Reactive oxygen species (ROS) in mycorrhizal fungi and symbiotic interactions with plants

Maaria Rosenkranz[a], Huili Shi[b], Johannes Ballauff[b], Jörg-Peter Schnitzler[a], and Andrea Polle[b,*]

[a]Research Unit Environmental Simulation, Institute of Biochemical Plant Pathology, Helmholtz Munich, Neuherberg, Germany
[b]Forest Botany and Tree Physiology, Georg-August University of Goettingen, Göttingen, Germany
*Corresponding author. e-mail address: apolle@gwdg.de

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Abstract

The interaction of plant roots with mycorrhizal fungi leads to the formation of a novel structure, the ectomycorrhiza. Ectomycorrhizal symbioses benefit both organisms by

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mutual nutrient exchange and improve the host´s stress resilience. Here, we review the roles of reactive oxygen species and antioxidative systems in the fungal partner and the host plant for the establishment of the symbiosis and for the induction of local and systemic mycorrhizal resistance. Ectomycorrhizal symbioses can be formed with different fungal species each equipped with a distinct set of extracellular oxidative enzymes. While one function of the extracellular enzymes is the access to recalcitrant nutrient sources, some examples suggest neofunctionalization to enable intraradical colonization. The colonization also involves small secreted proteins, NADPH oxidases, H_2O_2 production, and affects the plant catalase, superoxide dismutase and peroxidase-based defenses. The fungal antioxidative defense is mainly based on glutathione and thioredoxin-based systems but its role in host colonization is largely unexplored. Among signals likely involved in systemic responses are jasmonates and salicylic acid in the plant, and volatile organic compounds released by the fungus. An emerging player with a role in plant-fungal interactions is nitric oxide. Higher stress tolerance of the mycorrhizal fungal species generally confers higher stress tolerance to the host and can vary among different isolates of the same fungal species. This review emphasizes that many puzzle parts are present, but that a complete picture has not yet emerged. We discuss further studies required to increase our understanding of the inter-kingdom dialog of both organisms.

1. Introduction

Fungi occur globally in all biomes. They have versatile life-styles, colonizing soil, plants and animals with many astonishing shapes and properties (Bunyard, 2022). In terrestrial forest ecosystems, their most important functions are the biotrophic or necrotrophic interactions with plants as mutualists or pathogens and their saprotrophic activities leading to the degradation of organic matter and solubilization of minerals. Fungi occur as unicellular (yeasts) or multicellular (hyphae) organisms and have been used by humans as resource for baking, brewing, and food, for example, truffles (*Tuber melanosporum*, Pezizales), chanterelle (*Cantharellus cibarius*, Canthrellales), cep (*Boletus edulis*, Boletales), Shiitake (*Lentiluna edodes*, Agaricales), etc. Fungi are also a source for an enormous range of chemical compounds such as the well-known drugs and poisons of fly agaric (*Amanita muscaria*) and death cap (*Amanita phalloides*, Agaricales). An outstanding example involving reactive oxygen species (ROS) in fungal metabolism is bioluminescence. Several saprotrophic fungi in the genus *Mycena* (Agaricales) glow in darkness. They contain a luciferase-enzyme complex (Ke et al., 2020), which can use molecular oxygen to oxidize luciferin to a high-energy endoperoxide, which then releases CO_2 and chemiluminescence (Garcia-Iriepa, Marazzi, & Navizet, 2020). It is

obvious that different fungal species harbor highly diverse metabolism and biochemistry to engage in a wide array of ecological functions and trophic modes (Li et al., 2012). Recent advances in genome sequencing provided novel insights into their metabolic potentials (Kohler et al., 2015; Martin et al., 2008; Miyauchi et al., 2020) but many fungal species still await discovery (Martin et al., 2018). It is, therefore, important to note that even within a selected group of fungi, we can only scratch the surface of the many roles and functions of fungal ROS metabolism and their interaction with plants.

Here, we focus on ectomycorrhizal fungi (EMF). EMF form mutualistic associations with roots of woody plant species. The interaction of soil fungi with plant roots was initially observed by Hartig (1840) and later functionally described by Frank (1885) as "fungus root" (mýkēs (fungus), rhiza (root)); Frank postulated that the mycorrhiza was the sole organ for water and mineral supply to the tree and sustained by provision of carbon compounds from the host (cited after Trappe, 2005). A typical ectomycorrhiza consists of a hyphal mantle, which ensheathes the root tip and an extramatrical mycelium which emanates into the soil; inside the mantle facing the root surface, hyphae invade the walls between adjacent rhizodermal cells, forming a novel interface (Hartig net) for nutrient exchange between the fungus and the plant (Smith and Read, 2010) (Fig. 1). In addition to ectomycorrhizas, various other forms of mycorrhizal associations with woody and non-woody plants exist such as arbuscular mycorrhizal fungi (AMF), ericoid mycorrhizas, orchid mycorrhizas, etc. AMF (phylum Mucoromycota) (Bonfante & Venice, 2020) are the most ancient symbionts, whereas EMF are relatively recent evolutionary inventions of polyphyletic origin (Basidiomycota, Ascomycota) (Lebreton et al., 2021; Strullu-Derrien, Selosse, Kenrick, & Martin, 2018). Their ancestors are wood-degrading brown and white rot fungi as well as litter decomposers (Kohler et al., 2015). Wood degrading fungi rely on extracellular production of ROS, mainly hydroxyl radicals generated by Fenton chemistry, to access recalcitrant nutrient sources from lignin or cellulose (Castaño, Zhang, Anderson, & Schilling, 2018). EMF have retained a range of these molecular tools (Miyauchi et al., 2020), enabling many species to thrive as free-living hyphae without a host plant, whereas AMF are obligate biotroph (Bago & Bécard, 2002).

In this review, we compile an overview on ROS metabolism in EMF and ROS functions for nutrient acquisition and plant defense responses. Examples from other mycorrhizal symbioses have been interspersed occasionally to illustrate communalities or differences. We discuss the

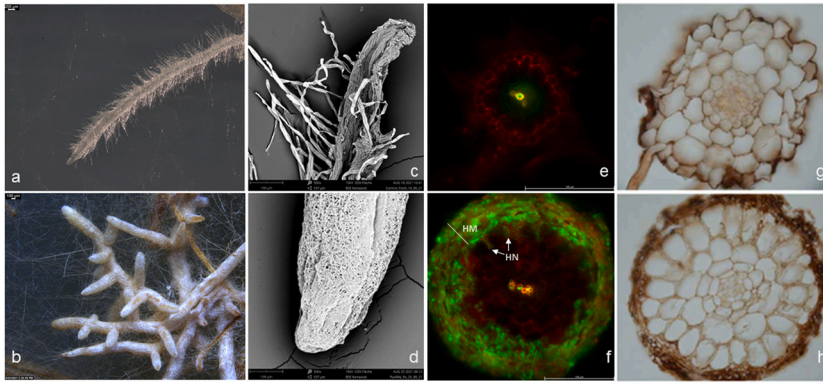

Fig. 1 Morphology and anatomical characteristics of non-mycorrhizal (A, C, E, G) and ectomycorrhizal (B, D, F, H) poplar root tips in association with *Paxillus involutus*. Photos of poplar root tips were taken under a compound microscope (A: non-mycorrhizal root tip, B: ectomycorrhizal root tips) and a scanning electron microscope (C: non-mycorrhizal root tip, D: ectomycorrhizal root tips). Plates A and C show abundant root hairs emanating from non-mycorrhizal roots and plates B and D the colonized root tips with very fine emanating hyphae (B) and the net-like structure formed around the root tip by the fungus (D). Cross sections of non-mycorrhizal (E) and ectomycorrhizal (F) poplar root tips were stained with propidium iodide to view plant cell walls (*red*) and with WGA-Alexa488 to view fungal hyphae (*green*). HM: hyphal mantle, HN: Hartig net.·H_2O_2 accumulation in cross-section of non-mycorrhizal (G) and ectomycorrhizal (H) poplar root tips is visualized by staining with diaminobenzidine.

question of how extra- and intracellular ROS-forming and detoxifying systems of EMF fungi are controlled. Since ROS and antioxidative systems also play important roles in plant development (Dat et al., 2003; Huang, Ullah, Zhou, Yi, & Zhao, 2019), we address the general role of ROS in the establishment of host-fungal interactions and their impact on host defenses.

2. ROS production and antioxidative systems in ectomycorrhizal fungi

2.1 Reactive oxygen species in ectomycorrhizal fungi

The production and detoxification of ROS is a hallmark of aerobic life. The main forms of ROS occurring in cellular metabolism are superoxide radicals ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). In the presence of transition metals (Mn, Fe, Cr), H_2O_2 and $O_2^{\cdot-}$ can drive the production of highly

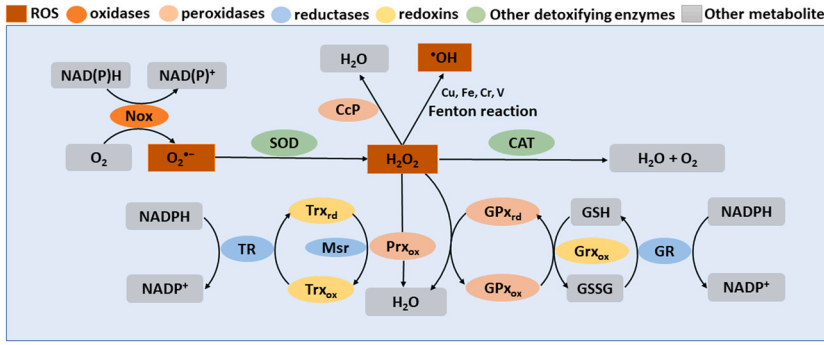

Fig. 2 Schematic representation of the intracellular ROS-producing and antioxidative system in EMF. NAD(P)H oxidases produce $O_2^{\cdot-}$. SODs convert $O_2^{\cdot-}$ to O_2 and H_2O_2. CATs catalyze H_2O_2 degradation to H_2O and O_2. EMF control intracellular ROS mainly by glutathione and thiol-related redox systems. GPx remove cellular H_2O_2 by oxidation of GSH to GSSG; the regeneration of GSH is achieved by GR with NADPH as the reductant. In thioredoxin systems, Prx reduce H_2O_2 to H_2O with Trx as donor; oxidized Trx is reduced by TR. Grx and Msr participate in antioxidant systems indirectly. Oxidized Grx is reduced by GSH non-enzymatically, whereas Msr reduces methionine sulfoxide to methionine and is regenerated by Trxs. Besides, H_2O_2 can be removed by CcP or utilized in the Fenton reaction. SOD, Superoxide dismutases; CAT, catalases; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; Grx, glutaredoxin; Prx, thioredoxin-dependent peroxiredoxin; Trx, thioredoxin; TR, thioredoxin reductases; Msr, methionine sulfoxide reductases; CcP, cytochrome c peroxidases.

reactive hydroxyl radicals (*OH) (Fig. 2). This reaction is particularly relevant in some clades of EMF, as described below. ROS have functions as signaling compounds and moreover, in the extracellular compartment, they help the fungus to mine the surroundings for nutrients. ROS levels increase in response to abiotic and biotic stress in free living EMF as well as in plant symbioses and induce antioxidative systems (Bellion, Courbot, Jacob, Blaudez, & Chalot, 2006; Chen, Hawighorst, Sun, & Polle, 2014; Chot & Reddy, 2022; Luo et al., 2014; Schützendübel & Polle, 2002). Unattended, excessive ROS production causes oxidation of membranes, DNA, and proteins, leading to lipid peroxides, DNA strand breaks and deregulation of the metabolic redox state (as described elsewhere in this volume). Under these conditions, plants activate programmed cell death (Gaspar & Pawlowska, 2022). Whether regulated ROS-induced cell death also occurs in fungi is unknown but ferroptosis-induced lipid peroxidation (the release of iron and ROS production by NADPH oxidases) has been found in fungi under stress (Gaspar & Pawlowska, 2022).

Like in other organisms, controlled $O_2^{\cdot-}$ formation is mediated by ectomycorrhizal NADPH oxidases (termed Nox in fungi or RBOH = respiratory burst oxidase homolog in plants). NADPH oxidases (Nox, EC 1.6.3.1) are membrane-bound enzyme complexes, which catalyze the reduction of O_2 into $O_2^{\cdot-}$ by transferring electrons from NADPH to oxygen (Fig. 2). $O_2^{\cdot-}$ are produced in the extracellular space and converted to H_2O_2 by dismutation, superoxide dismutases or by the activity of certain classes of peroxidases. NADPH oxidases are widely distributed in different kingdoms of life (Bedard, Lardy, & Krause, 2007). Fungal Noxs belong to three subfamilies NoxA, NoxB, and NoxC, in addition to NoxR, which encodes a regulatory subunit (Takemoto, Tanaka, & Scott, 2007). Mining for homologous genes encoding Nox enzymes indicated that there are at least two Noxs (NoxA or B and NoxR) in ectomycorrhizal fungal genomes (Mattila, Österman-Udd, Mali, & Lundell, 2022).

Noxs play major roles in plant immune responses, mediating biotic interactions (Wrzaczek, Brosché, & Kangasjärvi, 2013). In the beneficial *Tricholoma*-plant interaction, the expression of plant jasmonate-related defense was enhanced in the presence of fungal strains with deletions of Nox genes (Villalobos-Escobedo et al., 2020). However, the vitality of the fungal deletion strains was reduced, supporting that Nox activities are important for normal life and for plant-fungal interactions. A further indication for a role for ROS in plant-EMF interaction is a strong accumulation of H_2O_2 in the hyphal mantle of *Paxillus involutus* ectomycorrhizas compared with non-mycorrhizal poplar roots (Fig. 1G and H; Gafur, Schützendübel, Langenfeld-Heyser, Fritz, & Polle, 2004). Whether this ROS production is due to ectomycorrhizal Nox activities is unknown. It has been speculated that H_2O_2 in the ectomycorrhizal mantle may have antimicrobial properties, preventing overgrowth by other microbes and stimulating the host´s defense systems (Gafur et al., 2004; Langenfeld-Heyser et al., 2007). It has also been shown that growth of EMF is sensitive to H_2O_2 (Liu et al., 2022; Mucha, Napierała-Filipiak, Gabała, Pawłowski, & Zadworny, 2019) and therefore, ROS may be a host-controlled mechanism to attenuate fungal proliferation at the root surface.

2.2 Intracellular ROS control

2.2.1 Superoxide dismutases and catalases

As in all known aerobic organisms, superoxide dismutases (SOD, EC 1.15.1.1) are major antioxidant enzymes in EMF protecting cells from damage by $O_2^{\cdot-}$ (Fig. 2). SODs convert $O_2^{\cdot-}$ to O_2 and H_2O_2

(McCord & Fridovich, 1969). Recent genomic analyses of potential SOD genes predicted the existence of different types of SODs in wide range of EMF species from different phyla (Bellion et al., 2006; Bolchi et al., 2011; Mattila et al., 2022). Different SODs can be distinguished by their reactive center and were predicted to be predominantly localized to the cytoplasm and mitochondria or to other organelles like peroxisomes (SOD1 = Cu/Zn-SOD = generally cytosolic enzymes, SOD2/3 = Fe- or Mn-SOD = generally mitochondrial or peroxisomal enzymes). In addition, some SODs, present in the genomes of EMF were predicted to be localized in the extracellular space of the fungal cell wall, for example, SOD1 from *Laccaria bicolor* and SOD4–6 from *Tulasnella calospora* (Mattila et al., 2022).

The stress responses of SODs differ among EMF. For example, *Pisolithus tinctorius* showed a massive enhancement of SOD activities under very high Mn concentrations, whereas *Cenococcum geophilum* and *Suillus granulatus* lost their SOD activities entirely and showed strong growth impairment under excessive Mn (Qi, Zhao, Liu, & Huang, 2016). A comparison of ten *C. geophilum* isolates under salt stress showed differences in SOD responses, which were however, unrelated to the salt tolerance or susceptibility of the isolates (Li et al., 2022). Only a few studies established causal links between ectomycorrhizal SODs and stress. A Mn-SOD was cloned from *Paxillus involutus* (Jacob et al., 2001). Complementation of an *E.coli* null sod-mutant with the *P. involutus* Mn-SOD rescued the bacteria from oxidative stress imposed by paraquat (a $O_2^{\cdot -}$ producing chemical) or cadmium (Cd) (Jacob et al., 2001). Likewise, Cd and Zn stress enhanced Cu/Zn-SOD activities (Chiapello, Martino, & Perotto, 2015), while disruption of a Cu/Zn-SOD gene in the ericoid mycorrhizal fungus *Oidiodendron maius* enhanced fungal stress susceptibility to Cd, Zn and menadione (a radical producing agent) (Abba, Khouja, Martino, Archer, & Perotto, 2009). Although the *O. maius* strain with the loss-of-Cu/Zn-SOD still contained another SOD, i.e., SOD2, its vitality, conidia production and ability for mycorrhization was decreased compared with the wildtype (Abba et al., 2009). These studies underpin the importance of SODs for fungal fitness. Jacob et al., (2001) found that oxidative stress hardly affected the transcript abundance of SOD in *P. involutus* and speculated that the enzyme was post-transcriptionally regulated. It is now known that yeast SOD can be regulated by phosphorylation or other protein modifications (Tsang, Liu, Thomas, Zhang, & Zheng, 2014). However, regulatory details are unknown for SODs in EMF.

Catalases (CAT, EC 1.11.1.6), together with SODs function as the first line of antioxidant defense (Fig. 2). CATs convert two molecules of H_2O_2 into H_2O and O_2. Despite their importance for detoxification of H_2O_2 (Vandenabeele et al., 2004), to the best of our knowledge, there is currently no evidence for the purification and biochemical characterization of a CAT enzyme from an EMF species. Putative CAT encoding genes were found in all genomes of EMF species inspected to date (Bellion et al., 2006; Bolchi et al., 2011; Mattila et al., 2022) and CAT activities were measured in ectomycorrhizal mycelia (Kothamasi et al., 2019; Ott, Fritz, Polle, & Schützendübel, 2002). For instance, exposure to radioactive gamma radiation of pure cultures of *Suillus luteus*, *S. bovinus,* and *Rhizopon luteolus* resulted in increased enzymatic activities of CAT and SOD (Kothamasi et al., 2019). Cu and Cd also caused significant increases in CAT, SOD and other antioxidant enzyme activities in mycelia of *Lepista sodaria* (Dachuan & Jinyu, 2021). Ott et al. (2002) examined the time courses of SOD and CAT activities under different Cd levels in *P. involutus* cultures. They showed that CAT activities were relatively stress susceptible, declining with increasing Cd accumulation, whereas SOD activities showed time-dependent activity peaks. Therefore, Ott et al. (2002) suggested that other defense mechanisms than CATs might have taken over H_2O_2 removal under stress.

2.2.2 Glutathione and thiol-related redox systems

EMF contain multiple enzymatic and non-enzymatic systems for the control of H_2O_2, mainly based on thiol- and NADPH-dependent redox reactions. The NADPH-dependent glutathione system is composed of glutathione peroxidase (GPx, EC 1.11.1.9) and glutathione reductase (GR, EC 1.8.1.7), and requires glutathione (GSH) and NADPH as reductants (Fig. 2). GPxs remove cellular H_2O_2 or lipid peroxides by oxidation of GSH to glutathione disulfide (GSSG) and concomitant reduction of the peroxide to H_2O. The regeneration of GSH from GSSG is achieved by GR with NADPH as the reductant. Some studies also support that fungal GPxs use thioredoxin instead of GSH for the reduction of H_2O_2 (Adriani et al., 2021; Tanaka, Izawa, & Inoue, 2005). In vitro, GPx activity of *Paxillus involutus* can be measured with GSH in a coupled reaction consuming NADPH (Ott et al., 2002). However, whether GSH is replaced by thioredoxin in the cellular metabolism of an ectomycorrhizal fungus is not clear. Thioredoxins are also reduced by NADPH in a TR mediated reaction (Fernandez & Wilson, 2014). Since $NAD(P)^+$ reduction is

achieved by glucose degradation, surveillance and keeping adequate H_2O_2 levels is directly linked to the cellular energy metabolism of the fungus.

When the first genome of an ectomycorrhizal fungus was sequenced, Morel et al. (2008) conducted a comprehensive screening of the genes encoding GPxs and the thioredoxin system in *Laccaria bicolor* (Morel, Kohler, Martin, Gelhaye, & Rouhier, 2008). For *L. bicolor*, the following gene families (with the number of homolog genes) were reported: thioredoxin reductase (EC 1.8.1.9, TR: 1), thioredoxin (EC 1.8.1.9, Trx: 4), glutathione reductase (EC 1.8.1.7, GR:1), glutaredoxins (EC 1.20.4, Grx: 5), thioredoxin-dependent peroxiredoxin [Prx, EC 1.11.1.24,Tpx: 6, including 2-Cys Prx (1), 1-Cys Prx (1), PrxQ (2), class II peroxidases EC1.11.1.14, Prx II (2)], glutathione peroxidase (EC 1.11.1.9, Gpx: 1), methionine sulfoxide reductases (MsrA, EC 1.8.4.13 and MsrB, EC 1.8.4.14, Msr: 2) but no sulfiredoxins (Srx, EC 1.8.98.2, Srx: 0) (Morel, Kohler, Martin, Gelhaye, & Rouhier, 2008). Sulfiredoxins are oxidoreductases, which have been identified in yeast (*Saccharomyces cerevisiae*) (Biteau, Labarre, & Toledano, 2003) and which are involved in re-activating peroxiredoxins (2-Cys Prx) by reducing sulfinic acids formed on the peroxidatic cysteines under oxidizing conditions. Methionine sulfoxide reductases reduce methionine sulfoxide to methionine and are regenerated by thioredoxins (Hage, Rosso, & Tarrago, 2021). The expression of thiol-related antioxidative genes was supported by microarray analyses (Morel et al., 2008). To date, many fungal genomes have been sequenced and the homologs to genes described by Morel et al. (2008) were annotated in a wide array of EMF species (Bolchi et al., 2011; Mattila et al., 2022). EMF in the phylum of Basidiomycota (Mattila et al., 2022) generally contained numbers of homolog genes similar to those reported by Morel et al. (2008).

2.2.3 Other ROS handling systems

Fungi contain class I and class II peroxidases (discussed below) but not class III peroxidases, which are typical for plants (Maruta, Sawa, Shigeoka, & Ishikawa, 2016). Accordingly, guaiacol peroxidase activities (class III Prxs) could not be measured in mycorrhizal hyphal mantle tissue of *Laccaria amethysthea* collected from larch *(Larix decidu*a) and spruce (*Picea abies*) roots (Münzenberger, Otter, Polle, & Wüstrich, 1997).

EMF taxa contain cytochrome c peroxidases (CcP, EC 1.11.1.5), which are heme-containing enzymes removing H_2O_2 produced by the cell respiration. CcP was isolated and extensively characterized from aerobically grown yeast and is related to plant ascorbate peroxidases (APX1.11.1.11)

since they shared the PF00141 (peroxidase) PFAM domain (Lyall, Nikoloski, & Gechev, 2020; Zámocký, Gasselhuber, Furtmüller, & Obinger, 2014). While CcPs are present in photosynthetic and non-photosynthetic eukaryotes, APXs are almost exclusively found in plastid-containing photosynthetic organisms (Maruta et al., 2016). The genomes of several EMF taxa contain one CcP gene per species (Mattila et al., 2022), while APXs were not present. Furthermore, APX requires ascorbate as a reductant, which was not discovered in *Paxillus involutus* (Ott et al., 2002).

In animals and plants, the final step of ascorbate biosynthesis is catalyzed by L-gulono-1,4-lactone oxidase (GGLO) and L-galactone-1,4-lactone dehydrogenase (GLDH), respectively (Smirnoff, 2018). Fungi produce an analog, D-erythroascorbate, employing D-arabino-1,4-lactone oxidase (ALO) for the final biosynthetic step (Smirnoff, 2018). We blasted the sequences of the *Arabidopsis thaliana* GLDH, the mammalian (*Mus mus*) GGLO and the yeast (*Saccharomyces cerevisiae*) ALO against several EMF genomes using blastp v.2.9.0 (Altschul, Gish, Miller, Myers, & Lipman, 1990) (Supplementary Table S1). The basidomycetes *Laccaria bicolor, Amanita muscaria, Lactarius quietus, Russula ochroleuca,* and *Paxillus involutus* show high homology to the mammalian enzyme (e-values $< E^{-80}$, high bit score and overlap with query length), while the tested ascomycetes produced two hits under these conditions, one for the mammalian GGLO (e-values $< E^{-80}$) and one for the yeast ALO enzyme (e-values close to E^{-100}) (Supplementary Table S1). High similarities with the plant enzyme GLDH were not detected (Supplementary Table S1). Morel et al. (2008) did not discover genes for dehydroascorbate reductase and monodehydroascorbate radical reductase in *L. bicolor*. Thus, the potential role of erythroascorbate and its reduction for ROS detoxification in EMF is elusive.

Some reports indicated that heavy metals (Cd, Cu) or drought stress treatment induced ascorbate activities in EMF (Alvarez et al., 2009; Dachuan & Jinyu, 2021). In the absence of ascorbate peroxidase genes and most likely also of ascorbate, confounding non-enzymatic reaction might have mimicked erroneously the detected activities (Smirnoff, 2018). Reis et al. (2011) reported the presence of ascorbate in a range of EMF grown in pure culture. However, their analysis was based on a relatively unspecific method, which measures spectrophotometrically absorbance changes due to the reduction of 2,6-dichloroindophenol by fungal extracts. Therefore, antioxidative reactions of compounds other than ascorbate in these extracts cannot be excluded. Tocopherols, another important group of antioxidant compounds known from the plant metabolism, have been discovered by

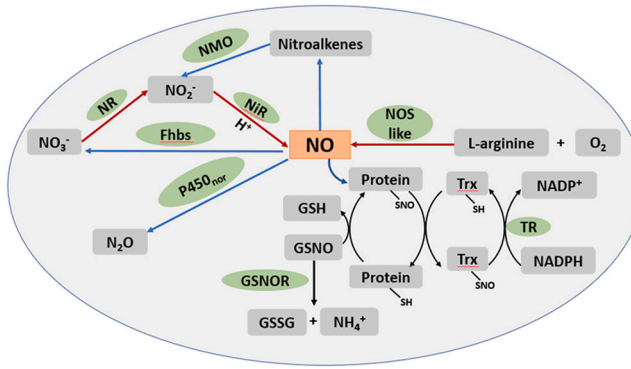

Fig. 3 Schematic, partly hypothetical overview of NO production and removal in fungi. NO may be produced by oxidative and reductive pathways. Oxidative pathway: L-arginine and O_2 catalyze NO formation by NOS-like proteins. Reductive pathways: NO_3^- is reduced by NR and NiR to NO_2^- and NO. The removal of NO is achieved by multiple reactions. Fhbs convert NO to NO_3^-, $P450_{nor}$ reduce NO to N_2O in an NADPH-dependent reaction. Higher levels of NO are converted to nitroalkenes during nitro-oxidative conditions, then further metabolized by NMO to NO_2^-. A specific NO-inducible nitrosothionein is capable of directly scavenging NO in coordination with the Trx/TR redox system. GSNOR is involved in the NO-dependent nitrosative signaling. Red arrows indicate NO production and blue arrows NO removal. NO_3^-, nitrate; NO_2^-, nitrite; NO, nitric oxide; NR, nitrate reductase; NiR, nitrite reductase; NOS, nitric oxide synthases; Fhbs, flavohemoglobins; P450nor, Cytochrome P450 nitric oxide reductase; NMO, nitronate monooxygenase; GSH, glutathione; GSNO, *S*-nitrosoglutathione; GSNOR, S-nitrosoglutathione reductase; GSSG, glutathione disulfide; Protein-SH, proteins with free thiols in cysteine; Protein-SNO, *S*-nitrosylated proteins; Trx, thioredoxin; TR, thioredoxin reductase.

high pressure liquid chromatographic and comparison with authentic standards in EMF species from various genera (Amanita, Chroogomphus, Cortinarius, Lactarius, Russula Suillus, and Tricholoma) (Reis et al., 2011). However, physiological studies on the role of tocopherols in EMF are lacking. Since antioxidative systems in mycorrhizal fungi are often investigated with the aim to select stress tolerant EMF species for phytoremediation, there is an urgent need to characterize the metabolism of mycorrhizal tocopherols, ascorbate-like compounds and related enzyme activities to better understand fungal ROS handling.

A further important reactive compound for redox regulation is nitric oxide NO* (Fig. 3). NO* participates in the redox cycle by accepting an electron (NO^-, nitroxyl anion) or donating an electron (NO^+, nitrosonium cation), while the radical NO* is uncharged (Correa-Aragunde, Foresi, & Lamattina, 2015). In plants, NO* can be produced by nitrate

reductase or NO synthase-like proteins through different pathways (Moreau, Lindermayr, Durner, & Klessig, 2010; Rekhter et al., 2019). NO* exerts its signaling effect by direct post-translational protein modifications, i.e., *S*-nitrosylation, metal nitrosylation, and tyrosine nitration (Lindermayr, Saalbach, & Durner, 2005) (Fig. 3). *S*-nitrosylation, the covalent binding of NO* to the thiol side of protein cysteine residues to form nitrosothiols (SNOs), is considered the major post translational modification of NO* signaling (Moreau et al., 2010; Vanzo et al., 2016). In plants, NO* is involved in the regulation of ROS levels (Lindermayr & Durner, 2015) by enhancing or decreasing the activities of ROS-metabolizing enzymes such as NADPH oxidase, CAT, APX, and GR (Correa-Aragunde et al., 2015; Dat et al., 2003; Davletova, Schlauch, Coutu, & Mittler, 2005; Lindermayr et al., 2005; Vandenabeele et al., 2004; Vanzo et al., 2016), thereby adjusting the ROS pool. Recently, Martínez-Medina et al. (2019) demonstrated that NO-dependent regulation of phytoglobins plays a key role in establishing AMF-plant interactions. Phytoglobins (Pgb), along with S-nitrosoglutathione reductase (GSNOR), are the major scavenging pathways for NO in plants. Phytoglobins comprise a group of non-symbiotic hemoglobins that exhibit high affinity for both oxygen and NO under certain conditions such as hypoxia (Berger et al., 2018). In this process, NO is converted to nitrate by the oxygen-enriched ferrous phytoglobin ($Pgb(Fe^{2+})$), which is thereby transformed to the metPgb form ($Pgb(Fe^{3+})$). metPgb is reduced by a NAD(P)H-dependent reductase and subsequently re-oxygenated (Kuruthukulangarakoola et al., 2017). Nitrate can serve as a substrate for NR to produce nitrite and thus, eventually also NO, driving again Pgb reactions. This cycle is referred to as the "Pgb-NO cycle" (Kumari, Pathak, Loake, & Gupta, 2019). To what extent the regulation of NO content by the Pgb-NO cycle also plays a role in the interaction of EMF species with plants during the formation of ectomycorrhizas is unknown.

Although fungal NO* production may be ecologically very important with regard to nitrogen cycling, we have little information on its role in EMF. Environmental contamination with Cr(IV) enhances nitrate and nitrite reductase gene expression and enzymes activities in *Pisolithus* species (Shi et al., 2020). Under these conditions, the production of NO* and ROS detoxification by GSH were stimulated (Shi et al., 2022). Suppression of NO* production by supplementation of the fungus with the NO* inhibitor PTIO (2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide) increased ROS production and antioxidative activities under stress (Shi et al., 2022).

When the NO* donor, sodium nitroprusside (SNP) was used for soil amendment, root colonization with *Tuber indicum* increased (Zhang et al., 2019). Thus, the current studies hint towards a role of NO* in redox regulation and root colonization. In natural EMF communities, considerable transcript abundances of nitrate reductase are present, although uptake of reduced nitrogen (NH_4^+, amino acids) is preferred (Rivera Pérez, Janz, Schneider, Daniel, & Polle, 2022). When nitrate reductase (NR, EC 1.7.1.1) was suppressed by genetic transformation (RNAi) in *L. bicolor*, viable ectomycorrhizas could not be formed anymore (Kemppainen, Duplessis, Martin, & Pardo, 2009). Therefore, either a direct or indirect nitrate signal via NO* radicals is important for the symbiosis. It is obvious that a better understanding of NO* in plant-fungal interactions is necessary.

2.3 Extracellular ROS producing and oxidative enzymes

Fungi produce an enormous variety of specialized extracellular enzymes, which reflect their abilities to thrive on a wide number of substrates and is due to different phylogenetic origins (Kohler et al., 2015; Miyauchi et al., 2020). Many of these enzymes are oxidases, resulting in ROS production when attacking recalcitrant potential nutrient sources. Since EMF have direct access to plant-derived simple carbohydrates when engaged in mutualistic symbiosis, the presence of multiple carbohydrate-active enzymes was surprising. However, comparative analyses of wood-degrading fungi and EMF in culture showed that the overall activities of EMF for degradation of recalcitrant compounds are lower than those of typical saprotrophs (Gramss, Kirsche, Voigt, Günther, & Fritsche, 1999). Nevertheless, substantial activities of cellobiose oxidase (cellobiose dehydrogenase, EC 1.1.99.18) and glucose oxidase (beta-D-glucose:oxygen 1-oxidoreductase, EC 1.1.3.4) along with H_2O_2 production were found when the EMF *Suillus variegatus, Pisolithus tinctorius,* and a *Cortinarius* sp. were grown on the corresponding substrates (Burke & Cairney, 1998). The ectomycorrhizal fungus *Paxillus involutus* is able to decompose organic matter by Fenton chemistry (Shah et al., 2015). In the Fenton reaction Fe^{2+} is oxidized by H_2O_2 to yield Fe^{3+}, OH* and HO^-. The re-reduction of Fe^{3+} to Fe^{2+} is driven by $O_2^{\cdot-}$ radicals and special metabolites, in the case of *P. involutus* probably by the diarylcyclopentenone involutin (Shah et al., 2015). Involutin production occurs only on organic but not on mineral substrates, thereby, enhancing nutrient accessibility to the host (Shah et al., 2015). Field studies on mycorrhizal root tips from forest trees also show environmental variation of carbohydrate-active enzyme activities and

variation among different EMF species, pointing to flexible adaptation to changing environmental conditions (Agerer, Schloter, & Hahn, 2000; Courty et al., 2010; Pritsch & Garbaye, 2011). How plant roots cope with varying enzyme activities and exposure to different types of ROS is not clear. It could be that ROS also have functions in nutrient signaling but this area is unexplored for mycorrhizal fungi, despite the importance of EMF for tree nutrition (Becquer, Guerreo-Galan, Eibensteiner Houdinet Bücking, Zimmermann & Garcia, 2019).

Progress has been made in the identification of carbohydrate-active enzymes in EMF by systematic screening of fungal genomes and classification of the enzymes in the Cazy data base (www.cazy.org; Drula et al., 2022). We used published information (modified from Supplementary Table S6 in Miyauchi et al., 2020) to generate an overview on AA enzymes, i.e., enzymes with "axillary activities" (AA1 to AA14) in 62 ectomycorrhizal fungi (Fig. 4). Among these categories AA1, AA2, AA3, AA9, and AA11 are of special interest because they explain differences among different phylogenetic and ecological mycorrhizal forms (Fig. 4).

High gene counts in the AA1 family are mainly characteristic of AMF but some basidiomycetes (*Hydnum rufescens, Piloderma croceum, Lactarius quietus, Suillus brevipes and Tricholoma matsutake*) were also present in this category. AA1 enzymes are multicopper oxidases (EC 1.10.3.-), e.g., laccases (EC 1.10.3.2) that use diphenols with oxygen as the acceptor. Furthermore, genes encoding AA3 enzymes, which comprise functionally diverse glucose–methanol–choline oxidoreductases (GMC oxidoreductase) were more enriched in AMF than in most other mycorrhizal life forms. GMC oxidases are a source of H_2O_2. They include aryl-alcohol oxidoreductase (AAO, EC 1.1.3.7), alcohol oxidase (AOx, EC 1.1.3.13), cellobiose dehydrogenase (CDH, EC 1.1.99.18), glucose dehydrogenase (GDH, EC 1.1.5.9), pyranose dehydrogenase (PDH, EC 1.1.99.29), and pyranose oxidase (Pox, EC 1.1.3.10).

The AA2 family contains class-II peroxidases such as manganese peroxidase (EC 1.11.1.13), versatile peroxidase (EC 1.11.1.16), lignin peroxidase (EC 1.11.1.14), peroxidase (EC 1.11.1.-), cytochrome-c peroxidase (EC 1.11.1.5), and ascorbate peroxidase (EC 1.11.1.11). These PODs are heme-containing enzymes, which use H_2O_2 as the electron acceptor, occurring in many EMF species (except ascorbate peroxidase) (Bödeker, Nygren, Taylor, Olson, & Lindahl, 2009; Floudas et al., 2012). For example, Chen et al. (2001) showed the presence of lignin peroxidase (LiP) and manganese peroxidase (MnP) genes in a broad taxonomic range of EMF by PCR with a

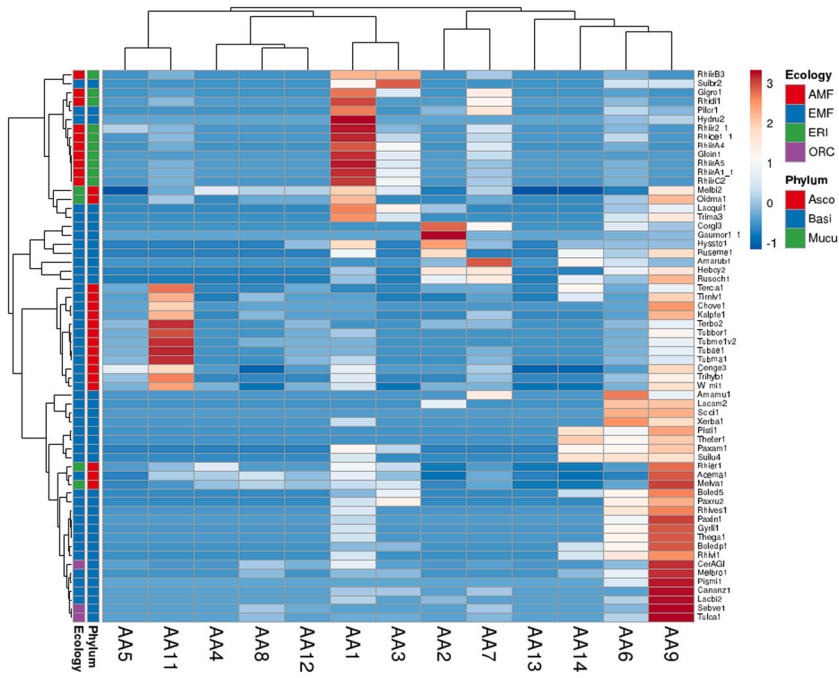

Fig. 4 Hierarchical clustering of gene abundances of carbohydrate-activing enzyme with axillary activities (AA). The heatmap was generated with the numbers of genes per genome and per AA family provided by Miyauchi et al. (2020). The AA families were taken from the Cazy classification system. The function of different families are described in the main text. Abbreviations and taxonomic information for the fungi is found in Supplementary Table S2.

specific primer and confirmed the identity of the amplified fragments by sequencing (Chen, Taylor, Burke, & Cairney, 2001). In our analysis, *Gautieria morchelliformis, Cortinarius glaucopus* and several Russulaceae stood out with regard to their AA2 gene inventory (Fig. 4).

The ectomycorrhizal Ascomycota were enriched with AA11 family members (Fig. 4). AA11 proteins are copper-dependent lytic polysaccharide monooxygenases (LPMOs), which have been detected just a decade ago (Courtade & Aachmann, 2019). They can cleave crystalline cellulose and chitin chains, thereby, complementing glycoside hydrolases, which act on amorphous cellulose (Courtade & Aachmann, 2019). Although the A11 gene counts clearly separated ascomycetes EMF from basidiomycetes EMF, we noted an exception: the AA11 gene inventory of Ascomycota forming ericoid mycorrhiza resembled more closely that of Basidiomycota EMF (Fig. 4).

The AA9 family also contains copper-dependent lytic polysaccharide monooxygenases but they were reported to catalyze oxidative cellulose degradation [lytic cellulose monooxygenase (C1-hydroxylating) (EC 1.14.99.54), lytic cellulose monooxygenase (C4-dehydrogenating) (EC 1.14.99.56)] (Drula et al., 2022). However, usage of other substrates cannot be excluded (Vandhana et al., 2022). Very high counts of genes encoding AA9 family members were present in *Pisolithus microcarpus, Melanogaster broomeianus, Cantharellus anzutake, Laccaria bicolor* and in the three orchid EMF (Basidiomycota) available in the list. Veneault-Fourrey et al. (2014) found that three AA9 LPMO genes in *Laccaria bicolor* were expressed during first root contact and during mycorrhizal maturation when the Hartig net is formed (Veneault-Fourrey et al., 2014). LPMO enzymes may be involved in remodeling of root cell walls by loosening the wall together with endoglucanases (Zhang et al., 2018) since they are localized at the interface between fungal hyphae and root cortex cells (Labourel et al., 2020). In as much LPMO activities from the AA9 and AA11 families functionally distinguish asco- and basidiomycetes should be studied in the future. The new insights open interesting avenues for further characterization of plant fungal interaction and their ecological consequences for carbon turnover.

3. Local and systemic defenses in plants induced by ectomycorrhizal fungi

3.1 ROS, volatile organic compounds and other signaling components involved in initiation of EMF-symbiosis

The establishment of a symbiotic association between EMF and plant root requires several coordinated events that are not yet completely understood. The initiation of an EMF symbiosis includes at least the secretion of small secreted proteins (SSPs) (Plett et al., 2011; Pellegrin, Morin, Martin, & Veneault-Fourrey, 2015), lipochitooligosaccharides (LCOs) (Cope et al., 2019) and other chitin-related compounds on the fungal side and exudation of flavonoids and phytohormones and chitinase activity (Garcia, Delaux, Cope, & Ané, 2015) on the plant side (Fig. 5).

The exudation of flavonoids and phytohormones is considered to initiate the chemical dialogue between the roots and the fungus. Plant chitinases release fungus-typical chitin-related compounds and are involved in plant response to pathogenic as well as beneficial fungi (Salzer, Hebe, & Hager, 1997; Schickler & Chet, 1997). Chitin-related compounds elicit

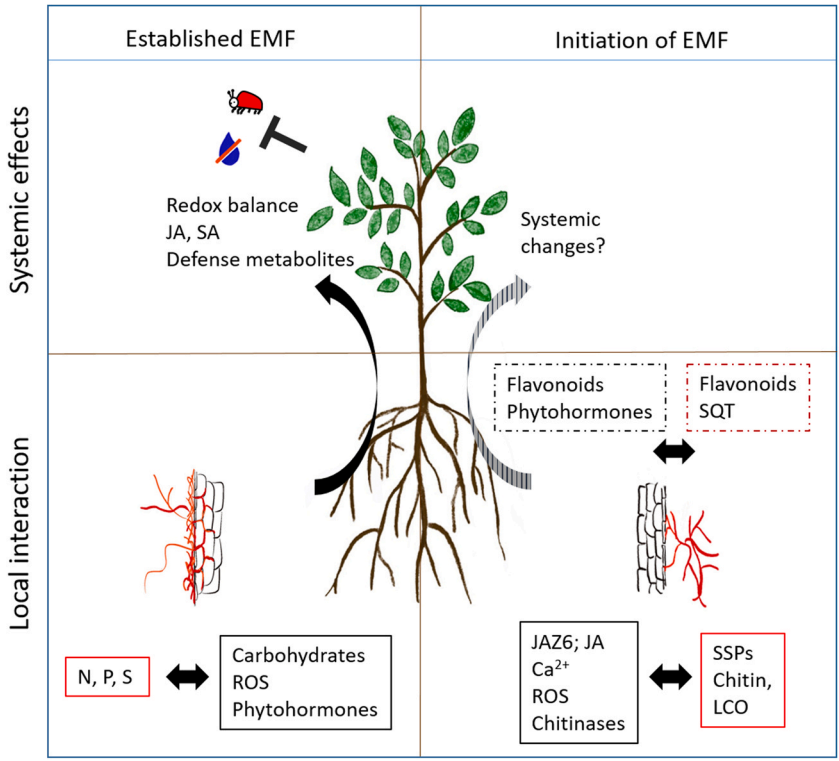

Fig. 5 Potential interaction signals involved in initiating and maintaining the ectomycorrhizal relationship. Systemic effects that are associated to the initiation stage and to established mycorrhiza are also shown. The dashed boxes include cues that are potentially involved in interaction through soil matrix, before the contact. JA: jasmonates; LCO: lipochitooligosaccharides; SA: salicylic acid; SQT: sesquiterpenes; SSPs: small secreted proteins; JAZ6: JASMONATE-ZIM-DOMAIN 6; N, P, S: nitrogen, phosphorus, sulfur.

further adjustments in plant signaling to prepare for the interaction (Punja & Zhang, 1993; Salzer et al., 1997; Sauter & Hager, 1989). Here, the extracellular oxidative enzymes could play important roles but general, overarching principles have not yet been clarified. The signal exchange between the partners leads to altered jasmonic acid (JA)-dependent signaling in roots and a locally reduced defense response (Garcia et al., 2015; Plett, Daguerre, et al., 2014; Plett, Khachane, et al., 2014). For *L. bicolor* it is known that fungal SSPs (specifically the mycorrhiza-induced SSP7 (MiSSP7)) function as effectors that interact with the poplar JASMONATE-ZIM-DOMAIN 6 (JAZ6)-protein. JAZ6 is a repressor of JA-

signaling and its binding to MiSSP7 prevents induction of local JA-related defense responses, thus, probably allowing the symbiotic relationship to develop (Fig. 5; for detailed review see Garcia et al., 2015). Also other SSPs are able to modulate the plant phytohormone balance similar to various further fungal effectors that can manipulate plant hormonal pathways and regulate host defenses (Dreischhoff, Das, Jakobi, Kasper, & Polle, 2020). In later stages of symbiosis, ethylene- and JA-pathways are again induced probably to limit fungal growth within the roots (Plett, Khachane, et al., 2014). In addition to altered JA signaling, the early interaction mechanisms between plant and EMF include Ca^{2+} spiking (Cope et al., 2019; Garcia et al., 2015) and local adjustments in the root's redox balance (Baptista et al., 2007). Together these initial adjustments might be involved in initiating cell-to-cell signaling and further systemic responses. JA signaling is considered as antagonistic to salicylic acid (SA) signaling and involved in cross-talk with other plant signaling pathways (Erb, 2018; Vlot et al., 2021). Therefore, already the initial changes necessary to allow mycorrhizal interactions can cause comprehensive adjustments in plant performance. In support of this idea, circumstantial evidence shows that contact to ecto-mycorrhizal cell wall fragments is sufficient to induce systemic immunity against a biotrophic leaf pathogen (*Pseudomonas syringae*) in the non-mycorrhizal plant *Arabidopsis thaliana* (Vishwanathan et al., 2020). Obviously, a functional mycorrhiza is not required to induce systemic effects in plants. Moreover, EMF-induced systemic effects may have an evolutionary signature that is conserved irrespective of the trophic mode of the interacting fungus.

3.1.1 Involvement of ROS in initiation of an EMF symbiosis

Though all the mechanisms in "the interaction dance" behind the initiation of EMF symbiosis are not elucidated, ROS (H_2O_2, O_2^-) are known to play a role in this process. An EMF-related ROS burst was detected when the initial contact between *Picea abies* and two different EMF fungi, *Amanita muscaria* and *Hebeloma crustuliniforme* was studied (Salzer, Hubner, Sirrenberg, & Hager, 1997; Salzer et al., 1996; Schwacke & Hager, 1992). Early contact between *Castanea sativa* roots and *Pisolithus tinctorius* triggered three sequentially occurring H_2O_2 bursts, which were additionally accompanied by two O_2^- bursts (Baptista et al., 2007). Interestingly, adjustments in ROS scavenging enzymes SOD and CAT were observed in association with ROS bursts, suggesting that during the early stages of EMF establishment the ROS burst results from an inhibition of these enzymes,

thus, underlining the tight regulation of the ROS signaling events. In *C. sativa,* the EMF-induced ROS bursts were followed by increased root exudation and subsequently induced hyphal growth of the EMF-fungus (Baptista, Martins, Pais, Tavares, & Lino-Neto, 2007). These transient adjustments in ROS pools upon EMF contact resemble those that were found for AMF during mycorrhization events. Dynamic ROS levels are known to play a central role in initial AMF colonization, subsequent symbiosis formation and eventually, in the degradation of arbuscules. The initial, transient ROS burst is overcome by accumulation of ROS scavenging enzymes (e.g., increased activity of SOD, CAT, APX) as well as by non-enzymatic antioxidants (carotenoids, α-tocopherol, proline, ascorbate and glutathione) in the plant (Zou, Wu, & Kuča, 2021). Also various antioxidative enzymes of AMF are known to contribute to regulation of the initial, dynamic oxidative burst, which is mainly restricted to AMF-containing cortical root cells (Zou et al., 2021). The exact function of ROS bursts and the possibility of an induction of ROS waves for communication between root cells and ectomycorrhizal fungus remain to be elucidated. Many different types of abiotic and biotic stresses can trigger ROS waves, which are considered as an essential signal to alert and prepare the plant for environmental changes (Fichman & Mittler, 2020). Likewise, ROS waves could also have a function in the initiation of a symbiotic relationship between plant and fungus.

Moreover, it is known that similar to rhizobia, AMF exude so-called Nod-factors, such as LCOs that are involved in the initiation process to allow plant roots to prepare for symbiosis. After perceiving LCOs from AMF, mevalonate biosynthesis is induced followed by activation of nuclear ion channels and Ca^{2+} flow. As Ca^{2+} is released from the nucleus and pumped again back by a calcium ATPase, LCOs trigger Ca^{2+} spiking (Bertoni, 2019). Further down-stream steps lead to an activation of transcription factors essential for the regulation of the "common symbiosis signaling pathway" (Bertoni, 2019). Recent transcriptomic analyses of EMF-colonized oak tree roots suggest that the "common symbiosis signaling pathway" of AMF is involved also in the initiation of ectomycorrhizal symbiosis (Bouffaud et al., 2020). Cope and colleagues revealed that *L. bicolor* produce LCOs that can trigger the "common symbiosis signaling pathway" and thus allow colonization of plant roots (Cope et al., 2019). *Laccaria bicolor* LCOs were shown to cause Ca^{2+} spiking in nuclei of *Populus* roots and enhance lateral root development and EMF colonization (Cope et al., 2019). While symbiotic organisms elicit the nuclear Ca^{2+} oscillations,

pathogens trigger cytoplasmic spiking, a difference which is considered as a key to specify plant responses (Tian, Wang, Gao, Li, & Luan, 2020). In *Phaseolus vulgaris*-rhizobia interactions, the transient increase of Ca^{2+} correlated spatially and temporarily with changing ROS concentrations (Cárdenas, Martínez, Sánchez, & Quinto, 2008; Cárdenas & Quinto, 2008). The function of ROS in initiation of different beneficial relationships might, however, strongly depend on the interacting species. For example, the *Phaseolus vulgaris* NADPH-oxidase functions as positive or negative regulator of biotic interactions depending on the interacting partner: enhanced plant ROS levels induced enhanced nodule formation by rhizobia, whereas the opposite, reduced colonization was observed for AMF (Arthikala et al., 2014). On the other hand, in arbusculated *Medicago truncatula* cells NADPH oxidase (MtRbohE) gene expression was enhanced compared to control roots and knock-down of the *MtRbohE*-gene lead to altered root cortex colonization pattern of *Medicago* (Belmondo et al., 2016). Together, these results suggest important roles of NADPH-oxidase and ROS concentrations in AMF mycorrhization. Whether exposure to EMF-released LCOs alter ROS in roots and what might be their final role in initiation of EMF symbiosis remains to be elucidated. The redox balance may however be a key regulating step for EMF colonization of roots. At least production of ROS has been linked to the specificity of the EMF interactions: plants produce ROS when exposed to EMF strains that are capable of forming ectomycorrhizas (Fig. 1), whereas no increase in ROS levels was observed upon exposure to a *Paxillus* strain that cannot develop a complete ectomycorrhiza (Gafur et al., 2004). The host specialization of the EMF *Suillus* spp. is also linked to various oxidative stress-associated domains such as thioredoxin reductase, glutathione, SOD, CATs, pyridine nucleotide-disulfide oxidoreductase and multiple aldehyde dehydrogenases in the ectomycorrhiza (Lofgren et al., 2021). Targeted ROS deactivation was suggested as a possible mechanism behind host specificity of this EMF-host combination (Lofgren et al., 2021).

3.1.2 Potential volatile signals exchanged before a contact

Volatile organic compound (VOC)-mediated interactions may prepare for symbiosis initiation already before the interacting partners are in wall-to-wall contact with each other. *Laccaria bicolor* VOCs were shown to alter the root redox balance already before the contact. ROS accumulated in poplar and *Arabidopsis* roots exposed to a volatile sesquiterpene from *L. bicolor* in an NADPH-oxidase dependent manner (Fig. 5; Ditengou et al.,

2015). The altered ROS concentration was associated to enhanced lateral root and root hair development. Lateral roots are the preferred target for mycorrhizal fungi to colonize roots (Sun, Bonfante, & Tang, 2015) and their stimulation can facilitate the establishment of symbiosis. Several other studies reported altered root architecture and root growth upon exposure to microbial VOCs (Garnica-Vergara et al., 2016; Moisan et al., 2021; Sun et al., 2015; Werner, Polle, & Brinkmann, 2016), even though none of these studies tested explicitly the involvement of ROS signaling in the plants` response. Some years ago Matsui (2016) suggested that perceiving and sensing specific external VOCs in plant cells might generally be associated with plant redox adjustments (Matsui, 2016). This hypothesis is supported by a few studies, which detected increased ROS levels in conjunction with altered plant performance upon perceiving an external VOC cue (Ameye et al., 2020; Riedlmeier et al., 2017). Further, isoprene, a root endogenous volatile hemiterpene, is involved in an NADPH-oxidase dependent accumulation of ROS and altered lateral root growth of poplar (*P.* x *canescens*) (Miloradovic van Doorn et al., 2020). Thus, collectively recent studies support a ROS-related function of VOCs in roots. An intriguing question to be tackled in the future is if specific VOCs released by EMF lead to adjustments in root performance in natural conditions and what their importance is in initiation of a symbiotic relationship. Since transient ROS bursts are involved in early events of mycorrhization (Salzer et al., 1996; Schwacke & Hager, 1992) and since H_2O_2 is present in the mature ectomycorrhizal mantle (Gafur et al., 2004), in vivo imaging of H_2O_2 and redox compounds by the recently developed whole-plant ROS live imaging (Fichman et al., 2019) or by Hyper-lines (Belousov et al., 2006) may help to view the dynamics of ROS upon VOC and EMF exposure. Thereby, essential information about the role of VOCs and ROS in early and later events of symbiosis establishment and functioning could be disentangled. Recent comparative genomics further supports the potential role of terpenes in EMF-host interaction. Terpene biosynthesis related gene clusters are enriched in the specialist EMF-genus *Suillus*, suggesting that terpenes might have a function in initiating interaction between a specialist fungus and the host. As specificity was associated with overrepresentation of oxidative stress related domains (Lofgren et al., 2021), it seems evident that the link between specificity, terpene profile and redox balance deserves more attention when exploring the mechanisms of ectomycorrhizal establishment in the future.

3.2 Established ectomycorrhiza alters plant internal signaling and alleviate stress

Signaling is not only crucial in initiation of EMF symbiosis, but also in the established mycorrhizal state and in alleviating plant stress responses. For example, *Paxillus involutus* colonization was related to increased concentrations of SA and ABA in roots, whereas JA and auxin concentrations decreased (Luo et al., 2009), suggesting alterations in internal signaling of EMF-poplar roots that were exposed to salinity. The EMF-host interaction does not only alleviate acute plant stress tolerance but seems to adjust plant internal signaling already before stress exposure. In a recent study, the effects of three different EMFs were investigated by analyzing the transcriptomes of EMF colonized roots, systemic non-colonized roots and leaves of oak trees (*Quercus robur*) (Bouffaud et al., 2020). In general, more common differentially expressed genes induced by all the three EMF species, *L. bicolor*, *Paxillus involutus* and *Pisolithus microcarpus* were detected in the colonized roots than in any of the systemic tissues. The data suggest that the local responses to EMF are more conserved than the EMF-induced systemic responses, which varied depending on the interacting species. All three fungal species altered expression levels of various disease resistance-related genes in the systemic roots and SOD copper chaperones and genes encoding ABA degrading enzymes in leaves. The symbiosis with *P. microcarpus* affected redox process-related genes in the systemic leaves (Bouffaud et al., 2020). Together the results suggest that redox balance can be altered in systemic tissues of EMF-colonized trees already before experiencing a stress. Although not all the mechanisms and prevailing factors leading to altered plant signaling in mycorrhizal stage are elucidated, several studies linked EMF-based improved stress tolerance with alterations in ROS, ROS scavenging enzymes and phytohormones (Alvarez et al., 2009; Bai, Hao, Hu, & Leng, 2021; Kaling et al., 2018; Pfabel et al., 2012; Vishwanathan et al., 2020). In order to alert the plant for stress adaptation, ROS can act also as long distance signals conferring information within plant tissues and activate defense related signaling pathways (Baxter, Mittler, & Suzuki, 2014; Gilroy et al., 2014; Mittler & Blumwald, 2015). To efficiently utilize ROS as internal signaling molecules and to mitigate toxic effects of ROS, plants need to sustain a delicate balance between ROS generation and ROS scavenging (Baxter et al., 2014). Such a balance is a central component of various within-plant signaling events, including local and systemic responses to EMF (Bai et al., 2021; Sun et al., 2022).

3.2.1 The interplay between ROS and ROS regulating enzymes in plants is adjusted by EMF

The balance between different antioxidative enzyme activities, such as SOD, APX and CAT, is essential for suppressing toxic ROS levels in a cell (Mittler, 2017). Furthermore, EMF-related attenuation of oxidative stress is regulated by ROS scavenging enzymes and metabolites. The link between EMF-based stress tolerance, ROS and ROS scavenging enzymes has often been studied in association with abiotic stresses, which enhance the production of ROS, such as drought. For example, Alvarez et al. (2009) found that the combination of effective ROS prevention and ROS detoxification in EMF-roots resulted in reduced cellular damage and increased *Nothofagus dombeyi* growth under drought. Different EMF species can trigger different protective mechanisms to reduce oxidative stress: *Descolea antartica* mycorrhization helped to maintain SOD activity at a steady-state level, whereas *Pisolithus tinctorius*-colonized roots showed high activities of several ROS scavenging enzymes. However, different defense strategies were differentially effective since only ROS detoxification through the synchronized action of antioxidative enzymes led to reduced cellular damage in the host (Alvarez et al., 2009). In accordance with these results, AMF-plants are well known to have lower levels of H_2O_2, O_2^- and less lipid peroxidation as the consequence of enhanced antioxidant enzyme activities and mitigation of the oxidative burst especially under abiotic stress conditions, such as drought (Nath et al., 2016; Zou, Wu, & Kuča, 2021). Reduced malondialdehyde (a marker for lipid peroxidation), H_2O_2 and O_2^- levels were detected in drought-stressed plants colonized with the AMF *Funneliformis mosseae* (Huang, Zou, & Wu, 2017). Interestingly, using the non-invasive micro-test technique, the authors detected increased efflux of H_2O_2 from roots, which could be a cause of the alleviated oxidative stress (Huang, Zou, & Wu, 2017).

3.2.2 EMF, ROS and plant systemic responses to abiotic stresses

The colonization by EMF can alleviate the plant abiotic stress response in systemic tissues by inducing a systemic acquired acclimation (SAA)-like state. SAA is distinguished from systemic acquired resistance (SAR) because SAA refers to the systemic acclimation of the plant to abiotic stresses, while SAR is associated with induced immunity against pathogen infection. Several rapid whole-plant systemic signals including Ca^{2+}, ROS and hydraulic and electric waves are associated with mediating SAA (Fichman & Mittler, 2020). Furthermore, EMF induced systemic plant acclimation is

associated with redox regulation (Bai, Hao, Hu, & Leng, 2021; Sun et al., 2022). For example, Bai et al. (2021) showed higher CAT and peroxidase activities, that were associated with the salt stress tolerance in leaves of EMF-colonized oak trees (*Quercus mongolica*) (Bai et al., 2021). The authors found more pronounced effects in the trees that were colonized by *Gomphidius viscidus* than by *Suillus luteus*, suggesting, like Bouffaud and colleagues (Bouffaud et al., 2020), that different EMF species induce different systemic responses (Bai et al., 2021). *Suillus luteus* colonization has also been shown to modulate ROS and the oxidative stress response of oak (*Quercus acutissima*) and pine trees (*Pinus massoniana*) (Liu, Chen, Ding, Li, & Ren, 2020; Sun et al., 2022). In leaves of EMF oak trees, SOD and GR were enhanced (Sun et al., 2022), whereas in pine trees the mycorrhization was connected with improved growth performance, enhanced antioxidant activities (SOD and POD) and reduced malondialdehyde accumulation (Liu et al., 2020). Recent transcriptomic analyses revealed, furthermore, an activated ROS scavenging machinery in ectomycorrhizal pine prior to the stress. Such EMF-induced primed state could allow rapid responses to abiotic and biotic stresses.

EMF can improve plant performance under salinity and enhance tolerance against heavy metals (Chen et al., 2014; Langenfeld-Heyser et al., 2007; Schützendübel & Polle, 2002; Zhang et al., 2017). The capability of EMF to exclude Na^+ (Chen et al., 2014) and enhance the uptake of nutrients (Fig. 5) can enhance the tolerance against high salinity. The EMF-induced SAA-like response has, for example, been associated with decreased unsaturated-to-saturated fatty acid ratios, which together with altered nutrient status and osmo-regulation reduced the salinity induced damage in the leaves of mycorrhizal poplars (Luo et al., 2011). In addition, the plant phytoremediation potential can be facilitated by ectomycorrhizas in a ROS-dependent manner. The colonization by the EMF-species *Paxillus involutus* was shown to enhance H_2O_2 production and a H^+-pumping activity in poplar that grew under high Cd^{2+} or salt concentrations (Deng et al., 2021; Li et al., 2012). This, in turn, activated plasma membrane Ca^{2+} channels that were employed for the enhanced uptake of Cd^{2+} (Zhang et al., 2017) and thus, improved the phytoremediation.

Together these studies reveal adjustments in ROS, several signaling pathways and plant metabolome and, thereby, provide a basis to better understand the EMF-induced improvements in abiotic stress tolerance. In several studies, however, the final cause of improved performance due to EMF has remained unexplained. For example, the mechanism behind the

improved growth and increased unsaturation level of membrane lipids in the *P. tinctorius* mycorrhizal oak trees under drought was not discovered (Sebastiana et al., 2018). The transient nature of the ROS signals and the concentration-dependent action of some signaling compounds make it challenging to detect the components involved in plant signaling and mounting an improved resistance.

3.2.3 ROS in mycorrhiza induced resistance (MIR) against biotic stresses

Colonization by EMF does not only improve plant tolerance against abiotic stresses, but it can also improve the resistance against aboveground biotic threats. Altered response of EMF-trees to leaf herbivores has been shown for poplar (*P.* x *canescens*), eucalyptus (*Eucalyptus urophylla*), American chestnut (*Castanea dentata*) and birch (*Betula pubescens*) (Gange, Gane, Chen, & Gong, 2005; Kaling et al., 2018; Oddsdottir, Eilenberg, Sen, Harding, & Halldorsson, 2010; Rieske, Rhoades, & Miller, 2003). EMF-caused a state of induced systemic resistance (ISR) is commonly referred to as "mycorrhiza induced resistance" (MIR) and is considered to share characteristics of ISR and systemic acquired resistance (SAR) (Cameron, Neal, van Wees, & Ton, 2013). Both, ISR and SAR induction in systemic tissues include adjustments in phytohormone-signaling and redox balance (El-Shetehy et al., 2015; Vlot et al., 2021; Yu et al., 2022). There are hints that induction of MIR requires similar adjustments. Recent studies on poplar – *L. bicolor* interactions, for example, revealed a MIR-like state of EMF poplar-trees and improved resistance towards the specialist poplar leaf feeding herbivore, *Chrysomela populi* (Kaling et al., 2018; Sivaprakasam Padmanaban et al., 2022). The improved resistance was associated with prominent changes in plant secondary metabolites and enzymes such as VOCs, nitriles, aldoximes and chitinases. Transcriptomic analyses of poplar leaves have shown that EMF can also trigger SA- and JA-signaling related changes in systemic tissues: *Laccaria bicolor* colonization altered the gene expression of the SA-signaling essential protein, NONEXPRESSOR OF PATHOGENESIS-RELATED PROTEINS1 (NPR1) and various transcription factors related to JA and JAZ1 (Kaling et al., 2018). SA concentrations were, moreover, altered in poplars that were colonized by *Hebeloma mesophaeum* (Pfabel et al., 2012). For AMF, it is known that adjustments in different phytohormones, including SA and JA, are involved in such systemic responses (Bedini, Mercy, Schneider, Franken, & Lucic-Mercy, 2018; Benjamin, Pandharikar, & Frendo, 2022). EMF-based induced immunity and altered phytohormone levels have been observed even in non-

host plants: in *Arabidopsis* heat-killed *L. bicolor* fragments triggered SA- and JA-signaling and immunity against the aboveground pest *Trichoplusia ni* (Vishwanathan et al., 2020). In general, ROS levels are interconnected to phytohormone adjustments, making it thus likely that ROS can also be essential in conferring immunity to systemic tissues. Leastwise, ROS is a central element of SAR induction in systemic tissues: The accumulation of pipecolic acid (Pip) depends on the interplay between ROS and NO* in addition to other signaling compounds such as azelaic acid and glycerol-3-phosphate (Rekhter et al., 2019; Vlot et al., 2021). In addition, adjustments in ROS levels are known to be involved in ISR (Yu et al., 2022) and even in plant responses to fungal volatile sequiterpenes (Ditengou et al., 2015). For example, *Laccaria bicolor* interacts with *Arabidopsis* through the air phase by volatile compounds in controlled laboratory set-ups. VOCs from *Laccaria bicolor* elicit ROS accumulation and alter the lateral root growth of both, the host plant poplar and the non-host *Arabidopsis* (Ditengou et al., 2015).

3.2.4 Potential signals in transmitting information to systemic tissues

How the information is transmitted from local to systemic tissues remains unknown at the moment. As MIR resembles both, ISR and SAR, further analyses of the mobile signals associated to these two routes of induced resistance to shed light on EMF-based resistance are needed. Various mobile signals have been suggested to transmit SAR-related information, e.g., SA and Pip having verified functions in phloem-based SAR fortification (Vlot et al., 2021). Pip and NHP have also been detected along the transport route from root to leaves in xylem sap (Kasper et al., 2022). Other suggested compounds that are related to propagation of SAR include methyl salicylate (MeSA), azelaic acid and glycerol-3-phosphate (Dreischhoff et al., 2020; Vlot et al., 2021). Regarding ISR, the involved signaling routes are less clear. In the last decade, evidence accumulated that not only JA/ethylene signaling related cues but also SA signaling are involved in inducing ISR (Yu et al., 2022). Both ISR and SAR require adjustments of ROS levels as discussed above (El-Shetehy et al., 2015; Vlot et al., 2021; Yu et al., 2022). ROS are considered essential in cell-to-cell propagation of SAR and ISR signals. The defense responses that are activated by SA and Pip to propagate SAR, are dependent on activation of CALCIUM-DEPENDENT PROTEIN KINASE 5 (CPK5). The activation of CPK5 is associated to altered Ca^{2+} levels and activation of NADPH-oxidases. The phosphorylation of NADPH-oxidase is followed by elevated ROS levels which, in turn, activate the same mechanisms in

the recipient cells to further propagate the signal (Vlot et al., 2021; Yu et al., 2022). The enhanced Ca^{2+} influx and related strong production of NADPH-mediated ROS is also essential for transmission of the ISR signal to systemic tissues (Yu et al., 2022). The role of ROS in transmitting EMF-based induced resistance is, however, unknown and even their final role in propagating SAR or ISR needs still to be validated.

Considering the speed of the movement of soluble signals and the rapidness of the response in the systemic tissues, volatile cues might also contribute to eliciting induced resistance. To date, there is no direct evidence of the involvement of VOCs in inducing EMF-based resistance in systemic tissues. However, it is possible that VOCs contribute to conferring systemic immunity to non-colonized roots or to aboveground tissues. Interestingly, also microbial VOCs have functions in inducing systemic resistance. Perception of distinct microbial VOCs can elicit ISR in *Arabidopsis* either in JA- or SA-dependent manner without wall-to-wall contact between the organisms (Naznin et al., 2014). Specific VOCs can also propagate SAR-related information in aboveground interactions (Frank et al., 2021; Wenig et al., 2019). It remains to be elucidated if EMF-based induced resistance might be transmitted to systemic tissues through the air or through the soil matrix.

4. Conclusions and outlook

This review highlights the composition of the antioxidative system of ectomycorrhizal fungi and identifies important gaps, such as a possible involvement of erythroascorbate in the defense system. Although several relevant metabolites and proteins required to mediate contact and establish a functional mycorrhiza have been identified, a clear mechanistic picture is yet to emerge. Genetic suppression of fungal SOD and NR established their roles in ectomycorrhizal formation and fitness, underpinning the crucial roles of reactive oxygen species in this interaction. These enzymes may control plant ROS burst and NO* signaling, which occur in response to biotic invasion. In the extracellular space, secreted oxidative enzymes drive not only oxidative degradation of potential nutrient sources but are also critical for intra-radical fungal growth. In mature mycorrhizas, plant-fungal interactions are characterized by mutual growth control, involving H_2O_2. Local and systemic responses of the plant to mycorrhizal colonization involve ROS, phytohormones, and Ca^{2+} signaling. Less is known

about the fungal metabolism in the interaction. It will be important to understand how mycorrhizal fungal species, able to produce *OH radicals via Fenton reaction, control these activities depending on host and nutrient source. A picture is now emerging that the genetic inventory of different mycorrhizal fungal species varies with their phylogenetic origin. How this affects fungal-plant interactions is not yet well understood but evidence is accumulating that different fungi evoke different systemic effects in their host. Mechanistic molecular understanding of mycorrhizal functions and their metabolic range in different host-fungal species interactions is an outstanding task for future research. These studies are of fundamental interest. They have also an important applied perspective since soil amended with ectomycorrhizal fungi has often been recommended to improve growth and yield. In climate-stressed habitats and on polluted soils, the selection of suitable mycorrhizal species to increase the establishment and stress tolerance of saplings is of high practical relevance. Deeper insights into the genetic make-up and the underlying processes that control the host-plant interaction and resilience can open new avenues for plant improvement.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/bs.abr.2022.11.001.

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