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Wickerhamomyces anomalus is a predator of the *Castanea* spp. ink disease-causing oomycetes *Phytophthora cinnamomi* and *P. xcambivora*. Based on morphological evidence, a model illustrating a specific mode of action is provided

Mariana Amorim-Rodrigues [a,b], Rogélio Lopes Brandão [c], Fernanda Cássio [a,b], Cândida Lucas [a,b,*]

[a] Molecular and Environmental Biology Centre (CBMA), University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal[1]
[b] Institute for Science and Innovation on Bio-Sustainability (IB-S), University of Minho, Campus de Gualtar, 4710-054, Braga, Portugal
[c] Cellular and Molecular Biology Laboratory, School of Pharmacy, Federal University of Ouro Preto, Rua Xavier da Veiga s/n, 1°, Centro, Ouro Preto, MG, CEP 35400-000, Brazil

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ABSTRACT

Phytophthora cinnamomi and *P. xcambivora* are serious phytopathogens, namely causing root rot/ink in chestnut trees, which severely threatens this historical crop. Oomycetes produce mycelium but are hard to control with traditional fungicides or BCAs. Recent research revealed *Wickerhamomyces anomalus*, a yeast endophyte, as a strong antagonist of key pre- and post-harvest fungal pathogens in laboratory settings. This study shows it also inhibits *Phytophthora* growth. Microscopy revealed that *W. anomalus* cells adhere to and are found inside *Phytophthora* hyphae, accumulating within collapsed areas, possibly nourishing on hyphal contents. These interactions occur without the intervention of volatile compounds, siderophores, or hydrolytic enzymes, leaving hyphal walls intact. SEM and TEM of hyphae from *P. cinnamomi* when co-cultured with *W. anomalus* showed numerous intrahyphal structures formed in response to the yeast-imposed stress. Otherwise, the yeast shows polarised cells, nuclei fusion, irregularly numbered spores and intercellular bridges, indicating a disrupted reproductive cycle. This is consistent with *P. cinnamomi* and *W. anomalus* recognising each other's pheromones, triggering a mating-like response leading to the yeast's attachment and internalisation without damaging the hyphal cell wall. This *W. anomalus* specific mode-of-action, different from the ones previously reported, suggests potential as a BCA for the pre-harvest management of *Phytophthora*.

1. Introduction

Phytophthora spp. are dangerous phytopathogens that have threatened agriculture and forestry for centuries and still lack economically and environmentally sustainable management tools. *Phytophthora cinnamomi* Rands (1928) and *P. cambivora* (Petri) Buisman (1927) pose serious threats to the world economy, infecting nearly 5000 different plant species (Hardham and Blackman, 2018; Bose et al., 2023; Mullett et al., 2023), and causing severe damage to forests in Europe, the USA and Australia (Tyler, 2001). Chestnut tree root rot/ink disease is primarily caused by *P. cinnamomi* and *P. xcambivora* (Jung et al., 2016, 2017a; Mullett et al., 2023). The symptoms induced by either species on chestnut are identical, although *P. cinnamomi* is recognisably more aggressive. Their co-infection leads to the trees' rapid decline and high mortality rate (Jung et al., 2017a).

Phytophthora spp. are oomycetes, a class of fungus-like protists from the *Stramenopiles* clade of *Eukaryota*. This group includes saprobes from aquatic communities and parasites that infect a diverse and extensive range of animals and plants. In terrestrial habitats, oomycetes can cause plant diseases, ranging from simple lesions to severe cankers, rots, blights, and wilts, which can eventually kill the host. *The life cycle of Phytophthora species* includes the development of a mycelium, which is diploid, unlike that of fungi. As part of their asexual reproductive cycle, under favourable conditions, the mycelium produces sporangia that

* Corresponding author. Molecular and Environmental Biology Centre (CBMA), University of Minho, Campus de Gualtar, 4710-057, Braga, Portugal.
E-mail address: clucas@bio.uminho.pt (C. Lucas).
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generate large quantities of motile zoospores (Hemmes & Wong, 1975; Hardham, 1987; Hyde et al., 1991), which migrate in the aqueous microenvironments of humid soils and attach to plant root walls, forming cysts. These cysts eventually develop a germination tube that penetrates the root, producing new mycelium that invades and feeds on the plant tissues and cells (Judelson and Blanco, 2005). Along with the growth of the mycelium, the roots progressively die, impairing the trees' basic physiological and biochemical life-supporting processes, such as water and nutrient transport. The initial infection by *P. cinnamomi* may also rely on behaviour reminiscent of biofilm formation, with multiple spores aggregating and attaching to the root wall, aided by the abundant secretion of a polysaccharidic extracellular matrix (Galiana et al., 2008; Theodorakopoulos et al., 2011; Hardham and Blackman, 2018), contributing to more efficient and massive zoospore encystment and germination. According to classical morphological classification, *P. cinnamomi* and *P. cambivora* belong to oomycetes Group VI (Waterhouse, 1963; Drenth et al., 2001), characterised by non-papillate sporangia. Based on molecular phylogeny, both species belong to Clade 7, with *P. Cinnamomi* in subgroup 7c, while *P. cambivora* belongs to subgroup 7a (Jung et al., 2017b; Abad et al., 2023). *P. cambivora is currently considered an allopolyploid hybrid of yet* unidentified parental origins (Jung et al., 2017b; Mullett et al., 2023) and is therefore redesignated as *P. xcambivora.* Regarding cultures' morphology, *P. cinnamomi* hyphae are coralloid, while *P. xcambivora* hyphae are smooth. *P. cinnamomi* displays thin walled, lightly pigmented hyphal swellings and chlamydospores aggregated into botryose and smooth oogonia. Conversely, oogonia from *P. xcambivora* are verrucous, and this species is not known to produce chlamydospores. Both *P. cinnamomi* chlamydospores and *P. xcambivora* oospores are resting structures that can survive dormant in the soil for long periods (Crone et al., 2013; Jung et al., 2017a; Boevink et al., 2020).

Castanea is a member of the Fagaceae family found in mid to low-altitude temperate ecosystems (Freitas et al., 2021). *Castanea* species have been for millennia an important asset for human nutrition and timber in most countries from the Northern Hemisphere while characterising mountain landscapes, supplying vital ecosystem services and biological functions, namely contributing to the preservation of autochthonous biodiversity (Pezzi et al., 2017; Roces-Diaz et al., 2018; Pérez-Girón et al., 2020). After decades of decline, mainly due to the abandonment and neglect of the traditional orchards and the general lack of investment in *Castanea* spp. agroforestry (Bellat et al., 2022), this crop appears to have lately regained interest (Pezzi et al., 2017; Freitas et al., 2021). Chestnut market englobes seven species, dominated by *Castanea sativa* Mill (1768) in Europe and Turkey, *Castanea dentata* (Marshall) Borkh (1800) in North America, and *Castanea mollissima* Blume (1851) in China. In 2019, approximately 596 $\times$ 10^3 ha of *Castanea* spp. orchards were registered worldwide (Freitas et al., 2021), and the chestnut global market is predicted to reach almost 5 billion USD by 2030 (Bellat et al., 2022; https://www.industryarc.com/Report/17804/chestnut-market.html). Demand is expected to increase significantly due to the increasing introduction of chestnut flour in gluten-free dietary products. Moreover, these numbers do not represent the whole economic scenario since they do not include chestnut-derived timber and derivatives production. Apart from the root rot/ink disease, *Castanea* is also severely endangered by the fungus *Cryphonectria parasitica* that causes chestnut blight/canker (Rigling and Prospero, 2018) and by the infestation with the Asian chestnut gall wasp, *Dryocosmus kuriphilus*, whose larvae develop inside galls that form on young leaves, petioles, and buds, causing severe losses in fruit production, and debilitating the trees that eventually die (https://planthealthportal.defra.gov.uk/pests-and-diseases/high-profile-pests-and-diseases/oriental-chestnut-gall-wasp/). Additionally, the climate change imposes further problems (Pérez-Girón et al., 2020; Bellat et al., 2022).

The management of *Castanea* spp. ink disease is complex and very ineffective. One reason for this ineffectiveness is the long duration that can elapse between the onset of infection and the appearance of symptoms, which typically emerge only in the late stages of the disease (Hardham and Blackman, 2018; Brown and Brasier, 2007; Boevink et al., 2020; Fernandes et al., 2022; Marzocchi et al., 2024). Trees may either die quickly or harbour latent infections for many years. The extraordinarily high number and variety of plants that *Phytophthora* spp. can infect (Hardham and Blackman, 2018), combined with the widespread use of contaminated soil substrates/conditioners and infected germplasm, contribute to a significant disease inoculum (Jung et al., 2016). Furthermore, the frequent low poor genetic diversity of the more recent *Castanea* orchards (Fernandes et al., 2022; Marzocchi et al., 2024) significantly aggravates the problem, exacerbates the issue, along with anthropogenic factors related to landscaping that impact soil compaction and tillage (Fonseca et al., 2004; Vannini et al., 2021) and inappropriate agroforest soil occupation and management strategies, including over-intense soil occupation, aggravated by the current rapidly changing climate conditions and lack of predictability (Pérez-Girón et al., 2020; Freitas et al., 2021; Dorado et al., 2023). These conditions promote the dispersal and proliferation of the *Phytophthora* species. Strategies to prevent excessive water or moisture accumulation in the soil (Freitas et al., 2021; Marzocchi et al., 2024) could help reduce the dispersal of *Phytophthora* spp. reproductive structures, therefore lessening the disease burden. However, eradicating *Phytophthora* spp. from the soil necessitates the ongoing use of systemic fungicides (Brown et al., 2019a, 2019b; Neupane et al., 2022; Brandano et al., 2023), although these are notoriously ineffective, likely due to the phylogenetic distance between oomycetes and fungi (Hardham and Blackman, 2018). Despite their potential efficacy, these agrochemicals face increasing restrictions or prohibitions in most European countries under the EU Forest Strategy for 2030, part of the European Green Deal, as well as Directive 2009/128/EC and Regulation CE/1107, which aim for a more judicious and sustainable use of pesticides. One possible exception may be phosphonate compounds (acids or salts), which target the development of *Phytophthora* spp. while promoting the formation of fine roots and minimally impacting tree-associated microbiota (Barret and Rathbone, 2018; Brandano et al., 2023). The long-term efficacy and safety of repeated treatments with these compounds for ecosystems and human health still require further evaluation.

As substitutes for agrochemicals, more products are being commercialised that contain active ingredients derived from microbes or microbial sources operating as plant defence immunity inducers or as antagonists of phytopathogens. These include microbial species originating from the plants' biome or living as endophytes (Joubert and Doty, 2018; Ling et al., 2020; Khunnamwong et al., 2020), as well as microbes from distant ecosystems (*e.g.,* Volynchikova and Kim, 2022). The biocontrol of *P. cinnamomi* (Ruiz-Gómez and Miguel-Rojas, 2021; Lourenço et al., 2022; García-Latorre et al., 2022), as well as that of *P. xcambivora* (Bourbos and Metzidakis, 2000; Frascella et al., 2022), has been proposed using products derived from *Trichoderma* fungi (Bourbos and Metzidakis, 2000; Lourenço et al., 2022; Frascella et al., 2022; Woo et al., 2023; Guzmán-Guzmán et al., 2023). These fungi are endophytes or root colonisers that establish complex relationships with the host plant, yielding numerous multifaceted benefits (Harman et al., 2004). They serve as active ingredients in biopesticides and biofertilisers and may enhance natural resistance (Woo et al., 2023). Other less-explored endophytes, that seem effective against *P. cinnamomi,* include the fungi *Drechslera biseptata* and *Epicoccum nigrum* (García-Latorre et al., 2022), *Piriformospora indica* and *Aspergillus flavipes* (Lourenço et al., 2022), along with the bacterium *Bacillus amyloliquefaciens* (Brown et al., 2019b). There are no records of any attempts or studies regarding the potential use of yeasts as biocontrol agents (BCAs) for *Phytophthora* spp., although the biocontrol of *Phytophthora infestans* by yeasts has been suggested, namely using *Aureobasidium pullulans* (di Francesco et al., 2017; Volynchikova and Kim, 2022), *Metschnikowia pulcherrima,* and *Curvibasidium pallidicorallinum* (Hadwiger et al., 2015; Volynchikova and Kim, 2022). The recent reversal of policies and investment in *Castanea* spp. at the EU level (Pezzi et al., 2017; Freitas et al., 2021; Bellat

et al., 2022), may contribute to the survival and expansion of this multipurpose species that will benefit agroforest ecosystems. However, to support this challenge, efficient BCAs and/or derived products need to be urgently developed and licensed.

Yeasts have been successfully used for decades to prevent post-harvest fungal decay in fruits and vegetables (Lucas and Cássio, 2022; Sellitto et al., 2021; Sipiczki, 2023; Oztekin et al., 2023). They easily adhere to the outer skin of fruits, sometimes developing a thin biofilm that benefits the fruit in several ways (Punja and Utkhede, 2003; Luna, 2017), mainly by making contact and colonisation by phytopathogens more difficult (Davies et al., 2021; Sellitto et al., 2021). Moreover, yeasts actively antagonise phytopathogens through various processes, including the secretion of VOCs, lytic enzymes, or other antifungal compounds and mycoparasitism (Lucas and Cássio, 2022). A more indirect action employed by yeasts is eliciting the plant's immune system (Freimoser et al., 2019; Rovetto et al., 2024). The efficacy of any of these actions varies considerably with the plant host-pathogen-yeast triangulation (Luna, 2017). Yeasts are naturally abundant in plant storage tissues, such as fruits, tubers, or seeds, found both in intercellular spaces and inside plant cells (Isaeva et al., 2010; Joubert and Doty, 2018; Ling et al., 2020). However, their biology as endophytes and their ecology are still poorly understood (Joubert and Doty, 2018; Ling et al., 2020). They exhibit several plant growth-promotion traits, which include the production of phytohormones like auxins and gibberellins, indole-3-acetic acid (IAA), or 1-aminocyclopropane-1-carboxylate (ACC) (Joubert and Doty, 2018). They also help alleviate stress, enhance nutrient intake, and protect against phytopathogens, primarily through siderophore production (Joubert and Doty, 2018).

Wicherhamomyces anomalus (Hansen) Kurjan and Herskowitz (1982) is a yeast species known to inhabit rice, corn, and sugarcane as an endophyte. Several strains of this species are notable for their effective antifungal activity against harmful phytopathogens. It strongly antagonises and eliminates the agents of rice dirty panicle and sheath blight diseases, respectively *Curvulata lunata* and *Rhizoctonia solani*, as well as the rice Bakanae, corn stalk rot and sugarcane stem red rot agent *Fusarium moniliforme* (formerly. *F. verticillioides*) (Khunnamwong et al., 2020), cacao Witches' Broom disease agent *Moniliophthora perniciosa* (Ferraz et al., 2021), and olive tree anthracnose *Colletotrichum nymphaeae, C. godetiae* and *Colletotrichum gloeosporioides* (Amorim-Rodrigues et al., 2024). *W. anomalus did not antagonise* other phytopathogenic fungal species infecting rice, corn or sugar cane (Khunnamwong et al., 2020), which suggests target specificity, making this species more suitable for future field use. Numerous modes of action have been reported for this yeast, which has been successfully used in post-harvest biocontrol for decades (Passoth et al., 2006; Haïssam, 2011; Kuchen et al., 2023; Godana et al., 2024). These include the secretion of killer toxins (Farkas et al., 2012), ethyl acetate, CO_2 or other VOCs (Khunnamwong et al., 2020) or hydrolytic enzymes (Hong et al., 2017; Khunnamwong et al., 2020), as well as inducing in the host a reduction in the production of stress-associated chemical species and metabolites (Zhu et al., 2023) and related enzymes (Zhao et al., 2021). Importantly, *W. anomalus* has been reported as potentially acting as a predator of *Colletotrichum* fungi, feeding on hyphal contents (Amorim-Rodrigues et al., 2024).

In the present work, *W. anomalus* is shown to be a potent antagonist of oomycetes. It adheres to and fuses with the cell walls of the vegetative hyphae *of P. cinnamomi* and *P. xcambivora*, accumulating inside the hyphae and likely consuming the cellular contents of the oomycetes. This occurs independently of the secretion of hydrolytic enzymes, VOCs, or siderophores. This mode of action supports previous claims that *W. anomalus* may act as a microbial predator against specific microorganisms. The evidence presented in this work gives rise to a model that provides a conceptual framework to explain this behaviour and specificity.

2. Materials and methods

2.1. Microorganisms culture conditions

W. anomalus LBCM1105 is an industrial yeast strain from the Cellular and Molecular Biology Laboratory of the Federal University of Ouro Preto, MG, Brazil (https://sites.ufop.br/lbcm) (da Conceição et al., 2015; da Cunha et al., 2019, 2020). *P. cinnamomi* CBS 145330 and *P. xcambivora* CBS 248.60 were purchased from WI-KNAW Collections, The Netherlands (https://wi.knaw.nl/Collection). Both yeast and oomycetes were stored at −80 °C in 30 % glycerol and at 4 °C on YPDA (10 g/l yeast extract (Panreac AppliChem), 10 g/l bacto peptone (BD Difco), 20 g/l D-glucose (LabChem), with 20 g/l agar (Cultimed)), and propagated prior to the assays at 25 °C in ME (malt extract, Sigma–Aldrich, 20 g/l), pH 5.5, with 200 rpm orbital shaking and a liquid/air ratio of 1:2.5. Assays were conducted under the same conditions for 8–14 days as mentioned.

2.2. Yeast-fungi antagonism assays

Dual agar diffusion antagonism assays were conducted in non-septate Ø 6 cm Petri dishes using MEA (malt extract (Sigma–Aldrich, 20 g/l), with 2 % agar (Cultimed)), with or without 0.03 % (v/v) methylene blue (MB) (Merck). Liquid media co-cultures for evaluating antagonism were carried out in 20 ml ME 2 %, in glass tubes (Ø 3 cm × 13 cm height) with a cotton plug, incubated at 25 °C with 200 rpm orbital shaking, as previously described (Ferraz et al., 2021; Amorim-Rodrigues et al., 2024). At the end of the incubation period, samples from solid or liquid cultures were stained with MB as mentioned (Ferraz et al., 2021; Amorim-Rodrigues et al., 2024). Observations were made using an Olympus BX63F2 light microscope equipped with an Olympus DP74 camera, utilising bright field and Differential Interference Contrast (DIC).

The activity of hydrolytic enzymes on cell-free supernatants from yeast-fungi co-cultures was tested as previously described (Amorim-Rodrigues et al., 2024). This was achieved by decanting, centrifuging (9500 rpm), and filtering (0.2 μm) the liquid growth medium of 8-day co-cultures. The activity of chitinase was assayed with colloidal chitin (10 mg/ml, Alfa Aesar), cellulase activity was evaluated with carboxymethil cellulose (CMC) (0.55 %, Acros Organics), and the activity of β-glucanase was tested using laminarin (4 mg/ml, Sigma–Aldrich). The resulting reducing sugars were quantified using a colorimetric assay with DNS (3,5-dinitrosalicylic acid, Alfa Aesar) (Miller, 1959). The potential secretion by yeasts of an agar-diffusible molecule or a volatile compound (VOC) affecting fungal development was assessed by repeating the dual culture assays in septate Petri dishes (Ø 9 cm, MEA 2 %), which were inoculated equidistantly with a mycelium plug (±Ø 6 mm) and a yeast streak, ensuring that the cultures shared only the atmosphere inside the dishes. The plates were incubated for up to 2 weeks at 25 °C. A blue agar CAS (chrome azurol S) assay for siderophore detection was performed by combining the procedures from Schwyn and Neilands (1987) and Nally et al. (2015), as protocolized by Louden et al. (2011), as described in Amorim-Rodrigues et al. (2024). In brief, the base medium was prepared with 20 g/l glucose (LabChem), 20 g/l agar (Cultimed), and 32.24 g/l PIPES buffer (Sigma–Aldrich) with the pH adjusted to 6.8 using 2 M NaOH. The Chrome Azurol S dye (TCI Chemicals) solution was made by carefully mixing three components under gentle manual stirring: (i) $FeCl_3{\cdot}6H_2O$ (Fisher, 1 mM in 10 mM HCl), (ii) CAS (TCI Chemicals, 60.5 mg/l), and (iii) HDTMA (Sigma–Aldrich, 72.9 mg/l), in a ratio of 1:5:4. Both the base medium and the dye solution were autoclaved and cooled to approximately 50 °C before combining with YNB (Yeast Nitrogen Base) with amino acids (BD Difco, 6.7 g/l), which was sterilised by filtration through a 0.2 μm pore-size membrane (Sartorius). The final mixture was aseptically poured into Ø 6 cm Petri dishes. After solidification, the medium was inoculated and cultured as described above for dual agar diffusion antagonism assays.

Siderophore production was indicated by the appearance of yellow to orange halos around the streaks, caused by iron being removed from the blue CAS–Fe^{3+} complex.

2.3. Scanning electron microscopy (SEM)

Samples containing either or both oomycete and yeast were prepared following the protocol previously described (Ferraz et al., 2021; Amorim-Rodrigues et al., 2024). *(i) Sampling.* The oomycete mycelia impregnated with yeast after the 8-day co-cultures were sampled by scraping the entire surface of the approximately Ø 6 mm mycelium plug. Control cultures of oomycetes without yeast were sampled by removing a small amount of grown mycelium with sterilised tweezers. The yeasts remaining in suspension in the co-cultures, as well as those from control overnight cultures (without oomycetes), were sampled by withdrawing 1 ml, which was centrifuged to remove the culture medium (9500 rpm, 10 min at room temperature). Oomycete and yeast samples were washed similarly with ultrapure water under gentle, manual shaking. *(ii) Fixation.* Washed samples were immersed in 1 ml of 2.5 % (v/v) glutaraldehyde (Sigma–Aldrich) in PBS (phosphate-buffered saline: NaCl, 8.0 g/L, Panreac; $Na_2HPO_4{\cdot}12H_2O$, 2.9 g/L, Panreac; KH_2PO_4, 0.2 g/L, Panreac; pH 7.4) for 48 h at 4 °C. Post-fixation. Samples were treated with 0.5–1 ml of osmium tetroxide (Agar Scientific) at 1 % (v/v) and kept at room temperature for 24 h. Subsequently, samples were dehydrated through sequential immersion for 20 min in ethanol–water solutions of increasing concentration (20, 30, 40, 55, 70, 80, 90, 95, and 100 % (v/v) ethanol), then air-dried at room temperature on top of a Ø 12 mm coverslip. *(iii) Observation.* This was carried out at the SEMAT Unit of the University of Minho (http://www.semat.lab.uminho.pt), using a NanoSEM FEI Nova 200 scanning electron microscope at 5/10 kV with a through-lens detector (TLD).

2.4. Transmission electron microscopy (TEM)

Sampling was conducted in the same manner as for SEM analysis. The subsequent procedures were adapted from Murtey and Ramasamy (2016) and Graham and Orenstein (2007) as follows. *(i) Primary fixation.* (a) Oomycetes. Mycelium samples were immersed in 1 ml of 2.5 % (v/v) glutaraldehyde in PB (phosphate buffer; $Na_2HPO_4{\cdot}12H_2O$, 7.16 g/L, Panreac; $NaH_2PO_4{\cdot}H_2O$, 2.72 g/L, Panreac; pH 7.4) for 48 h at 4 °C. This was then aspirated and replaced with an equal volume of fresh PB, followed by incubation at room temperature without shaking for 20 min. The buffer was then aspirated again and replaced with fresh buffer. This process was repeated six times. (b) Yeast. Control overnight cultures (without oomycetes) were sampled by removing 1 ml, which was centrifuged at 9500 rpm for 10 min at room temperature to discard the culture medium. The pellets were fixed by suspending them in glutaraldehyde, as described in (a). Afterwards, samples were centrifuged again for 2 min at 9500 rpm at room temperature, the supernatant was decanted, and an equal volume of PB was added. This process was repeated six times. *(ii) Immobilisation of yeast samples in agarose.* The yeast suspension (1 ml) was centrifuged (9500 rpm, 10 min, room temperature), and the pellet was mixed with 2 % liquid (warm) agarose, just enough to cover the pellet. The mixture was gently vortexed so the yeasts integrated with the agarose and then centrifuged once more (9500 rpm, 10 min, room temperature) to form a new pellet of yeasts immobilised in agarose, which was left to solidify at 4 °C. Blocks of approximately 1–2 mm^3 were cut. *(iii) Secondary fixation.* Samples were post-fixed by incubating for 48 h at room temperature with 1 ml of 1 % (v/v) aqueous OsO4 (Agar Scientific), which was then aspirated, and the sample was immersed in 1 ml of PBS for 30 min. This was repeated six times. *(iv) Dehydration.* Dehydration was obtained by first immersing the samples for 30 min in 1 ml of 20 % (v/v) ethanol–water solution, followed by successive immersions in 1 ml of ethanol–water solutions (20, 30, 40, 55, 70, 80, 90, 95 % (v/v)). Finally, the samples were immersed twice in 1 ml of 100 % ethanol for 20 min, and in 1 ml of 100 % acetone for 30 min. The acetone was then decanted, and the process was repeated three times. *(v) Inclusion and trimming.* Samples were embedded in a 1:1 (v/v) mixture of acetone and Araldite resin (10 ml Araldite CY 212, 11 ml DDSA, 550 μL BDMA, from Agar Scientific) for 2 h, placed in the bottom of BEEM® capsules, which were three-quarters filled with Araldite, and left to cure at 60 °C for 48 h. Ultra-thin sections (70 nm) were cut using the PowerTome PC Ultramicrotome RMC Boeckeler. *(vi) Contrasting.* Sections on copper grids (400 mesh) were stained sequentially for 5 min with UranyLess EM Stain (#22409) and Lead Citrate Reynolds Stain (3 %) (#22410-01) from Electron Microscopy Sciences/Biolyst Scientific. *(vii) Observation.* Electron micrographs were taken with a Jeol JEM-2100-HT transmission electron microscope operating at 80 kV, equipped with a fast-readout.

3. Results

The antagonistic ability of the yeast *W. anomalus* was tested against the oomycetes *P. cinnamomi* and *P. xcambivora.* Oomycetes are known to spread through the dispersal of zoospores or oogonia in humid soil, while the disease results from the proliferation of mycelium within the tree tissues/cells (Brasier et al., 2022). Therefore, this study focused on the effect of the yeast on the true causal agent of the disease, *namely*, the mycelium of *P. cinnamomi* and *P. xcambivora.*

3.1. Antagonism assays of W. anomalus x P. cinnamomi and P. xcambivora

Dual agar diffusion assays were performed in MEA-MB. Results were recorded after an 8-day incubation at 25 °C. The yeast inhibited the mycelial growth of both *Phytophthora* species, particularly that of *P. xcambivora,* which was nearly completely impaired (Fig. 1A). Additionally, the oomycetes' mycelia were deeply stained, as was the yeast strikeout colony (not shown). In liquid yeast/oomycete co-cultures, after incubating for 8 days at 25 °C and 200 rpm, both *Phytophthora* inoculum mycelium plugs remained undeveloped (Fig. 1B right panels), confirming that *W. anomalus* exhibited strong antagonistic ability. Samples of these ungrown plugs and samples of the yeasts in suspension from the same co-cultures were stained with MB and observed under a light microscope. The yeast cells mostly did not stain, indicating that the culture remained healthy throughout the liquid medium antagonism testing period, similar to the control yeast cultures (not shown). On the other hand, hyphae from oomycetes were heavily and extensively stained, suggesting that *Phytophthora* mycelia died upon contact with *W. anomalus* (not shown). To verify this possibility, the original mycelium plugs from co-cultures were washed and re-incubated in fresh MEA for another 8 days at 25 °C. Generous growth of both *Phytophthora* was achieved (Fig. 1C right panels), showing that a portion of the ungrown and MB-stained mycelium was still viable.

The hyphal cell walls of *P. cinnamomi* and *P. xcambivora* are primarily composed of variable amounts of cellulose and (1 → 3)-β-D/(1 → 6)-β-D-glucan, with virtually no chitin. For the present work, the nonspecific activity of β-glucanase, chitinase and cellulase was measured using cell-free extracts from liquid medium co-cultures. Results (Fig. 2A) showed statistically invariant, very weak, or no activity from these hydrolytic enzymes. These findings are similar to those previously obtained with the same yeast strain facing *Colletotrichum* spp. (Amorim-Rodrigues et al., 2024), suggesting that the role of these enzymes in the antagonism mode of action, if any, may be residual and indirect.

W. anomalus did not produce VOCs (Fig. 2B) or siderophores (Fig. 2C) that could be detrimental to *Phytophthora* survival. The absence of inhibition observed in septate Petri dishes (Fig. 2B), compared with the inhibition noted in dual agar diffusion assays (Fig. 1A), suggests that in solid medium, antagonism may arise from the action of an agar-diffusible molecule. Alternatively, in liquid medium, either this phenomenon is occurring, or direct contact between the yeast and hyphal cells is necessary to impede the proliferation of *Phytophthora* spp.

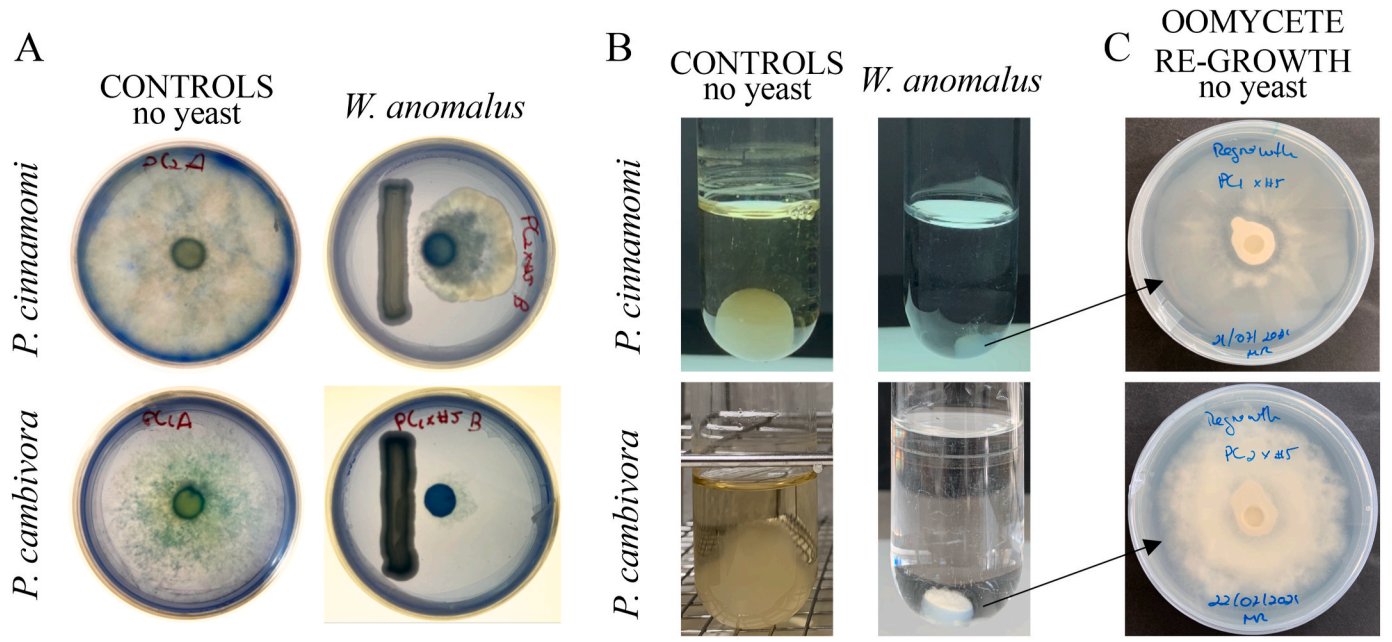

Fig. 1. Antagonism assays of *W. anomalus* against *P. cinnamomi* and *P. xcambivora*. Co-culture antagonism assays were conducted on MEA supplemented with 0.015 % Methylene Blue (A) and on ME (B), incubating for 8 days at 25 °C with 200 rpm orbital shaking. Samples of residual inoculum from ME co-cultures were washed and transferred to fresh MEA, as shown in (A), to assess remaining cell viability. Results were recorded after an additional 8 days of incubation under identical conditions (C). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Previous results indicated that *W. anomalus* acts as a necrotrophic mycoparasite/predator towards phytopathogenic filamentous fungi (Ferraz et al., 2021; Amorim-Rodrigues et al., 2024). To determine if *W. anomalus* interacts with *Phytophthora* in the same manner, samples of co-cultures were analysed in detail using several microscopy methods.

3.2. Light microscopy and SEM analysis of the W. anomalus antagonism of P. cinnamomi and P. xcambivora

Light microscopy observations of samples from liquid medium co-cultures stained with MB were extremely revealing. As mentioned above, abundant heavily stained hyphae and empty hyphae from both oomycetes were observed (Fig. 3A). Many yeasts were found adhering to the hyphae cell walls of both oomycetes. Additionally, large amounts of yeast cells were packed inside *P. cinnamomi* empty hyphae (Fig. 3B e-h). Yeasts were not stained with MB, which indicates they are thriving. Empty hyphae were also not stained, as they were devoid of cellular remains. Accordingly, yeasts were observed in contact with *P. cinnamomi* intracellular debris from partially emptied hyphae, suggesting they could be feeding on the oomycetes' hyphal content (Fig. 3B i, j).

SEM observations also revealed numerous yeast cells adhering to or appearing to penetrate *Phytophthora* hyphae (Fig. 4B c, d, f). SEM micrographs also showed what seemed to be yeast cells within the hyphae of *P. cinnamomi* (Fig. 4B e). To confirm whether these structures corresponded to yeast cells, measurements of yeast cells from SEM micrographs of yeasts cultivated alone were compared with those presumably residing inside the hyphae. Cell volume was estimated under the assumption that yeast cells are oblate structures (Amorim-Rodrigues et al., 2024). The results were, respectively, $15.63 \pm 9.87\ \mu m^3$ ($n = 12$) and $21.23 \pm 19.88\ \mu m^3$ ($n = 10$), indicating no statistical difference between the two groups (p-value >0.05). It was also noted that although hyphae could be emptied, their cell walls remained intact (Fig. 5), aligning with the absence of significant activity of yeast hydrolytic enzymes (Fig. 2A).

3.3. TEM analysis of the W. anomalus antagonism of P. cinnamomi

The detailed observation of *P. cinnamomi* hyphae ultrastructure was performed using TEM, which revealed, as expected, a highly pleomorphic phenotype, exhibiting a wide variety of hyphal shapes and sizes along with several distinctive features. Hyphae were highly vacuolated, presenting numerous small, irregularly shaped and sized vacuoles (V) (Fig. 6A) or a large central vacuole (cV) (Fig. 6B). Occasionally, they also displayed many small vesicles (ve) partially filled with electron-dense material (Fig. 6B). Furthermore, numerous lipid droplets (Ld) of variable size, along with some sparse glycogen rosettes (Faoro et al., 2022), were visible (Fig. 6). The cell walls of the hyphae varied in thickness (Fig. 6C) and typically appeared double-layered, with some irregular interspaces between the layers (Fig. 6D). Additionally, adjacent hyphae could appear side by side (Fig. 6E) or may locally fuse (Fig. 6F). Some of these characteristics were previously described for other *Phytophthora* species (Ehrlich & Ehrlich, 1966; Hohl & Hamamoto, 1967; Hemmes & Wong, 1975; Hardham, 1987; Hyde et al., 1991). When *P. cinnamomi* was cultured alongside *W. anomalus,* these characteristics persisted. However, a notable new feature emerged: the presence of numerous distinctive intrahyphal hyphae (IhH) or endohyphae (Fig. 7A) (Miller & Anderson, 1961; Lowry & Sussman, 1966; Chan & Stephen, 1967; Farley et al., 1975; Lim et al., 1983; Kim & Hyun, 2007). The light microscope micrographs had previously revealed this feature (Fig. 3B), albeit less conspicuously. The inner hyphal cells were identical to those described above, while the outer hyphae displayed only residual or no cytoplasm.

Similar to the results obtained with light microscopy and SEM, samples from *W. anomalus/P. cinnamomi* co-cultures displayed yeasts residing within hyphae (Fig. 7B). Yeast cells exhibited a healthy cytology, with numerous organelles and intracellular structures. The outer hyphae showed only residual or no cytoplasm, but the cell wall appeared intact and of regular thickness (121 ± 32 nm ($n = 84$)). No fusion was observed between the yeast and the hyphal walls. Another important feature extensively observed in samples from *W. anomalus/P. cinnamomi* co-cultures was the shedding of *W. anomalus* cell walls, whose outer layers were seen to peel off, while the yeast cultured alone

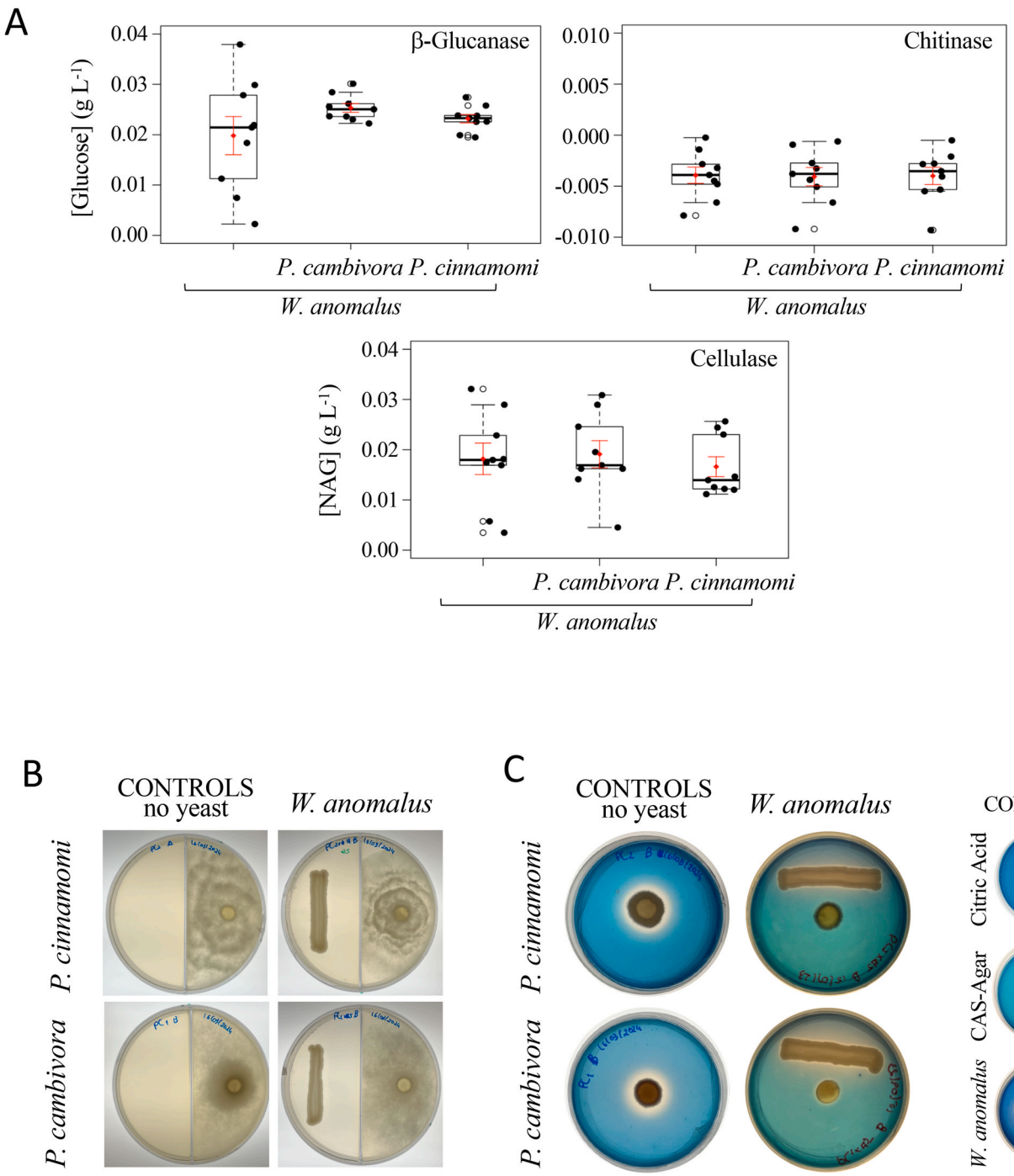

Fig. 2. Assays to detect the activity of hydrolytic enzymes, VOC secretion, and siderophore production. (A) Hydrolytic enzyme activity tested on cell-free extracts of *W. anomalus* cultivated alone or with *P. cinnamomi* or *P. xcambivora,* over 8 days at 25 °C in liquid ME with 200 rpm orbital shaking. Assays were performed three times, each in triplicate: p-value >0.05. (B) MEA agar diffusion assays in septate petri dishes to identify VOC secretion. (C) CAS (Chrome Azurol S) assay for detecting secreted siderophores, with appropriate controls.

showed only very occasional shedding (Fig. 8A–F). This was also occasionally noted in TEM micrographs (not shown). In the present work, *W. anomalus* was shown to extensively display these inter-cell connections when cultured alone, apparently less frequently when cultured with *P. cinnamomi* (Fig. 8B–G). Additionally, many polarized cells (Fig. 8C–H) and numerous fusing nuclei were observed (Fig. 8D–I) that would align with a conjugation process during meiotic sporulation. Nevertheless, only two hat-shaped spores were occasionally observed per cell in either culture (Fig. 8E–J).

4. Discussion

W. anomalus has long been reported as a strong antagonist of several filamentous fungi that cause severe deterioration of fruits during the post-harvest transport, storage and commercialisation phases. These include *Botryodiplodia theobromae* (Hashem and Alamri, 2009), *Botrytis cinerea* (Parafati et al., 2015; Pretscher et al., 2018; Lanhuang et al., 2022), *Magnaporte oryzae*, *Roesleria subterranea* (Pretscher et al., 2018) and *Monilinia fructicola* (Czarnecka et al., 2019). Yet, these spoilage fungi are presently counteracted with commercially available products

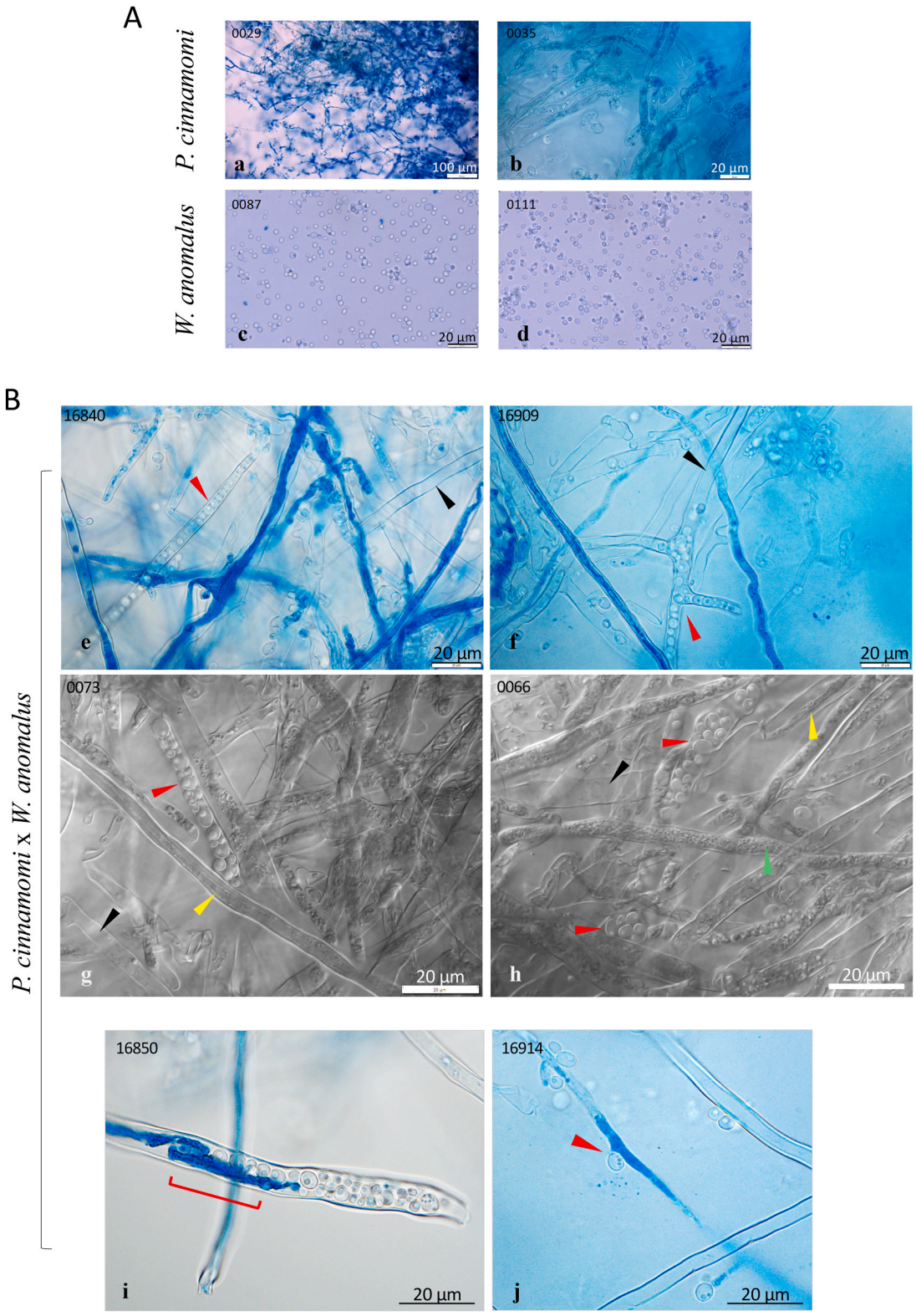

Fig. 3. Representative optical microscope images of *W. anomalus* cells and *P. cinnamomi* hyphae cultured separately or **together.** Samples were retrieved from 8-day cultures in ME at 25 °C with 200 rpm orbital shaking, stained with MB. (A) Bright field micrographs of control samples. (a, b) Different magnifications of *P. cinnamomi* cultured alone. (c) *W. anomalus* cultured alone. (d) *W. anomalus* from a supernatant of an 8-day co-culture with *P. cinnamomi*. (B) Bright field and Differential Interference Contrast (DIC) micrographs of *W. anomalus/P. cinnamomi* co-cultures. Black arrows indicate empty hyphae. The green arrow points to a regular, healthy hypha. Yellow arrows indicate intrahyphal hyphae. Red arrows point to yeast cells packing inside hyphae (e–h), seemingly feeding on the heavily MB-stained hyphal intrahyphal remains (i) or attaching and pushing the hyphal cell, while the hyphal intracellular remains are heavily MB stained and appear to be being drawn into the yeast (j). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

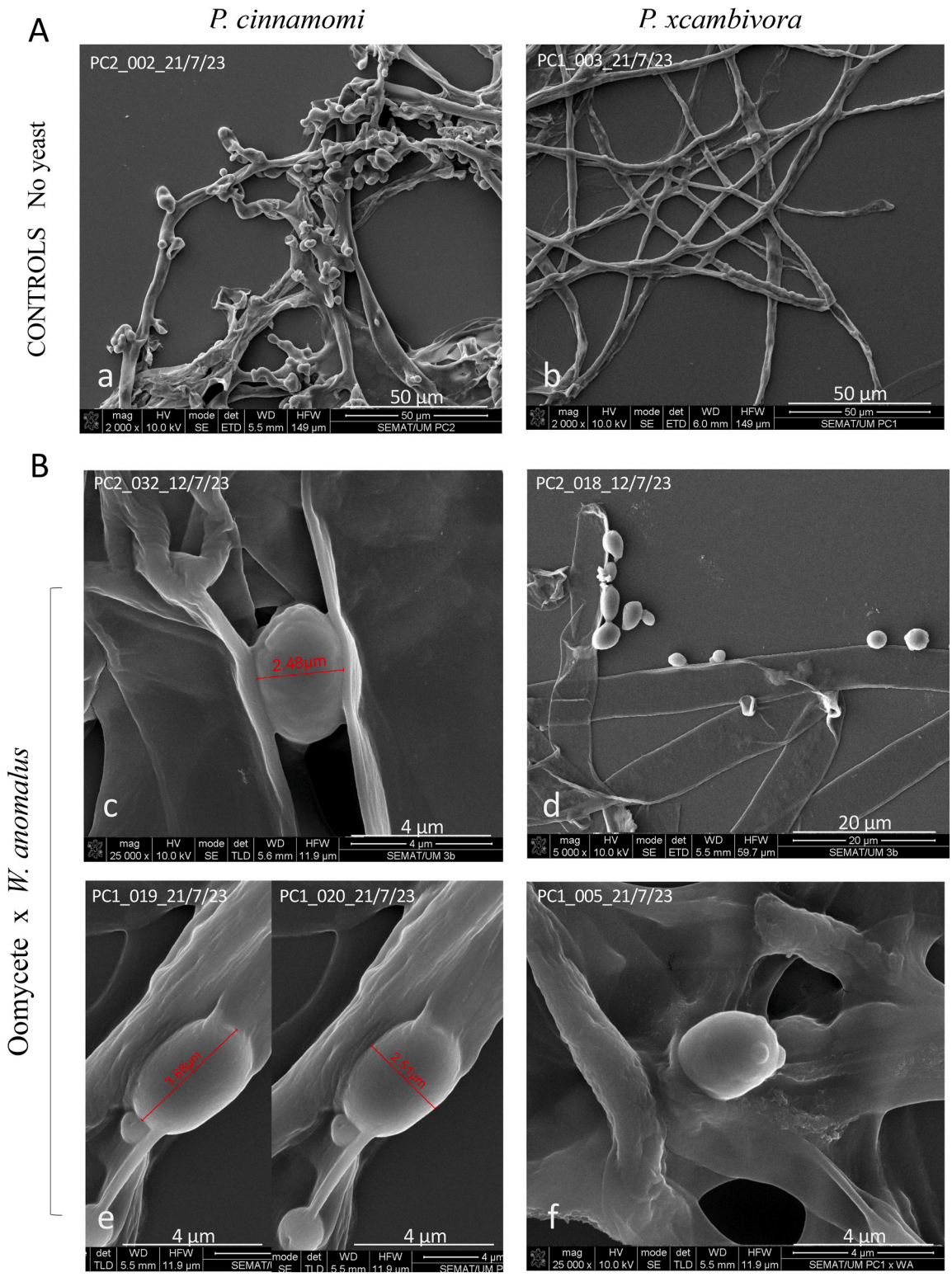

Fig. 4. Representative SEM micrographs of hyphae of oomycetes from single cultures or co-cultures with *W. anomalus*. (A) *P. cinnamomi* (a) and *P. xcambivora* (b) grown alone or (B) co-cultured with *W. anomalus,* all in liquid ME at 25 °C with orbital shaking at 200 rpm for 8 days. Magnifications are 2000 x (a and b), 5000 x (d), and 25,000 x (c, e, f). Yeast cells are shown fused with hyphae (c, d, f), inside the hyphae of *P. cinnamomi* (e), and appearing to enter the hyphae of *P. xcambivora* (f).

which are based on BCAs other than *W. anomalus*, including bacteria (*e.g.* Bio-Save® from Lallemand Plant Care, or Serenade®, Rhapsody® and Cease® from Bayer), or other yeasts (reviewed by Lucas and Cássio, 2022). Plant phytopathogens of the pre-harvest phase were also identified as targets of *W. anomalus* BCA activity, including species from several genera, like *Monilinia* (Czarnecka et al., 2019), *Aspergillus, Cladospaorium, Talaromyces, Penicillium* (Solairaj et al., 2020; Zhao et al., 2021), *Curvularia, Fusarium* and *Rhizoctonia* (Khunnamwong et al., 2020), *Moniliophthora* (Ferraz et al., 2021), *Alternaria* (Zhu et al., 2023) and *Colletotrichum* (Amorim-Rodrigues et al., 2024). *W. anomalus* action as a BCA has been reported frequently in association with the secretion of hydrolytic enzymes. This species was reported to secrete amylase,

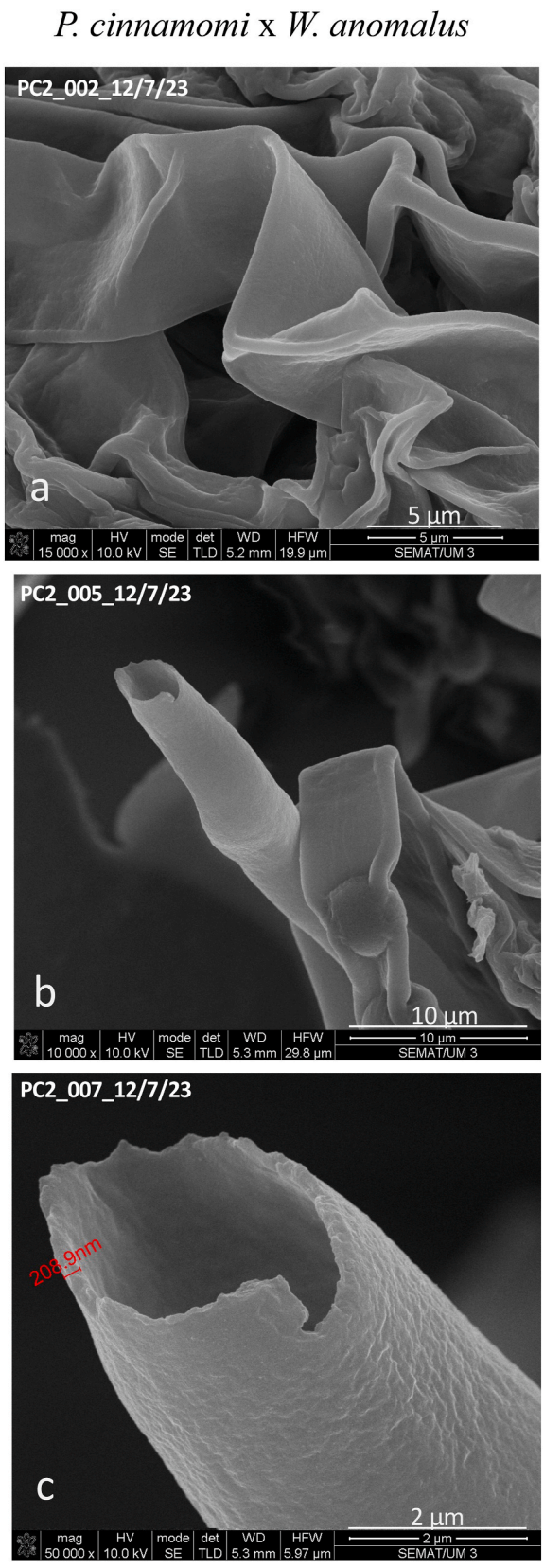

Fig. 5. *P. cinnamomi* intact hyphal cell wall after co-culturing with *W. anomalous*. SEM micrographs of *P. cinnamomi* empty hyphae were retrieved from a co-culture with *W. anomalous* for 8 days in liquid ME at 25 °C and 200 rpm orbital shaking. (a to c) Sequentially higher magnification: 15,000 x (a); 10,000 x (b) and 50,000 x (c).

cellulase, glucosidase, chitinase and protease (Pretscher et al., 2018). The secretion of these enzymes was shown to vary significantly with the strain (Pretscher et al., 2018), and to depend on nutrients' availability (Sandhya et al., 2005; Passoth et al., 2006; Parafati et al., 2017). Importantly, the expression of exoglucanase-encoding *WaEXG1* and *WaEXG2* was found to be stimulated by the presence of cell wall extracts from target phytopathogens. However, this was not replicated when these were confronted with the yeast in co-cultures (Parafati et al., 2017). Further attempts to demonstrate the antifungal activity of *W. anomalus* hydrolytic enzymes-containing culture filtrates were unfruitful, despite the several methods used to increase the enzyme concentration (Pretscher et al., 2018). The authors provided several justifications, including the low stability of the enzymes, and their possible inactivation by the target phytopathogen. Nevertheless, in yeasts, (1 → 3) and (1 → 6)-β-glucanases are supposedly constitutively expressed and secreted to participate in constant wall remodelling processes (Wessels and Sietsma, 1981), which, in agreement with the present results, excludes a BCA-related purpose for their secretion. On the other hand, chitinases from yeasts (2–4 according to Hartl et al., 2012) are under the control of the cell cycle, which in turn responds to nutrient availability (*e.g.* Zhang et al., 2023), while in filamentous fungi there are 10–20 chitinase-encoding genes generally repressed by glucose, inducible by chitin and upregulated during starvation (Hartl et al., 2012). The number of chitinases might relate to the amounts of chitin of the respective cell walls, which are 0.5–5 % and ±20 % in yeasts and filamentous fungi, respectively. However, it could also relate to each species' ecological niche since insect mycoparasitic fungi were described to have more than 30 chitinases (Hartl et al., 2012). This evolutionary relation concurs with the absence of activity of this enzyme in *W. anomalus*, not only because, in general, yeasts have limited chitin in their cell walls, but also because oomycetes most often have none. Instead, oomycetes present cellulose in different percentages, and (1 → 3)-β-D/(1 → 6)-β-D-glucan, both compounds extremely alkali-insoluble and putatively forming strong covalent networks (Sietsma et al., 1969; Wessels & Sietsma, 1981). Accordingly, *P. infestans* contains ±30 % cellulose and no chitin (Mélida et al., 2013). Other oomycetes may still present up to 1 % chitin. Cellulolytic activity, on the other hand, is more frequent in microbes thriving in plant decay environmental niches, which is not the case of *W. anomalus,* justifying, through the same rationale, the absence of the correspondent activity now found despite the *Phytophthora* cell walls' composition (Sohail et al., 2022).

Important results from the present work pertain to the absence of the secretion of VOCs or siderophores. This, along with the secretion of hydrolytic enzymes, has often been shown to vary with the strain and the growth conditions and is routinely associated with the BCA function of yeasts, including *W. anomalus* (Parafati et al., 2015, 2017; Hong et al., 2017; Khunnamwong et al., 2020; Zhu et al., 2023). However, this assumption originates from the detection of these secreted compounds, which does not necessarily account for their actual contribution to the antagonistic processes. Moreover, in liquid medium, the evidence from the present work confirms that *W. anomalus* acts as a necrotrophic parasitic agent of oomycetes, similar to what was previously shown with phytopathogenic filamentous fungi (Hashem and Alamri, 2009; Ferraz et al., 2021; Amorim-Rodrigues et al., 2024). Considering the large number of microorganisms documented in the literature that are antagonized by *W. anomalus,* along with the frequent mentions of this yeast's ability to secrete enzymes, VOCs, or siderophores, as well as other types of proteins/peptides that are commonly classified as killer toxins (Giovati et al., 2021), a necrotrophic parasite mode of action cannot be generalized to all cases of antagonism, suggesting specificity in action depending on the organism being antagonized.

According to results from co-cultures of *W. anomalus* with *P. cinnamomi,* the yeast adheres to the hyphal cell wall, as previously observed with *M. perniciosa* (Ferraz et al., 2021) and *C. godetiae* (Amorim-Rodrigues et al., 2024). However, no evidence was found of the secretion of a viscous extracellular matrix, which would contribute

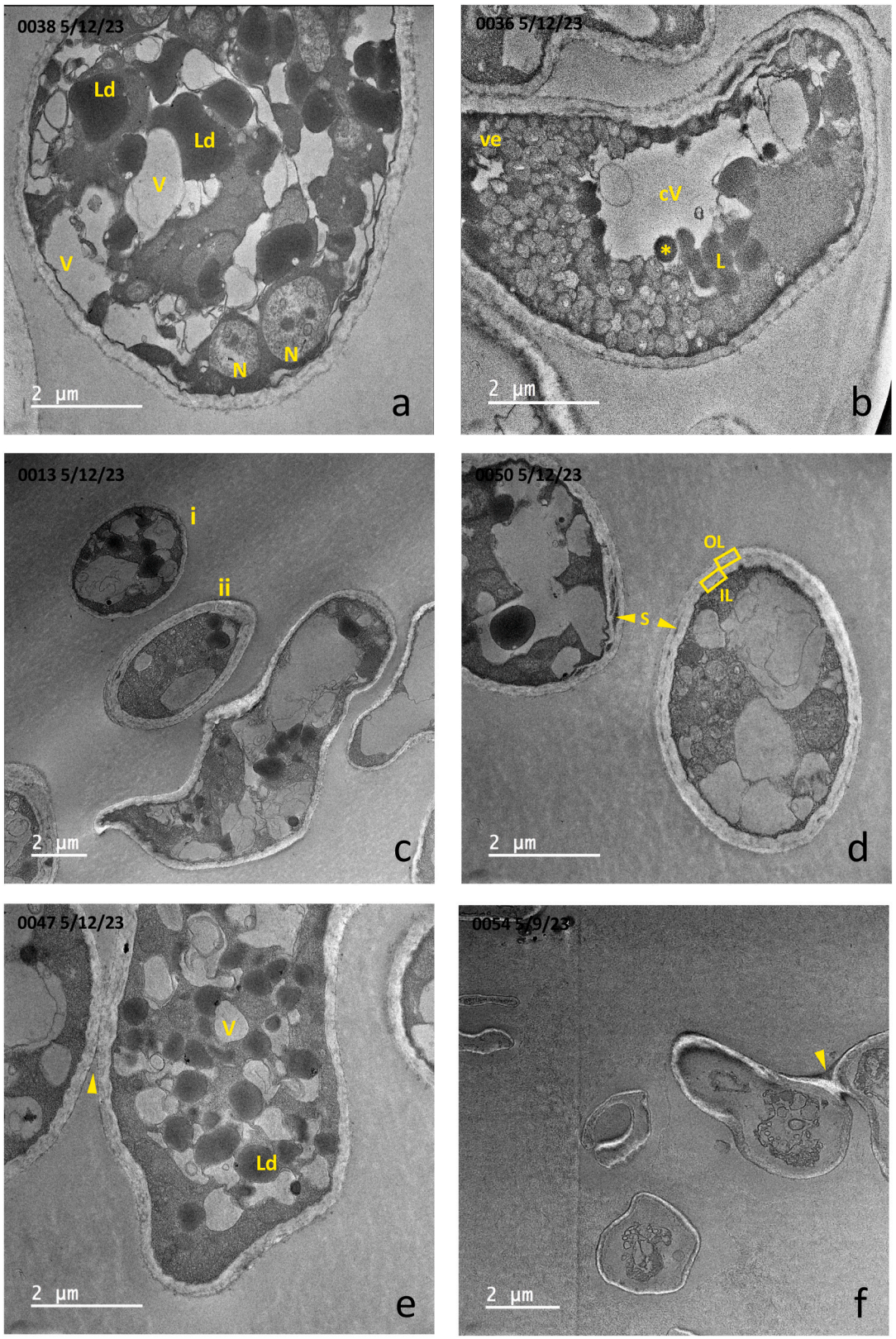

Fig. 6. Representative TEM micrographs of the cellular structures of *P. cinnamomi* hyphae. Samples were taken from 8-day cultures in liquid ME at 25 °C with 200 rpm orbital shaking. (a, b) Hyphae show extensive vacuolisation. Vacuoles (V) and the central vacuole (cV) are irregular in shape. Hyphae often contained two nuclei (N), as well as significant amounts of lipid droplets (Ld) and a few glycogen rosettes (*). (c, d) The hyphal cell wall was thin (i) or thick (ii), and it was frequently made up of two layers: inner (IL) and outer (OL), with a small space between (S). Two adjacent hyphae laid side by side, their walls only touching (e). Yeast cell walls fused (f). Magnification: 6000 x (a, b, d, e), 3000 x (c), and 4000 x (f).

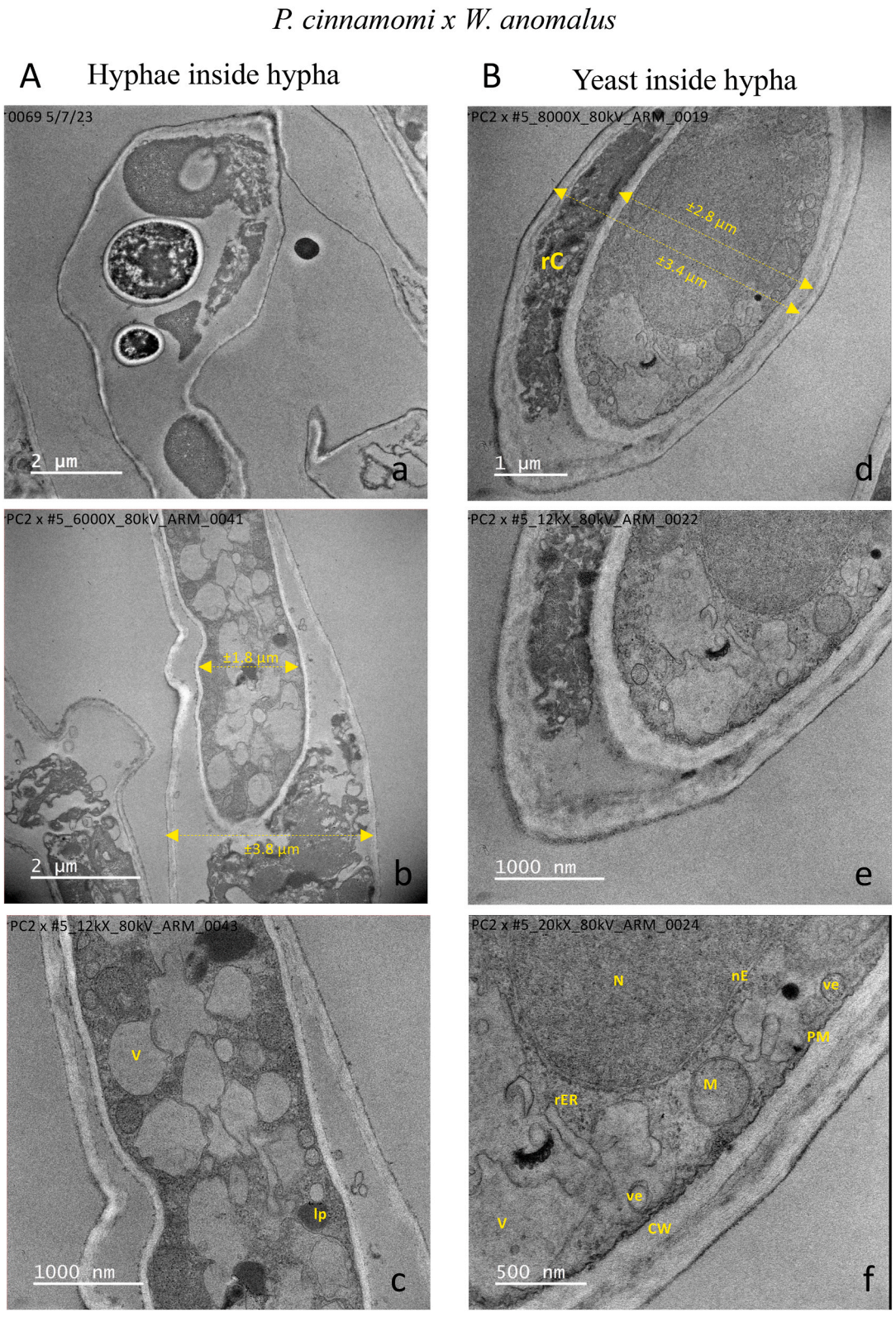

Fig. 7. Representative TEM micrographs of *P. cinnamomi* showing intrahyphal hyphae or yeasts inside hyphae. Samples were collected from a co-culture with *W. anomalus* for 8 days in liquid ME at 25 °C and shaken at 200 rpm. (A) *P. cinnamomi* hyphae displaying intrahyphal hyphae (IhH). (a) The intracellular contents of the outer hypha are disassembled, with two small IhH present. (b, c) Outer hyphae contain disaggregated remnant cytoplasm (rC), while the inner hypha features numerous vacuoles (V) and lipid droplets (Ld), typical of hyphae. (B) Increasing magnifications of *P. cinnamomi* hyphae showing yeast cells inside. Yeast cells exhibit a nucleus (N) with a well-defined nuclear envelope (Ne), rough ER (rER), plasma membrane (PM), cell wall (CW), mitochondria (M), vacuoles (V), and vesicles (ve).

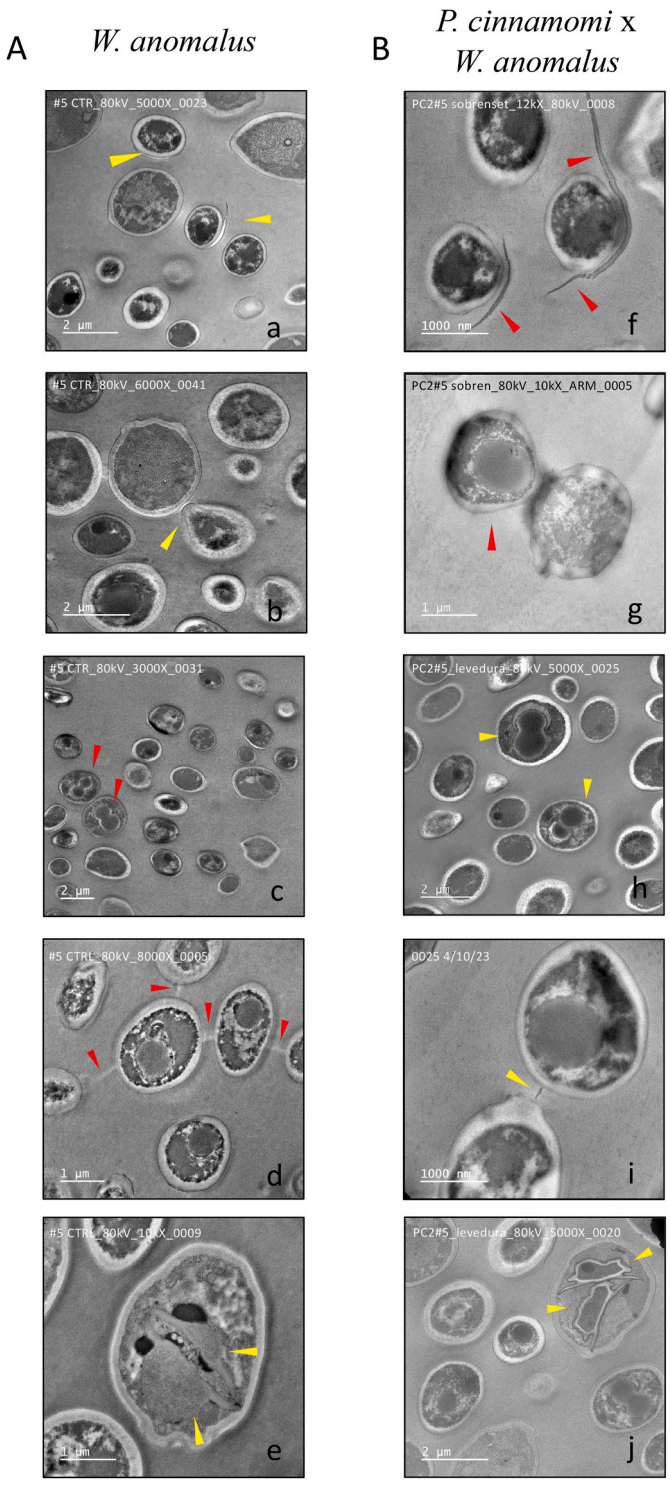

Fig. 8. Representative TEM micrographs of *W. anomalus* cellular features when cultured alone or with *P. cinnamomi*. Samples were collected from single (A) and co-cultures with *P. cinnamomi* (B) after 8-day incubation in liquid ME at 25 °C and 200 rpm orbital shaking. (a, f) Extensive shedding of the outer layer of the cell wall, resulting in a considerably reduced thickness. (b, g) Polarised yeasts. (c, h) Fusing yeast cell nuclei. (d, i) Ligations connecting yeast cells, with estimated sizes of 20–40 nm in width and 300–400 nm in length. (e, j) Yeast hat-shaped spores, two per cell. Red arrows highlight abundant features. Yellow arrows indicate features observed infrequently. Magnification: 5000 × (a, h, j), 6000 × (b), 3000 × (c), 8000 × (d), 10,000 × (e, g), 12,000 × (f, i). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

to the adhesion between the two antagonists. New evidence corroborates that *W. anomalus* antagonism is independent of the secretion of VOCs, siderophores, or hydrolytic enzymes, occurring instead in a cell-to-cell contact-dependent manner, as previously suggested for the antagonism of two other *Colletotrichum* species (Amorim-Rodrigues et al., 2024). Yeast cells were found to pack inside *P. cinnamomi* hyphae, like the previous findings with *C. godetiae* (Amorim-Rodrigues et al., 2024), thriving on their intracellular contents and emptying them without destroying the intact cell walls. This behaviour could, at least in part, correspond to an opportunistic strategy derived from extensive hyphae breaking that can be observed in mature oomycete cultures. However, this does not account for the large number of yeasts attached to the lateral hyphae cell walls and their apparent hyphal penetration efforts. Such occurrences suggest that the yeast and the hyphae cell walls may locally and transiently fuse, as observed in SEM micrographs from this study. Cell–cell fusion is part of the sexual cycle of yeasts and filamentous fungi, whose walls are very plastic and dynamic structures. Although TEM analysis did not reveal the yeast-to-hypha fusion, it did show fusion between different hyphae cell walls, indicative of an active enzyme ensemble necessary for wall remodelling essential for fusion to occur (Gow et al., 2017).

The dual agar diffusion antagonism assays in non-septate and septate Petri dishes, revealed that the inhibition of the growth of *Phytophthora* relies on some unknown agar-diffusible non-volatile molecule or peptide/protein. Some of the most segregated proteins by yeasts and fungi are cell fusion-inducing pheromones (Clark-Cotton et al., 2022). Depending on the mating type, the culture conditions and the growth phase, yeasts secrete the peptides α- and a-factors, the latter of which is prenylated (Sieber et al., 2023). *Phytophthora* spp. also release pheromones, designated as α1 and α2, but these are not proteic; they are acyclic diterpenes which differ in their oxygenated state (Ojika et al., 2011). These are highly insoluble compounds whose structure resembles that of the highly hydrophobic a-factor prenyl residue. In *Saccharomyces cerevisiae*, the substitution of the prenyl group from a-factor by the longer chained geranylgeranyl, whose hydrocarbon chain is the same size as the *Phytophthora* α2 and α1, did not significantly affect the activity or specificity of the a-factor receptor activation (Caldwell et al., 1994), suggesting that the a-factor receptor (Ste3) has the flexibility to accept peptides with different lapidated structures. This flexibility is also evident in the activity of the terpene-pheromones from *Phytophthora,* which can signal species other than the one producing them, thus mediating interspecies crosstalk (Ojika et al., 2011; Tomura et al., 2017).

Regardless of the type of molecule that the pheromones may be, the processes triggered are basically the same in yeasts, fungi, and oomycetes. Once the hormones are sensed by specific receptors in the confronting cell, a chain of events is induced through the activation of MAPK cascades, which eventually lead to cell cycle arrest, polarization of the cells by remodelling the cytoskeleton, induction of agglutinins and other proteins, and increased traffic of vesicles carrying cell wall hydrolytic or synthetic enzymes towards a polar tip, resulting in the fusion of the cell with the other mating type. Eventually, a localized balance between the processes of degrading and building the cell wall is achieved, allowing fusion of the cells without lysis (Huberman and Murray, 2014). Although this process appears to be more consistent with solid media-growing cultures and the establishment of a hormone gradient in the agar, conjugation may also occur in liquid cultures. In this case, the shaking turbulence causes cells to randomly bump into each other, which locally triggers the same processes that lead to cell wall fusion (Clark-Cotton et al., 2022). Cell–cell fusion may thus occur either in dependence on or independently of a pheromone gradient.

Given all this, a plausible model for *W. anomalus* mode of action is suggested (Fig. 9). *P. cinnamomi* pheromones are unknown, but they should not be too unsimilar to those secreted by other *Phytophthora* species. The secretion of acyclic terpenes varies considerably with the species/strain and the culture conditions (Tomura et al., 2017). Complex

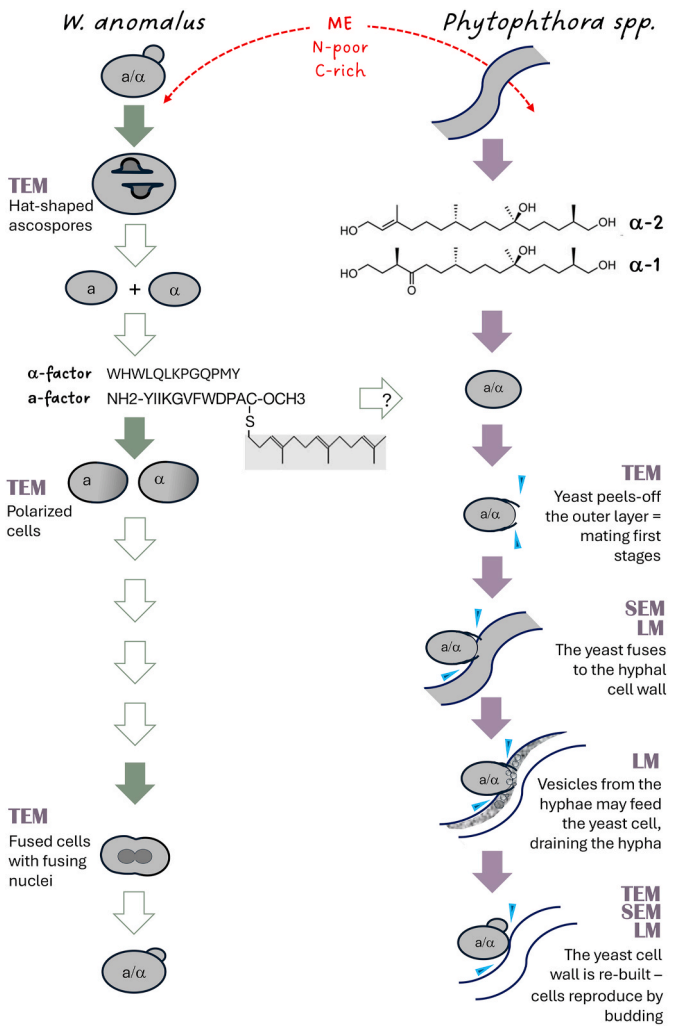

Fig. 9. Scheme representing an interpretative model of how *W. anomalus* antagonises *Phytophthora spp*. The shown a and α-factors peptide sequences are those from *S. cerevisiae* (https://ximbio.com/reagent/153998/a-factor-yeast; https://www.uniprot.org/uniprotkb/P01149/entry; Kurjan and Herskowitz, 1982; Michaelis and Barrowman, 2012). The α1 acyclic terpene and α 2 oxygenated acyclic terpene are the pheromones produced by *Phytophthora* spp. (Ojika et al., 2011; Tomura et al., 2017). Blue arrows indicate the target areas on the cell surface where the hydrolytic and wall-building enzymes should act together. LM, SEM, and TEM refer to the microscopy techniques that revealed the corresponding structures. White arrows indicate the path a possible mating cycle from *W. anomalus* might follow. However, most of the downstream spores' germination intermediate forms that should appear were either not observed or not fully confirmed. Purple arrows indicate the proposed model pathway. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

undefined media with low nitrogen availability, like V8, allied to low pH, apparently should be the best combination. ME is also an undefined N-poor medium, though richer in carbon sources than V8. It is composed of 52 % maltose, 20 % glucose, 15 % dextrin, 6 % other carbohydrates and 5 % protein. Moreover, the growth of the yeast population in the co-cultures lowers the medium pH, making it more favourable for pheromone production. The model postulates that the α2 and α1 *Phytophthora* pheromones can replace the yeast a-factor and trigger in the yeast cells the early stages of the cell fusion process from the mating programme, even if the cells did not undergo meiosis beforehand. At the same time, the same growth medium triggers the sporulation of *W. anomalus*, whose ascospores were observed carrying only two hat-shaped spores. These should not be haploid because they are not four. Subsequent germination of these spores into mature cells is probable, proceeding towards their polarization and fusion with another cell. Plenty of polarised cells were observed, as well as their tentative ligation to regular-shaped yeast cells. Also, the fusing nuclei of cells that theoretically should have emerged from the haploid cells' conjugation were observed. Still, other structures characteristic of the yeast conjugative cell cycle (like shmoos, heterokaryons or budding zygotes) were not observed.

The hypothesis that *Phytophthora* pheromones trigger cell fusion processes in yeasts relies on the a-factor receptor's ability to recognise terpenes. In *S. cerevisiae,* the a-factor receptor, Ste3, is a classical G-protein-coupled receptor that commands intracellular signal transduction, promoting the synthesis of adhesins, agglutinins, and hydrolytic enzymes (Michaelis and Barrowman, 2012). In turn, the a-factor induces the transcription of the *STE3* gene (Hagen and Sprague, 1984). This means that transcription, even if only at basal levels, must occur independently of the a-factor, justifying how cells at any stage of the cell cycle can respond to its presence and induction. The hydrophobicity of the a-factor suggests it should easily ligate non-specifically to the plasma membrane (Michaelis and Barrowman, 2012). However, there is no information on whether this type of ligation could trigger any signalling effects. The same could apply to the *Phytophthora* terpenes, which are also very hydrophobic/lipophilic. The present model thus proposes that the *Phytophthora* pheromones are either sensed by the yeast's a-factor receptor or simply bind to the plasma membrane, in either case contributing to the induction of adhesins, agglutinins, and wall-enzymes (Clark-Cotton et al., 2022). This would facilitate the attachment of the yeast to the hyphae when they meet by chance, subsequently triggering the complete process leading to cell–cell fusion. The inherent localised degradation of the *W. anomalus* cell wall observed, as before (Lane et al., 1969; Lopes-Bezerra et al., 2018), would expose the inner glucans layer (Klis et al., 2002; Latgé, 2007), which would then be readily compatible with the matching (1 → 3)-β-D and (1 → 6)-β-D-glucans in the oomycete's cell walls (Mélida et al., 2013), thereby strengthening the attachment of the two cells. The transient and localised balance of hyphal cell wall hydrolysis and synthesis processes (Clark-Cotton et al., 2022) would promote the recovery of wall integrity, explaining why it was never possible to observe the complete penetration of the hyphae by the yeast using TEM.

Considering all this, the model postulates that *W. anomalus* packs inside hyphae after these are broken or physically compromised, rather than directly penetrating through the wall and membrane. This, in turn, introduces further complexity. Not only do the yeasts contribute to emptying the hyphae of their contents, possibly through a vesicle-mediated process as suggested, but more importantly, they must do so in response to a driver that promotes their attraction. Since yeast cells lack locomotion structures, the only means by which they may "move" is through chemotropism, which has been shown to be induced by pheromones preceding cell–cell fusion (Clark-Cotton et al., 2022). Consistently, the diffusion of the yeast and the oomycete pheromones through the agar in dual agar diffusion assays would contribute to the arrest of the cell cycle of both cultures (Clark-Cotton et al., 2022; Sieber et al., 2023) in the vicinity of each other's grown biomasses. Additionally, these pheromones would also promote cell death (Huberman and Murray, 2014), justifying the MB staining of the yeast colony in dual agar diffusion assays. This model, although derived from the experimental evidence of the present work, will need confirmation in the future. If the entire process could be triggered by the *Phytophthora* spp. pheromones as suggested, antagonism by *W. anomalus* would be a highly specific process, allowing for multiple modes of action when opposing different species.

Climate change is reshaping the spread and severity of *Phytophthora* plant infections, which are occurring at an unprecedented pace (Fry et al., 2015; Jung et al. 2016, 2017a; Dorado et al., 2023; Seidel et al., 2024). Their control using agrochemicals has proven inefficient (Brown et al., 2019a; Neupane et al., 2022), sometimes causing undesired side

effects on plants, and is increasingly restricted or prohibited. Furthermore, it is unsustainable in the long run due to its damaging effects on human and animal health and the environment. The use of BCAs might be the solution (Sellitto et al., 2021; Lourenço et al., 2022; Volynchikova et al., 2022), particularly yeasts (Pretscher et al., 2018; Lucas and Cássio, 2022), noting that these microorganisms are generally less aggressive colonizers of plant ecosystems (Luna, 2017). In particular, the actual application of *W. anomalus*-derived products in the field still requires specific health and environmental security checks. On one hand, attention has been drawn to the ability of *W. anomalus* to act as a human pathogen (Ioannou et al., 2024), reflecting a global trend of increasing fungal and yeast infections in humans (Seidel et al., 2024). This is probably not a cause for concern given how limited the manifestation of the disease has been, although it must be considered that true epidemiology is not fully understood. More importantly, the regular introduction of *W. anomalus,* like any other BCA in agroecosystems, requires knowledge of the long-term impacts it could cause. In this context, *W. anomalus* is promising, not only because it is a strong antagonist of *Phytophthora* and other phytopathogens, but also because its apparent specificity decreases the likelihood of collateral effects.

CRediT authorship contribution statement

Mariana Amorim-Rodrigues: Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization. **Rogélio Lopes Brandão:** Writing – review & editing, Validation, Resources. **Fernanda Cássio:** Writing – review & editing, Supervision, Resources, Funding acquisition. **Cândida Lucas:** Writing – review & editing, Writing – original draft, Validation, Supervision, Formal analysis, Data curation, Conceptualization.

Ethics approval

This article does not contain any studies with human participants or animals, other than nematodes, performed by any of the authors.

Declaration of generative AI in scientific writing

Grammar and spelling were checked using Grammarly Pro (https://www.grammarly.com/).

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Declaration of competing interest

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

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Data availability

The data generated during the current study are available from the corresponding author on reasonable request.

References

Abad, Z.G., Burgess, T.I., Bourret, T., Bensch, K., Cacciola, S.O., Scanu, B., Mathew, R., Kasiborski, B., Srivastava, S., Kageyama, K., Bienapfl, J.C., Verkleij, G., Broders, K., Schena, L., Redford, A.J., 2023. *Phytophthora*: taxonomic and phylogenetic revision of the genus. Stud. Mycol. 106, 259–348. https://doi.org/10.3114/sim.2023.106.05.

Amorim-Rodrigues, M., Brandão, R.L., Cássio, F., Lucas, C., 2024. The yeast *Wickerhamomyces anomalus* acts as a predator of the olive anthracnose-causing fungi, *Colletotrichum nymphaeae*, C. godetiae, and C. gloeosporioides. Front. Fung. Biol. 5, 1463860. https://doi.org/10.3389/ffunb.2024.1463860.

Barret, S., Rathbone, D., 2018. Long-term phosphite application maintains species assemblages, richness and structure of plant communities invaded by *Phytophthora cinnamomi*. Austral Ecol. 43, 360–374. https://doi.org/10.1111/aec.12574.

Bellat, J.-L., Dasque, J., Guen, B., Laranjo, J.G., Castelloti, T., Beccaro, G.L., Forget, L., 2022. EUROCASTANEA. European chestnut white paper 2022. https://issuu.com/areflh/docs/livre-blanc-2019-en-jl.

Boevink, P.C., Birch, P.R.J., Turnbull, D., Whisson, S.C., 2020. Devastating intimacy: the cell biology of plant-*Phytophthora* interactions. New Phytol. 228 (2), 445–458. https://doi.org/10.1111/nph.16650.

Bose, T., Spies, C.F.J., Hammerbacher, A., Coutinho, T., 2023. *Phytophthora*: an underestimated threat to agriculture, forestry, and natural ecosystems in Sub-Saharan Africa. Mycol. Prog. 22, 78. https://doi.org/10.1007/s11557-023-01926-0.

Bourbos, V.A., Metzidakis, I., 2000. Biological control of *phytophthora xcambivora* (Petri) Buisman in chestnut tree (*Castanea sativa* Mill.). Ecol. Mediterr. 26, 123–127. https://doi.org/10.3406/ecmed.2000.1896.

Brandano, A., Serra, S., Hardy, G.E.S.J., Scanu, B., 2023. Potassium phosphonate induces resistance in sweet chestnut against ink disease caused by *Phytophthora* species. Pathogens 12 (3), 365. https://doi.org/10.3390/pathogens12030365.

Brasier, C., Scanu, B., Cooke, D., Jung, T., 2022. *Phytophthora*: an ancient, historic, biologically and structurally cohesive and evolutionarily successful generic concept in need of preservation. IMA Fungus 13, 12. https://doi.org/10.1186/s43008-022-00097-z.

Brown, A.V., Brasier, C.M., 2007. Colonization of tree xylem by *Phytophthora ramorum, P. kernoviae* and other *Phytophthora* species. Plant Pathol. 56, 227–241. https://doi.org/10.1111/j.1365-3059.2006.01511.x.

Brown, M.S., Baysal-Gurel, F., Oliver, J.B., Addesso, K.M., 2019a. Comparative performance of fungicides, biofungicides, and host plant defence inducers in suppression of *Phytophthora* root rot in flowering dogwood during simulated root flooding events. Plant Dis. 103, 1703–1711. https://doi.org/10.1094/PDIS-09-18-1597-RE.

Brown, M.S., Baysal-Gurel, F., Oliver, J.B., Addesso, K.M., 2019b. Evaluation of fungicides and biofungicide to control *Phytophthora* root rot (*Phytophthora cinnamomi* Rands) and ambrosia beetles (*Coleoptera: curculionidae: scolytinae*) on flowering dogwoods exposed to simulated flood events. Crop Prot. 124, 104834. https://doi.org/10.1016/j.cropro.2019.05.028.

Caldwell, G.A., Wang, S.H., Naider, F., Becker, J.M., 1994. Consequences of altered isoprenylation targets on a-factor export and bioactivity. Proc. Natl. Acad. Sci. U. S. A. 91 (4), 1275–1279. https://doi.org/10.1073/pnas.91.4.1275.

Chan, C., Stephen, R.C., 1967. Intrahyphal hyphae in the genus *Linderina*. Can. J. Bot. 45, 1995–1998. https://doi.org/10.1139/b67-217.

Clark-Cotton, M.R., Jacobs, K.C., Lew, D.J., 2022. Chemotropism and cell-cell fusion in fungi. Microbiol. Mol. Biol. Rev. 86 (1), e0016521. https://doi.org/10.1128/mmbr.00165-21.

Crone, M., McComb, J.A., O'Brien, P.A., Hardy, G.E., 2013. Survival of *Phytophthora cinnamomi* as oospores, stromata, and thick-walled chlamydospores in roots of symptomatic and asymptomatic annual and herbaceous perennial plant species. Fungal Biol. 117 (2), 112–123. https://doi.org/10.1016/j.funbio.2012.12.004.

Czarnecka, M., Żarowska, B., Połomska, X., Restuccia, C., Cirvilleri, G., 2019. Role of biocontrol yeasts *Debaryomyces hansenii* and *Wickerhamomyces anomalus* in plants' defence mechanisms against *Monilinia fructicola* in apple fruits. Food Microbiol. 83, 1–8. https://doi.org/10.1016/j.fm.2019.04.004.

da Conceição, L.E., Saraiva, M.A., Diniz, R.H., Oliveira, J., Barbosa, G.D., Alvarez, F., Correa, L.F., Mezadri, H., Coutrim, M.X., Afonso, R.J., Lucas, C., Castro, I.M., Brandão, R.L., 2015. Biotechnological potential of yeast isolates from cachaça: the Brazilian spirit. J. Ind. Microbiol. Biotechnol. 42 (2), 237–246. https://doi.org/10.1007/s10295-014-1528-y.

da Cunha, A.C., Gomes, L.S., Godoy-Santos, F., Faria-Oliveira, F., Teixeira, J.A., Sampaio, G.M.S., Trópia, M.J.M., Castro, I.M., Lucas, C., Brandão, R.L., 2019. High-affinity transport, cyanide-resistant respiration, and ethanol production under aerobiosis underlying efficient high glycerol consumption by *Wickerhamomyces anomalus*. J. Ind. Microbiol. Biotechnol. 46 (5), 709–723. https://doi.org/10.1007/s10295-018-02119-5.

da Cunha, A.C., Santos, R.A.C.D., Riaño-Pachon, D.M., Squina, F.M., Oliveira, J.V.C., Goldman, G.H., Souza, A.T., Gomes, L.S., Godoy-Santos, F., Teixeira, J.A., Faria-Oliveira, F., Rosse, I.C., Castro, I.M., Lucas, C., Brandão, R.L., 2020. Draft genome sequence of *Wickerhamomyces anomalus* LBCM1105, isolated from cachaça fermentation. Genet. Mol. Biol. 43 (3), e20190122. https://doi.org/10.1590/1678-4685-GMB-2019-0122.

Davies, C.R., Wohlgemuth, F., Young, T., Violet, J., Dickinson, M., Sanders, J.-W., Vallieres, C., Avery, S.V., 2021. Evolving challenges and strategies for fungal control in the food supply chain. Fungal Biol. Rev. 36, 15–26. https://doi.org/10.1016/j.fbr.2021.01.003.

di Francesco, A., Milella, F., Mari, M., et al., 2017. A preliminary investigation into *Aureobasidium pullulans* as a potential biocontrol agent against *Phytophthora infestans* of tomato. Biol. Control 114, 144–149. https://doi.org/10.1016/j.biocontrol.2017.08.010.

Dorado, F.J., Alías, J.C., Chaves, N., Solla, A., 2023. Warming scenarios and *Phytophthora cinnamomi* infection in chestnut (*Castanea sativa* Mill.). Plants 12, 556. https://doi.org/10.3390/plants12030556.

Drenth, A., Sendall, B., Australian Centre for International Agricultural Research, ACIAR, 2001. Practical Guide to Detection and Identification of *Phytophthora*. CRC for Tropical Plant Protection, Brisbane Australia.

Ehrlich, M.A., Ehrlich, H.G., 1966. Ultrastructure of the hyphae and haustoria of *Phytophthora Infestans* and hyphae of *P. Parasitica*. Can. J. Bot. 44 (11), 1495–1503. https://doi.org/10.1139/b66-164.

Faoro, F., Faccio, A., Balestrini, R., 2022. Contributions of ultrastructural studies to the knowledge of filamentous fungi biology and fungi-plant interactions. Front. Fungal Biol. 2, 805739. https://doi.org/10.3389/ffunb.2021.805739.

Farkas, Z., Márki-Zay, J., Kucsera, J., Vágvölgyi, C., Golubev, W.I., Pfeiffer, I., 2012. Characterization of two different toxins of *Wickerhamomyces anomalus* (*Pichia anomala*) VKM Y-159. Acta Biol. Hung. 63 (2), 277–287. https://doi.org/10.1556/ABiol.63.2012.2.9.

Farley, J.F., Jersild, R.A., Niederpruem, D.J., 1975. Origin and ultrastructure of intra-hyphal hyphae in *Trichophyton terrestre* and *T. rubrum*. Arch. Microbiol. 106 (3), 195–200. https://doi.org/10.1007/BF00446523.

Fernandes, P., Colavolpe, M.B., Serrazina, S., Costa, R.L., 2022. European and American chestnuts: an overview of the main threats and control efforts. Front. Plant Sci. 13, 951844. https://doi.org/10.3389/fpls.2022.951844.

Ferraz, P., Brandão, R.L., Cássio, F., Lucas, C., 2021. *Moniliophthora perniciosa*, the causal agent of cacao *Witches' Broom Disease* is killed *in vitro* by *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* yeasts. Front. Microbiol. 12, 706675. https://doi.org/10.3389/fmicb.2021.706675.

Fonseca, T.F., Abreu, C.G., Parresol, B.R., 2004. Soil compaction and chestnut ink disease. For. Pathol. 34, 273–283. https://doi.org/10.1111/j.1439-0329.2004.00371.x.

Frascella, A., Sarrocco, S., Mello, A., Venice, F., Salvatici, C., Danti, R., Emiliani, G., Barberini, S., della Rocca, G., 2022. Biocontrol of *Phytophthora xcambivora* on *Castanea sativa*: selection of local *Trichoderma* spp. isolates for the management of ink disease. Forests 13 (7), 1065. https://doi.org/10.3390/f13071065.

Freimoser, F.M., Rueda-Mejia, M.P., Tilocca, B., Migheli, Q., 2019. Biocontrol yeasts: mechanisms and applications. World J. Microbiol. Biotechnol. 35, 154. https://doi.org/10.1007/s11274-019-2728-4.

Freitas, T.R., Santos, J.A., Silva, A.P., Fraga, H., 2021. Influence of climate change on chestnut trees: a review. Plants 10 (7), 1463. https://doi.org/10.3390/plants10071463. Erratum in: Plants (Basel), 2022. 11(11), 1518.

Fry, W.E., Birch, P.R., Judelson, H.S., Grunwald, N.J., Danies, G., Everts, K.L., Gevens, A. J., Gugino, B.K., Johnson, D.A., Johnson, S.B., McGrath, M.T., Myers, K.L., Ristaino, J.B., Roberts, P.D., Secor, G., Smart, C.D., 2015. Five reasons to consider *Phytophthora infestans* a reemerging pathogen. Phytopathology 105, 966–981.

Galiana, E., Fourré, S., Engler, G., 2008. *Phytophthora parasitica* biofilm formation: installation and organization of microcolonies on the surface of a host plant. Environ. Microbiol. 10 (8), 2164–2171. https://doi.org/10.1111/j.1462-2920.2008.01619.x.

García-Latorre, C., Rodrigo, S., Santamaria, O., 2022. Protective effects of filtrates from fungal endophytes on *Phytophthora cinnamomi* in *Lupinus luteus*. Plants 11, 1455. https://doi.org/10.3390/plants11111455.

Giovati, L., Ciociola, T., De Simone, T., Conti, S., Magliani, W., 2021. *Wickerhamomyces* yeast killer toxins' medical applications. Toxins 13 (9), 655. https://doi.org/10.3390/toxins13090655.

Godana, E.A., Edo, G.S., Yang, Q., Zhang, X., Zhao, L., Wang, K., Legrand, G., Ngea, N., Zhang, H., 2024. *Wickerhamomyces anomalus*: a promising yeast for controlling mold growth and diverse biotechnological applications. Trends Food Sci. Technol. 151, 104649. https://doi.org/10.1016/j.tifs.2024.104649.

Gow, N.A.R., Latgé, J.P., Munro, C.A., 2017. The fungal cell wall: structure, biosynthesis, and function. Microbiol. Spectr. 5 (3), 1–25. https://doi.org/10.1128/microbiolspec.FUNK-0035-2016.

Graham, L., Orenstein, J., 2007. Processing tissue and cells for transmission electron microscopy in diagnostic pathology and research. Nat. Protoc. 2, 2439–2450. https://doi.org/10.1038/nprot.2007.304.

Guzmán-Guzmán, P., Kumar, A., de los Santos-Villalobos, S., Parra-Cota, F.I., Orozco-Mosqueda, M.dC., Fadiji, A.E., Hyder, S., Babalola, O.O., Santoyo, G., 2023. *Trichoderma* species: our best fungal allies in the biocontrol of plant diseases - a review. Plants 12 (3), 432. https://doi.org/10.3390/plants12030432.

Hadwiger, L.A., McDonel, H., Glawe, D., 2015. Wild yeast strains as prospective candidates to induce resistance against potato late blight (*Phytophthora infestans*). Am. J. Potato Res. 92 (3), 379–386. https://doi.org/10.1007/s12230-015-9443-y.

Hagen, D.C., Sprague Jr., G.F., 1984. Induction of the yeast alpha-specific *STE3* gene by the peptide pheromone a-factor. J. Mol. Biol. 178 (4), 835–852. https://doi.org/10.1016/0022-2836(84)90314-0. PMID: 6436496.

Haïssam, J.M., 2011. *Pichia anomala* in biocontrol for apples: 20 years of fundamental research and practical applications. Antonie Leeuwenhoek 99 (1), 93–105. https://doi.org/10.1007/s10482-010-9541-2.

Hardham, A.R., 1987. Ultrastructure and serial section reconstruction of zoospores of the fungus *Phytophthora cinnamomi*. Exp. Mycol. 11 (4), 297–306. https://doi.org/10.1016/0147-5975(87)90018-1.

Hardham, A.R., Blackman, L.M., 2018. Pathogen profile update. *Phytophthora cinnamomi*. Mol. Plant Pathol. 19 (2), 260–285. https://doi.org/10.1111/mpp.12568.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I., Lorito, M., 2004. *Trichoderma* species - opportunistic, avirulent plant symbionts. Nat. Rev. Microbiol. 2 (1), 43–56. https://doi.org/10.1038/nrmicro797.

Hartl, L., Zach, S., Seidl-Seiboth, V., 2012. Fungal chitinases: diversity, mechanistic properties and biotechnological potential. Appl. Microbiol. Biotechnol. 93 (2), 533–543. https://doi.org/10.1007/s00253-011-3723-3.

Hashem, M., Alamri, S., 2009. The biocontrol of post-harvest disease (*Botryodiplodia theobromae*) of guava (*Psidium guajava* L.) by the application of yeast strains. Postharvest Biol. Technol. 53 (3), 123–130. https://doi.org/10.1016/j.postharvbio.2009.04.001.

Hemmes, D.E., Wong, L.D.S., 1975. Ultrastructure of chlamydospores of *Phytophthora cinnamomi* during development and germination. Can. J. Bot. 53 (24), 2945. https://doi.org/10.1139/b75-325, 2957.

Hohl, H.R., Hamamoto, S.T., 1967. Ultrastructural changes during zoospore formation in *Phytophthora parasitica*. Am. J. Bot. 4 (0), 1131. https://doi.org/10.2307/2440538, 1130.

Hong, S.H., Song, Y.S., Seo, D.J., Kim, K.Y., Jung, W.J., 2017. Antifungal activity and expression patterns of extracellular chitinase and β-1,3-glucanase in *Wickerhamomyces anomalus* EG2 treated with chitin and glucan. Microb. Pathog. 110, 159–164. https://doi.org/10.1016/j.micpath.2017.06.038.

Huberman, L.B., Murray, A.W., 2014. A model for cell wall dissolution in mating yeast cells: polarized secretion and restricted diffusion of cell wall remodelling enzymes induces local dissolution. PLoS One 9 (10), e109780. https://doi.org/10.1371/journal.pone.0109780.

Hyde, G.J., Gubler, F., Hardham, A.R., 1991. Ultrastructure of zoosporogenesis in *Phytophthora cinnamomi*. Mycol. Res. 95 (5), 577–591. https://doi.org/10.1016/S0953-7562(09)80072-5.

Ioannou, P., Baliou, S., Kofteridis, D.P., 2024. Fungemia by *Wickerhamomyces anomalus*-A narrative review. Pathogens 13 (3), 269. https://doi.org/10.3390/pathogens13030269.

Isaeva, O.V., Glushakova, A.M., Garbuz, S.A., Kachalkin, A.V., Chernov, I.Y., 2010. Endophytic yeast fungi in plant storage tissues. Biol. Bull. 37 (1), 26–34. https://doi.org/10.1134/S1062359010010048.

Joubert, P.M., Doty, S.L., 2018. Endophytic yeasts: biology, ecology, and applications. In: Pirttilä, A., Frank, A. (Eds.), Endophytes of Forest Trees. Forestry Sciences, second ed., vol. 86. Springer, Cham. https://doi.org/10.1007/978-3-319-89833-91.

Judelson, H.S., Blanco, F.A., 2005. The spores of *Phytophthora*: weapons of the plant destroyer. Nat. Rev. Microbiol. 3 (1), 47–58. https://doi.org/10.1038/nrmicro1064.

Jung, T., Jung, M.H., Cacciola, S.O., Cech, T., Bakonyi, J., Seress, D., Mosca, S., Schena, L., Seddaiu, S., Pane, A., di San Lio, G.M., Maia, C., Cravador, A., Franceschini, A., Scanu, B., 2017a. Multiple new cryptic pathogenic *Phytophthora* species from *Fagaceae* forests in Austria, Italy and Portugal. IMA Fungus 8 (2), 219–244. https://doi.org/10.5598/imafungus.2017.08.02.02.

Jung, T., Jung, M.H., Scanu, B., Seress, D., Kovács, G.M., Maia, C., Pérez-Sierra, A., Chang, T.-T., Chandelier, A., Heungens, K., van Poucke, K., Abad-Campos, P., Léon, M., Cacciola, S.O., Bakonyi, J., 2017b. Six new *Phytophthora* species from ITS Clade 7a including two sexually functional heterothallic hybrid species detected in natural ecosystems in Taiwan. Persoonia 38, 100–135. https://doi.org/10.3767/003158517X693615.

Jung, T., Orlikowski, L., Henricot, B., Abad-Campos, P., Aday, A.G., Aguín Casal, O., Bakonyi, J., Cacciola, S.O., Cech, T., Chavarriaga, D., Corcobado, T., Cravador, A., Decourcelle, T., Denton, G., Diamandis, S., Dogmus-Lehtijärvi, H.T., Franceschini, A., Ginetti, B., Green, S., Glavendekic, M., Hantula, J., Hartmann, G., Herrero, M., Ivic, D., Jung, M.H., Lilja, A., Keca, N., Kramarets, V., Lyubenova, A., Machado, H., Magnano di San Lio, G., Vázquez, P.J.M., Marçais, B., Matsiakh, I., Milenkovic, I., Moricca, S., Nagy, Z.Á., Nechwatal, J., Olsson, C., Oszako, T., Pane, A., Paplomatas, E.J., Pintos Varela, C., Prospero, S., Rial Martínez, C., Rigling, D., Robin, C., Rytkönen, A., Sánchez, M.E., Sanz Ros, A.V., Scanu, B., Schlenzig, A., Schumacher, S., Slavov, A., Solla, E., Sousa, J., Stenlid, V., Talgø, Z., Tomic, P., Tsopelas, A., Vannini, A.M., Vettraino, J., Wenneker, M., Woodward, S., Peréz-Sierra, A., 2016. Widespread *Phytophthora* infestations in European nurseries put forest, semi-natural and horticultural ecosystems at high risk of *Phytophthora* diseases. For. Pathol. 46, 134–163. https://doi.org/10.1111/efp.12239.

Khunnamwong, P., Lertwattanasakul, N., Jindamorakot, S., Suwannarach, N., Matsui, K., Limtong, S., 2020. Evaluation of antagonistic activity and mechanisms of endophytic yeasts against pathogenic fungi causing economic crop diseases. Folia Microbiol. 65, 573–590. https://doi.org/10.1007/s12223-019-00764-6.

Kim, K.W., Hyun, J.W., 2007. Nonhost-associated proliferation of intrahyphal hyphae of citrus scab fungus *Elsinoe fawcettii*: refining the perception of cell-within-a-cell organization. Micron 38 (6), 565–571. https://doi.org/10.1016/j.micron.2006.10.007.

Klis, F.M., Mol, P., Hellingwerf, K., Brul, S., 2002. Dynamics of cell wall structure in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 26 (3), 239–256. https://doi.org/10.1111/j.1574-6976.2002.tb00613.x.

Kuchen, B., Paroldi, E.H., Azcona, M.S., Groff, M.C., Pera, L., Vasquez, F., 2023. Evaluation of the application of *Wickerhamomyces anomalus* supernatant for the control of relevant spoilage yeasts in wines. OENO One 57 (1). https://doi.org/10.20870/oeno-one.2023.57.1.7165.

Kurjan, J., Herskowitz, I., 1982. Structure of a yeast pheromone gene (MF alpha): a putative alpha-factor precursor contains four tandem copies of mature alpha-factor. Cell 30 (3), 933–943. https://doi.org/10.1016/0092-8674(82)90298-7.

Lane, J.W., Grrison, R.G., Field, M.F., 1969. Ultrastructural studies on the yeastlike and mycelial phases of *Sporotrichum schenckii*. J. Bacteriol. 100 (2), 1010. https://doi.org/10.1128/jb.100.2.1010-1019.1969, 943.

Lanhuang, B., Yang, Q., Godana, E.A., Zhang, H., 2022. Efficacy of the yeast *Wickerhamomyces anomalus* in biocontrol of gray mold decay of tomatoes and study of the mechanisms involved. Foods 11 (5), 720. https://doi.org/10.3390/foods11050720.

Latgé, J.P., 2007. The cell wall: a carbohydrate armour for the fungal cell. Mol. Microbiol. 66 (2), 279–290. https://doi.org/10.1111/j.1365-2958.2007.05872.x.
Lim, L.L., Fineran, B.A., Cole, A.L.J., 1983. Ultrastructure of intrahyphal hyphae of *Glomus fasciculatum* (Thaxter) Gerdemann and Trappe in roots of white clover (*Trifolium repens* L.). New Phytol. 95 (2), 231–239. http://www.jstor.org/stable/2432695.
Ling, L., Tu, Y., Ma, W., Feng, S., Yang, C., Zhao, Y., Wang, N., Li, Z., Lu, L., Zhang, J., 2020. A potentially important resource: endophytic yeasts. World J. Microbiol. Biotechnol. 36, 110. https://doi.org/10.1007/s11274-020-02889-0.
Lopes-Bezerra, L.M., Walker, L.A., Niño-Vega, G., Mora-Montes, H.M., Neves, G.W.P., Villalobos-Duno, H., Barreto, L., Garcia, K., Franco, B., Martínez-Álvarez, J.A., Munro, C.A., Gow, N.A.R., 2018. Cell walls of the dimorphic fungal pathogens *Sporothrix schenckii* and *Sporothrix brasiliensis* exhibit bilaminate structures and sloughing of extensive and intact layers. PLoS Neglected Trop. Dis. 12 (3), e0006169. https://doi.org/10.1371/journal.pntd.0006169.
Louden, B.C., Haarmann, D., Lynne, A.M., 2011. Use of blue agar CAS assay for siderophore detection. J. Microbiol. Biol. Educ. 12 (1), 51–53. https://doi.org/10.1128/jmbe.v12i1.249.
Lourenço, D.A., Branco, I., Choupina, A., 2022. A systematic review about biological control of phytopathogenic *Phytophthora cinnamomi*. Mol. Biol. Rep. 49 (10), 9947–9962. https://doi.org/10.1007/s11033-022-07547-2.
Lowry, R.J., Sussman, A.S., 1966. Intra-hyphal hyphae in "clock" mutants of *Neurospora*. Mycologia 58 (4), 541–548. https://doi.org/10.1080/00275514.1966.12018345.
Lucas, C., Cássio, F., 2022. Antagonistic yeasts as biocontrol agents: a novel and safe alternative to chemical fungicides for controlling plant pathogens. In: Abd-Elsalam, K.A., Alghuthaymi, M.A. (Eds.), Biofungicides: Eco-Safety and Future Trends, vol. 5. CRC Press, Boca Raton, USA, pp. 94–143. https://doi.org/10.1201/9781003287575.
Luna, E.P.I., 2017. Endophytic yeast and host: a mutualistic association friendly to the environment, 2017. In: Lucas, C., Pais, C. (Eds.), Old Yeasts - New Questions, vol. 8. Intech Open, pp. 169–188. https://doi.org/10.5772/intechopen.70326.
Marzocchi, G., Maresi, G., Luchi, N., Pedori, F., Gionni, A., Longa, C.M.O., Pezzi, G., Ferretti, F., 2024. 85 years counteracting an invasion: chestnut ecosystems and landscapes survival against ink disease. Biol. Invasions 26, 2049–2062. https://doi.org/10.1007/s10530-024-03292-8.
Mélida, H., Sandoval-Sierra, J.V., Diéguez-Uribeondo, J., Bulone, V., 2013. Analyses of extracellular carbohydrates in oomycetes unveil the existence of three different cell wall types. Euk. Cell 12 (2), 194–203. https://doi.org/10.1128/EC.00288-12.
Michaelis, S., Barrowman, J., 2012. Biogenesis of the *Saccharomyces cerevisiae* pheromone a-factor, from yeast mating to human disease. Microbiol. Mol. Biol. Rev. 76 (3), 626–651. https://doi.org/10.1128/MMBR.00010-12.
Miller, C.W., Anderson, N.A., 1961. Proliferation of conidiophores and intrahyphal hyphae in *Aspergillus niger*. Mycologia 53, 433–436. https://doi.org/10.2307/3756587.
Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. J. Anal. Chem. 31, 426–428. https://doi.org/10.1021/ac60147a030.
Mullett, M.S., van Poucke, K., Haegeman, A., Focquet, F., Cauldron, N.C., Knaus, B.J., Jung, M.H., Kageyama, K., Hieno, A., Masuja, H., Uematsu, S., Webber, J.F., Brasier, C.M., Bakonyi, J., Heungens, K., Grünwald, N.J., Jung, T., 2023. Phylogeography and population structure of the global, wide host-range hybrid pathogen *Phytophthora xcambivora*. IMA Fungus 14, 4. https://doi.org/10.1186/s43008-023-00109-6.
Murtey, M.D., Ramasamy, P., 2016. Sample preparations for scanning electron microscopy – Life Sciences. In: Janecek, M., Kral, R. (Eds.), Modern Electron Microscopy in Physical and Life Sciences, vol. 8. https://doi.org/10.5772/61720, 2016.
Nally, M.C., Pesce, V.M., Maturano, Y.P., Rodriguez Assaf, L.A., Toro, M.E., Castellanos de Figueroa, L.I., Vazquez, F., 2015. Antifungal modes of action of *Saccharomyces* and other biocontrol yeasts against fungi isolated from sour and grey rots. Int. J. Food Microbiol. 204, 91–100. https://doi.org/10.1016/j.ijfoodmicro.2015.03.024.
Neupane, K., Alexander, L., Baysal-Gurel, F., 2022. Management of *Phytophthora cinnamomi* using fungicides and host plant defence inducers under drought conditions: a case study of flowering dogwood. Plant Dis. 106, 475–485. https://doi.org/10.1094/PDIS-04-21-0789-RE.
Ojika, M., Molli, S.D., Kanazawa, H., Yajima, A., Toda, K., Nukada, T., Mao, H., Murata, R., Asano, T., Qi, J., Sakagami, Y., 2011. The second *Phytophthora* mating hormone defines interspecies biosynthetic crosstalk. Nat. Chem. Biol. 7 (9), 591–593. https://doi.org/10.1038/nchembio.617.
Oztekin, S., Dikmetas, D.N., Devecioglu, D., Acar, E.G., Karbancioglu-Guler, F., 2023. Recent insights into the use of antagonistic yeasts for sustainable biomanagement of postharvest pathogenic and mycotoxigenic fungi in fruits with their prevention strategies against mycotoxins. J. Agric. Food Chem. 71 (26), 9923–9950. https://doi.org/10.1021/acs.jafc.3c00315.
Parafati, L., Cirvilleri, G., Restuccia, C., Wisniewski, M., 2017. Potential role of exoglucanase genes (WaEXG1 and WaEXG2) in the biocontrol activity of *Wickerhamomyces anomalus*. Microb. Ecol. 73 (4), 876–884. https://doi.org/10.1007/s00248-016-0887-5.
Parafati, L., Vitale, A., Restuccia, C., Cirvilleri, G., 2015. Biocontrol ability and action mechanism of food-isolated yeast strains against *Botrytis cinerea* causing post-harvest bunch rot of table grape. Food Microbiol. 47, 85–92. https://doi.org/10.1016/j.fm.2014.11.013.
Passoth, V., Fredlund, E., del Druvefors, U.Ä., Schnürer, J., 2006. Biotechnology, physiology and genetics of the yeast *Pichia anomala*. FEMS Yeast Res. 6 (1), 3–13. https://doi.org/10.1111/j.1567-1364.2005.00004.x.
Pérez-Girón, J.C., Álvarez-Álvarez, P., Díaz-Varela, E.R., Mendes Lopes, D.M., 2020. Influence of climate variations on primary production indicators and on the resilience of forest ecosystems in a future scenario of climate change: application to sweet chestnut agroforestry systems in the Iberian Peninsula. Ecol. Indic. 113, 106199. https://doi.org/10.1016/j.ecolind.2020.106199.
Pezzi, G., Lucchi, E., Maresi, G., Ferretti, F., Viaggi, D., Frascaroli, F., 2017. Abandonment or survival? Understanding the future of *Castanea sativa* stands in function of local attitude (Northern Apennine, Italy). Land Use Policy 61, 564–574. https://doi.org/10.1016/j.landusepol.2016.10.049.
Pretscher, J., Fischkal, T., Branscheidt, S., Jäger, L., Kahl, S., Schlander, M., Thines, E., Claus, H., 2018. Yeasts from different habitats and their potential as biocontrol agents. Fermentation 4, 31. https://doi.org/10.3390/fermentation4020031.
Punja, Z.K., Utkhede, R.S., 2003. Using fungi and yeasts to manage vegetable crop diseases. Trends Biotechnol. 21 (9), 400–407. https://doi.org/10.1016/S0167-7799(03)00193-8.
Rigling, D., Prospero, S., 2018. *Cryphonectria parasitica*, the causal agent of chestnut blight: invasion history, population biology and disease control. Mol. Plant Pathol. 19 (1), 7–20. https://doi.org/10.1111/mpp.12542.
Roces-Diaz, J.V., Díaz-Varela, E.R., Barrio-Anta, M., Álvarez-Álvarez, P., 2018. Sweet chestnut agroforestry systems in North-western Spain: classification, spatial distribution and an ecosystem services assessment. Forest Syst 27 (1), e03S. https://doi.org/10.5424/fs/2018271-11973.
Rovetto, E.I., la Spada, F., el Boumlasy, S., Taguali, S.C., Riolo, M., Pane, A., Cacciola, S. O., 2024. Biological control of green mold in simulated post-harvest chain of citrus fruit: efficacy of *Candida oleophila* strain O and molecular insight into elicitation of host immune system. Biol. Control 193, 105531. https://doi.org/10.1016/j.biocontrol.2024.105531.
Ruiz-Gómez, F., Miguel-Rojas, C., 2021. Antagonistic potential of native *Trichoderma* spp. against *Phytophthora cinnamomi* in the control of holm oak decline in dehesas ecosystems. Forests 12 (7), 945. https://doi.org/10.3390/f12070945.
Sandhya, C., Binod, P., Nampoothiri, K.M., Szakacs, G., Pandey, A., 2005. Microbial synthesis of chitinase in solid cultures and its potential as a biocontrol agent against phytopathogenic fungus *Colletotrichum gloeosporioides*. Appl. Biochem. Biotechnol. 127 (1), 1–15. https://doi.org/10.1385/abab:127:1:001.
Schwyn, B., Neilands, J.B., 1987. Universal chemical assay for the detection and determination of siderophores. Anal. Biochem. 160, 47–56. https://doi.org/10.1016/0003-2697(87)90612-9.
Seidel, D., Wurster, S., Jenks, J.D., Sati, H., Gangneux, J.P., Egger, M., Alastruey-Izquierdo, A., Ford, N.P., Chowdhary, A., Sprute, R., Cornely, O., Thompson 3rd, G. R., Hoenigl, M., Kontoyiannis, D.P., 2024. Impact of climate change and natural disasters on fungal infections. Lancet Microbe 5 (6), e594–e605. https://doi.org/10.1016/S2666-5247(24)00039-9.
Sellitto, V.M., Zara, S., Fracchetti, F., Capozzi, V., Nardi, T., 2021. Microbial biocontrol as an alternative to synthetic fungicides: boundaries between pre- and postharvest applications on vegetables and fruits. Fermentation 7, 60. https://doi.org/10.3390/fermentation7020060.
Sieber, B., Coronas-Serna, J.M., Martin, S.G., 2023. A focus on yeast mating: from pheromone signalling to cell-cell fusion. Semin. Cell Dev. Biol. 133, 83–95. https://doi.org/10.1016/j.semcdb.2022.02.003.
Sietsma, J.H., Eveleigh, D.E., Haskins, R.H., 1969. Cell wall composition and protoplast formation of some oomycete species. Biochim. Biophys. Acta 184 (2), 306–317. https://doi.org/10.1016/0304-4165(69)90033-6.
Sipiczki, M., 2023. Identification of antagonistic yeasts as potential biocontrol agents: diverse criteria and strategies. Int. J. Food Microbiol. 406, 110360. https://doi.org/10.1016/j.ijfoodmicro.2023.110360.
Sohail, M., Barzkar, N., Michaud, P., Jahromi, S.T., Babich, O., Sukhikh, S., Das, R., Nahavandi, R., 2022. Cellulolytic and xylanolytic enzymes from yeasts: properties and industrial applications. Molecules 27 (12), 3783. https://doi.org/10.3390/molecules27123783.
Solairaj, D., Legrand, N.N.G., Yang, Q., Zhang, H., 2020. Isolation of pathogenic fungi causing postharvest decay in table grapes and *in vivo* biocontrol activity of selected yeasts against them. Physiol. Mol. Plant Pathol. 110, 101478. https://doi.org/10.1016/j.pmpp.2020.101478.
Theodorakopoulos, N., Govetto, B., Industri, B., Massi, L., Gaysinski, M., Deleury, E., Mura, C., Marais, A., Arbiol, G., Burger, A., Gourgues, M., Panabières, F., Ponchet, M., Galiana, E., 2011. Biology and ecology of biofilms formed by a plant pathogen *Phytophthora parasitica*: from biochemical ecology to ecological engineering. Procedia Environ. Sci. 9, 178–182. https://doi.org/10.1016/j.proenv.2011.11.027.
Tomura, T., Molli, S.D., Murata, R., Ojika, M., 2017. Universality of the *Phytophthora* mating hormones and diversity of their production profile. Sci. Rep. 7 (1), 5007. https://doi.org/10.1038/s41598-017-05380-3.
Tyler, B.M., 2001. Genetics and genomics of the oomycete–host interface. Trends Genet. 17 (11), 611–614. https://doi.org/10.1016/s0168-9525(01)02517-3.
Vannini, A., Natili, G., Thomidis, T., Belli, C., Morales-Rodriguez, C., 2021. Anthropogenic and landscape features are associated with ink disease impact in Central Italy. For. Pathol. 51, 1–8. https://doi.org/10.1111/efp.12722.
Volynchikova, E., Kim, K.D., 2022. Biological control of oomycete soilborne diseases caused by *Phytophthora capsici, Phytophthora infestans,* and *Phytophthora nicotianae* in Solanaceous crops. MYCOBIOLOGY 50 (5), 269–293. https://doi.org/10.1080/12298093.2022.2136333.
Waterhouse, G.M., 1963. Key to the Species of Phytophthora De Bary. Mycol. Papers 92. Commonwealth Mycological Institute, Kew, Surrey, England. https://books.google.pt/books?id=fcz-xgEACAAJ.
Wessels, J.G.H., Sietsma, J.H., 1981. Fungal cell walls: a survey. In: Tanner, W., Loewus, F.A. (Eds.), Plant Carbohydrates II, Encyclopedia of Plant Physiology, 13/B. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-68234-615.

Woo, S.L., Hermosa, R., Lorito, M., Monte, H., 2023. *Trichoderma*: a multipurpose, plant-beneficial microorganism for eco-sustainable agriculture. Nat. Rev. Microbiol. 21, 312–326. https://doi.org/10.1038/s41579-022-00819-5.

Zhang, Y., van der Zee, L., Barberis, M., 2023. Two-way communication between cell cycle and metabolism in budding yeast: what do we know? Front. Microbiol. 14, 1187304. https://doi.org/10.3389/fmicb.2023.1187304.

Zhao, L., Wang, Y., Dhanasekaran, S., Guo, Z., Chen, S., Zhang, X., Zhang, H., 2021. Efficacy of *Wickerhamomyces anomalus* yeast in the biocontrol of blue mold decay in apples and investigation of the mechanisms involved. BioControl 66, 547–558. https://doi.org/10.1007/s10526-021-10088-5.

Zhu, M., Qiya, Y., Abiso, G.E., Yaning, H., Shiyao, H., Hongyin, Z., 2023. Efficacy of *Wickerhamomyces anomalus* in the biocontrol of black spot decay in tomatoes and investigation of the mechanisms involved. Biol. Control 186, 105356. https://doi.org/10.1016/j.biocontrol.2023.105356.