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Fungal biology reviews technical advances in extracellular vesicle isolation from fungi and oomycetes: Insights from plant-pathogenic species

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ABSTRACT

Research on fungal extracellular vesicles (EVs) has gained a lot of attention due to their role in plant-microbe interaction and intercellular and cross-kingdom communication. However, the isolation and characterization of EVs from plant pathogenic fungi and oomycetes still face challenges. We provide a comprehensive overview of the most recent methods for EV isolation, such as density gradient, ultracentrifugation size exclusion chromatography and differential ultracentrifugation. Quality control measures, such as dynamic light scattering, nanoparticle tracking analysis and transmission electron microscopy to ensure purity and integrity, are discussed. EVs from various organisms display heterogenicity in size and cargo. To ensure reproducibility and cross-study comparisons, we highlight the importance of standardized protocols for EV isolation and characterization. Identification of pan-fungal and pan-oomycetal EV marker proteins are needed to improve our knowledge of their function in plant-pathogen interactions. This work provides a methodological framework for the comparative analysis of EVs from fungi and oomycetes based on approaches from plant pathogens and highlights their potential relevance as targets or tools in the development of innovative plant protection strategies.

1. Background

Over the past decade there has been increasing evidence that plants and phytopathogens can communicate via small RNAs (sRNAs) (Cai et al., 2021; Huang et al., 2019; Weiberg et al., 2015). This new level of cross-kingdom communication was first described in 2013, demonstrating that the fungal pathogen *Botrytis cinerea* produces sRNAs that mimic plant sRNAs and bind to *Arabidopsis thaliana* ARGONAUTE 1 to antagonistically silence important plant immunity genes (He et al., 2023; Weiberg et al., 2013). Since then, subsequent studies revealed sRNA effector-mediated manipulation of plant immunity in several pathosystems, such as *B. cinerea-A. thaliana* (Cai et al., 2018; He et al., 2023; Wang et al., 2017), *Blumeria graminis*-barley (Kusch et al., 2018), *Hyaloperonospora arabidopsidis-A. thaliana* (Dunker et al., 2020), and *Fusarium graminearum*-barley (Werner et al., 2021). sRNA-mediated silencing of host genes appears to be another virulence strategy of plant pathogenic fungi and oomycetes, essential to promote disease progression. However, the mechanism that facilitates the delivery or exchange of sRNAs between plants and their microbiome requires further research. Plant extracellular vesicles (EVs) were shown to inhibit fungal growth through their antifungal cargoes (Bleackley et al., 2020; De Palma et al., 2020; Regente et al., 2017; Schlemmer and Lischka, 2020). Moreover, plant EVs have been demonstrated to contain sRNAs which can induce gene silencing of fungal target genes (Cai et al., 2018; Schlemmer et al., 2021b; Wang et al., 2017; Zhang et al., 2016). Notably, plant pathogenic fungi, such as *Sclerotinia sclerotium* and *B. cinerea*, were found to take up EVs derived from sunflower (*Helianthus annuus*) and *A. thaliana*, respectively (Cai et al., 2018; Regente et al., 2017; Wang et al., 2017). Interestingly, *F. graminearum* hyphae was found to be unable to take up plant-derived EVs in vitro (Schlemmer et al., 2021a). A recent study showed that EVs from the oomycete *Phytophthora capsici* contained elicitors and induced brown lesions on *Capsicum annum* leaves (Fang et al., 2021). In addition, He et al. (2023) showed that *B. cinerea* delivers sRNA effectors into *A. thaliana* via EV-mediated transport. However, further research is needed to determine whether phytopathogenic fungi and oomycetes use EVs as vehicles to deliver their plant immunosuppressive cargoes, such as sRNA or protein effectors.

2. EVs in oomycetes and fungi

Oomycetes, thought to be fungi until the late 20th century, resemble true fungi in their nutrition and in forming tip-growing branching

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mycelial hyphal systems, but are closely related to brown algae and belong to the kingdom of Chromista (Fry, 2020). In eukaryotes EVs serve as lipid bilayer-enclosed vehicles that contain proteins, nucleic acids, polysaccharides and pigments, mediating intra- and inter-organismal communication (Rizzo et al., 2020). Eukaryotic cells secrete three main types of EVs: apoptotic bodies (1000–5000 nm), microvesicles (100–1000 nm), and exosomes (10–150 nm), each distinguished by its biogenesis (Akers et al., 2013; György et al., 2011 ; Rutter and Innes, 2017; Van der Pol et al., 2012). Apoptotic bodies are formed during programmed cell death, microvesicles bud directly from the plasma membrane, and exosomes are released via fusion of multivesicular bodies with the plasma membrane. However, overlapping size ranges and shared protein markers complicate the classification of isolated EVs. Although fungal EVs have been shown to mediate the transfer of virulence-associated molecules in studies involving yeast and other human pathogens (Rizzo et al., 2020), research on their role in plant-microbial interactions is only beginning to emerge. Here we discuss latest studies demonstrating EV isolation and analysis from the plant pathogenic fungi: *Zymoseptoria tritici* (Hill and Solomon, 2020), *Fusarium oxysporum* f. sp. *vasinfectum* (Bleackley et al., 2020; Garcia--Ceron et al., 2021), *F. graminearum* (Schlemmer and Lischka, 2020), *Ustilago maydis* (Kwon et al., 2021), *Alternaria infectoria* (Silva et al., 2014), *B. cinerea* (He et al., 2023), and *Colletotrichum higginsianum* (Rutter et al., 2022) (Table 1), as well as two plant pathogenic oomycetes: *Phytophthora sojae* (Zhu et al., 2023) and *P. capsici* (Fang et al., 2021) (Table 2). We compare different EV isolation techniques, provide technical insights, and discuss future perspectives to stimulate further research on EVs in plant-microbial interactions, including those involving oomycetes and fungi.

While the number of publications regarding EVs from plant pathogenic fungi has increased, only two studies on EVs derived from oomycetes have been reported to date. As fungi and oomycetes can manipulate their host plants through EV cargoes, research on their role, including proteins and RNAs, in plant-pathogen interactions has expanded significantly over the past decade (Bleackley et al., 2020; Fang et al., 2021; Garcia-Ceron et al., 2023; Hill and Solomon, 2020; Kwon et al., 2021; Schlemmer and Lischka, 2020; Zhu et al., 2023). In addition to the identification of immune-related cargos (e.g., proteases, effector proteins), the infiltration of *F. oxysporum* f. sp. *vasinfectum*, *F. graminearum*, and *P. capsici* EVs into leaves has been shown to induce phytotoxic effects (Bleackley et al., 2020; Fang et al., 2021; Schlemmer and Lischka, 2020). Notably, this progress contrasts with the field of plant EV research, which has largely been driven by the adaptation of EV isolation protocols originally developed for human pathogenic fungi, which were subsequently modified for use with plant pathogenic fungi and oomycetes (Hill and Solomon, 2020). The techniques discussed here might be applicable to a broader range of mutualistic or saprotrophic fungi and oomycetes, when grown in vitro.

3. EV isolation techniques

Isolation methods for EVs derived from a wide range of biological matrices include differential (ultra)centrifugation (UC), sequential (ultra)filtration (UF), size exclusion chromatography (SEC) and density gradient ultracentrifugation (DG-UC) (Li et al., 2017). A combination of these techniques is often used to isolate EVs from plant pathogenic fungi and oomycetes (Fig. 1). As a first step the fungus or oomycete is grown in an appropriate liquid medium (Fig. 1 (1)). Subsequently, prefiltration or centrifugation is chosen to remove cells and cell debris (Table 1; Fig. 1 (2)). Multiple studies mention miracloth filtration to remove mycelia. Miracloth is a rayon polyester filter material with an acrylic-binder that has a pore size of 22–25 μm and mechanically retains debris. It efficiently removes most of the mycelia in a cost-effective and straightforward way while preventing clogging of the filter membrane. However, subsequent centrifugation or filtration remains necessary to ensure complete purification. Subsequently, EVs are purified from culture supernatants by a size-based ultrafiltration (0,45 μm) (Fig. 1 (3)) followed by final ultracentrifugation (100,000 *g*) (Fig. 1 (4)) (Table 1). Notably, ultracentrifugation can be used in combination with density gradients (e.g., sucrose (Schlemmer and Lischka, 2020) or OptiPrep (Bleackley et al., 2020)) to float the relatively low-density EVs away from other vesicles and particles (Table 1), which can increase the quality and purity of EVs. OptiPrep is a density gradient medium based on jodixanol that ensures the extraction of intact and functional vesicles or other cell compartments. Ultracentrifugation-based isolation protocols are simple and inexpensive but have been discussed as comparatively time-consuming, laborious, and inefficient due to high material required, resulting in low output quantities (Jia et al., 2022; Li et al., 2017). According to Monguió-Tortajada et al. (2019) additional rounds of UC increase the amount of EVs, underlining the low recovery of EVs and the inefficiency of this method. Moreover, loss of EV integrity and aggregation has been reported upon high centrifugal forces (Jia et al., 2022; Linares et al., 2015; Monguió-Tortajada et al., 2019). Compared to that, sequential UF enables a precise size-based separation while retaining biological function. Sequential UF requires less time and specialized equipment, but sample loss and decreased purity can occur due to adhesion to the filtration membrane (Jia et al., 2022). SEC presents another step forward, as it simplifies the isolation process to a single-step. However, it requires larger sample amount and can be more time-consuming, especially when first optimizing and characterizing EV fractions (Monguió-Tortajada et al., 2019). SEC and DG-UC can be used as an alternative to, or in conjunction with, ultracentrifugation (Fig. 1 (5)). Both methods enable a separation of various EVs based on their size or density, respectively. Recently, SEC was applied to isolate EVs from *F. oxysporum* f. sp. *vasinfectum* using a lipophilic fluorescent dye (FM5-95 (*N*-(3-Trimethylammoniumpropyl)-4-(6-(4-(Diethylamino)phenyl)hexatrienyl)Pyridinium Dibromide), Thermo Fisher) (Garcia-Ceron et al., 2021). The authors isolated three times more EVs per isolation run compared to the yield of EVs from UC isolation which allowed the proteomic analysis of independent biological replicates without the need for pooling. However, although SEC proves promising, potential non-specific interactions of the FM5-95 dye with soluble particles remain to be verified. Additional concentration of EV samples by tangential flow filtration (TFF), UF or spin columns is optional (Fig. 1 (6)) but recommended when dealing with low amounts of EVs.

4. Quality control measures

After isolation, EVs must be characterized to confirm the presence of membrane-bound vesicles (Fig. 1 (8)). Recommended analyses include particle size and concentration measurements using techniques such as dynamic light scattering (DLS) or nanoparticle tracking analysis (NTA), visual inspection by transmission electron microscopy (TEM), and molecular analysis for EV biomarker proteins via immunoblotting. However, the latter method relies on the availability of specific biomarkers which are still insufficiently identified for many fungi and oomycetes. According to the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines (Théry et al., 2018; Welsh et al., 2024), at least two independent yet complementary methods should be used to ensure accurate EV characterization.

5. Sample treatments

A major challenge in EV research is to ensure sample purity and avoid the co-purification of contaminants (Rutter and Innes, 2020). Treatments such as RNase A digestion are often used to eliminate extravesicular RNA, which could otherwise lead to misinterpretations (Kwon et al., 2021). This step is particularly important when RNA sequencing is performed after isolation. Similarly, protease treatments can be applied to increase purity by removing non-vesicular proteins, although they may result in quality losses upon exposure to incubation temperature (37 °C) required for many proteases (Kwon et al., 2021).

Table 1
EV isolation protocols for phytopathogenic fungi.

EV isolation	*Zymoseptoria tritici* (Hill and Solomon, 2020)	*Fusarium oxysporum* f. sp. *vasinfectum* (Bleackley et al., 2020)	*Fusarium oxysporum* f. sp. *vasinfectum* (Garcia-Ceron et al., 2021)	*Ustilago maydis* (Kwon et al., 2021)	*Fusarium graminearum* (Schlemmer et al., 2021a)	*Alternaria infectoria* (Silva et al., 2014)	*Botrytis cinerea* (He et al., 2023)	*Colletotrichum higginsianum* (Rutter et al., 2022)
Growth medium Concentration	YSA & transferred to Fries 3 broth 2×10^5 blastospores/mL	Half-strength Potato-dextrose agar	Czapek Dox liquid culture or Saboraud's dextrose broth 3.8×10^4 conidia/mL	Sporidial cultures switched to nitrated minimal medium	Synthetic nutrient poor broth	Yeast malt extract liquid media	YEPD	Mathur's liquid culture
Removal of cell debris (supernatant preperation)	4500×*g*, 25 min, 4 °C; 15,000×*g*, 45 min, 4 °C	Miracloth filtration 4000×*g*, 15 min 15,000×*g*, 30 min	Miracloth filtration; 0.45 μm filters	6000 rpm/ 3951×*g*, 10 min	Miracloth filtration; 10,000×*g*, 20 min, 4 °C	15,000 rpm, 30 min	3000×*g*, 15 min, 4 °C; 70 μm cell strainer, 10,000×*g*, 30 min twice	Cell wall digestion solution. Protoplasts, 2X 2000 rpm, 8 min, 4 °C
Filtration	0.45 μm MF-Millipore membrane filter		0.45 μm MF-Millipore membrane filter	0.45 μm filters	0.45 μm & 0.22 μm polyvinylidenfluorid membrane filter	0.45 μm polyvinylidene difluoride filter	0.45 μm filter	0.22 μm filter
Sample concentration		When needed concentrated by pelleting	1000 times using centrifugal filter units (MWC 30 kDa)	with vivaspin-500 (MWC 1000 kDa)	Concentrated 10 times by Tangential flow filtration (TFF)	About 50-fold with a 100 kDa exclusion filter.		
EV pelleting	100,000×*g* 75 min, 4 °C 75 min	100,000×*g*	SEC with a fluorescent lipophilic dye	100,000×*g*, 60 min, 4 °C	150,000×*g*, 22 h, 4 °C	60,000 rpm, 60 min, 4 °C	100,000×*g*, 1 h	10,000×*g*, 30 min, 4 °C 60,000×*g*, 90 min, 4 °C 40,000×*g*, 60 min, 4 °C
Purify EV pellets	PBS (pH 7.4) 100,000×*g*, 75 min, 4 °C	Further fractionation by OptiPrep		PBS (pH 7.2) 100,000×*g*, 60 min, 4 °C SEC (qEVorginal/ 70 nm)	Sucrose gradient 150,000×*g*, 2 h, 4 °C		Sucrose gradient centrifugation 100,000×*g*, 16 h, 4 °C & final centrifugation 100,000×*g*, 1h, 4 °C	Further purified by OptiPrep 100,000×*g*, 17 h
EV treatments	none	none	none	RNase A (0.1 μg/μL) and Triton X-100 (0.1 %) at 4 °C 10 min	none	none	Micrococcal nuclease (MNase) with and without Triton X-100	none
Resuspension & storage	PBS (pH 7.4); −80 °C	PBS; Storage −80 °C		−80 °C	PBS	PBS	PBS	20 mM Tris-HCL, pH 7.5
EV amount	1.45-2.43 × 10^8 particles/mL culture medium	1.0 × 10^{12} particles/mL culture medium	In SDB 1,1 × 10^{11} or 6 × 10^{10} particles/mL in CD 1.4 × 10^{11} or 7.6 × 10^{10} particles/mL		1.97 × 10^{10} particles/mL (fraction 45 %)	SEM (473) in 5 microscopic fields/TEM 1110 vesicels		
EV size	<50 to >300 nm; majority 50–150 nm Mean 91.8 nm	Mean 155.1 nm	100–300 nm Mean 120 nm	100–200 nm	90–230 nm Mean 123 nm	SEM 20–40 nm mean size 28.36 nm TEM mean. 21.29 nm DLS 50–100 nm	DUC 113 nm sucrose gradient 93.5 nm	100–106 nm
EV content	210 *Z. tritici* EV proteins identified	482 *F. oxysporum* f. sp. *vasinfectum* EV proteins identified and EVs caused phytotoxic effects upon leaf infiltration	From the CD medium 465 EV proteins identified and 658 EV proteins from SDB	mRNAs were enriched inside EV; thousands of mRNAs associated with EVs; and rRNA were detected. Most RNA detected were under 200 nt	EVs caused phytotoxic effects upon leaf infiltration	20 proteins of which 7 were described as vesicular	sRNA, tetraspannin protein PUNCHLESS1	In low density population of EV 253 proteins and 198 proteins in high density were detected

Overview of EV isolation steps for phytopathogenic fungi, including *Zymoseptoria tritici*, *Fusarium oxysporum* f. sp. *vasinfectum*, *Ustilago maydis*, *Fusarium graminearum*, *Botrytis cinerea*, *Alternaria infectoria* and *Colletotrichum higginsianum*. Key aspects such as culture media, debris removal, sample concentration, purification, storage conditions, and EV characterization are compared across studies.
Definition of abbreviations.
CD Czapek Dox liquid.

DUCDifferential Ultracentrifugation.
EVExtracellular vesicles.
MWCMolecular weight cut-off.
PBSPhosphate-buffered saline.
SDBSaboraud's dextrose broth.
SECSize exclusion Chromatography.
SEMScanning electron microscopy.
TEMTransmission electron microscopy.
YEPDYeast extract peptone dextrose.
ÝSA Yeast sucro se agar.

Table 2
EV isolation protocols for phytopathogenic oomycetes.

EV isolation	*Phytophthora sojae* (Zhu et al., 2023)	*Phytophthora capsica* (Fang et al., 2021)
Growth medium Concentration	Synthetic liquid medium	V8 juice plates and subcultured in potato dextrose broth
Removal of cell debris (supernatant preperation)	Miracloth filtration 10,000×*g*, 30 min	Miracloth filtration 0.22 μm filter 4000×*g*, 15 min 15,000×g,15 min
Filtration	0.22 μm membrane	4 °C Aaicon ultrafiltration system (MWC 100 kDa) 4000×*g*, 15 min 15,000×g,15 min
Sample concentration	Concentrated to 100 ml by ultrafiltration (MWC 100 kDa)	Concentrated 20-fold amicon ultrafiltration
EV pelleting	100,000×*g*	100,00×g, 60 min, 4 °C
Purify EV pellets	Sucrose gradient 160,000×*g*, 16 h, 4 °C	
EV treatments	none	none
Resuspension & storage	PBS Storage: short term 4 °C; long-term −80 °C	PBS
EV amount		0.5 μg/μl
EV size	60–200 nm	40–120 nm
EV content		208 proteins

Overview of EV isolation steps for phytopathogenic oomycetes, including *Phytophthora sojae* and *Phytophthora capsici*. Key aspects such as culture media, debris removal, sample concentration, purification, storage conditions, and EV characterization are compared across studies.
Definition of abbreviations.
EV Extracellul ar vesicles.
MWCMolecular weight cut-off.
PBSPhosphate-buffered saline.
SEM Scanning electro n microscopy.

The use of cold-active (20 °C) proteases may represent a promising alternative to mitigate these effects (Perfumo et al., 2020). Detergent treatment (Triton X 100 or Tween) can serve as a positive control to confirm whether RNAs or proteins are protected within intact EVs or externally associated. For downstream omics analyses and EV cargo identification, treatments like RNase and protease digestion, or combinations of these, are highly recommended to ensure accurate characterization of EV-associated molecules (Fig. 1 (9, 10)).

6. EV heterogeneity

Remarkably, and confirming to what is known from human fungal pathogens (Bielska and May 2019), EV populations isolated from plant pathogenic fungi and oomycetes are heterogenous in size (Table 1). The size range of EVs from fungi was found to be < 50 to >300 nm with the majority exhibiting mean sizes ranging from ~90 nm (*Z. tritici*) and to ~150 nm (*F. oxysporum* f. sp. *vasinfectum*) (Table 1), while the size range of oomycete EVs varies between 40 and 200 nm (Table 2). For both fungi and oomycetes, the identified EV cargo differs among different species. Proteomic analysis (gene ontology) of phytopathogenic fungi further revealed significant differences in EV protein content depending on the growth medium used, such as Czapek Dox versus Saboraud's dextrose broth (Garcia-Ceron et al., 2021) versus potato dextrose broth (Bleackley et al., 2020), underlining the importance of selecting appropriate nutrient conditions for each study (Table 1). The composition of the growth medium has a significant effect on EV production and protein content. While the nutrient requirements of different pathogens necessitate the use of different media, the lack of a standardized medium poses a challenge for cross-study comparisons. It is therefore essential to ensure consistency of experimental conditions.

7. EV markers

EV markers enable immunoaffinity-based EV extraction methods, which are standard in mammalian systems where EV biomarkers are used to differentiate between various subclasses of EVs. Identifying EV markers for fungi and oomycetes is essential for quality control, allowing researchers to assess EV purity and investigate the role of distinct EV subclasses. The heat shock protein 70 (Hsp70), suggested as a biomarker for *F. graminearum* EVs, was also detected in *P. capsici* and *Z. tritici* proteome data (Fang et al., 2021; Garcia-Ceron et al., 2021; Hill and Solomon, 2020). Interestingly, the common plant EV marker tetraspanin 8 (TET8) is not considered an EV marker for fungi but is recognized for the oomycete *P. sojae*. Remarkably, plants were able to differentiate between microbe-derived EVs and plant-derived EVs using TET8 (Zhu et al., 2023).

Aiming at the identification of pan-fungal EV markers, claudin-like Sur7 family proteins - previously identified from an EV marker screening in *Candida albicans* (Dawson et al., 2020) - were consistently detected in *Z. tritici,* and *F. graminearum* (Bleackley et al., 2020; Garcia-Ceron et al., 2021; Hill and Solomon, 2020) suggesting their potential as reliable fungal EV markers. However, in EVs from *C. higginsianum* Sur7 was detected in the EV proteome, but not at levels high enough to consider them robust EV markers. Interestingly, Sur7 family proteins were absent in EVs from the Fusarium species *F. oxysporum* f. sp. *vasinfectum*, when EVs were extracted with SEC. This discrepancy may indicate species-specific differences in EV composition, or variations introduced by the isolation method. These findings highlight the critical need for standardized EV isolation and characterization protocols, as they are essential for consistent marker identification, enabling reproducibility and reliability across fungal and oomycete studies.

8. Conclusion and future perspectives

The various isolation techniques enable EV isolation, however post-isolation content analysis can vary significantly based on the instruments used, databases referenced, and data processing methods applied. This inconsistency can make cross-study comparisons difficult. To ensure the robustness and reliability of EV research, it is crucial to establish gold standards for both pre- and post-isolation protocols. These standards would allow for more accurate comparisons across studies, especially within the same species. Such efforts will improve reproducibility, minimize biases, and allow for more consistent identification of EV markers, thereby improving the overall reliability of EV-related findings across different research groups.

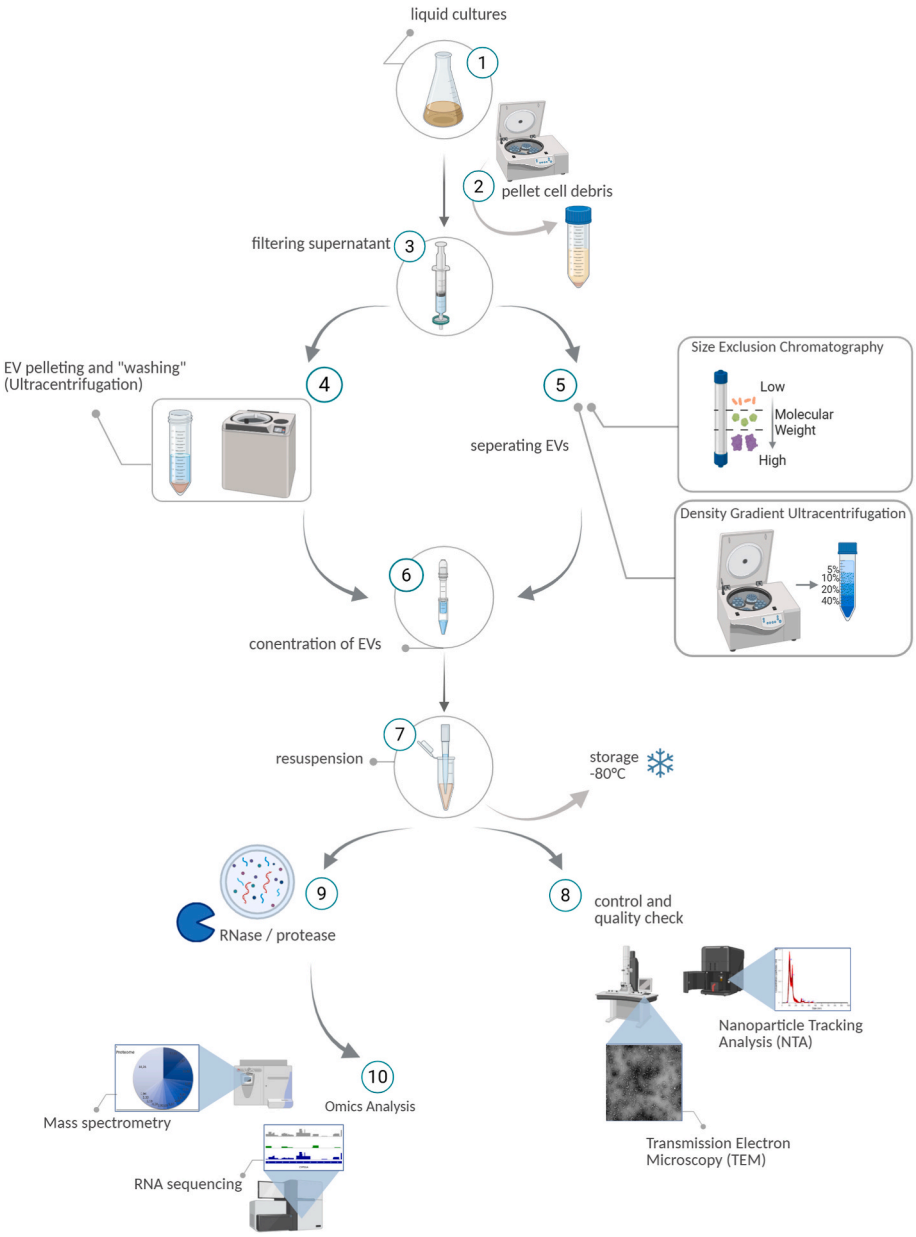

Fig. 1. Workflow for the isolation, purification and analysis of extracellular vesicles (EVs) of Phytopathogenic Fungi and Oomycetes gown in liquid cultures

Caption: Workflow summarizing EV isolation steps for phytopathogenic fungi and oomycetes. (1) Cultures are grown in appropriate liquid media until the desired growth stage is reached. (2) Cell debris is pelleted by low-speed centrifugation, and (3) the supernatant is filtered to eliminate smaller impurities while retaining the EVs debris. (4) Ultracentrifugation is used to pellet and wash the EVs, ensuring improved purity. (5) Separation of EVs is further refined through methods such as density gradient ultracentrifugation or size exclusion chromatography to ensure purity. (6) The purified EV fractions are concentrated and collected for downstream applications. (7) Following this, the EVs are resuspended in an appropriate buffer for storage at −80 °C or for immediate analysis. (8) Quality control and characterization are performed using techniques like nanoparticle tracking analysis (NTA) and transmission electron microscopy (TEM). (9) RNase and protease treatments may be applied to remove contaminating RNA and proteins. (10) The final steps involve omics-analyses, such as RNA sequencing and mass spectrometry, to characterize EV-associated biomolecules. (Created with BioRender).

The summarized studies lay the foundation for future research on EV content of fungi and oomycetes. They represent a good starting point for further development of isolation and characterization protocols (Cai and Jin, 2021). Standardized isolation protocols and consistent EV biomarkers are crucial to distinguish between plant and pathogen derived EVs, especially important for plant interacting biotrophic fungi and oomycetes. Advances in EV research from other fields, such as mammalian or bacterial systems, are likely to stimulate EV studies in fungi and oomycetes. New isolation techniques and omics-based approaches can lead to simpler, faster, and more efficient protocols on a standard base, facilitating cross-study comparisons. Identifying reliable EV biomarkers for fungal and oomycetal EVs, allows to further investigate their role in plant-pathogen interaction. Gaining knowledge on the importance of EVs for pathogen virulence and host manipulation could improve the development of novel approaches in plant protection e.g., fungal EV biogenesis may represent suitable targets for RNA spray applications ("RNA spray fights fungus," 2016). By integrating basic and applied research, future studies on plant pathogen EVs could not only uncover fundamental biological processes but also contribute to sustainable and innovative approaches for crop protection.

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Declaration of competing interest

None.

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