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Capítulo 1

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ARBUSCULAR MYCORRHIZAL FUNGI AND MYCORRHIZAL SYMBIOSIS: PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR ASPECTS.

Durinézio José de Almeida

*Universidade Estadual do Centro Oeste
(UNICENTRO)*

Odair Alberton

Universidade Paranaense (Unipar)

Rosilaine Carrenho

Universidade Estadual de Maringá (UEM)

Abstract: This review analyzes the physiological, biochemical and molecular implications related to the interaction between arbuscular mycorrhizal fungi (AMF) and plants in the pre-symbiotic and symbiotic stages. Factors involving spore germination, germ tube growth, signaling and recognition of mycorrhizal fungi are discussed from a macro context, in which the plant interacts with the environment, to a most intrinsic context, in which genes are involved in this interaction. The colonization stages, from the formation of hyphopodium to the differentiation of arbuscules, are described according to cellular changes and molecular mechanisms. The dynamics of nutrient exchanges are discussed bidirectionally, from the fungus to the plant and from the plant to the fungus. The regulation processes of absorption and transport of water, phosphorus, nitrogen and calcium, and carbohydrates via the periarbuscular membrane are described in cellular, functional and molecular terms. The most common changes in the secondary metabolism of plants associated to AMF are also presented, based on biochemical processes. The conjuncture analysis showed that in the last decade, studies made efforts to identify the mechanisms of communication and symbiosis functioning, differently from studies carried in the past decades, which extensively investigated the aspects related to the growth responses of symbionts, especially in plants.

Keywords: Arbuscular mycorrhiza; genes; proteins; germination; root colonization

1. INTRODUCTION

Most of the terrestrial plants have their roots associated with arbuscular mycorrhizal fungi (AMF); forming a symbiosis that allowed their dissemination in the most varied environments and maintenance in different biomes (Smith and Read, 2008). The AMF symbiosis, this is a mutualistic association, in which the plant by photosynthesis, provides energy and carbon sources for the survival and multiplication of the fungi; The fungus, in its turn, absorb minerals from the soil solution, through extraradical mycelium, and transfer them to the plant roots; via arbuscules, in the radical cortex (Smith and Read, 2008). The nutritional increment, is the most evident benefit of this association. What making the plants more vigorous and resistant to environmental stresses (Gu et al., 2014). Therefore, the establishment of the arbuscular mycorrhizal association is importance in different contexts; agronomic, ecological and evolutionary.

This interaction results in intense physiological changes for both partners and has been intensively study. Since the discovery of the symbiosis between plants and AMF, several studies have aimed to identify its regulatory mechanisms (molecular, physiological and biochemical). Due to its importance, the knowledge about the mycorrhizal symbiosis has increased considerably in the last decade. Especially regarding the understanding of the physiological, genetic and biochemical aspects, related to the mechanisms of recognition and nutrient exchange (Hamiaux et al., 2012; Cheng et al. 2013; Phillips, 2017.)

This review, addresses different stages of the AMF association, from the spore germination to the effective establishment of symbiosis and aims to make explicit the constituted knowledge, and reveal the points that need to be further deepened.

2. PRE-SYMBIOSIS

2.1 SPORE GERMINATION

Germination of AMF spores occurs simply by the presence of water (Harrison, 2005), but, spore germination is influenced by the need for a period of dormancy; soil moisture; temperature; pH (Daniels, and Trappe, 1980; Maia and Iano Melo , 2001); light; CO₂ levels and presence of bacteria (Xavier, and Germida, 2003; Chen et al, 2008). However, the most responsive variables are root exudates. Strigolactone, a plant hormone, induces spore germination and hypha branching in AMF (Akiyama et al., 2005). More recently, it was observed a class of strigolactone, (methyl

phenlactonoates), what inhibit AMF spore germination, but promote the intra-radical root colonization (Kountche et al., 2018).

Some authors have reported inhibitory activity in the germination of AMF spores in contact with metanoic extracts from roots of different plants, (David-Schwartz et al., 2001; Gadkar et al., 2003; Ellouze et al., 2012), these authors suggest that glycoproteins rich in hydroxyproline (Ellouze et al., 2012) together with the flavonoids biochanin A, genistein and hesperetin (Chabot et al., 1992), and the isoflavone formononetine (Tsai and Phillips, 1991) are responsible by this inhibition. However, the biochemical mechanisms responsible for this inhibition, have not yet been clarified.

According to Bentivenga et al. (2013) germ tubes of all species are extremely sensitive to manipulation, which makes them difficult to study. Healthy germ tubes have a rapid growth and bi-directional cytoplasmic movement. Inside them, there is, commonly a set of 10-20 spherical bodies, possibly lipid bodies moving rapidly (Bentivenga et al., 2013). During AMF germination, considerable amounts of lipids should be; generated, modified and moved, promoting the elongation of the cytoplasmic membrane, which would explain the vesicles observed by Bentivenga et al. (2013). However the endoplasmic reticulum would be well developed.

It is important, that the biochemical mechanisms, and the cellular modifications during the germination of the AMF, should be the object of studies in the future, characterizing a gap in the study the AMF symbiosis.

2.2 BRANCHING FACTOR

Although germination of glomeromycetes spores is independent of the presence of plant compounds in the soil, the presence of root exudates stimulates her. Besides directing germination tube growth (Gianinazzi-Pearson et al., 1989). AMF hyphae grow faster and have an intense branching when they develop near roots of host mycorrhizal plants. Suggesting that the development of hyphae is related to compounds produced by the plant, called branching factors (Gianinazzi-Pearson et al., 1989).

Akiyama et al. (2005) isolated and identified a sesquiterpene from *Lotus japonicus* L. (Fabaceae), able to induce hyphae branching. This sesquiterpene was characterized as a strigolactone (Figure 1). Stringolactone is a hormone derived from carotenoids. The hormone contain an ether linkage easily hydrolyzable in the rhizosphere; that which triggers the formation of a concentration gradient between, areas near the root and those further from it.

Figure 1. Metabolic pathway of the 5-deoxystrigol synthesis: beta-carotene is converted to 9 cis-beta-carotene by the beta-carotene isomerase (A); then, to carlactone by the carlactone synthase (B), and from carlactone to strigolactone by an enzyme still unknown. Strigolactone converted to 5-deoxystrigol by the P450 mono-oxygenase (E) enzymes. Based on Alder et al. (2002) and Kanerisa laboratories (2014).

All natural strigolactone contain a tricyclic ring system (ABC) linked to a butenolide (ring D) through an enol ether bridge. The C-D ring part has a germination stimulation activity and different substituents in rings A and B affect not only the germination stimulation activity but also the stability of the strigolactonemolecule (Lin et al., 2009).

As described by Alder et al., (2002), for the formation of 5-deoxystrigol, the beta-carotene is converted by the beta-carotene isomerase [EC: 5.2.1.14]; to 9-cis-beta-carotene. Which is converted by the 9-cis-beta-carotene 9-10-dioxygenase cleavage [EC: 1.13.11.68] to 9-cis-10-apo-beta-carotenal; and then to carlactone by the carlactone synthase or all-trans-10'-apo-carotenal 13,14-beta-cleavage dioxygenase [EC: 1.13.11.69 1.13.11.70], and finally to 5-deoxystrigol by the cytochrome P450 monooxygenase enzyme.

In the plant, the strigolactone regulate the concentration of auxins and other plant hormones (Makhzoum et al., 2015; Rameau et al., 2019). The auxins are produced in the apical meristem and are transported by the phloem to the primary meristem of the root (Akiyama et al., 2005; Rameau et al., 2019). The formation of a concentration gradient from the apex to the roots probably positively regulate the biosynthesis of strigolactone (Akiyama et al., 2005; Janssen and Snowden, 2012; Rameau et al., 2019; Vam Rougen et al., 2019). Strigolactone are transported by the xylem from the root to the aerial part, where they regulate the level of free auxins in young leaves (Rameau et al., 2019). Strigolactone controls the architecture of the plant and is a key player in symbiotic and parasitic interactions. For this reason, since its discovery, publication has focused on this plant hormone. Its importance is such that it is the subject of international patents and development of biotechnological products. Like this, a simple search on <https://patents.google.com/> with the term Strigolactone, reveals that from 2016 to the present date 442 new patents.

2.2.1 STRIGOLACTONE, SOIL PHOSPHORUS AND AMF

Phosphorus (P) is one of the essential macronutrients for plants, However only some forms of phosphate in the soil are absorbed by the roots; of which the main and, inorganic phosphate (Pi) (Wang et al., 2010). Soils with a low concentration of Pi increase the sensitivity of roots to auxin (Wang et al., 2010), leading an elongation of the lateral and main roots. Under these conditions, the production of strigolactone is increased (Etemadi et al., 2014). The expression of proteins (PDR1) that export strigolactone, is also induced, by the deprivation of Pi; and therefore, part of the excess strigolactone is released into the rhizosphere, forming a concentration gradient to rhizosphere. The PDR1 are located on the plasma membrane of epidermal cells and absorbent hair cells, and facilitates the exudation of strigolactone in the rhizosphere, positively affecting the interaction of roots with glomeromycetes (Etemadi et al., 2014; Abdelhalim et al., 2020).

When, the strigolactone, meet the spore already germinated, they stimulate the growth and modify the morphology of the AMF hyphae, changing the density and the activity of mitochondria (Etemadi et al., 2014; Abdelhalim et al., 2020). In response to strigolactone, hyphae growth and branching is stimulated, by the cell proliferation and increased mitochondrial metabolism in the fungus. The increase in respiratory activity in the fungus; regulated by the strigolactone, occurs before the beginning of the branching process. First, the GrosPyc 1 and Gros(RE)-60 genes are activated; these are which are responsible for the formation of a mitochondrial pyruvate carboxylase and an ADP/ATP translocase, respectively, occurring between 30 and 60 minutes after contact with the strigolactone

(Tamasloukh et al., 2003). Subsequently, at a physiological level (120 minutes to 360 minutes), there is an increase in the production of mitochondria, and finally, as a morphological response (after 360 minutes), the hyphae branching occurs (Tamasloukh et al., 2003).

Phosphate-deficient plants contain more strigolactone than well-nourished plants, which demonstrates a relationship between plant nutritional deficiency and symbiosis development (Marzec et al., 2013).

Strigolactone play multiple roles in plant response to the low concentration of Pi. They favor the communication between roots and AMF, benefiting the establishment of the association, in addition to the acquisition of Pi from the soil, increasing the symbiosis efficiency. Moreover, mycorrhizal plants produce less 5-deoxystrigol, possibly in response to the increase of phosphorus in root tissues (López-Raez et al., 2011; Müller and Harrison, 2019), which may directly interfere with the maintenance of symbiosis.

2.3 MYC FACTOR

As described plants influence the development of MFA, in the other way; when the glomeromycetes germinate, they release compounds that are perceived by host plants (Maillet et al., 2011). These compounds act as growth regulators in the plant roots, modifying their developmental pattern (Harrison, 2005). These compounds, were initially isolated in exudates from carrot (*Daucus carota* L.), roots colonized by *G. intraradices*, and, in germinated spores of the same fungus; and today are known as Myc-factors. These exudates are composed of a simple mixture of sulfated and non-sulphated lipochitooligosaccharides (LCOS) composed of four or five residues of glucosamine, structurally resembling the Nod factors of *Rhizobium*, although with a simpler structure (Maillet et al. 2011). The induction of lateral roots is the main response observed, which significantly increases the total area of the root system (Parniske, 2008; Fusconi, 2013; Qiang-Shenga et al. 2016).

These compounds are recognized by two proteins (DMI1 and DMI2) of the common symbiosis signaling pathway (SYM), a molecular recognition pathway also found in the symbiosis of leguminous with *Rhizobium* (Maillet et al., 2011; Fusconi, 2013). However, the mechanisms involved in internal signaling in plant root cells are not yet known.

Although it is known that fungal exudates stimulate lateral root formation, some aspects of this response have not yet been fully elucidated, and the relationship between MYC factors and plant hormones has yet to be studied. It is possible that the common pathway (SYM), induced by the MYC

factors, is activated only in plants that accept symbiosis, and, may be related to the choice host process of symbiotic partners. Morcilo et al. (2015) suggested the oxylipin 9-LOX enzyme is the regulator for the SYM pathway since the expression of this molecule was increased during mycorrhization. It is also possible that there are different signs of fungal origin that may be involved in the induction of lateral root development and in the species-specific host by the plant (Fusconi, 2013).

Mateus et al. (2019) describe that, a large number of cassava genes exhibited altered transcriptional responses to the AMF but transcription of most of these plant genes responded in a different direction or magnitude depending on the plant genotype. Key genes illustrated that the two organisms jointly program their cytoskeleton organization during growth of the fungus inside roots what reveals that plant and fungal genetic variation has a strong role in shaping the genetic reprogramming in response to symbiosis, indicating considerable genotype $\times$ genotype interactions in the mycorrhizal symbiosis (Mateus et al., 2019). Perhaps MYC factors are signaling promoter molecules.

Figure 2. Presumptive model of the mechanism of communication between symbionts in the presymbiotic phase: in the soil, the strigolactone form a gradient from the plant root to the fungus, directing the hyphae growth. When the fungus germinates, it initiates the release of symbiotic initiation factors (Myc Factor) that alter the metabolism of the host plant. The blue frame on the left illustrates the flow of regulators and nutrients in the plant; the downward flow of carbohydrates and strigolactone, and upward flow of auxins, phosphate, and ammonia. The frame on the right shows the functioning of auxin regulation by strigolactone, through the SCF-max2 protein complex.

3. SYMBIOSIS

3.1 HYPHOPODIUM FORMATION

After the spore germination, the strigolactone direct the growth of hyphae to the roots of the host plant. When these hyphae meet the epidermis, the hyphopodium is formed, a structure that allows the penetration of hyphae into the cells of the root epidermis. The changes that generate the hyphopodium occur soon after the contact of the hyphae with the root surface and involve exchanges of biochemical signals between the symbionts, and the recognition of specific topographic signals by the AMF (Gianinazzi-Pearson et al., 2000).

The hyphopodium formation does not require intact cells since AMF can form it on cell walls without protoplast. However, in this case, the hypha penetrates the root but does not develop towards the cortex, indicating that the signaling between the symbionts is necessary to form the intraradical mycelium (Amiour et al., 2006).

Hyphopodium development only occurs on the epidermal cell wall of host plants (Nagahashi and Douds, 1997) and does not seem to be related to the presence of strigolactone, as observed by Harrison and Van Buuren (1995). This structure is not formed on the wall of cortical and vascular cells (Gadkar et al., 2002). Hyphopodia formed in fragments of cell wall develop hyphae that do not completely penetrate the cell and do not differentiate into arbuscules, demonstrating their development requires intact cells (Harrison, 2005). Thus, the molecular signals for the formation of hyphopodium are in the cell wall of the epidermal cell, but for the establishment of symbiosis, intact cells are necessary (Gadkar et al., 2002).

The hyphopodium development is associated with the induction of lectin genes, which possibly identify the compatibility of mycorrhizal partners (Bapaume et al., 2012; Czaja et al., 2012; Garg et al, 2019).

Russo et al. (2019) observed the TPLATE promoter is activated in colonized cells of the root cortex where we also observed the appearance of cells that are half the size of the surrounding cells developing cell plates highlighted ectopic cell division events in the inner root cortex during early AM colonization. Lastly, transcripts of TPLATE , KNOLLE and Cyclinlike 1 (CYC1) are all upregulated in the same context, alongside endocytic markers Adaptor Related Protein complex 2 alpha 1 subunit (AP2A1) and Clathrin Heavy Chain 2 (CHC2), known to be active during cell plate formation. This pattern of

gene expression was recorded in wild type *Medicago truncatula* roots, but not in a common symbiotic signalling pathway mutant where fungal colonization is blocked at the epidermal level.

These signaling molecules develop a molecular dialogue between AM fungi and plant roots, which generates a cascade of co-evolutionary events leading to the preparation of both the partners for successive root colonization (Dhanker et al., 2020).

This fact demonstrates that cell wall interactions can modulate the compatibility and, therefore, affect the host/symbiont specificity (Figure 3).

3.2 PENETRATION APPARATUS

The adhesion of hyphopodium to the root surface is regulated by ligands present in the cell wall (lectins), which signal internally and induce the formation of a penetration apparatus in the epidermal cell (Genre et al., 2005; Garg et al., 2019). The epidermal cell nucleus is moved by the cytoskeleton, directly positioning below the contact site of the hyphopodium in approximately 2 hours, from the fixation of the hypha to the complete migration of the nucleus (Genre et al., 2005). Microtubule aggregation and calcium concentration fluctuations, known as calcium spiking, are initially observed (Grunwald et al., 2014; Garg et al., 2019). Then, the nucleus is directed to the other end of the cell, and in this pathway, the microtubule aggregation accompanies the nucleus, forming a hollow column, which corresponds to the penetration apparatus, formed between 4 and 5 hours (Genre et al., 2005). The primary wall is perforated as the secondary wall invaginates into the cellular lumen. The fungus penetrates the primary wall, and the inner layers of the secondary wall are polymerized in the penetration apparatus. A thick layer of interstitial matrix material is accumulated on the outer wall and penetrates the invaginated inner wall. The invaginated secondary wall remains continuous with the initial secondary wall forming a lumen between the penetrating hypha and the protoplast; the hypha is lodged in the penetration apparatus, and its growth is delimited by this "pathway" created by the cell (Rich et al., 2014).

3.3 ROOT PENETRATION AND COLONIZATION

After colonization of the epidermal cells, the intra-radicular hypha migrates into the cells of the cortex, where the symbiosis will become functional. Plasmodesmata are structures that allow the exchange of signals and nutrients between cells of the colonized epidermis and cells of the cortex (Casieri, et al. 2013).

The migration of the epidermal cell nucleus to the proximity of the cortical cell generates a signaling via plasmodesmata and a penetration apparatus in the underlying cortical cell; such apparatus is like the one formed in the epidermis. Fungal penetration occurs precisely in the region of the initial cytoplasmic aggregation in the epidermal cell, and the growth of the hypha is directed by the intracellular columnar structure formed by the penetration apparatus (Genre et al., 2005; Garg et al., 2019).

The growth of the hypha in the cortex is directed by the apparatus, cell to cell, according to the nuclear repositioning and cytoplasmic aggregations. The penetration of the cortex cells is preceded by the formation of large penetration apparatus, which precede the development of the arbuscules (Genre et al., 2005; Garg et al., 2019), in which there are nutrient exchanges among the mycorrhizal partners.

3.4 ARBUSCULE FORMATION

Once inside the cortical parenchyma, the hyphae form arbuscules, which are branched structures of exchange of carbon, water, and nutrients between the plant and the fungus (Van Creij et al., 2020).

Not all cortical cells are colonized, and the differentiation of terminal hyphae into arbuscules occurs only in some of the cells (Genre et al., 2005; Garg et al., 2019).

According to Blee and Anderson (1998) the arbuscules are formed in the cells in the ideal position to exchange metabolites, to guarantee the flow of minerals from the fungus to the root vascular system, the protoxylem must be formed, thus guaranteeing access to the xylem. Continuity with phloem and xylem is essential for effective exchange with mycorrhizal hyphae, likewise, since symplastic continuity with phloem is limited, the maintenance of the bush would be useless. This creates an ideal region for the formation of the arbusculus.

Arbuscules can originate in two ways: i) the hyphae longitudinally run through the cortex by the middle lamella, and after the penetration apparatus formation by the cells to be colonized, several branches of the hyphae penetrate into these cells and become into the arbuscule with numerous fine branches; or ii) the hyphae penetrates into the cortical cell and after the penetration apparatus development, they enter into the adjacent cells; in this second condition, the hypha is thicker and less branched (Genre et al., 2005; Garg et al., 2019).

The success of mycorrhization depends on the synthesis of a membrane related to the formation of the interface between the hypha that originates the arbuscule and the protoplasm of the cortical cell (Genre et al., 2005, Van Creij et al., 2020). The mechanism of synthesis of this membrane

(periarbuscular) is the same one associated with cell division in plants, including cell plate formation during cytokinesis, extensive plastid modifications and mitochondrial proliferation and enlargement (Genre et al., 2011).

The formation of arbuscule depends on the fungus ability to supply the phosphate to the host plant, which should have active genes of phosphate carrier protein (*MtPT4*), located in the periarbuscular interface. These genes are expressed only in cells that have arbuscules and are transcribed by the activation of a signal of the lysophosphatidylcholine that is associated with the cell membrane (Grunwald et al., 2014; Van Creij et al., 2020). The expression of *MtPT4* seems to be a highly versatile mechanism that can modulate the formation of the arbuscule according to the specific needs of each cell.

Figure 3. Representation of the current knowledge of the mechanisms of exchange among symbionts. Green arrows represent the flow of nitrogen (it enters as nitrite, is transported in the extraradical hypha as urea and is released to the periarbuscular interface in the form of ammonia). Blue arrows represent the flow of glucose (the sucrose is transferred to the periarbuscular interface, degraded into glucose, which is transferred into the arbuscule, where it is converted into glycogen and migrates from the intraradical to the extraradical mycelium, in which it is transformed into triacylglycerol). Red arrows refer to phosphate flow (phosphorus is absorbed as phosphate by extraradical hyphae, converted to polyphosphate and transported to the arbuscule; then, this polyphosphate is converted to phosphate and transferred to the periarbuscular space to be assimilated by the plant cell). The model also shows the proteins involved in the process, the formation of hyphopodium and penetration apparatus, the spiking of calcium, and the transference of calcium from the fungus to the plant.

3.5 GENES AND PROTEINS INVOLVED IN THE ESTABLISHMENT OF SYMBIOSIS

A small group of genes, originally identified because of their role in the early stages of rhizobium nodule formation, is responsible for root penetration. These genes have a function in the transduction of the specific signal (factor Nod and factor Myc) perceived in the cell membrane, being essential for the mycorrhizal symbiosis. They were named genes common to symbiosis (*SYM*). In plants, mutations in genes of this group (*SYM2*, *SYM3*, *SYM4*, and *SYM15*) result in the absence of symbiosis (Demchenko et al., 2004; Van Creijl et al., 2020) due to a complete or partial blockade of epidermal penetration.

Some genes have also been identified as responsible for preventing the colonization of leguminous roots by rhizobium; they are known as non-colonization genes, *DMI1*, *DMI2*, and *DMI3* (Demchenko et al., 2004). Molecular studies have shown that the genes responsible for nodulation in leguminous, *ENOD* genes (*ENOD2*, *ENOD5*, *ENOD11*, *ENOD12*, and *ENOD40*) are also transcribed during AMF root colonization (Journet et al., 2001; Van Creijl et al., 2020).

Hogekamp and Küster, (2013) described changes in the transcription pattern in metallothionein genes (*Mt*) during the early stages of mycorrhization (*MtBcp1*, *MtScp1*, *MtFlot4*, *MtCbf1*, *MtCbf2* and *MtDxs2*). Metallothioneins are proteins rich in cysteine, located on the membrane of the Golgi apparatus, able to bind with metals (such as zinc, copper, selenium, cadmium, mercury, silver, and arsenic) through cysteine residues of the thiol group, which account for about 30% of their constituents, and their transcription is probably associated with the formation of the penetration apparatus.

In addition to these genes, Groth et al. (2013) identified the primordial function of the *SYMrk* gene and of those relating to the transcription of Castor and Póllux proteins. The first one transcribes a Kinase-like receptor, consisting of transmembrane proteins with amino-terminal extracellular domains and intracellular kinase domains in the terminal carboxyl. Castor and Póllux proteins are ionic channels responsible for the spiking of calcium, which occurs about 10-30 minutes after the plant detecting the presence of Nod factors, which are identified in symbiosis with rhizobium and in symbiosis with AMF (Journet et al., 2001; Van Creijl et al., 2020). Both Castor and Póllux act on the depolarization of the membrane that triggers the opening of calcium release channels.

Previously, Parniske (2008) had also reported the presence of genes involved in transcription of two nuclear proteins: the calcium/calmodulin-dependent kinase (CCAMK), which acts together with the calcium spiking, and the cyclops phosphorila of which function is still unknown. In addition to these

genes, there is a transcription of nucleoporins genes (*NUP85 and NUP133*), which are responsible for nuclear import and export mediated by signals external to the nucleus, allowing the flow through the nuclear pore complex (NPC) (Groth et al., 2013)

Symbiosis and intracellular hyphae lodging require the activation of the *Vapyrin* gene, which is a gene induced by mycorrhization encoding the "vesicle-associated membrane proteins" (VAMPs), an integral part of endoplasmic reticulum membrane proteins, primarily related to the fusion of vesicles (Pumplin and Harrison, 2009). The *Vapyrin* gene is indispensable for the development of mycorrhizae is directly related to the formation of the penetration apparatus (Pumplin and Harrison, 2009).

The arbuscule development is accompanied by the activation of enzymes that lead to the synthesis and accumulation of fatty acids and apocarotenoids (products from the methylerythritol phosphate pathway) (Walter et al., 2000). The methylerythritol phosphate pathway seems to be important for the development of the arbuscule because it reduces the expression of 1-deoxy-D-xylulose-5-phosphate synthase (DXS2), a gene that encodes a key enzyme of the methylerythritol phosphate pathway, and affects the development and induction of the genes responsible for the arbuscule formation (Blee and Anderson, 1998; Groth et al., 2013).

In addition to these genes, which are essential for the process of establishing symbiosis, it is important to highlight the presence of genes responsible for the communication between the fungus and the plant. Among these, the most expressive are: *MtPt4*, responsible for the transport of inorganic phosphate; *MtLec7* and *MtLec5*, precursors of lectin, which are proteins responsible for the adhesion of the hypha to the cell wall, and *MtHa1*, responsible for the transcription of an H⁺-ATPase that polarizes the membrane, allowing the phosphate flow (Pumplin and Harrison, 2009; Van Creijl et al., 2020).

Etemadi et al. (2014) investigated the role of microRNA393 (*miR393*), which acts on several auxin receptors. In root colonization by AMF, these authors observed that the overexpression of *miR393* in root tissues reduced the expression of auxin receptor genes, culminating in underdeveloped arbuscules; and they demonstrated that *miR393* is a negative regulator of the arbuscule development, making difficult the perception of auxin in colonized cells (Etemadi et al., 2014).

Pimprikar et al. (2016) reported that AM, CYCLOPS is necessary for the beginning of shrubs and expression of colonization marker genes, such as *SbtM1*, *PT4* in *Lotus japonicus*, or *AM10* and *PT11* in rice. And that an overexpression of an active dominant version of CCaMK (*CCaMK314*) can induce a transcription of marker genes related to AM, such as *SbtM1*, *RAM1*, *RAM2* and *Vapyrin* in the absence

of AM fungi and spiking content, and even that the expression of *SbtM1*, *RAM1*, and *Vapyrin* have been shown to depend on *CCaMK*. Taken together, this suggests that the complex gene regulates CCaMK-CYCLOPS during AM symbolism.

Several transcription factors involved in transcriptional reprogramming of cortical cells are present during the development of arbuscules (Luginbuehl and Giles, 2017), among these we can highlight, gens *MtCCaMK*, that transcribes a calcium and calcium/calmodulin-dependent serine/threonine-protein kinase DMI-3; genes related to *GRAS family transcription factor*, involved in the regulation of the expression of symbiotic genes and the specificity of symbiosis signaling; and *SERCA-type calcium-translocating P-type ATPase transcriptin factor*, involved in calcium transport (Hogekamp and Kuster, 2013; Oldroyd, 2013).

3.6 PERIARBUSCULAR MEMBRANE AND TRANSPORT PROTEINS

Arbuscules are temporary structures with a complete life cycle estimated in 7 to 10 days, varying according to the symbionts involved (Alexander et al., 1989). Although the arbuscules are formed within the cortical cells, they remain separated from the plant cell cytoplasm by a membrane, the periarbuscular membrane. The resulting interface, delimited by this membrane, is characterized by a network of phosphate carriers (MtPT4) that are expressed exclusively in cells with arbuscules (Harrison et al., 2002). Moreover, transport ATPases of hydrogen proton (MtHa1) create a proton gradient necessary for the adequate action of MtPT4 carriers (Gianinazzi-Pearson et al., 2000).

Currently, the mechanisms and cellular machinery involved in biogenesis of the periarbuscular membrane are largely unknown. Zhang et al (2015) we identify an EXO70I protein present exclusively in plants forming AM symbiosis. Roth et al (2018) identify the periarbuscular membrane -specific Arbuscular Receptor-like Kinase 1 (ARK1) from maize and rice to condition sustained AM symbiosis.

Plants control the density of arbuscules by the functioning of the periarbuscular membrane, depending on their nutritional status and it has repeatedly been reported that the high supply of phosphate represses the differentiation and the functioning of the arbuscules (Menge et al., 1978; Balzergue et al., 2013). In addition to phosphate regulation, the useful life of an arbuscule is also regulated by nitrogen (Javot et al., 2011). The suppressive effect caused by excess phosphate is partially inhibited by the lack of nitrogen and, to a lesser extent, by the lack of potassium, calcium or iron (Nouri et al., 2014). MtBcp1, a protein that has copper as a prosthetic group, is specifically induced in the root cortex in regions colonized by AMF. It is on the opposite side of the cell from the MtPT4

and is restricted around the arbuscular stem (Genre et al., 2008; Genre et al., 2005). In addition, its functional dependence on copper makes possible to infer that this micronutrient also acts as a regulator of the symbiosis.

It is important to report that the flow of these nutrients in the periarbuscular membrane is perhaps the most important factor in the maintenance of the useful life of an arbuscule, but the regulation for the formation of new arbuscules and the degeneration of the existing ones can be centered in proteins regulated by the variation of these nutrients in non-colonized regions in the plant.

The possible long-distance phosphate signaling compounds can be from the miR399 family since they play a well-established role in signaling nutritional deficiency for this nutrient (Gu et al., 2011). The expression of miR399 is increased after formation of the periarbuscular membrane (Gu et al., 2014) and, therefore, after the phosphate input resulting from the symbiosis.

The collapse of an arbuscule seems to be associated with the accumulation of peroxisomes (Pumplin and Harrison, 2009), which are organelles involved in the catabolism of fatty acids, D-amino acids, and polyamines. About 10% of their function are focused on two enzymes (glucose-6-phosphate dehydrogenase "G6PD" and 6-phosphogluconate dehydrogenase "6PGD") in the pentoses phosphate pathway; such enzymes are important for energy metabolism of eukaryotes.

The mechanism of phosphate transfer in symbiosis has been studied extensively, whereas the process of reciprocal transfer of carbon is less well known. Nowadays, it is known that there is a monosaccharide (MST2) carrier protein, in the fungal membrane, necessary for colonization and development of the arbuscules (Kosuta et al., 2003). MST2 has an increase in expression positively correlated with the expression of MtPT4, and both proteins are repressed by high phosphate availability (Kosuta et al., 2003). Therefore, the phosphate supply seems to be the main regulator of the carbohydrate flow from the plant to the symbiotic fungus. Initiation, development, and maintenance of the symbiosis are largely under the control of the host plant and strongly influenced by the plants' P status.

Recent advances reveal, according to Müller and Harrison, (2019) that mycorrhizal symbiosis modifies the expression of several miRNA genes, exemplifying: miR393 is expressed in roots grown with a low level of phosphorus where it negatively regulates the development of arbuscules in the cell by means of regulation of auxin receptors; miR171h negatively regulates symbiosis in response to the nutritional and symbiotic status of plants, avoiding over-colonization and miR171a; miR171c; miR171 d; miR171 e; miR171 fe miR171 g silence the GRAS MtLOM1 important proteins thatThese play diverse roles in

root and shoot development, gibberellic acid signals, *ing* and phytochrome A, signal transduction transcriptional regulator in all root cells, except for colonized cortex cells, while *miR171b* is specifically expressed in colonized cells.

3.7 SUMMARY OF SYMBIOSIS ESTABLISHMENT

In the first phase of colonization, the exudates released by the fungus activate the common symbiosis pathway (SYM), generating a morphological response in the plant demonstrated by the increase in the lateral root.

Physiologically, there is an increase in the production of strigolactone, which are released into the rhizosphere, leading to a concentration gradient that induces the branching of the AMF hyphae. When the hypha meets the root surface, it interacts with lectins specific for the epidermal cell, modifies and forms the hyphopodium. This process generates structural changes in the epidermal cell, and the penetration apparatus is formed, directing the growth of the hyphae from the epidermis to the cortex. After recognizing the cortex root cells, the hyphae grow and initiates the formation of the arbuscule, which is accompanied by the formation of a membrane (periarbuscular) rich in phosphate transport proteins (MtPT4).

The increase in the phosphate flow to the plant changes the root architecture through different mechanisms and plays a central role in the arbuscule maintenance and symbiosis implementation.. Once the symbiosis has been established, there are increases in the concentration of auxin and cytokinin and decrease in the concentration of strigolactone, stabilizing the symbiosis.

3.8 DYNAMICS OF SYMBIOSIS FUNCTIONING

3.8.1 WATER ABSORPTION

The symbiosis with glomeromycetes increases the resistance of phytobionts to drought by promoting an increase in water absorption capacity, which prevents dehydration and maintains stability in hydraulic conductivity, eliminating the generation of ROS, such as hydroxyl radicals, singlet oxygen, hydrogen peroxide and radical superoxide anion (Bapaume et al., 2012; Rodrigues, 2017; Huang, 2017). Mycorrhization generates an induced resistance in plants after their exposure to abiotic stresses but also activates stress tolerance mechanisms implemented by the fungus (Lenoir et al., 2016). Improved control of water use by the plant results in a better overall nutritional status, and this improvement is due to the fungus's ability to absorb and transport substances (Egerton-Warburton et al., 2007).

Bárzana et al., (2014) describe an expression of different protein types of aquaporins in mycorrhizal plants; these different types transport different nutrients and organic compounds together with water, so according to the authors, a mycorrhizal symbiosis helps not only in the supply of water but also without a flow of nutrients and in the mobilization of nitrogen compounds, glycerol, signaling molecules or metalloids with role in tolerance to abiotic stress. In non-mycorrhizal plants, the water flow through the membranes passively occurs through pores (aquaporins) that facilitate the transfer of water through the membranes along an osmotic gradient, but by this pathway, it is not possible to actively pump water against a potential osmotic gradient (Egerton-Warburton et al., 2007; Ruiz – Lozano and Arouca, 2017). The aquaporins in superior plants are classified into five groups, according to their subcellular location, genetic expression pattern. and protein structure: plasma membrane intrinsic proteins (PIP), tonoplast intrinsic proteins (TIP), nodulin26-like intrinsic proteins (NIP), simple intrinsic proteins (SIP), and intrinsic X proteins (IXPs) (Danielson and Johanson, 2008; Ruiz–Lozano and Arouca, 2017).

Porcel et al., (2006) characterized the influence of mycorrhizal symbiosis in the expression of PIP genes, related to aquaporin transcription; these authors describe a negative regulation in the expression of these genes, in comparison with non-mycorrhizal plants, in response to water stress. In this context, symbiosis, in addition to ensuring a greater influx of water to the plant, allows for a greater regulation of the response to hydraulic stress. According to Bahadur et al., (2019) differential expression of genes that code for aquaporins occurs both in plants and in mycorrhizal partner when symbiosis is established. This expression is regulated, in both partners, by the hydric status of the root allowing grounding in the hydraulic conductivity and tolerance under water deficiency.

The aquaporins besides acting as channels for water, they facilitate the diffusion of neutral compounds with low molar weight, such as glycerol, ammonia and carbon dioxide through the membranes (Uehlein et al., 2007; Ruiz–Lozano and Arouca, 2017).

The PIP, TIP and NIP proteins have increased expression in mycorrhizal plants (Bapaume et al., 2012; Nehls et al., 2014; Chitarra et al., 2016; Guerrero-Galan et al., 2018), and in addition to their transport activity, these proteins can mediate interactions near the periarbuscular membrane, but the exact biochemical mechanisms and functions in mycorrhizal symbiosis are still unknown.

3.8.2 Phosphate absorption

The absorption of phosphate by the fungus from the soil solution is probably associated with a co-transport system, in which the H^+ and Na^+ ions are transported together with the inorganic phosphate

(Tatsuhiro et al., 2016; Kameoka et al., 2019). After absorption of phosphate from the soil solution, it is transferred from the cytoplasm to vacuoles within the extraradical hyphae, through an electric potential gradient created by the vacuolar H^+ -ATPase (V-ATPase) (Fellbaum et al., 2014).

Orthophosphate and polyphosphate are the major forms of inorganic phosphate in fungal vacuoles, but organic phosphate compounds may also be found, as described by Tatsuhiro et al. (2016). According to these authors, polyphosphate is a linear polymer of P_i residues linked to each other by high energy phosphoanhydride linkages, which has a temporary storage role of P_i in fungi. And they also reported that phosphate translocation from the absorption zone to the arbuscule is probably carried out by vacuoles, both by cytoplasmic motions and by vacuolar fusion (Tatsuhiro et al., 2016).

The polyphosphate in the intraradical hyphae is longer than that in the extraradical hyphae or spores, indicating that the molecule is depolymerized before the transference in the periarbuscular space (Ohtomo and Saito, 2005; Kameoka et al., 2019).

According to Smith et al, (2001) inside the root, P is transferred from the arbuscule to the interfacial apoplast by a still unknown mechanism and is absorbed by the cortical cells of the root. according to the authors, the expression of a high affinity P and H^+ -ATPase transporter in cells containing arbuscules indicates that these are probably the phosphorus / plant transfer sites; however this is a gap yet to be answered.

3.8.3 NITROGEN ABSORPTION

Nitrogen is absorbed by AMF extraradical hyphae as nitrate or ammonium. Studies have been suggested that the capture system has five times more affinity with NH_4^+ (Bücking and Kafle, 2015; Kameoka et al., 2019), and within the hyphae, they are converted into glutamine compounds (Tatsuhiro et al., 2016).

The produced glutamine is directed to the urea cycle, promoting the synthesis of arginine, which is transferred to the vacuoles. Then, the vacuoles filled with arginine are transported to the arbuscule. Arginine is taken out of the vacuole and transformed into urea, which is stored at high concentrations in the arbuscule (Tatsuhiro et al., 2016).

Finally, the nitrogen is transferred to the plant root as ammonium (Fellbaum et al., 2014), probably due to the different concentration in the intracellular medium, and NH_4^+ transporters located in the periarbuscular membrane of the plant (.

3.8.4 CALCIUM ABSORPTION

Vacuolar calcium is the main source of Ca^{2+} storage in AMF hyphae (Tatsuhiko et al., 2016). Two carriers perform complementary functions in sequestering Ca^{2+} in the vacuole: **Vcx1p**, a low-affinity $\text{Ca}^{2+}/\text{H}^{+}$ exchanger, which rapidly sequesters Ca^{2+} in the vacuole, and **Pmc1p**, a high-affinity Ca^{2+} ATPase required to maintain the low calcium concentration in the cytosol (Liu et al., 2013; Kameoka et al., 2019). This element is essential for the establishment and maintenance of mycorrhization by mediating intracellular signaling in perinuclear membranes, forming a rhythmic flow of calcium in the nuclear membrane (calcium spiking), which triggers the transcriptional reprogramming in host cells (Oldroyd and Downie, 2006).

The calcium spiking occurs around the nucleus, suggesting that the calcium channels are in the nuclear membrane and that it probably comes from the nucleus. Several components of the nuclear pore complex (NPC) are required for symbiotic signaling (Parniske, 2008). Mutations in nucleoporins affect calcium spiking and prevent the establishment of symbiosis (Fellbaum et al., 2014; Groth et al., 2013).

The common pathway (SYM) involves cation channels (DMI1, Castor and Pollux), located in the nuclear envelope (Parniske, 2008). These channels mediate the potassium flux to compensate for the imbalance of charge resulting from the calcium flux (Charpentier et al., 2008). Whereas calcium efflux from the nucleus to the cytoplasm is mediated by nucleoporins; a calcium ATPase regulates the influx of this same calcium back into the nucleus while maintaining the rhythmicity of calcium spiking (Bapaume et al., 2012).

3.8.5 TRANSFERENCE OF CARBOHYDRATES

Plants colonized by AMF have a high respiration rate (Fahey et al., 2016), caused by the flow of carbohydrates to their symbiont. Carbohydrates are transported in the plant as sucrose, which is converted into fructose and glucose in the colonized cells; then, these two monosaccharides cross the membrane to the periarbuscular space and then into the mycobiont.

In the hyphae, these hexoses are converted into trehalose (Bécard et al., 1991) and glycogen (Bonfante-Fasolo and Grippiolo, 1984), which are two carbon pools that can be rapidly synthesized and degraded, regulating carbohydrate concentrations in fungal plasma. Also, the hexoses enter the phosphate pentoses pathway, making possible the formation of pentoses for nucleic acid formation (Bécard et al., 1991). A high percentage of the carbon obtained is converted into lipids (the dominant

form of carbon stored in the fungi) and exported to the extraradical hyphae, serving as fuel for spore germination (Beilby and Kidby, 1980; Wewer et al., 2014).

There are several enzymes active in the symbiosis, such as fungal lipases, phosphatases (Wewer et al., 2014), succinate and malate dehydrogenases, trehalases, and peroxidases, involved in the metabolism of lipids and carbohydrates in the intra- and extraradical hyphae (Bago et al., 2003)

At the level of molecular genetics, fungal genes were identified for glyceraldehyde-3-P dehydrogenase, chitin synthetase, and phosphoglycerate kinase (Grunwald et al., 2014), which allows inferring the glycolysis, tricarboxylic acid cycle, phosphate pentose pathway, and synthesis of lipids are regulated by the development of the fungus. It is also possible to infer that lipid metabolism involves β -oxidation and the glyoxylate cycle (Lammers et al., 2001).

3.8.6 TRANSFERENCE OF LIPIDS.

Since the periarbuscular membrane is of phospholipid origin, it is presumed that the materials resulting from its fragmentation are used by the fungus as an energy source.

Many plant lipid metabolism genes are up-regulated during AMS (Tang et al., 2016). Furthermore, several genes involved in lipid metabolism are conserved exclusively in plants that engage in AMS (Bravo et al., 2016). Various jobs reveals extensive changes in lipid content during AMS with, which could include lipid biosynthesis (Wewer et al., 2014). According to Bravo et al (2017), during arbuscular mycorrhizal symbiosis, considerable amounts of lipids are generated, modified and moved within the cell to accommodate the fungus in the root, and it has been suggested that lipids are delivered to the fungus. Thus, during AMS, high-level, specific expression of key lipid biosynthetic enzymes located in the plastid and the endoplasmic reticulum enables the root cell to fine-tune lipid biosynthesis to increase the production of β -monoacylglycerols, Bravo et al. (2017).

This may be the explanation for the variations observed in the production of fatty acids, and essential oils in mycorrhizal plants, which contradicts the current statement, that the increase in nutrients and water is the reason for a pool in the production of these compounds by plants.

3.9 SUMMARY OF SYMBIOSIS FUNCTIONING

The H^+ -ATPases of the plant and the fungus generate in the inter-symbiotic space a difference of concentration of H^+ that energizes the membrane, promoting the transport of phosphate, ammonium, and calcium from the fungus to the plant, and glucose from the plant to the fungus. However, the

continuous flow of nutrients and H^+ protons acidifies the periarbuscular space and part of these protons must be removed to make the process viable. Calcium is responsible for the signaling for differential transcription that results in the penetration apparatus, indispensable for symbiosis.

The carbohydrate produced in the photosynthesis migrates to the colonized cortex cells and is made available to the fungus, which converts it to more complex compounds, which are stored and later used in spore germination. Phosphate is absorbed from the soil solution by the fungus and converted into polyphosphate compounds, which migrate through the vacuole to be made available to the plant, via MtPT4 in the periarbuscular space. Similarly, nitrogen is obtained from the soil solution by the fungus and converted to urea, which is converted into ammonium, and transferred to the plant by the periarbuscular pathway. In this way, the flow of nutrients from the fungi and photoassimilates from the plant regulates the symbiosis.

4. CHANGES IN SECONDARY METABOLISM OF PLANTS PROMOTED BY ARBUSCULAR MYCORRHIZAL SYMBIOSIS

The mycorrhizal symbiosis can influence the secondary metabolism of plants (Smith and Read, 2008), altering the levels of the main groups of metabolites, terpenoids, phenolics and alkaloids (Andrade et al., 2013; Zubek et al., 2015; Welling et al., 2016), including flavonoids involved in recognition mechanisms between roots and endosymbiotic microorganisms (Larose et al., 2002; Ponce et al., 2004).

Such interference results, secondarily, in changes in the production of structural, reserve and defense components. Singh et al. (2010) carried out studies on mycorrhizal and non-mycorrhizal green tea plants [*Camellia sinensis* (L.) Kuntze, Theaceae] for two years and they found that mycorrhization significantly increased plant growth and amino acid and total protein levels (Up to 100%), total sugars (16%), reducing sugars (45%) and non-reducing sugars (69%). Regarding the secondary metabolism compounds, these authors verified an increase in the concentrations of total polyphenols (15%) and caffeine (34%).

Geneva et al. (2010) evaluated the quantitative pattern of essential oil in *Salvia officinalis* L. (Lamiaceae) inoculated with *G. intraradices*, and they obtained increases in dry mass production and in the content of antioxidant metabolites (ascorbic acid and reduced glutathione). These authors also observed changes in the activities of antioxidant catalase, ascorbate peroxidase, and superoxide

dismutase enzymes, with increased amounts of 1,8-cineole and α -thujone of other essential oils in mycorrhizal plants.

Zubek et al. (2012) evaluated specimens of *Inula ensifolia* L. (Asteraceae) after inoculation with *G. intraradices* (UNIJAG PL-Bot and UNIJAG PL-Kap isolates), *G. clarum* (UNIJAGPL13-2) and native communities of natural populations of the species and they found increases in thymol content in mycorrhizal plants. Barto et al. (2010), by evaluating the allelopathic potential of *Alliaria petiolata* Bieb. (Brassicaceae), found higher levels of glucosinolates and flavonoids in mycorrhizal plants. Wewer et al. (2014) found that the symbiosis between *L. japonicus* (Fabaceae) and *Rhizophagus irregularis* resulted in the suppression of galactolipid synthesis and increased the levels of various compounds, such as free sterols and sterol esters, phospholipids (phosphatidylcholine), triacylglycerides and some sphingolipids (glucosylceramides).

Lingua et al. (2013) reported a significant increase in the proportion of anthocyanins in fruit strawberry (*Fragaria x ananassa*, Rosaceae) mycorrhizal plants. Barley plants inoculated with *Glomus intraradices* had higher levels of jasmonic acid (JA) and of its conjugated amino acid JA-isoleucine (Hause et al., 2007). Zubek (2012) reported that *Hipericum perforatum* L. (Hipericaceae) mycorrhized with a mixture of AMF genera had a higher concentration of hypericin and pseudohypericin in the aerial part.

Tarraf et al., (2017) suggest that AM symbiosis is a good candidate for promoting plant growth and essential oil composition and for improving P uptake in low fertility soils. Thus, mycorrhiza can be considered as a sustainable strategy based on natural resources in order to influence the manool and α -thujone content in sage essential oils composition

Cruz et al. (2020) reported a significant increase the total sugars, reducing sugars, phenolic content, flavonoids, antioxidant DPPH (2,2-diphenyl-1-picrylhydrazyl) activity and variation of the essential oil (EO) chemical composition of the *lemon grass* inoculated with AMF *Rhizophagus clarus* and *Claroideoglomus etunicatum* and with plant growth promoting bacteria *Azospirillum brasilense*. Variation in EO, it was also reported for Morelli et al. (2017) in *Ocimum basilicum* L. inoculated with for *C. etunicatum*; and by Cruz et al. (2019) in *Salvia officinalis*, inoculated with *R. clarus* and *C. etunicatum*; and also for Almeida et al. (2020) in chamomile plants inoculated with the soil-inoculum were composed of 22 AMF species. These authors describe that a possible increase in plants' total metabolic rate is explained by symbiotic interaction between the AMF to improve root development and nutrition.

Xie et al. (2019) suggested that improved P nutrition by AM symbiosis was of primary importance for facilitating glycyrrhizin and liquiritin accumulation in *G. uralensis* plants. This confirmed the role of AM symbiosis improving plant P uptake in the regulation of secondary metabolite biosynthesis.

5. FINAL CONSIDERATIONS AND PROSPECTS

After analyzing the general context of the topics presented in this review, it is verified that the knowledge of symbiosis quantitatively increased in the last years, mainly regarding mechanisms involved in the communication between symbionts as well as on molecular approach of this communication. On the other hand, it was observed a low number of studies related to the secondary metabolism of plants, indicating a lack of interest in this subject by the researchers, who focused more on the symbiosis than on the its interference with the host plant. Also, it was evidenced that information on the physiological and molecular aspects related to the development of mycobiont is still scarce, so further studies are necessary.

The spore germination mechanism has not yet been fully elucidated, as well as the mechanisms involved in internal signaling in plant root cells in response to MYC factors. Factors that lead to host/symbiont specificity are also unknown, and the maintenance and collapse of an arbuscule need further scientific evidence for a complete understanding, especially regarding the function of the metallothioneins found in the arbuscule.

Although the activation of the Vapyrin gene is essential for the arbuscule formation, the function of the VAMP proteins in the communication between symbionts has not yet been evidenced and must be later investigated.

In addition, mycorrhizal symbiosis interferes with secondary metabolism, increasing the concentration of secondary compounds, interfering with the host plant's relationship with other biotic components.

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Capítulo 2

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UTILIZAÇÃO DE FUNGOS NA DEGRADAÇÃO DE COMPOSTOS FENÓLICOS DE DERIVADOS DE PETRÓLEO

thariny faria borges de assis

Instituto Federal de Educação, Ciência e Tecnologia do Amazonas

igor ramon nascimento hoshihara

Instituto Federal de Educação, Ciência e Tecnologia do Amazonas

Libertalarimar Bilhalva Saraiva

Instituto Federal de Educação, Ciência e Tecnologia do Amazonas

Renato Costa Mena Barreto

Instituto Federal de Educação, Ciência e Tecnologia do Amazonas

Sonia Maria de Melo Lima

Instituto Federal de Educação, Ciência e Tecnologia do Amazonas

Elton Alves de Souza Filho

Instituto Federal de Educação, Ciência e Tecnologia do Amazonas

Resumo: As atividades em postos de abastecimento de combustível além dos efluentes gerados em lavagens de automóveis e outras limpezas podem constituir fontes de materiais tóxicos como o benzeno e seus derivados. Os compostos fenólicos são acumulados nos corpos de água receptores e causam aumento da demanda de oxigênio. Os fungos representam uma alternativa no tratamento de efluentes que possuem compostos aromáticos recalcitrantes, como fenol e derivados. O presente trabalho propôs estudar a degradação de compostos fenólicos em efluente de postos de gasolina pela ação dos fungos *Aspergillus sp.* e *Trichoderma sp.* em um reator em batelada. O reator foi operado por 60 dias, com tempo de retenção de quatro dias, atingindo em sessenta dias 43% de eficiência de remoção em DQO. Os resultados obtidos, mesmos ainda incipientes em função do tempo de operação do reator, mostram o potencial dos microrganismos na degradação de efluente de posto de combustível. E a adaptabilidade ao meio, que foi observada pelo grande crescimento dos microrganismos.

Palavras-chave: Compostos Fenólicos, Efluente, Petróleo, *Aspergillus sp.*, *Trichoderma sp.*

1. INTRODUÇÃO

O envolvimento de postos revendedores de combustíveis com poluição ambiental refere-se, geralmente, a vazamentos dos tanques de armazenagem subterrâneos. No entanto, as próprias atividades de abastecimento de veículos, transferência do combustível dos caminhões para os tanques subterrâneos e troca de óleo também podem constituir fontes de contaminantes, além dos efluentes gerados em lavagens de automóveis e outras limpezas que podem conter óleos, graxas, sabões, material argiloso em suspensão.

Dentre os diferentes tipos de poluentes tipicamente atribuídos às atividades humanas, os produtos de petróleo são um dos mais relevantes em razão de seu potencial tóxico. Esta toxicidade é atribuída à presença de substâncias como benzeno e seus derivados, além de hidrocarbonetos policíclicos aromáticos, como os compostos fenólicos (MARTINS *et al*, 2007).

Os compostos aromáticos apresentam características recalcitrantes e são altamente tóxicos e acumulam-se nos corpos de água receptores e causam aumento da demanda de oxigênio. A problemática do descarte é decorrente do fato de que muitos desses poluentes se encontram dissolvidos no efluente e, portanto, não podem ser removidos por processos físicos convencionais.

Estes compostos são encontrados em diferentes concentrações em diversos tipos de águas residuárias de indústrias, tais como, as de azeite de oliva, química, farmacêutica, corantes, pesticidas, solventes, destilarias de vinhos, de resinas, de papel e outros (ALBUQUERQUE, 2000).

Os compostos fenólicos são encontrados no meio ambiente como resultado da decomposição de substância húmicas, lignicas e taninos em concentração menores que os gerados pelas atividades antrópicas, TIMUR *et al* (2004). Além disso, os resíduos de compostos fenólicos são os de maior potencial poluidor, em virtude de suas características ácidas, tóxicas e bactericidas. Estes compostos fenólicos, geralmente apresentam-se em efluentes industriais em altas concentrações e sua remoção é onerosa e de difícil depuração pelo meio ambiente.

Os compostos fenólicos são acumulados nos corpos de água receptores e causam aumento da demanda de oxigênio. A problemática do descarte é decorrente do fato de que muitos desses poluentes se encontram dissolvidos no efluente e, portanto, não podem ser removidos por processos convencionais. A oxidação biológica tem se revelado como uma forma efetiva para a remoção de compostos fenólicos de efluentes industriais e domésticos. A atividade microbiana destaca-se como importante fator na eliminação de poluentes químicos, pois tanto bactérias quanto fungos têm

desenvolvido mecanismos de sobrevivência na presença de compostos aromáticos em concentrações consideradas tóxicas. Os fungos representam uma alternativa no tratamento de efluentes que possuem compostos aromáticos recalcitrantes, como fenol e derivados (TRABULSI, 2002; RODRIGUES, 2004).

Embora os fungos tenham um crescimento mais lento possuem a vantagem de se ramificarem rapidamente no meio através das enzimas secretoras, além de tolerarem a maioria das substâncias tóxicas. Uma espécie fúngica é capaz de produzir vários tipos de enzimas, o que torna este microrganismo de interesse relevante para remoção de compostos fenólicos. Além disso, os fungos são organismos capazes de suportar mudanças bruscas na concentração de matéria orgânica, são capazes de tolerar grandes variações no pH, temperatura, além de suportar a escassez de umidade e de oxigênio, dando viabilidade ao trabalho.

Este trabalho teve por objetivo a utilização dos fungos *Aspergillus sp.* e *Trichoderma sp.* na degradação de efluente de posto de abastecimento de combustíveis em reator em batelada.

2. MATERIAL E MÉTODOS

O projeto foi desenvolvido nos laboratórios de Análise e Controle Ambiental e Microbiologia da Gerência de Química no Campus Manaus Centro do IFAM. E dividiu-se em etapas experimentais:

CONSTRUÇÃO DO REATOR DE BANCADA

Na construção utilizou-se um tubo de acrílico de 100 mm de diâmetro, 500 mm de altura, 3 mm de espessura, com volume útil total de 3,7 L (Figura 1).

O sistema operacional constituiu-se de reservatórios para o afluente e o efluente, válvula de saída de água (tipo lavadora), mangueiras flexíveis. E, foi controlado utilizando o software Kemo Relais Timer (Figura 1) por porta paralela (é uma interface de comunicação utilizada para enviar e receber dados entre o computador e um periférico), sistemas de placa de circuito com leds e cabo conector instalados em um computador. O reator foi protegido com insulfilm para evitar fotodegradação dos compostos fenólicos.

Figura 1. Reator; Software Kemo Relais.

EFLUENTE SEMI-SINTÉTICO

O preparo do efluente semi-sintético foi realizado nos Laboratórios de Controle Ambiental e Físico-Química do IFAM, Campus Manaus Centro, com concentração baseada em Silva 2002, conforme Tabela 1. Modificando-se o valor em fenol puro por uma concentração de 2% de gasolina e 1% de etanol simulando um efluente de posto de gasolina.

Em um intervalo de 4 dias retirava-se 2L do efluente e ajustava-se o pH 4 ± 5 com ácido clorídrico e a glicose era adicionada numa porção de 100 mg/L.

Foram feitas as análises em um período de 60 dias, com as mesmas condições e concentrações iniciais, para facilitar a observação da degradação nesse período de tempo.

Tabela 1 - Composição do efluente semi-sintético

Compostos	Concentração mg/L
NH ₄ Cl	76,1
NaCl	10,1
KCl	4,7
Na ₂ HPO ₄ .12H ₂ O	46,2
NaHCO ₃	243,3
MgSO ₄ .7H ₂ O	16,7
ZnSO ₄	0,2
MnSO ₄ . H ₂ O	0,2
CuSO ₄	0,2
FeCl ₃ .6H ₂ O	0,2
Gasolina	2%
Etanol	1%
Clorofórmio	1%
Glicose	100

INOCULAÇÃO DOS MICRORGANISMOS

Os microrganismos escolhidos para o trabalho foram as espécies *Aspergillus sp.* (Figura 2) e *Trichoderma sp.* (Figura 3), por já terem sido empregados com êxito na remoção de efluentes contendo fenol e compostos derivados e por haver cepas disponíveis no acervo do IFAM. As cepas foram cultivada em meio sólido Ágar Batata, a temperatura de 28°C, durante 7 dias em tubos inclinados, para então serem utilizados como fonte de esporos, no reator. Os conídios da linhagem de *Aspergillus sp.* e *Trichoderma sp.* foram suspensos em água destilada esterilizada e a suspensão obtida foi ajustada para 2×10^6 esporos/mL para ser inoculada no reator.

Figura 2 - *Trichoderma sp.*Figura 3 - *Aspergillus sp.*

CONTROLE OPERACIONAL

O reator foi operado em batelada com leito submerso, tempo de detenção hidráulica de quatro dias, com vazão de 2L e com ajuste de pH entre 4 e 5, valores ótimos para os microrganismos. A temperatura ambiente foi mantida $28\pm 2^{\circ}\text{C}$. O oxigênio dissolvido dentro do reator foi mantido em 2mg/L por meio de compressor tipo aquário.

Os parâmetros foram avaliados segundo a variação de concentração de Demanda Química de Oxigênio (DQO) no afluente e no efluente mantendo-se as mesmas condições do processo. As análises seguiram as metodologias do Standard methods for the examination of the water and wastewater (APHA, 1998).

CONTROLE DE BIOMASSA

Adicionou-se clorofórmio ao efluente para inibir o crescimento de bactérias, e após o término das análises retirou-se amostras das partes sólidas, líquidas e da biomassa para verificar a incidência ou não de contaminantes no reator. As amostras foram cultivadas em meio sólido Ágar Batata.

3. RESULTADOS E DISCUSSÃO

O reator foi operado por 60 dias, sendo alimentado com 2L a cada quatro dias coletando-se as amostras de entrada e saída em DQO, já que a utilização de um efluente semi-sintético com derivados do petróleo contém compostos orgânicos mais complexos que o fenol. Observou-se também a formação de espuma e grande crescimento de fungos. Isto comprova que as espécies utilizadas no experimento conseguem sobreviver e degradar o resíduo avaliado. Cunha *et al* (2007) observaram uma boa adaptação quando trabalharam com várias espécies de *Candida*.

Pelos resultados mostrados nas figuras 4 e 5 pode-se observar que a aclimação dos microrganismos ao resíduo líquido foi de quatro dias. A partir daí observa-se o crescimento gradativo de degradação atingindo em sessenta dias 43% de eficiência de remoção (Figura 5).

Passos (2006) obteve degradação total de fenol por uma nova linhagem de *Aspergillus sp* até uma concentração de $989\pm 15\text{mg/L}$, com diferentes velocidades de reação com 72 horas para degradar $320\pm 0,57\text{ mg/L}$ de fenol.

Saraiva et al (2011) obteve em uma concentração de 600 mg/L de fenol uma degradação média de 73% em reator sequencial em batelada com *Aspergillus sp.* e *Trichoderma sp.*

Figura 4 - Resultados afluente e efluente na análise de DQO.

Figura 5 - Eficiência da remoção de DQO

No controle de biomassa retirou-se amostras das fases líquida, sólida e do biofilme e foi observada contaminação (Figura 6), que também pode ser observada em trabalhos anteriores.

Figura 6 - Placas com amostras das fases sólida, líquida e biofilme, respectivamente da esquerda para a direita.

Na placa com amostra sólida pode-se observar a amostra e o contaminante; na placa com amostra líquida é bem visível a contaminação; e na amostra do biofilme, como o esperado houve crescimento dos fungos *Aspergillus sp.* e *Trichoderma sp.* juntamente com alguns resquícios do contaminante.

Os resultados da caracterização microbiológica dos contaminantes levam a crer que os fungos encontrados no biorreator são leveduras pertencentes ao gênero *Candida*. Porém, vale ressaltar que tais resultados não são definitivos, pois a identificação laboratorial de leveduras compreende várias provas fisiológicas (assimilação de açúcares e diferentes fontes de nitrogênio) que não foram realizadas nessa pesquisa. Adicionalmente, é possível que outros microrganismos (incluindo fungos filamentosos) possam ser isolados dessas mesmas amostras, caso sejam modificadas algumas condições de cultivo.

Através da análise destas amostras, foi possível verificar que as diferentes amostras apresentam microbiota mista, porém com algumas semelhanças entre si. É provável que ocorra uma sucessão de microrganismos no efluente ao longo do tempo.

6. CONCLUSÕES

Os resultados obtidos, mesmos ainda incipientes em função do tempo de operação do reator, mostram o potencial dos microrganismos na degradação de efluente de posto de combustível. E a adaptabilidade ao meio, que foi observada pelo grande crescimento dos microrganismos.

Há que observar na continuação do trabalho a formação de espuma o que em reatores industriais podem ser considerados problemas e as contaminações por outros microorganismos.

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