Fungi and their Environmental Micropredators

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Abstract

Predator–prey interactions are obvious drivers in the evolution among higher animals, but until today, there is little known about such relationships in the microverse. Like any other microbes, fungi can be prone to predation by bacteria, amoebae, or nematodes. While the boundaries between parasitism, biotrophy, and predation appear to be less sharp for bacteria, highly sophisticated mechanisms to feed on are obvious for unicellular amoebae and nematodes. Even phagocytic amoebae are not restricted to the ingestion and intracellular killing of single yeast cells or conidia but they can also attack and invade entire hyphae of filamentous fungi. Nematodes, in turn, can specifically open hyphae via injection-needle like stylets. In this chapter, we provide a selected overview of examples from recent years on how fungal preys exploit a variety of strategies to either escape or survive attacks by these specialized environmental predators. At least some of these factors have been shown to fulfil a dual function, serving in predator defence but also against innate immune cells when colonizing higher animals. Whether predatory defence has thus acted as a selection pressure towards virulence determinants in fungi is a matter of ongoing research.

Keywords

Amoeba · Predation · Phagocytosis · Virulence

9.1 Introduction

What is long known for higher eukaryotes becomes increasingly clear also on the microbial scale: Predator–prey relationships are abundant in natural environments and can impose selection pressure on both partners. More and more examples show that not only bacteria but also filamentous fungi and yeasts are exposed to micropredators. While insects and even vertebrates feed mainly on fungal fruiting bodies, this chapter will focus on micropredators, unseen

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bacteria, nematodes, and especially the ubiquitous amoebae that have adapted highly specific tools to find, engulf, or open fungal cells. While predatory interactions between fungi and eukaryotic micropredators were described half a century ago, laboratory- controlled bipartite interactions studies aiming to reveal the mechanistic cellular, biochemical, and molecular details were only initiated in the last 20 years. Apart from unique killing mechanisms on the predator side, these studies have also provided accumulating evidence that coevolution between the two partners has triggered fungal defence mechanisms that not only allow predator escape, but may also be exploited against immune cells and thus favour the emergence of traits considered to be virulence determinants in higher animals.

9.2 Fungal Micropredators

There are numerous examples for highly evolved mutualistic interactions between fungi and other taxonomic groups. The most famous are the highly complex symbioses in lichens or the formation of fungal roots called mycorrhizae and are known for several decades. Without any doubt, these cross-kingdom partnerships have driven the evolution of the fungal kingdom, but frequently occurring antagonistic interactions have also shaped the fungal tree. The two most simple antagonisms between two fungal species occur when one partner simply feeds on or acquires nutrients from other fungi in close proximity (Fig. 9.1). Such a lifestyle is considered necrotrophic when nutrients are derived from a dead fungal host cell. Necrotrophic mycoparasites are less specific to fungal prey and therefore exhibit a rather broad host range. Members of the *Trichoderma, Escovopsis, Tolypocladium,* and *Clonostachys* genera are the most common examples (Karlsson et al. 2017). When in contrast, a living organism is directly attacked, a fungal lifestyle becomes biotrophic and can be considered mycopredatory or mycoparasitic (Barnett and Binder 1973). Since biotrophic mycoparasites usually coevolve with their hosts, they generally exhibit a narrow host range and thus, rather few organisms have been studied in detail. *Ampelomyces quisqualis*, a natural fungal opponent of Erysiphales fungi, the causative agents of powdery mildew disease, is one of them (Németh et al. 2019). Such mycoparasites detect the existence of prospective hosts, move towards, and attach to them, followed by the frequent formation of coils around the prey to produce appressoria-like infectious structures that aid host penetration (Chet et al. 1981; Elad et al. 1983; Lu et al. 2004). Furthermore, numerous cell-wall disintegrating enzymes and antifungal metabolites are released throughout necrotrophic or biotrophic mycoparasitism to digest and destroy fungal hosts and utilize nutrients (Lu et al. 2004; Almeida et al. 2007; Druzhinina et al. 2012). In addition to fungal predation, the best-known examples of mycoparasites have the ability to facilitate plant growth through rhizosphere competence (Harman et al. 2004; Chatterton and Punja 2010; Dubey et al. 2014). Therefore, their use as an active ingredient in biocontrol formulations is not surprising. A prominent example of such a biocontrol agent (BCA) is *Coniothyrium minitans*, a fungal parasite whose conidia are used as a bio-fungicide against the white mould causing fungus *Sclerotinia sclerotiorum* (de Vrije et al. 2001).

A wide range of bacteria are also fungivorous and have the capability to feed on fungal cells (Boer et al. 2005; Fritsche et al. 2006). Like mycoparasites, bacteria exhibit three phenomenal mechanisms of acquiring nutrients, namely exo- and endocellular biotrophy and exocellular necrotrophy (Fig. 9.1). In exocellular biotrophic interactions, bacteria coexist with fungal hyphae or may colonize their surfaces to assimilate the nutrients released by active fungal cells. The synthesis of antibacterial compounds by fungal cells can either be tolerated or suppressed by biotrophs, and they may be able to modify fungal metabolism to facilitate the release of nutrients (Leveau and Preston 2008). However, endocellular mycoparasitic bacteria reside in the living fungal host and actively absorb the nutrients from the cytoplasm, such as *Burkholderia mallei* and *Burkholderia pseudomallei* (Levy et al. 2003).

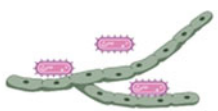
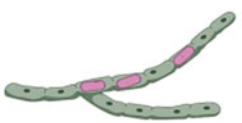
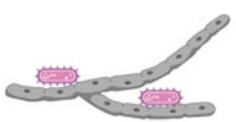
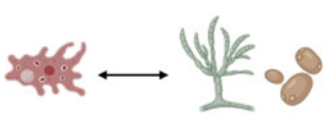
Parasitic and predatory interactions	Organisms	Description	Micropredators
Necrotrophic mycoparasitism		Nutrients are released from dead fungal host cell. Less specific to fungal prey, broad host range	*Trichoderma, Escovospsis, Tolypocladium, Clonostachys* (Karlsson et al. 2017)
Biotrophic mycoparasitism		Living fungal cell is directly attacked by another fungus. Mycoparasites usually coevolve with their host, therefor exhibit a narrow host range.	*Ampelomyces quisqualis* (Nemeth et al. 2019) *Coniothyrium minitans* (de Vrije et al. 2001)
Exocellular biotrophy		Bacteria coexist with or on the surface of fungi and utilise the nutrients released by the fungus. Often able to modify fungal metabolism.	*Pseudomonas aeruginosa* (Davis-Hanna et al. 2008; Mowat et al. 2010; Rella et al. 2012)
Endocellular biotrophy		Bacteria reside in the living fungal host and actively absorb nutrients from cytoplasm.	*Burkholderia mallei, B. pseudomallei* (Levy et al. 2003)
Exocellular necrotrophy		Bacteria release antifungal metabolites to kill their fungal prey and feed on them.	*Pseudomonas tolaasii* (Rainy et al. 1993; jolivet et al. 1999; Lo Cantore et al. 2006) *janthinobacterium agaricidamnosum* (Graupner et al. 2012)
Nematode predation		Mycophagous nematodes feed on fungi whereas, nematophagous fungi feed on nematodes using trapping systems.	*Aphelenchus avenae,* Bursaphelenchus okinawaensis, Caenorhabditid elegans (Zhang et al. 2020; Tayyrov et al. 2018; Fischer and Requena 2022)
Amoeba predation		Fungivorous amoeba feed predominantly on the fungal cells via phagocytosis or ruphocytosis.	*Acanthamoeba castellanii* (Goncalves et al. 2019; Steenbergen et al. 2004), *Allovahlkampfia spelaean, Vermamoeba vermiformis* (Albuquerque et al. 2019) *Protostelium aurantium* (Hillmann et al. 2018; Radosa et al. 2019; Ferling et al. 2020)

Fig. 9.1 Overview table of major micropredatory interactions among the fungal kingdom

In contrast, exocellular necrotrophic bacteria release antifungal metabolites to kill their fungal prey and feed on them (Leveau and Preston 2008). Actinobacteria, β-proteobacteria, and myxobacteria are only a few of the diverse bacterial groups that have been shown to necrotize fungal hosts (Boer et al. 2005). These mycophagous bacteria produce a variety of distinct hydrolytic enzymes, toxins, and antibiotics. Nevertheless, most of the bacterial–fungal interaction studies have identified low molecular weight bacterial toxins as the primary causative agents of fungal necrosis. *Pseudomonas tolaasii*'s toxin tolaasin is one of the prominent examples of such predation factors causing brown blotch disease in the button mushroom *Agaricus bisporus* (Rainey et al. 1993; Jolivet et al. 1999; Lo Cantore et al. 2006). Tolaasin's ability to disrupt the cell membrane by pore formation or biosurfactant activity has been extensively investigated by Jourdan and others (Scherlach et al. 2013; Jourdan et al. 2003). A similar mode of action has been shown for Jagaricin from *Janthinobacterium agaricidamnosum*, another membrane active toxin against a major causative agent in soft rot disease of mushrooms like *Agaricus bisporus* (Graupner et al. 2012). In addition to toxins, necrotizing bacteria may subsequently secrete an array of hydrolytic enzymes such as chitinases, lipases, glucanases, and proteases to disintegrate cell components and absorb fungal nutrients (Lorito et al. 1994; Fogliano et al. 2002; Woo et al. 2002; Someya et al. 2007). Moreover, biotrophic mycophagous bacteria are anticipated to modify three key aspects of fungal physiology, i.e., growth, membrane permeability, and metabolism (nutrient efflux) to feed on the fungal host (Leveau and Preston 2008). For example, *Pseudomonas aeruginosa* quorum sensing molecules (QSM) 3-oxo-C12 homoserine lactone (HSL) and 2-heptyl-3,4-dihydroxyquinoline have the ability to inhibit filamentation in *Candida albicans* (Davis-Hanna et al. 2008), biofilm formation in *Aspergillus fumigatus* (Mowat et al. 2010), and growth in *Cryptococcus neoformans* (Rella et al. 2012), respectively. This might help the bacteria to benefit from the fungal host.

Single cell eukaryotes from the kingdom Amoebozoa are dominant environmental micropredator that feeds on a variety of microorganisms, including fungi (Chakraborty et al. 1983; Old and Darbyshire 1978; Magnet et al. 2015). However, only few of them such as *Acanthamoeba castellanii*, *Allovahlkampfia spelaea, Protostelium aurantium,* and *Vermamoeba vermiformis* have been well documented as true fungal predators (Albuquerque et al. 2019; Tosetti et al. 2014; Hillmann et al. 2015; Radosa et al. 2019; Radosa et al. 2021).

Interestingly, these fungivorous amoebae also display distinct predatory tendencies based on the species and environment they live in. For example, *P. aurantium,* a widespread inhabitant of plant leaves, can feed on many members of *Candida* clade but not on any of those of the *Saccharomyces* clade (Radosa et al. 2019). Consequently, their role is crucial in determining the composition of the microbial communities in the environment (German et al. 2013; Siddiqui and Khan 2012). When considering that fungi and their amoeba predators may have interacted for millions of years it appears obvious that predatory selection pressure could have boosted the ramification of the fungal evolutionary tree.

The amoeba *P. aurantium* utilizes phagocytosis and ruphocytosis mechanisms to feed on the opportunistic human pathogenic yeast *Candida parapsilosis* and the filamentous fungus *A. fumigatus*, respectively. Following phagocytosis, the yeast cells of *C. parapsilosis* encounter oxidative stress and are lyzed in acidified phagolysosomes of *P. aurantium.* A similar mechanism of intracellular killing was observed for swollen conidia of *A. fumigatus*. However, before swelling, dormant conidia of *Aspergillus* and a variety of other fungi are covered by protective coats of amorphous polymers and hydrophobin proteins. These melanins and hydrophobin layers can mask the pathogen (or prey) associated molecular patterns (PAMPs), thereby protecting the dormant conidia from predation by *P. aurantium* and other amoebae (Ferling et al. 2020; Geib et al. 2016; Radosa et al. 2019; Radosa et al. 2021).

Germlings and hyphae have either none or drastically reduced melanin layers, allowing amoebae like *P. aurantium* to perforate the cell membrane of *A. fumigatus.* Germlings and hyphae are further invaded by the amoebae and the complete cytoplasmic fluid is resorbed within minutes. This mechanism was coined ruphocytosis (Fig. 9.2)., based on the Greek word *ρουφω,* "to suck", or "slurp" (Radosa et al. 2019).

The first observation of fungivorous amoeba dates back to the original discovery of a predation on *Cryptococcus neoformans* (Castellani 1930). *A. castellanii* can phagocytose a wide variety of fungal species, but feeding in laboratory conditions depends on environmental factors, more precisely on balance of divalent cations Mg^{2+} and Ca^{2+} (Fu and Casadevall 2018). The phospholipid-dependent expression of polysaccharide capsule in the yeast *C. neoformans* and temperature-mediated filamentous growth of dimorphic fungi also protect them from intracellular killing by *A. castellanii* (Steenbergen et al. 2004; Chrisman et al. 2011; Guimaraes et al. 2016). To further analyse the impact of fungal predation, Gonçalves and colleagues infected the wax moth *Galleria mellonella* with *C. neoformans*, *C. albicans*, *Paracoccidioides brasiliensis*, *Sporothrix brasiliensis*, *Histoplasma capsulatum,* and *Saccharomyces cerevisiae* cells recovered from amoeba and in all cases the larvae were killed quicker than controls (Gonçalves et al. 2019). Hence, predation studies with fungivorous amoebae such as *A. castellanii* and *P. aurantium* support the hypothesis that environmental interactions trigger the evolution of pathogenic traits or resistance to immune cells.

9.3 Dual-Use of Fungal Predatory Defence Mechanisms as Virulence Factors

Environmentally acquired human pathogenic fungi naturally occur as saprophytes involved in the processing of decayed organic matter, nutrient, and carbon recycling, intensely shaping the microbiome they are inhabiting. In this hostile milieu, they are constantly exposed to changing conditions such as temperature, light, oxygen, salinity, or humidity, as well as to competition for nutrients with other microorganisms and more importantly, they often represent a central dietary resource for soil protists, nematodes, or arthropods (Bahram and Netherway 2021).

In this respect, fungi have evolved sophisticated strategies to enhance their competitiveness for nutrient acquisition and to protect themselves against eukaryotic predators (Künzler 2018). In turn, predators continuously evolved new strategies to secure food from their preys. This process of an evolutionary arms race has widely been described for fungi and nematodes. The latter even includes fungivorous nematodes as well as nematophagous fungi (Zhang et al. 2020; Bleuler-Martínez et al. 2011; Tayyrov

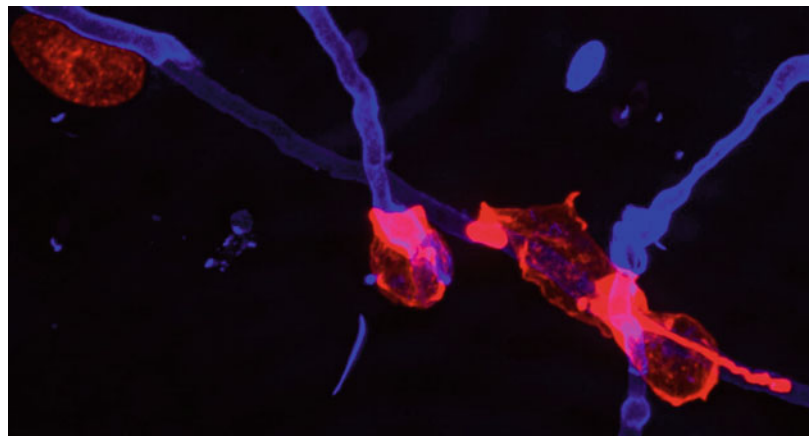


Fig. 9.2 Ruphocytic interactions of *Protostelium aurantium* with the filamentous fungus *Aspergillus fumigatus.* The amoebae attack and invade hyphal filaments. F-actin filaments of *P. aurantium* and chitin of the fungal cell wall are stained in red and blue, respectively

et al. 2018; Fischer and Requena 2022; Yang et al. 2020; Youssar et al. 2019). Both antagonists can contribute significantly to the immune status of surrounding plants and gain a lot of interest due to their possible role in biocontrol applications mainly in agriculture and forestry (Zhang et al. 2020).

For several human pathogenic fungi it has been demonstrated that some of these predator defence factors fulfil a so-called *dual-use* in the sense that they can be equally relevant for the survival in the natural environment as well as within the human host (Novohradská et al. 2017). This appears convincing since an infection of humans or mammals with a fungal conidium or an environmental yeast like *C. neoformans* seems to be only accidental and any pathogenic outcome depends almost exclusively on the host susceptibility. Most of the environmentally acquired pathogenic fungi like *A. fumigatus* or *C. neoformans* have defined ecological niches as decomposers of organic material in natural environments, such as in compost heaps or decaying wood and thus, lack the specific need for a human or animal host (Casadevall and Pirofski 2007). This idea has already been extensively studied in bacteria like *Legionella* spp. or *Mycobacterium* spp., classic example of intracellular pathogens, which can employ amoebae as reservoirs and exploit similar mechanisms during replication and survival in human phagocytes (Molmeret et al. 2005).

9.3.1 Melanins

The chemically diverse and irregularly structured polymers are among the most prominent examples of dual-use factors contributing to the defence against phagocytic immune cells and amoebae. An important role for melanins in protecting fungi against a variety of abiotic stresses is known for a long time and includes UV light protection, metal binding, energy harvesting, thermoregulation, protection against oxidative stress, but also inhibitory activities against microbial lytic enzymes and defensins, thereby enhancing the fungal survival in the environment (Heinekamp et al. 2013).

In *A. fumigatus*, green pigment dihydroxynaphthalene (DHN) melanin regulates host proinflammatory cytokine response by physically masking fungal PAMPs from immune recognition (Chai et al. 2010). During the germination of fungal conidia, the melanin layer is gradually degraded, exposing fungal PAMPs and allowing recognition by pathogen recognition receptors (PRRs). A pigment-less mutant of *A. fumigatus*, Δ*pksP*, was taken up at significantly higher rates by human macrophages than a respective wild type strain (Luther et al. 2007). Interestingly, similar outcomes have resulted from the interaction of *A. fumigatus* with amoeboid soil predator *Dictyostelium discoideum* (Hillmann et al. 2015). Here, fungal spores were efficiently phagocytosed by *D. discoideum*, but ingestion was higher when conidia were devoid of the DHN-melanin. What is more, the same study proved the co-occurrence of *A. fumigatus* and *Dictyostelids* sharing the same micro-habitats indicating that such predatory–prey interactions could be abundant in nature. The biosynthesis of DHN-melanin is, in fact, restricted to the fruiting bodies, suggesting its possible protective role against environmental predators.

Further, by dissecting the endocytic pathway using confocal microscopy, it was found that DHN-melanin highly reduced acidification of phagolysosomes of murine-derived alveolar macrophages, human monocyte-derived macrophages, human neutrophils, and epithelial cells (Thywißen et al. 2011; Amin et al. 2014). In *D. discoideum*, phagocytosis of fungal conidia was followed by the formation of the nascent phagosome, but here the phagolysosome was rapidly, but only transiently acidified, then neutralized, before the final exocytosis of the fungal spore. However, melanized conidia displayed an extended retention time within the phagocytic cells. As a repercussion, the swelling of the fungal spore induced enhanced phagolysosomal damage. At the same time, the membrane repair machinery of the amoeba was inefficiently recruited to the damage sites, thereby interfering with the canonical autophagy pathway of the amoeba (Ferling et al. 2020).

The cinnamon-brown conidia of the closely related *Aspergillus terreus* are known to be covered by non-ribosomally synthetized Asp-melanin. *A. terreus* lacks the highly conserved naphthopyrone synthase, and as a consequence, it is not able to inhibit phagolysosomal acidification in macrophages (Slesiona et al. 2012). Nevertheless, Asp-melanin accounts for resistance against UV light and even hampers the phagocytosis by amoeba *D. discoideum*, thus likely contributing to the survival of this fungus in its natural environment (Geib et al. 2016).

As a sign of convergent evolution, a comparable protective mechanism has also been engaged by the basidiomycetous yeast *C. neoformans*. Like in *A. fumigatus*, melanin of *C. neoformans* provides protection from ultraviolet light and scavenges ROS generated by phagocytes (Liu et al. 2021). *C. neoformans* has long been studied as a typical example of an environmentally acquired fungal pathogen whose virulence determinants for humans have evolved as a consequence of selection pressure imposed by amoeboid predators (Derengowski Lda et al. 2013). Striking parallels in phagocytic processing by amoeba can be found when comparing to fungus–macrophage interactions (Gaylord et al. 2020). Melanin and polysaccharide capsules (mentioned later) are major virulence factors of *C. neoformans*. When phagocytosed by the amoeba *Acanthamoeba castellanii*, both melanized and non-melanized strains survived and replicated inside of the host cell which has led to the death of amoeba cells. However, a difference in survival was observed when cells devoid of melanin also lacked a capsule. These cells were rapidly killed (Steenbergen et al. 2001). Hence, melanin significantly contributes to the resistance against amoeba predation, but the capsule seems to play an even more important role in this interaction.

9.3.2 Capsule

The characteristic polysaccharide capsule of *C. neoformans* consists of two large repeating polymers, glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal). Although the capsule itself is weakly immunogenic, it masks the underlying cell wall PAMPs, such as β-glucans, α-mannans, and chitin, from the recognition by the innate immune system (Gaylord et al. 2020).

Incubation of *C. neoformans* with *A. castellanii* elicited an approximately four-fold increase in the cryptococcal capsular volume compared to yeast cells grown in the absence of amoeba (Chrisman et al. 2011). Similar capsular enlargement has been observed when yeast cells were confronted with macrophages. Environmental strains of *C. neoformans* have in general smaller capsule, in contrast with the situation found during infection whereby cells with very large capsules are commonly found in mammalian tissue (Zaragoza and Casadevall 2004). While in human cells, the capsular enlargement arises from high CO_2 and iron deprivation, in amoeba, this effect occurs in response to phospholipids of amoeba origin. *C. neoformans* can cause enzymatic damage to the cell membrane of amoebae and macrophages thus releasing membrane phospholipids. These are subsequently cleaved by fungal extracellular phospholipase B, releasing their polar heads that are in turn sensed by *C. neoformans*, triggering capsule enlargement and the formation of giant cells (Chrisman et al. 2011).

9.3.3 Phospholipase

Phospholipase activity is another important virulence factor and a factor of dual-use in *Cryptococcus* survival. Phospholipase B-deficient mutant, Δ*plb1*, was found to be dramatically impaired in virulence in both the mouse inhalational model and the rabbit meningitis model. Furthermore, Δ*plb1* strains exhibited a growth defect in a J774 macrophage-like cell line (Cox et al. 2001).

This mutant was also unable to growth in the presence of the amoeba *A. castellanii,* suggesting a significance of this virulence factor in

environment as well as during infection (Steenbergen et al. 2001).

In addition, phospholipase B seems to be implicated in the process of non-lytic exocytosis, also known as non-lytic phagosomal extrusion or vomocytosis, as mutants deficient in Plb1 secretion are impaired in non-lytic exocytosis (NLE) (Gaylord et al. 2020). Identical mechanisms of WASH-mediated constitutive exocytosis and non-lytic exocytosis are employed by the fungus to escape both amoeba and human macrophages (Watkins et al. 2018; Ma et al. 2006).

9.3.4 Mannoprotein Coat

In a first line of fungal defence stands the hiding from this recognition, and fungi have, in this respect, covered their PAMPs under the thick layers of cell wall composites, such as aforementioned capsule in *C. neoformans*. In the human commensal *Candida albicans,* the functional role of the capsule is at least partially fulfilled by the presence of a thick surface coating with mannoproteins (Erwig and Gow 2016).

Over the past decade, it has clearly been demonstrated that the mannoprotein coat is essential for *C. albicans* to escape the recognition of innate immune cells (McKenzie et al. 2010; Bain et al. 2014). The uptake and phagocytosis by macrophages were significantly reduced in mutants defected in phosphomannan biosynthesis and *O*- and *N*-linked mannosylation.

However, only lately, the role of this protective coat has been proven to be effective also in the context of the fungivorous amoeboid predator *Protostelium aurantium* (Radosa et al. 2019). Treatment with sub-inhibitory concentrations of caspofungin or mannosidase turned the previously unrecognizable *C. albicans* to a readily ingested food source.

In accordance with this, two mannose-binding proteins on the surface of *A. castellanii* have been identified to mediate the recognition of the fungal prey (Gonçalves et al. 2019). Besides, the interaction between *A. castellanii* and several fungi was inhibited by the addition of mannose, while no inhibition was observed with N-acetylgalactosamine (GalNac) and galactose.

9.3.5 Reactive Oxygen Species

Apart from recognition-shielding function, melanin and capsule structures on the surface of many fungi were thought to be antioxidants against host reactive oxygen and nitrogen species (ROS, RNS) (Zaragoza et al. 2008; Liu et al. 2021). However, the idea that melanins would serve a direct protective role against ROS was recently challenged for DHN-melanin of *A. fumigatus*, as conidia of mutants lacking DHN-melanin biosynthesis were as sensitive to ROS as melanized wild-type conidia (Keizer et al. 2022). Reactive oxygen species, such as superoxide ($O2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are by-products of normal aerobic metabolism, and together with more reactive singlet oxygen($^1O_2$), peroxide radical ($HO^{\bullet}{}_2$), peroxide ion ($HO^{-}{}_2$), hydroxyl radical ($HO^{\bullet}$), and nitric oxide ($NO^{\bullet}$) cause DNA damage, protein inactivation, and lipid peroxidation via oxidation of iron sulphur centre and cysteine residues in virtually any cell molecule (Aguirre et al. 2006; Gessler et al. 2007). Cells, in turn, evolved a number of mechanisms to reduce the intracellular ROS levels, like superoxide dismutases, catalases, peroxidases, glutathione peroxidases, or peroxiredoxins. Activation of this antioxidant response is not only an important virulence trait, but also many fungi produce and handle ROS as a signal transduction mechanism in multicellular development. In turn, innate immune cells may exploit ROS to activate the fungal apoptosis-like pathway, thereby killing ingested conidia of the human pathogen *A. fumigatus* (Shlezinger et al. 2017). Besides immune cells, the generation of ROS as a key component of cell autonomous defence against various pathogens has also been observed for environmental predators, such as insects (Bergin

et al. 2005), nematodes (McCallum and Garsin 2016), or amoebae (Dunn et al. 2018).

The insect immune response possesses a multitude of structural and functional similarities to the innate immune response of mammals. Haemocytes of the greater wax moth *G. mellonella* were found to be capable of phagocytosing *C. albicans* cells in a manner similar to human neutrophils via the production of superoxide (Bergin et al. 2005). The study further identified several proteins being homologous to the NADPH oxidase complex of human neutrophils.

The innate immune response of a nematode *Caenorhabditis elegans* is also accompanied by an increase of reactive oxygen species. The genome of *C. elegans* encodes for two NOX family enzymes, however ROS production has been functionally characterized only for the BLI-3 enzyme. BLI-3 generated ROS play an important defensive role against both epidermal and intestinal fungal infection (McCallum and Garsin 2016).

The amoeba *D. discoideum* encodes for three NADPH oxidase catalytic subunits, i.e. noxA to noxC, with NoxA and NoxB being even homologous of the mammalian gp91phox subunit (Lardy et al. 2005). In co-incubation experiments with *A. fumigatus,* ROS production coincides with neutralization of *A. fumigatus*-containing phagolysosomes, as previously shown for dendritic cells, having a proposed function in DHN-melanin degradation (Ferling et al. 2020).

In another example, the fungivorous amoeba *P. aurantium* directly targets cell redox homeostasis upon predation on the yeast *Candida parapsilosis* (Radosa et al. 2021). Yeast cells were exposed to host ROS shortly after uptake and resided within an acidic compartment, a process widely conserved in phagocytic cells of immune system.

These studies suggest that soil fungi have been constantly exposed to the ROS generated by their environmental predators and conversely, must have evolved strategies to counter this biotic stress. Further functional genomics of *C. parapsilosis* confronted with *P. aurantium* revealed the highly expressed *PRX1* gene in *C. parapsilosis*, encoding a thioredoxin-linked peroxidase accountable for cell redox homeostasis and response to oxidative stress (Radosa et al. 2021). A *C. parapsilosis PRX1* mutant was found to be not only impaired in the growth when subjected to organic hydroperoxide but also displays lower survival rate when confronted with amoeba as well as primary macrophages.

Finally, however, the production of reactive oxygen species by fungi as a direct response to environmental predators has not been clearly observed yet. Even more, it seems plausible that host-derived ROS act rather as a signal molecule in phagosome maturation (Ferling et al. 2020).

9.3.6 Nutritional Immunity

Every living organism requires a trace amount of metals like copper, iron, zinc, and manganese for its cellular functions. In human pathogenic fungi, maintenance of metal homeostasis has been extensively studied as a possible virulence factor, since many host cells, as a form of defence mechanism, actively sequestrate the amount of these micronutrient for the pathogen availability—a process collectively called nutritional immunity (Malavia et al. 2017).

The mechanisms how fungi deal with the lack of trace metals seems to be highly adapted to the environment in which they have evolved. Environmentally acquired fungi like *A. fumigatus* have developed more generic siderophore systems to acquire the iron, unlike human-adapted fungi like *C. albicans*, which relies on mechanisms to obtain iron from host-specific sources like heme or ferritin (Siscar-Lewin et al. 2022).

Amoeba cells are able to modulate zinc and copper homeostasis in an effort to attenuate microbial pathogenicity. This process has been described for bacterial cells, in fact, the same strategy has been now observed in fungi (German et al. 2013; Hao et al. 2016).

The acidic environment of amoebal phagolysosomes moreover increases the protease activity and enhances the toxicity of trace metals such as Cu(I) and Zn(II), and respectively

decreases their bioavailability. Transcriptional profiling of *C. neoformans* during ingestion by *A. castellanii* and macrophages revealed a common set of upregulated genes involved in nutrient uptake (Derengowski Lda et al. 2013). An experimental study of Ribeiro et al. provided further evidence that amoebae can reduce the zinc availability for *Cryptococcus gattii* in order to reduce fungal proliferation (Ribeiro et al. 2017). In parallel, the P1-type copper exporter CRP1 of *C. parapsilosis* was found to be the highest upregulated gene in response to amoeba predation, whereas an orthologue to CTR1 copper importer was the second most downregulated gene (Radosa et al. 2021). Additionally, sensitivity of the *C. parapsilosis* deletion mutant *Δ/Δcrp1* towards high copper concentrations was only apparent when cells were exposed to high Cu at acidic pH, suggesting that intoxication of fungal cells inside of the phagosomal compartments represents an evolutionary antimicrobial activity evolved by phagocytic cells.

9.3.7 Production of Secondary Metabolites and Peptides

Production of secondary metabolites and peptides (either ribosomally or non-ribosomally synthesized) acts as a direct killing factor and main chemical defence of fungi.

These bioactive compounds are involved in numerous inter-species interactions such as communication, chemical defence, endosymbiotic relationship, or pathogenicity (Boysen et al. 2021).

Interestingly, it has been proposed that the effector molecules directed against microbial competitors are secreted by fungal hyphae or mycelium, whereas molecules against metazoan predators are usually stored intracellularly within the fungal hyphae or in the fruiting bodies and are taken up during predation (Künzler 2018). Gliotoxin, aflatoxin B1, cyclosporin A, lovastatin, or penicillin are all autonomously secreted examples of fungal defence effectors, naturally meant to target either fungi, bacteria, or insects living in close proximity and competing for the same source of nutrients. Several secondary metabolites have been already identified to act effectively against environmental predators, possessing either amoebicidal or insecticidal activity (Boysen et al. 2021). For example, the model mushroom *Coprinopsis cinerea* produces a mycelium-localized toxic compound upon challenge with the fungivorous nematode *Aphelenchus avenae* (Plaza et al. 2016). Moreover, the functional analysis identified a specific induction of several genes encoding previously characterized nematotoxic lectins with high toxicity towards the bacterivorous nematode *C. elegans*, and several putative antibacterial proteins (Plaza et al. 2016).

One of the best known fungal secondary metabolite is aflatoxin, produced mainly by filamentous fungus *Aspergillus flavus*, contaminating grain, seeds, spices, edible nuts, and even milk. Aflatoxin is extremely hepatocarcinogenic in humans, further causing immune suppression, acute aflatoxicosis, and underdevelopment in small children (Wild and Gong 2009). However, not all strains of *A. flavus* produce aflatoxin, and it has been shown that selection for aflatoxin production is driven by interaction with insects (Drott et al. 2017). Aflatoxin greatly reduced the fitness of *Drosophila melanogaster* and the mortality of its larvae increased when infected with toxicogenic fungal strains, in comparison to non-toxicogenic strains. Interestingly, toxicogenic strains showed even slightly higher fitness during the larvae infection but not in the absence of larvae. Moreover, the addition of external aflatoxin greatly increased fungal fitness but only in the presence of *D. melanogaster* larvae. These data suggest that aflatoxin production is selected for through an interaction with insects and the cost of aflatoxin production in the absence of susceptible insects will favour non-toxigenic isolates (Drott et al. 2017).

The dual-use of cyclosporin A produced by *Tolypocladium inflatum* has been shown, as it blocks a crucial step in calcium dependent signal transduction in T-cells via inhibition of calcineurin, resulting in immunosuppression, but at the same time it can also suppress insect

defence cells (Bushley et al. 2013) and poses even antifungal properties (Cruz et al. 2000).

Gliotoxin is a non-ribosomal peptide derived toxin of several fungal genera such as *Aspergillus, Penicillium, Trichoderma,* and *Leptosphaeria*, whose production is furthermore tightly regulated by a plethora of exogenous biotic and abiotic factors (Boysen et al. 2021). Together with the spore-borne toxin trypacidin and meroterpenoid fumagilin, these secondary metabolites are not only cytotoxic to humans but play parallel roles in natural environment against amoeboid predators such as *D. discoideum* and *Entamoeba histolytica*, or against eukaryotic parasites such as *Trypanosoma* and *Plasmodium* (Hillmann et al. 2015; Mattern et al. 2015; Arico-Muendel et al. 2009).

Spore diffusates of non-germinated *A. fumigatus* conidia, of both clinical and environmental isolates, inhibited certain functions of neutrophil phagocytes as well as the growth of the amoeba *Naegleria gruberi* (Hobson 2000), suggesting that dormant conidia might have to defend against phagocytic predators.

In this sense, it is worth mentioning that co-cultivation experiments of fungi with other species often lead to the activation of silent gene clusters and thus, to the discovery of new metabolites with antimicrobial properties (Rutledge and Challis 2015). For example, grazing by larval, *D. melanogaster* induced the expression of several putative resistance genes in *Aspergillus nidulans*, including the secondary metabolite master regulator LaeA (Caballero Ortiz et al. 2013). *A. nidulans*, in fact, relies on secondary metabolite production to avoid predation by the fungivorous springtail *Folsomia candida*. Moreover, arthropod-induced production of secondary metabolites further accounted for a fungal phenotype that showed enhanced resistance to fungivory (Döll et al. 2013).

9.3.8 Filamentous Growth

A key component of fungal virulence is admittedly phenotypical switching from yeast to hyphae and filamentous growth. Despite the large number of fungal species that have the ability to grow filamentously, only a few of them switch to hyphae upon infection. These include *C. albicans*, *Candida dubliniensis*, *Malassezia* spp., or *Trichophyton rubrum* (Brand 2012). In the case of *C. albicans*, the combination of body temperature and serum is a key activator of hyphae formation, suggesting the adaptation of this fungus to commensal lifestyle. In fact, it seems very reasonable that the mechanism of hyphae formation in many fungi serve other purposes, such as substrate adherence, translocation between environments, nutrient acquisition, but induced filamentation or even the formation of biofilms may again serve a dual use to defend against natural predators.

Several dimorphic fungi, such as *Histoplasma capsulatum, Blastomyces dermatitidis,* and *Sporothrix schenckii* switch to a hyphae formation upon interaction with the amoeba *A. castellanii* occurring even under the condition where the yeast form in preferred (Steenbergen et al. 2004). *C. albicans* forms filaments when ingested by nematode *C. elegans* (Breger et al. 2007). Hyphae of the filamentous fungus *A. fumigatus* are directly targeted by fungivorous amoeba *P. aurantium* via "ruphocytosis", a strategy of amoeba to perforate the tip of the hyphae, invade it, and feed on the cytoplasmic content (Radosa et al. 2019).

An incubation of *D. discoideum* with non-filamentous *C. albicans* mutants defected in the hyphal growth, such as Δ*cph1*/Δ*efg1* or Δ*hgc1* mutant, resulted in reduced resistance towards amoeba predation. Besides, co-incubation with the amoeba at 22 °C induced the hyphae formation in *C. albicans* wild type even without the presence of any known hyphae-inducing substance in the medium in the contact-independent manner (Koller et al. 2016).

9.3.9 Dual-Use Virulence Factors of Phytopathogenic and Entomopathogenic Fungi

The number of studies concerning tripartite interaction of soil microorganism as an evolutionary

driver underlying the acquisition of virulence traits and establishment of endosymbiosis between fungi and bacteria is recently increasing. The phytopathogenic mucoromycete *Rhizopus microsporus* was found to harbour different bacterial endosymbionts that are necessary for full fungal virulence and serve the fungal host in fending off protozoan and metazoan predators (Itabangi et al. 2022; Richter et al. 2022).

The endosymbiotic beta-proteobacterium *Mycetohabitans rhizoxinica*, residing within the fungal hyphae, produces the extremely potent toxin rhizoxin. At least one ecological role of this phytotoxin seems to be the inhibition of microtubule polymerization in fungal micropredators, such as the amoeba *P. aurantium* and nematodes like *Aphelenchus avenae*, and thereby protecting the host fungus from these two fungivores (Richter et al. 2022).

This strategy to defend against environmental micropredators seems to be a common trait for Mucormycota fungi, since all toxic compounds identified in Mucormycota fungi are produced by their bacterial endosymbionts. On the other hand, the important plant-growth promoting fungus *Mortierella verticillata* forms an endosymbiotic relationship with the toxin-producing bacterium *Mycoavidus necroximicus*, which protects the fungus, and thereby also the plant, from nematode attacks (Büttner et al. 2021).

A so far unknown compound is also produced by endosymbiotic bacterium *Ralstonia pickettii* of *R. microsporus*. Conditioned media from endosymbiont-containing fungal cultures inhibited antiphagocytic activity of macrophages as well as of *D. discoideum* amoeba. Besides, the presence of the endosymbiotic bacterium was necessary for the virulence of *R. microsporus* in the zebrafish model (Itabangi et al. 2022).

Last but not least, even the virulence of entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* could be explained as a consequence of fungal adaptation to avoid amoeba grazing (Bidochka et al. 2010). As mentioned earlier, phagocytic haemocytes, circulating in the hemolymph of insect, are analogous to mammalian macrophages. Interestingly, the authors further proposed the idea, that the ability of fungi to avoid amoeboid predators seems to be restricted to zoopathogenic fungi, including the invertebrate pathogens, potentially due to the absence of phagocytic cells that would reinforce such an adaptation.

Hence, studies on fungal interactions with non-mammalian phagocytes shed the light on the origin and evolution of fungal virulence determinants. Predator-induced resistance to fungivory therefore significantly contributes to the understanding of the evolutionary distinct strategies of fungal pathogenicity.

9.4 Experimental Efforts on Evolution of Fungal Virulence

The major fungal lineages are very ancient and have emerged approximately 1.5–two million years ago, long before the appearance of innate immunity, which is estimated to have emerged 300–400 million years ago (Hedges et al. 2004). From over 3–five million fungal species, approximately only 500 are able to cause disease in humans (Siscar-Lewin et al. 2022). Thus, to establish the infection or commensal stage within the human host, fungi must have already been endowed with the ability to counteract with innate immune system. These properties they have either acquired earlier during the common evolution in the environment or lately by within the host selection.

A commonly accepted idea for the evolution of microbial virulence, known as continuum hypothesis, proposes that pathogens should always evolve towards avirulence or reduced virulence, especially if there is a higher possibility for vertical transmission. However, when there is a trade-off between virulence and vertical transmission, selection may favour horizontal transmission and higher virulence. In other words, virulence factors are maintained to support the exploitation, proliferation, and transmission of the parasite (Alizon et al. 2009). This seems to be typical for fungal plant pathogens. Less virulent fungal strains displayed a lower rate for vertical transmission, probably due to their slow

growth, thereby providing enough time for more chemical defences to be mobilized by the plant before fungal hyphae reached the seed. *Atkinsonella hypoxylon* is a fungal pathogen of the grass *Danthonia compressa*. Fully virulent strains reduce fitness of the grass by 50% or more by castrating the inflorescence, despite reducing its own change for vertical transmission by seeds. Less virulent strains of *A. hypoxylon* are causing less damage to the plant inflorescence but interestingly, those have only rarely been found in the nature (Kover and Clay 1998). Since the 90 s, the "trade-off" hypothesis has been significantly challenged, suggesting that the trade-offs exist, but may not be as simple as it was usually considered. Consequently, at least two alternative hypotheses have been proposed to explain the evolution of microbial virulence: the "short-sighted" and the "coincidental" hypothesis.

The coincidental evolution hypothesis states that factors responsible for virulence were not generated for virulence per se, but rather to increase the fitness of the pathogen in a non-parasitic context (Levin and Svanborg Edén 1990; Adiba et al. 2010). This idea was strongly supported by a series of microevolution experiments, in which continuous passage of an avirulent or low virulent strain of a certain pathogen through an environmental predator, would subsequently led to the generation of such a trait, that would increase the resistance of fungus in either murine infection or enhance the survival when confronted with innate immune cells.

The avirulent strain of *Histoplasma capsulatum*, CIB 1980, was passaged with and without amoeba *A. castellanii* and then used to infect mice. While no colony-forming units were recovered from mice infected with non-passaged strain, mice infected with amoeba-passaged strain suffered from persistent fungal infection. Exposure of *H. capsulatum* to *A. castellanii,* moreover resulted in an increase in hyphal cells (Steenbergen et al. 2004). Similarly, yeast cells of *H. capsulatum* are under normal condition, not able to kill the larvae of *G. mellonella*. However, the same cells recovered from an *A. castellanii* interaction, killed 100% of larvae within 6 days. A similar enhanced virulence phenotype against *Galleria* larvae after co-culture with *A. castellanii* was observed for the dimorphic fungi *Sporothrix brasiliensis* and *Paracoccidioides brasiliensis* as well as for *C. neoformans, C. albicans,* and *S. cerevisiae* (Gonçalves et al. 2019).

An encapsulated strain of *C. neoformans* increased its virulence by the formation of a larger capsule and faster time to melanization when continuously incubated with *D. discoideum* (Steenbergen et al. 2003). Even here, the mice infected with *C. neoformans* grown with live *D. discoideum* had significantly shorter survival time than mice infected with *C. neoformans* grown either alone or in the presence of dead amoeba. Furthermore, this study also proved the concept that even an avirulent strain can turn out to be virulent in a susceptible host, as the incubation of acapsular *C. neoformans* with amoeba impaired in phagocytosis resulted in infection.

Interestingly, exposing the environmental isolates of *C. neoformans* to another amoeba *A. castellanii* over the prolonged period resulted in complex pleiotropic changes such as pseudohyphal growth, larger capsule thickness, increased urease activity, enhanced macrophage toxicity but reduced melanin production. However, this in turn was accompanied by less virulence in mice because the strain elicited a strong antifungal immune response (Fu et al. 2021). Variations in phenotypic and genetic changes between evolved strains suggested that the microevolution happens frequently and rapidly when exposed to amoeba.

In another study, previous interaction of *C. neoformans* with *A. castellanii* generated fungal cells more efficient in killing the wax moth *G. mellonella* and more resistant to oxygen- and nitrogen-derived molecular species. Here, increased urease activity and amoeba-induced changes in cell wall architecture accounted proposedly for the enhanced survival in this invertebrate model (Rizzo et al. 2017).

The thermodimorphic fungus *Paracoccidioides* spp., endemic to Latin America, is a causative agent of a neglected tropical disease called paracoccidioidomycosis despite the fact for its life cycle does not need to infect humans or other animals. Cultivation of soil

samples revealed the presence of numerous amoeba species, such as *Allovahlkampfia spelaea*, *Vermamoeba vermiformis,* and *Acanthamoeba* sp. All of these efficiently ingested and killed yeast cells of *P. brasiliensis*. Later, a sequential co-cultivation of *Paracoccidioides* with *A. castellanii* selected for the induction of virulence transcripts and accumulation of cell wall alpha-glucans, polysaccharides that mask the recognition of fungal PAMPs. Survived fungal cells were further confronted with amoeba and J774 murine macrophages and used to infect BALB/c mice and *G. mellonella.* When compared to non-passaged cells, the proportion of dead amoeba increased and the number of fungal colony-forming units was significantly higher in both amoeba and murine macrophages. Additionally, evolved strains were also able to kill both animal models significantly faster (Albuquerque et al. 2019).

Noticeably, in the centre of these experiments stands an amoeboid predator, giving the evidence that amoeboid predators act as an important mediator for the maintenance of fungal virulence. The idea that environmentally acquired pathogens evolved their virulence factors upon interaction with soil amoeba in the so-called environmental school of virulence, has been studied over decades. However, only recently it has been summarized as "Amoeboid predator-animal virulence" hypothesis (Casadevall et al. 2019). In summary, this hypothesis states that constant amoeboid predation on fungal species during evolution selected for the virulence traits that supported the virulence in certain animal hosts. Given the countless number of potential soil predators, this hypothesis can also be universally extended to other microorganisms and their soil predators. The whole phenomenon seems to follow a similar pattern and seems to have occurred many times during the evolution: first, amoeba "feast" on the microorganism for its own nutrient acquisition; prey microorganisms adapt to the host cell and became more "fit" in terms of selecting for and maintenance of virulence factors; the fitness adaptation to the evolutionary more complex hosts results in the "fist" for the survival within the host milieu and consequently the host damage as "feat" outcome and returning the pathogen back to the environment; comprehensively named as "feast-fit-fist-feat" or "4F" hypothesis (da Silva Ferreira et al. 2021).

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