleleki, 2021). It is supposed that bacteria may use OMVs to destroy their co-infector to reduce competition over nutrients (Li et al., 1998), and this was

further supported by a study that showed that *Lysobacter* sp. secrete endopeptidase L5, which is a bacteriolytic enzyme that degrades competitors by targeting the peptidoglycan (Vasilyeva *et al.*, 2008, Jan, 2017).

*Pbr*1692 OMVs did not show inhibitory effects on *P. atrosepticum* and *P. carotovorum* (**Fig 3.2**). Similarly, *Pbr*1692 OMVs do not inhibit the growth of *P. parmentieri* (**Fig 3.2**). These results also suggest that the mechanism *Pbr*1692 cells employ in the inhibition cannot exist independently in OMVs out of the *Pbr*1692 cells.

Notably, the study was conducted *in vitro*, and OMVs were not isolated from *Pbr*1692 cells that were introduced with either SREs or isolated from the natural environment before the isolation. Therefore, it is plausible that the OMVs produced were not specifically to target these species of interest. Furthermore, OMVs concentration is essential to exhibiting a killing ability (Li *et al.*, 1998), and we cannot eliminate the possibility that the concentration of the *Pbr*1692 OMVs used against SREs might have been inadequate to exhibit antimicrobial activity.

The role played by *Pbr*1692 OMVs in interkingdom interactions

Since *Pbr*1692 OMVs did not inhibit some SRES, we further investigated the role of *Pbr*1692 OMVs on another group of potato pathogens, namely the oomycetes. Studies have shown that bacteria can inhibit oomycetes germination (van Dijk & Nelson, 2000). *Phytophthora parasitica* INRA 310 and *Pbr*1692 are both soilborne pathogens and have shown occurrence as potato pathogens; therefore, we first tried to establish whether there is a competitive interaction between the two pathogens. The results showed that *Pbr*1692 and *P. parasitica* INRA 310 could grow together on a CMA plate (**Fig 3.3**). Furthermore, *Pbr*1692 does not inhibit the INRA 310 germination of *P. parasitica* INRA 310 zoospores (**Fig 3.4**). Interestingly, a slowed motility was observed when *P. parasitica* INRA 310 zoospores and *Pbr*1692 cells were co-inoculated (**Sup Fig 3.13a**). In microbial communities, some bacteria produce signalling molecules that balance the zoospore gradient, thus promoting pathogen infection (van Dijk & Nelson, 2000, Joint *et al.*, 2002), which could have influenced the pattern of the motility observed. Oomycetes produce and utilise various molecules to mediate zoospores aggregation and plant infection. Furthermore, it is reported that bacteria promote *Phytophthora* plant infection by enhancing some stages of the *Phytophthora* infection cycle (Kong & Hong, 2016).

Bacterial OMVs have antimicrobial activities on interspecies, including eukaryotes (Wang et al., 2020). However, to this date, most of the studies have focused more on bacterial OMVs

carrying antimicrobial agents that target bacteria compared to bacterial OMVs on other species, such as fungi and oomycetes. The characteristic ability of bacterial OMVs to contain enzymes showing antimicrobial activity has also been reported in *Lysobacter* spp., *Pseudomonas aeruginosa*, and *Burkholderia thailandensis* (Kadurugamuwa & Beveridge, 1996, Vasilyeva *et al.*, 2008, Meers *et al.*, 2018, Wang *et al.*, 2020). Moreover, it was shown that *Pbr*1692 OMVs inhibit the growth of *D. dadantii*, suggesting that *Pbr*169*2* OMVs play an essential role in competition and may have antimicrobial activity; however, not against all SREs or *P. parasitica* INRA 310.

Although results from **Fig 3.3** did not show competition between *Pbr*1692 cells and *P. parasitica* INRA 310, we continued to test whether *Pbr*1692 OMVs would show non-inhibitory effects on three *P. parasitica* isolates. The antimicrobial test showed that three *P. parasitica* isolates did not show mycelial inhibition after the treatment with *Pbr*1692 OMVs (**Fig 3.5**); moreover, *P. parasitica* INRA 310 zoospores showed germination after *Pbr*1692 OMVs treatment (**Fig 3.6**). The results suggest that *Pbr*1692 OMVs do not have antimicrobial activity against *P. parasitica* INRA 310.

Interestingly, zoospores showed the ability to assimilate OMVs and remain viable (**Fig 3.7** and **3.8**). Due to the aforementioned results, we generated the question; could there be a different interaction between *Pbr*1692 and *P. parasitica* INRA 310 zoospores? In hindsight, the slowed *P. parasitica* INRA 310 zoospore motility in **Sup Fig 3.13a** may further support a possible interaction between the two plant pathogens, *Pbr*1692 and *P. parasitica* INRA 310.

*Pbr*1692 OMVs aid *P. parasitica* INRA 310 in carbon utilisation and response to chemical treatment

As a result of the OMV uptake by *P. parasitica* INRA 310 zoospores, we investigated the effect of the *Pbr*1692 OMVs on *P. parasitica* INRA 310 metabolism using PM. The preliminary PM results showed that about 85 carbon sources were utilised by both the zoospores treated with *Pbr*1692 OMVs and the untreated zoospores, and seven carbon sources were only utilised by the treated zoospores (**Fig 3.9**, **Table 3.1**). In addition, of the 85 carbon sources, the treated zoospores showed better growth and metabolism in 31 carbon sources compared to the untreated zoospores (**Table 3.2**). Based on the KEGG pathways database, these carbon sources are primarily involved in metabolic pathways that benefit the survival of microorganisms.

Oomycetes, such as *Pythium* spp. obtain their nutrients from the stems and roots of the host, including various carbon sources (Donaldson & Deacon, 1993, Walker & van West, 2007), which supports the utilisation of different carbon sources by *P. parasitica* INRA 310 (**Fig 3.9** and **Table 3.2**). Furthermore, zoospores are attracted to various carbon sources, including amino acids that serve as attractants during chemotaxis (Orpin & Bountiff, 1978). These carbon sources may aid in the invasion of the host. Similarly, *P. cryptogea* and *P. capsici* have utilised a list of carbon sources that the *P. parasitica* INRA 310 zoospores used in this study (Khalil & Alsanius, 2009).

Carbon sources like sucrose contribute to complex pathways such as the Tricarboxylic cycle, and the ability of micro-organisms to adapt to diverse environments (Judelson, 2017). Moreover, the carbon sources D-sorbitol, maltose, L-malic acid, and fumaric acid listed in **Table 3.2** have been predicted to play a role in phosphorylation pathways, which might contribute to the pathogenic fitness (Blanco & Judelson, 2005). However, there is no outstanding distinction between the pathways of the carbon sources showing improved carbon utilisation and growth in the untreated zoospores. Based on the proteomic analysis conducted by Maphosa & Moleleki (2021), *Pbr*1692 OMVs carry enzymes that are predicted for carbohydrate metabolism and may participate in the TCA cycle, such as Ribose-phosphate-phosphorylation-kinase.

Studies have shown that OMVs package enzymes such as hydrolases that can break down polysaccharides for consumption by other bacteria (Elhenawy *et al.*, 2014). Therefore, this suggests that OMVs can package enzymes that can catabolise complex compounds, which may benefit the surrounding microbes. Interestingly, *Pbr*1692 OMVs proteomics showed that OMVs enclose pectate lyase, endoglucanase, and aspartate lyase (Maphosa & Moleleki, 2021) and may play a role in synergistic interactions by degrading complex carbon sources that are beneficial to the microbial communities. However, it is unknown whether the relation of OMV-producing bacteria with other microbes is an exploitative or reciprocal benefit during nutrient utilisation (Caruana & Walper, 2020).

The comparison of the chemical sensitivity of the treated and untreated zoospores showed that OMVs might influence the sensitivity of zoospores to certain chemicals. As a result, it may promote the growth and metabolism of the zoospores in those chemicals or increase the inhibition of the zoospores in the chemicals. There was a decrease in the metabolism and

growth of the treated zoospores and untreated zoospores in 1-Hydroxypyridine-2-thione, sodium selenite, nickel chloride, and thiourea; however, at the early stages of incubation, treated zoospores showed better growth compared to the untreated zoospores in 1-Hydroxypyridine-2-thione and nickel chloride (**Table 3.3**). Meaning that the treated zoospores were less sensitive to 1-Hydroxypyridine-2-thione during the early hours of incubation. The chemical 1-Hydroxypyridine-2-thione, also reported as zinc pyrithione, is an antimicrobial agent (Schwartz, 2016, Kudera *et al.*, 2020). Furthermore, the Biolog software predicted that 1-Hydroxypyridine-2-thione is an antifungal and can inhibit biofilm formation. OMVs are reported to induce antimicrobial resistance by carrying enzymes that may inactivate antimicrobial activity or enclose antibiotics themselves (Allan & Beveridge, 2003, Roszkowiak *et al.*, 2006, Liao *et al.*, 2015, Kim *et al.*, 2018). From these observations, it can be assumed that *Pbr*1692 OMVs might induce antibiotic resistance at the early stages of the incubation.

Some chemicals, nystatin, sodium dichromate, and D-cycloserine, showed an increase in the metabolism of the treated and untreated zoospores. According to Lawrence *et al.* (2017), the inhibitory studies on *P. cinnamomi* and *P. agathidicida* showed that D-cycloserine does not inhibit the germination and motility of zoospores, which may suggest the viability of the zoospore and may further correspond to the increase in growth and metabolism that was observed in **Fig 3.10**. Furthermore, we may assume that the difference in the growth rate of the treated and untreated zoospores could result from the OMVs. D-cycloserine is an antibiotic that targets cell wall biosynthesis in bacteria (Kurokawa *et al.*, 2009), and the structure of the cell wall in oomycetes and bacteria varies, hence the lack of inhibitory effect on the *P. parasitica* INRA 310 zoospores.

Interestingly, nystatin showed better growth in the untreated zoospores compared to the treated (**Table 3.3**). Nystatin is an antifungal that has been shown to inhibit some fungi more actively on the vegetative cells compared to the spores, and according to the Biolog software, nystatin increases the membrane permeability of the cells (Lampen *et al.*, 1957). Reports have indicated that nystatin does not inhibit the growth of *P. agathidicida* (Armstrong, 2018), which may suggest that nystatin does not exhibit inhibitory effects against *Phytophthora* spp.; however, in this case, *Pbr*1692 OMVs may have negatively affected the metabolism of the treated zoospores.

Conclusion

To conclude, *Pbr*1692 OMVs have antimicrobial activity since they were able to inhibit the growth of *D. dadantii*. However, the OMVs do not take part in the competition of *Pbr*1692 interaction with *P. atrosepticum* and *P. carotovorum*. Contrary to our hypothesis, there was no competition between Pbr1692 cells and *P. parasitica* isolates, which stated that there might be competition between *Pbr*1692 and *P. parasitica.* Instead, *P. parasitica* INRA 310 zoospores showed the assimilation of *Pbr*1692 OMVs, suggesting a different interaction between the two pathogens. Based on the PM preliminary results, *Pbr*1692 OMVs assist *P. parasitica* INRA 310 zoospores in utilising specific carbon sources. The results also suggest that *Pbr*1692 OMVs could induce antibiotic resistance in zoospores. However, repeating this experiment will give clarity and also verify the observations that have been made. The validation results further substantiate the PM results that *Pbr*1692 OMVs may assist *P. parasitica* INRA 310 with carbon source utilisation.

3.3 Future work

The results presented on the phenotypic microarray were conducted in duplicates for PM1 and or single plate for PM1 may affect the reliability of the results, especially for PM21. Therefore, for our future work, we will repeat the phenotypic microarray in duplicate to verify and validate the preliminary results obtained. Furthermore, we will also conduct synergism assays to investigate the time of interaction between *Pbr*1692 cells and *P. parasitica* INRA 310. Since we have observed that *Pbr*1692 OMVs inhibit *D. dadantii*, we also plan to conduct membrane interaction assays to investigate the OMV action on *D. dadantii* membrane and select possible OMV proteins that may contribute to the observed phenotypes to investigate the mechanisms responsible.

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3.5 Supplementary data

a

https://drive.google.com/file/d/1Ha3hkbypRqUbR_N36ZvcEHwKTj_1p-JJ/view?usp=sharing *P. parasitica* INRA 310 zoospore with *Pbr*1692 cells first hour incubation

https://drive.google.com/file/d/1HM7hJ2wELc8N1RlHhOAa2vh4QDAdRyrq/view?usp=sharing *P. parasitica* INRA 310 zoospores with *Pbr*1692 cells third hour incubation

https://drive.google.com/file/d/1HM2Fvnz3snO07bt0rDSbMclDTkJo_Kep/view?usp=sharing *P. parasitica* zoospores with ddH_2O third hour incubation

b

https://drive.google.com/file/d/1cNU_T1ZL-BehBuvPnfuVruC8gR9yL_ab/view?usp=sharing *Pbr*1692 OMVs and *P. parasitica* zoospores at first hour of incubation

https://drive.google.com/file/d/1cNCkdDhTjkL9s89dbBMD2l6P3dHocScp/view?usp=sharing *Pbr*1692 OMVs and *P. parasitica* INRA 310 zoospore at 2 hours 30 mins of incubation

https://drive.google.com/file/d/1cQsu4llz2bX17BySgrkgpzkmWBUlcl_H/view?usp=sharing PBS buffer and *P. parasitica* INRA 310 zoospore at 2 hours 30 mins of incubation

Supplementary Figure 3. 13: Motility test links of *P. parasitica* INRA 310 zoospores treated with *Pbr*1692 cells, ddH2O used as the negative control (**a**), and *Pbr*1692 OMVs with PBS buffer used as a negative control (**b**).