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Monografia apresentada à Universidade Federal de Lavras, como parte das exigências do Curso de Agronomia, para a obtenção do título de Bacharel.

Dr. Marco Aurélio Carbone Carneiro
Orientador

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ABSTRACT

Soil symbiosis relationships are extremely important to the planet functioning, since they contribute to the cycle of chemical elements and promote benefits among live being. Plants of the *Leguminosae* family can symbiotically associate with nodulating nitrogen-fixing bacteria (NNFB) and arbuscular mycorrhizal fungi (AMF), simultaneously, culminating in an interaction called tripartite symbiosis. Several benefits have been described about tripartite symbiosis, with emphasis on the root extension of AMF provide to legumes, which in turn acquire nutrients with greater efficiency and expand the area for nodule formation by NNFB. Many authors report tripartite symbiosis evolving to a more specific interaction, developing a direct contact (face to face) between microorganisms, that is, fungal structures that colonize the nodular tissue, however, without absolute confirmation. The synchrotron light is a technology able to research into nano and micrometric materials and analyzing the structures present in microorganisms. Through the X-ray microtomography line of synchrotron laboratory, it is possible to scan a microorganism, without changing its structure, generating images in three dimensions. The aim of this study was check the singularities and gaps in the C.emicromorphological investigations of nodules performed by tripartite symbioses. The experiment was carried out in a greenhouse at the Soil Science Department pots of 5 kg capacity with soil filled up, using plants of soybean (*Glycine Max* (L.) Merrill) and Lima bean (*Phaseolus lunatus* L.), singly. The NNFB *Bradyrhizobium japonicum* was inoculated and for each culture respectively, also was inoculated the following AMF treatments: Without Mycorrhizal fungi; *Claroideoglomus etunicatum*; *Dentiscutata heterogama*; *Acaulospora morrowiae*; *Claroideoglomus etunicatum + Dentiscutata heterogama*; *Claroideoglomus etunicatum + Acaulospora morrowiae*; *Dentiscutata heterogama + Acaulospora morrowiae*; and *Claroideoglomus etunicatum + Dentiscutata heterogama + Acaulospora morrowiae* for each culture. After three months, nodules, roots and shoot were harvest to proceed the dry mass weight. Mycorrhizal colonization was also accessed and during the harvest ten nodules of each plant were immediately and carefully helded to nitrogenase enzyme analysis by the acetylene reduction method. The IMX analyses were carried out in Campinas, SP at Brazilian Synchrotron Light Laboratory. Statistical analyses were performed in SISVAR software and the images nodules were obtained through AVIZO and PARAVIEW. The results showed the treatments *C.etunicatum, C.. etunicatum + D. heterogama* and the mix provided significant increases in dry mass weight of soybean plants, as well as the treatments *C.etunicatum + D.heterogama*, *C.etunicatum + A. morrowiae* and the mix provided the same benefit for lima-bean plants. All evaluated treatments roots were highly colonized with averages greater than 60% to both plant species. The nodules weight evaluated indicated in soybean plants only one superior treatment, the *C. etunicatum + D. heterogama,* reaching 0.2 g and differing significantly from the other treatments. The Lima-bean nodules weight obtained in the treatments *C.etunicatum, D. heterogama* and in the mix the highest averages. Almost all the nodules submitted to nitrogenase enzyme analysis showed ethylene measurements, indicating the nitrogenase activity. The Lima-bean nodules were very small and could not reach ethylene measurements to all treatments. The uninoculated soybean nodules treatment presented the highest nitrogenase activity followed by *D.heterogama* and *A. morrowiae* treatments, and for Lima-bean nodules, *D.heterogama* had the greatest values followed by *D.heterogama + A. morrowiae* treatments. Tripartite symbiosis was not so efficient for soybean plants. The IMX synchrotron analyses allowed observed a complete soybean nodule structure and verify the bacteroids inside then. We conclude the IMX beamline is satisfactory for morphological identification of structures on legume nodules; There is not mycorrhizal colonization in an active soybean nodule;

Mycorrhizal colonization in soybean nodule reduces the efficiency of biological N_2 fixation under nutritional stress of P and N.

Key-words: Soybean*;* Lima-bean; *Claroideoglomus etunicatum*; *Dentiscutata heterogama*; Acaulospora *morrowiae*; *Bradyrhizobium japonicum*.

SUMMARY

1. INTRODUCTION

Soil symbiosis relationships are extremely important to the planet functioning, since they contribute to the cycle of chemical elements and promote benefits among live being. There are two main symbionts in soil that are the nitrogen-fixing bacteria (NFB) and arbuscular mycorrhizal fungi (AMF), which in addition to being individually associated with plants, can also perform a triple association, called tripartite (CARVALHO; MOREIRA, 2010)

The NFB are noted for their efficiency in fixing nitrogen in legumes, since nitrogen fertilization can be completely suppressed, such as in soybeans plants, generating considerable monetary and energy savings. AMF are known mainly for their efficiency in providing phosphorus to plants, and for colonizing up to 80% of existing plant species (MOREIRA; SIQUEIRA, 2006a). AMF symbiosis wide existence provided the discovery of other benefits such as: nutrient uptake, regulation of the trace elements uptake, greater reach of water, among others (BERBARA; SOUZA; FONSECA, 2006; MELLONI; SIQUEIRA; MOREIRA, 2003; MOREIRA; SIQUEIRA, 2006a; SOARES; CARNEIRO, 2010). Arbuscular mycorrhiza (AM) is the symbiosis between AMF and plants, and can be extremely versatile. It is used in biofortification programs, degraded rehabilitation areas, phytostabilization and phytoremediation (NOGUEIRA; SOARES, 2010).

The association between leguminous plants, AMF and NFB constitute a tripartite symbiosis. It is an important ecological and nutritional view point of special interest of researchers, because they offer beneficial characteristics for their host plants and for the ecosystem resilience. These benefits are conferred to the development of specialized structures, such as nodules in plant roots, formed by the expansion of nodular NFB growth cells and a network of fungal hyphae, which increases the area of nutrient and water uptake in the soil.

Several authors report that this symbiosis can evolve to a more specific interaction, developing a direct contact (face to face) between microorganisms, that is, fungal structures that colonize the nodular tissue. The first reports of this interaction between microorganisms were made with light microscopy, being observed only in senescent nodules - with no activity (BAIRD; CARUSO, 1994; DUHOUX et al., 2001a; KUCEY; DIAB, 1984; SCHEUBLIN; VAN DER HEIJDEN, 2006). With advances in visualization techniques, particularly in scanning electron microscopy (SEM), gains in spatial resolution and contrast have helped some authors to test the hypothesis that active nodules could also be colonized (PADAMSEE

et al., 2016; SILVA et al., 2016), however without absolute confirmation. Despite these reports, there are still many gaps to be closed, especially with regard to which tissues are colonized and which fungal structures can be formed.

All mycorrhizal colonization studies in NFB nodules needed to use nodule fragmentation in their methodology to evaluate this structure. Once there is a disruption of this morphological structure, there may be interfere in the characterization of the colonization of fungal hyphae in the nodules, such as the disruption of the hyphae or displacement of this structure over the nodule.

The synchrotron light use for research into nano and micrometric materials has been shown to be an efficient and reliable methodology, especially for analyzing and characterizing the structures and substance present in microorganisms (STAEDLER; MASSON; SCHÖNENBERGER, 2013; SZCZEPANOWSKA; JHA; MATHIA, 2015). Through the X-ray microtomography (IMX) line, it is possible to scan a micrometric object, without changing its structure, generating images in three dimensions (LNLS).

Observing the singularities and gaps in the micromorphological investigations of these soil symbionts, the IMX line of the national synchrotron light laboratory (LNLS) shows to be an alternative of high technological support and reliability to identify the specificities of nodular colonization by AMF in legumes. Thus, justifying the use of these techniques in microbiological investigations in soil symbionts.

The objective of this work was to evaluate the existence of mycorrhizal colonization in active nodules of legumes, without fragmenting this structure, and to investigate the efficiency of the tripartite interaction in fixing nitrogen.

2. THEORETICAL REFERENCE

2.1. Soil Symbiosis

Relationship between living beings in which both organisms receive benefits, even in non-equal proportions are called symbiosis. In the soil ecosystem, there are two main symbioses due to their occurrence, ecological and economic importance. Biological nitrogen fixation (NFB) and AM are indispensable for the functionality of the soil ecosystem, since it's nutritionally benefiting to vegetative species and they contribute to the environment resilience (MOREIRA; SIQUEIRA, 2006b).

These symbioses are also known as mutualism, as they promote benefits for host plants, which receive water and nutrients from the fungi and these, in turn, receive photoassimilates to perform their functional role. Among these soil microorganisms used in agriculture, the diazotrophic bacteria (FNB) stand out, providing N to the plant from atmospheric N_2. AMF also play an important role, as they increase the volume of soil explored by the roots, helping the plant to obtain nutrients such as P and other compounds from soil. There is also the double inoculation of these microorganisms, which can increase the benefit of leguminous plants by giving them greater capacity for uptaking nutrients, contributing to their establishment and growth. These triple interaction are called tripartite symbiosis (VAN DER HEIJDEN et al., 2016).

2.2. Biological Nitrogen Fixation

NFB is the process by which N_2 is assimilated by diazotrophic bacteria through breaking the triple bond of atmospheric N, and then been available to plants in assimilable forms by them (FREITAS et al., 2011; MOREIRA et al., 2010). This conversion is characterized as the main route of incorporation of N to terrestrial biomass.

Due to the nutritional importance of N for plants and the high cost of producing nitrogen fertilizers, FBN becomes essential for the planet's sustainability. This biological acquisition of N is responsible for reducing 258 million tons of atmospheric N in ammonia per year, avoiding energy costs for the production of nitrogen fertilizers and economic costs for the acquisition of these inputs (FREIBERG et al., 1997; MOREIRA; SIQUEIRA, 2006a).

NFB is characterized by the relationship that plants establish with diazotrophic bacteria, which are: free-living, associative or noduliferous (NNFB). In the latter case, when bacteria of the genera *Rhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Phylorhizobium*, *Bradyrhizobium* and *Azorhizobium* establish a relationship of mutualism with legumes (MOREIRA et al., 2010; MOREIRA; SIQUEIRA, 2006b; SANTOS; REIS, 2008).

The mechanisms used by NNFB to reduce the N_2 to NH_3 include breakdown of the triple bond catalyzed by the nitrogenase enzyme, which is produced by these microorganisms.

2.3. Nitrogenase

Nitrogenase is an enzymatic complex responsible by biological nitrogen fixation (BNF) in leguminous root nodules. Inside the root nodules tissue, are found the bacteroids,

which are the NNFB that living in these internal structures. These endo-symbiotic bacteria form are able synthesize the proteins of this enzyme complex, as well as reduce the molecular nitrogen for NH_3 within its cytoplasm (Equation 1), which is then quickly converted to amides and or ureides, to nourish the host plant (BROUGHTON et al., 2006; SANTOS; REIS, 2008).

(1) $$N_2 + 8H^+ + 16\,ATP + 8e^- \xrightarrow{Nitrogenase} 2NH_3 + H_2 + 16A\,DP + 16\,P_i$$

The enzyme complex is composed of different proteins: the iron protein or dinitrogenase; Molybdenum-iron protein or dinitrogenase reductase; Vanadium-iron protein, which occurs when vanadium replaces molybdenum atom structure, found in the bacteria *Clostridium, Rhodobacter, Anabaena, Rhodospirillum, Heliobacterium and Azospirillum*; and there is still a fourth type of nitrogenase, found in the bacterium *Streptomyces thermoautotrophicus*, whose most notable property is the dependence on oxygen and the superoxide radical, differing from the other proteins, which are extremely sensitive to the presence of oxygen (MOREIRA; SIQUEIRA, 2006b; VRUBEL, 2007).

Due to being present in most species of diazotrophic, Fe-protein and FeMo-protein are considered basic units of nitrogenase. Fe-protein is the electron donor for MoFe-protein, which in turn reduces the substrate through a very energetic process. In addition other substrates also can be catalyzed by nitrogenase, such as acetylene, cyanide, nitrous oxide and methyl isocyanide (BODDEY et al., 2007; BONFANTE; ANCA, 2010; EDSON, 2017).

Because it is non-toxic and readily available, acetylene (C_2H_2) is often used to measure nitrogenase activity. In the test procedure, the root nodules of the legumes are exposed to 25% acetylene in the air mixture and incubated at 25 - 30 ° C. The ethylene (C_2H_4) produced by the reduction of acetylene is measured by gas chromatography (BODDEY et al., 2007; COSTA, 2011).

2.4. Morfological Characterization

The NNFB are characterized by the presence of the nod gene and have the ability to colonize leguminous roots endophytically, forming highly specialized structures known as nodules (BROUGHTON et al., 2006; MOREIRA; SIQUEIRA, 2006b). Nodules are new structures that consist mainly of infected plant cells with bacteroids that promote biological nitrogen fixation (SANTOS; REIS, 2008).

The nodules formation starts by the exudation and chemotaxis stage, through the emission of flavonoids from the roots, which can activate the genetic expression of the NNFB NodD genes, which in turn direct the synthesis of lipo-chito-oligosaccharide of the Nod factor and secrete them, allowing the recognition by the cells of the legume root and consequently providing the rhizobia adhesion to the root hair, as well as its deformation and curving for the penetration of bacteria and formation of an infection channel (BROUGHTON et al., 2006; FREIBERG et al., 1997; PERRET; STAEHELIN; BROUGHTON, 2000).

After that through the parenchyma layers of the root cortex, the bacterial development to nodule formation keep continues until they reach the cells outside the endoderm. The cell division and the branching of the infection cord into adjacent cells occur, which, through bacterial multiplication, causes the cell cavity of the affected tissues to be filled. In this way, the cortical parenchyma and the epidermis are forced outwards, forming a lateral swelling called the nodule at the root (CARVALHO, 1946; COSTA, 2011; FREIBERG et al., 1997).

2.5. Arbuscular Mycorrhizae

The mycorrhizae are symbiotic, non-pathogenic and beneficial relationships between mycorrhizal fungi and higher plants (MIRANDA; MIRANDA, 1997). These associations are the most common kind of mutualism in nature, thus having a wide variety of fungal species and conditions for the establishment of symbiosis (SIQUEIRA; LAMBAIS; STÜRMER, 2002).

The symbiosis happen through the supply of plant photoassimilates from photosynthesis for fungi, which in turn uptake mineral nutrients and water from the soil into the plants (BERBARA; SOUZA; FONSECA, 2006). The AM develop intra and intercellular structures in the cortex of plant roots and thus form specific structures within the cortical cells, which made able the communication between fungus and plant (MIRANDA; MIRANDA, 1997; MOREIRA; SIQUEIRA, 2006a). Through the fungal hyphae emission, the AMs can exploit a larger soil volume and consequently reach more nutrients and minerals for plants (HART; FORSYTHE, 2012).

AMF do not require a rule or specificity to choose the host plant, however, there are studies showing a preferential association between species of fungi and plants (POUYÚ-ROJAS; SIQUEIRA, 2000). AMF occurrences are wide and can be found in all latitudes and in almost all terrestrial ecosystems. The plant colonization by AMF occurs in more than 97%

of phanerogams in tropical environments, therefore being the fungi group with greater practicality and possibility for the propagation of spores (NOVAIS DE et al., 2014).

There are several studies showing the efficiency of AMF in uptaking elements such as P, Zn and Cu (BRESSAN et al., 2001; SIQUEIRA et al., 1998; SIQUEIRA; SAGGIN-JÚNIOR, 2001). Marschner & Dell, 1994 found up to 100% efficiency in the P uptake in mycorrhizal plants, as well as 25% for Zn and 60% for Cu. The efficiency of MAs in the greater nutrient uptake can be attributed due to the small diameter of their hyphae, which allow a greater exploration of the spaces of the volume of the soil, unreachable by the roots, consequently increasing the rates of influx in the plants per unit of area (BERBARA; SOUZA; FONSECA, 2006).

Having been the target of research for over 40 years, AMs have been shown to be quite efficient, mainly due to their diversity, occurrence and benefit in plant nutrition, thus, they have a high agronomic and ecological interest.

2.6. Tripartite Symbiosis

Plants of the *Leguminosae* family can symbiotically associate with NNFB and AMF, simultaneously, culminating in an interaction called tripartite symbiosis. The beginning of this process is caused by exudations of several metabolites by legumes, including flavonoids, that will induce the nodulation process of NNFB and the AMF spore germination (ANTUNES; RAJCAN; GOSS, 2006; BROUGHTON et al., 2006; CARVALHO, 1946).

Several benefits have been described about tripartite symbiosis, with emphasis on the root extension of AMF provide to legumes, which in turn acquire nutrients with greater efficiency and expand the area for nodule formation by NNFB (FERROL; TAMAYO; VARGAS, 2016; PADAMSEE et al., 2016).

Another important aspect of this triple symbiosis is its nutritional functionality in tropical soils. Brazilian soils have low P availability, mainly in oxisols. The NNFB process demands high energy to nodule formation, thus the AM favor the demand and P uptake in the tripartite system (CARVALHO; MOREIRA, 2010). According to Vadez et al. (1997), the nodules are considered a strong P drain, due to the content of this element in this structure, which in comparison with the other organs of the legume it is about three times higher.

The self-regulation is a process relevant to the symbiosis of NFB and AM. It is understood by the non-infinite formation of nodules or fungi colonization. When the plant is supplied with N or P or when there is sufficient availability and quantity of these elements in

the soil, these microorganism cease and keep inactive (RAMOS, A.C. & MARTINS, 2010). Until the mid-1980s, it was believed that the formation of nodules could inhibit mycorrhization through self-regulation or vice versa, resulting in antagonism (BETHEMFALVAY; YODER, 1981). In 2005, a study with a soybean mutant, which did not self-regulate with rhizobium and AMF, showed that there was a beneficial dynamic between the regulatory processes of the establishment of both symbioses (MEIXNER et al., 2005). Since then, several other studies have shown the establishment of tripartite with synergistic effects was achieved when colonization were made simultaneously or even that the presence of NNFB was sufficient to promote the mycorrhization process (ABD-ALLA et al., 2014; ANTUNES; RAJCAN; GOSS, 2006; BONFANTE; ANCA, 2010; CARVALHO; MOREIRA, 2010).

A morphological aspect of tripartite that has not been well elucidated by science is the direct colonization by AMF in non-senescent nodules. Several studies show the effective colonization in non-active nodules, such as Baird and Caruzo (1994), who observed, through light microscopy, a plentiful AMF colonization *in Phaseolus vulgaris* nodules. Vidal-Dominguez et al. (1994) also observed with stereomicroscope an intense colonization in alfalfa (*Medicago sativa L.)* and clover (*Trifolium repens L*.) nodules, preferentially occurring in the distal part of the nodule and in the intercellular spaces between the cortical cells, they were unable to distinguish whether the nodules were active or senescent . In 2001, Duhoux et al., (2001b), through fluorescence microscopy and DNA analysis, showed mycorrhizal colonization occurred in sections of *Gymnostoma nodiforum* and *G. deplancheanum* nodules in the intercellular spaces of the cortex of active nodules. Scheublim et al. (2004) and Scheublin & Van der Heijden (2006) showed the existing of tripartite colonization, however AMF colonize only old senescent nodules and after nitrogen fixation is finished, furthermore this colonization could inhibit N fixation in active nodules. Recently, Padamsee et al., (2016) used light electron microscopy, scanning and transmission to characterize the tripartite colonization in *Agathis australis* and show that there is colonization of nodules by AMF arbuscules in the central cortex, hyphae in the first two rows of cells adjacent to the epidermis (external cortex) and degenerate arbuscules in the central cortical cells.

During the process of AMF colonization is possible that mycorrhization occurs in non-senescent nodules, it means the active ones, as reported by Silva et al. (2016) in soybean (*Glycine max*) with the presence of mycorrhizal stimulant formononetin and low P availability, being analyzed in electron microscopy.

Such clarifications regarding colonization in nodules still need further investigation, since none of the analysis carried out to date have kept the nodule structure intact for such evaluations.

2.7. Synchrotron Ligth

Synchrotron light, or radiation, is a high-flux and high-brightness electromagnetic radiation that extends over a wide range of the electromagnetic spectrum from infrared light, through ultraviolet radiation, to X-rays. It is produced when charged particles, accelerated to speeds close to the speed of light, have their trajectory deviated from direction by magnetic fields. Its use is extremely important, as it is able to penetrate into matter and reveal characteristics of its molecular and atomic structure (TASCH; DAMIANI, 2000).

This radiation is exponentially more intense than the radiation produced by conventional X-ray sources and covers a wide spectral range, in which there are no other devices or lasers available. Characteristics such as high intensity brightness, small spatial dimensions, and spectral amplitude make it necessary and desirable to use synchrotron light in scientific research involving nanoscale and micrometric materials (LNLS, 2017; SCHUBERT et al., 2017).

The synchrotron accelerator works from a beam of charged particle, which is accelerated in an intensifier and then guided to a large ring structure, moving in circular orbits through a set of electromagnets, generating a magnetic field. Synchrotron light is obtained when the electrons lose energy when passing through magnetic fields of electromagnets and it is directed to the beam lines, where it will be channeled and filtered according to the experimental need (LNLS, 2017).

2.7.1 IMX

Through microtomography and or propagation of phase contrast it is possible to obtain several images in different orientation angles of a biological sample or not, without causing its destruction resulting in a 3D projection (LNLS, 2017; SCHUBERT et al., 2017).

The X-ray microtomography (IMX) beamline collects synchrotron radiation emitted by the D6 dipole magnet with a 1.67 T magnetic field and a radius of curvature of 2.736 m (LNLS, 2017).

3. MATERIALS AND METHODS

The experiment was carried out in a greenhouse at the Soil Science Department of the Federal University of Lavras, Brazil during ninety days, december 2016 until march 2017. Soil samples were taken from the 0-20 cm layer of an Oxisol (LVAd) from the 'Cerrado' region. After air drying, the soil was sieved through a 2 mm mesh to perform physical and chemical analyses as stated in Embrapa (1997), with the follow results: pH(water) = 4.9; H+Al = 3.20 $cmol_c\ dm^{-3}$; Al = 0.6 $cmol_c\ dm^{-3}$; Ca = 0.1 $cmol_c\ dm^{-3}$; Mg = 0.1 $cmol_c\ dm^{-3}$; K = 48.0 mg dm^{-3}; Na = 0 mg dm^{-3}; P = 0.8 mg dm^{-3}; MO = 1.40 g dm^{-3}; sand = 73 g dm^{-3}; silt = 2.0 g dm^{-3} and clay = 25.0 g dm^{-3}. The soil was sterilized in autoclave for two consecutive days at 120° C for one hour in order to eliminate all the living beings, and then it was stored for 15 days for chemical stabilization. Limestone was realized in order to increase 70% of base saturation during 30 days.

Were used pots of 5 kg capacity with soil filled up, in which received four seeds in each one of soybean (*Glycine Max* (L.) Merrill) and Lima bean (*Phaseolus lunatus* L.), singly, inoculated with the NFB *Bradyrhizobium japonicum* and for each culture respectively. The fertilization was performed, according Malavolta (1980), suppressing the P supply until the thirtieth day, in order to induce stress in the plant to favor fungal colonization, and N supply in order to stimulate the nodules formation. As soon the plants emergency, thinning was performed allowing two plants per pot, and then three AMF species were inoculated individually and mixed with three hundred propagules of each one. *Acaulospora morrowiae* (UFLA 469), *Claroideoglomus etunicatum* (UFLA 217) and *Dentiscutata heterogama* were chosen to test its different genes in tripartities symbiosis.

At the end of cultivation, nodules, roots and shoot were harvest and washed in distilled water, placed in a paper bag and oven dried at 60º C until constant weight to be taken on a precision scale. Mycorrhizal colonization was accessed through gridline intersect method (GIOVANETTI; MOSSE, 1980), in which one gram of fresh roots of each plant was taken, clarified and colored with methyl blue (0.05%).

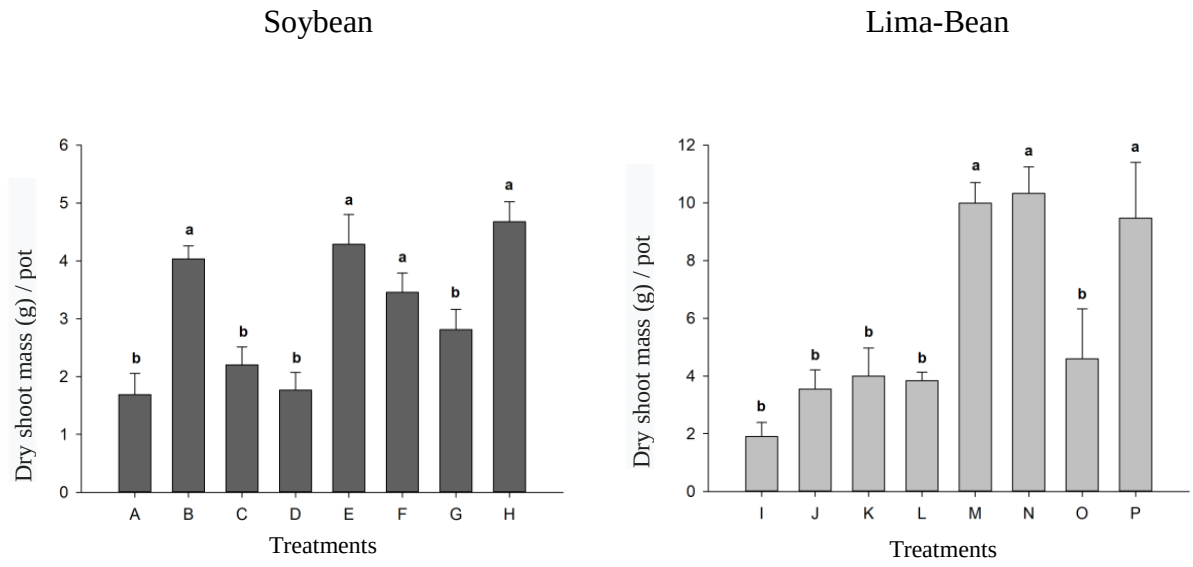
During the harvest, ten nodules of each plant were immediately and carefully helded to nitrogenase enzyme analysis by the acetylene reduction method (MORA et al., 2014; PERALTA et al., 2004). After that, these nodules, before synchrotron light analysis, were submitted to the following procedure: fixation in Karnovsky, washing twice in cacodylate buffer during ten minutes, immersed in 1% iodine solution (I_2) and then dehydrated in a

sequence of acetone solution gradients in the order of 25%, 50%, 75% and 100%, for ten minutes each, repeating the last concentration (personal statement, unpublished data). Afterwards, the samples were fitted on a tip with pure acetone inside it, and to close the sample and avoid bubbles a burned end at the lower and a plunger at the top were placed.

The IMX analyses were carried out in Campinas, SP at Brazilian Synchrotron Light Laboratory. The IMX beamline setup and the image detection system were configured as described by FIDALGO et al., 2018. The 3D images were generated by one thousand one images and the sample was exposure by six hundred mili seconds each one. Imagens acquired by IMX beamline were visualized and analyzed in AVIZO and PARAVIEW software application. Statistical results were obtained by averages of components of the treatments and were compared by Scott-Knot test at 5% probability variance using SISVAR (FERREIRA, 2006).

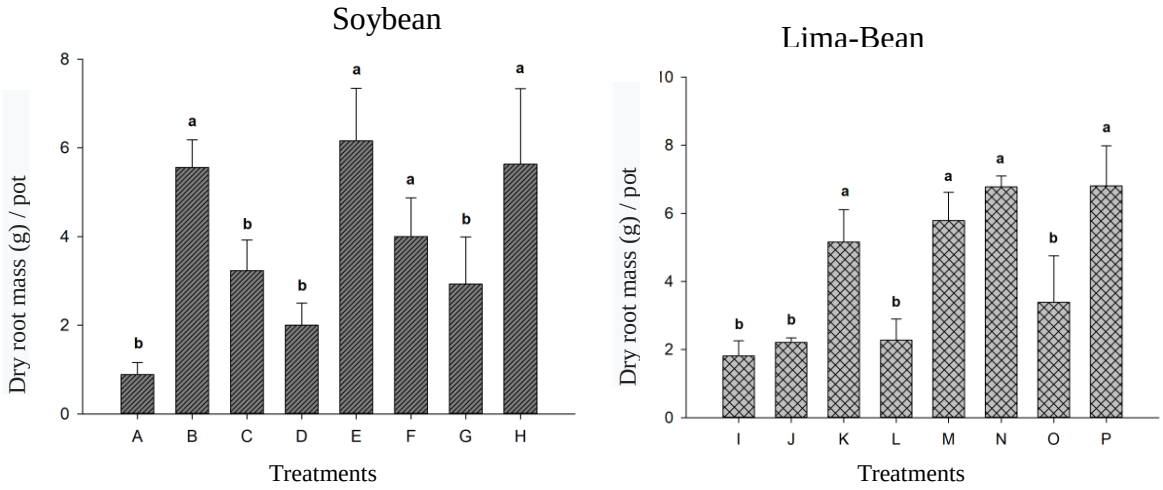
4. RESULTS AND DISSCUSSION

The treatments *C.etunicatum*, *C.etunicatum* + *D.heterogama* and the mix provided significant increases in dry mass weight of soybean plants, as well as the treatments *C.etunicatum* + *D.heterogama*, *C.etunicatum* + *A. morrowiae* and the mix provided the same benefit for lima-bean plants. In addition, the treatments *D.heterogama* increased the dry mass weight of root in lima-bean and *G.etunicatum* in shoot on soybean plants (Figure 1 and 2). We observed an effective symbiosis in all *C.etunicatum* consortiation with the others AMF and this fungus appears to be most efficient than the others, increasing the shoot dry mass even in consortiation. What occurs to AMF symbiotic efficiency is, mainly, attributed to the ability to galore colonize the roots and to promote beneficial responses for the host plant's growth (NOVAIS DE et al., 2014). Is well know the AMF effects vary according to the fungal isolate at the same plant species resulting in differences in the symbiotic efficiency (NOVAIS DE et al., 2014; VAN DER HEIJDEN; KUYPER, 2001). A study carried out at Iran on soybean plants with NFB and AMF inoculation, individually inoculated and mixed with *G. fasciculatum*, *C.etunicatum*, *Glomus intraradices*, *G. mosseae*, *G. versiforme*, also showed an increase on dry mass plants, 59%, when all fungi were inoculated together (HEMMAT JOU; BESALATPOUR, 2018). It is knew that AMF support nitrogen fixation through P providing other soil elements, however, these effects are depended on the specific microsymbiont combination (CLARK; ZETO, 2000).



* Averages followed by the same letter do not differ by the F test at 5%.

Figure 1. Shoot dry mass of Soybean and Lima-bean to the following treatments inoculation: A- Without Mycorrhizal fungi (control); B- *Claroideoglomus etunicatum*, C- *Dentiscutata heterogama*, D- *Acaulospora morrowiae*, E- *Claroideoglomus etunicatum* + *Dentiscutata heterogama*, F- Claroideoglomus etunicatum + Acaulospora morrowiae, G- *Dentiscutata heterogama* + *Acaulospora morrowiae*, H- *Claroideoglomus etunicatum* + *Dentiscutata heterogama* + *Acaulospora morrowiae*, I- Without Mycorrhizal fungi (control), J- *Claroideoglomus etunicatum*, K- *Dentiscutata heterogama*, L- *Acaulospora morrowiae*, M- *Claroideoglomus etunicatum* + *Dentiscutata heterogama*, N- *Claroideoglomus etunicatum* + *Acaulospora morrowiae*, O- *Dentiscutata heterogama* + *Acaulospora morrowiae* and P- *Claroideoglomus etunicatum* + *Dentiscutata heterogama* + *Acaulospora morrowiae*.

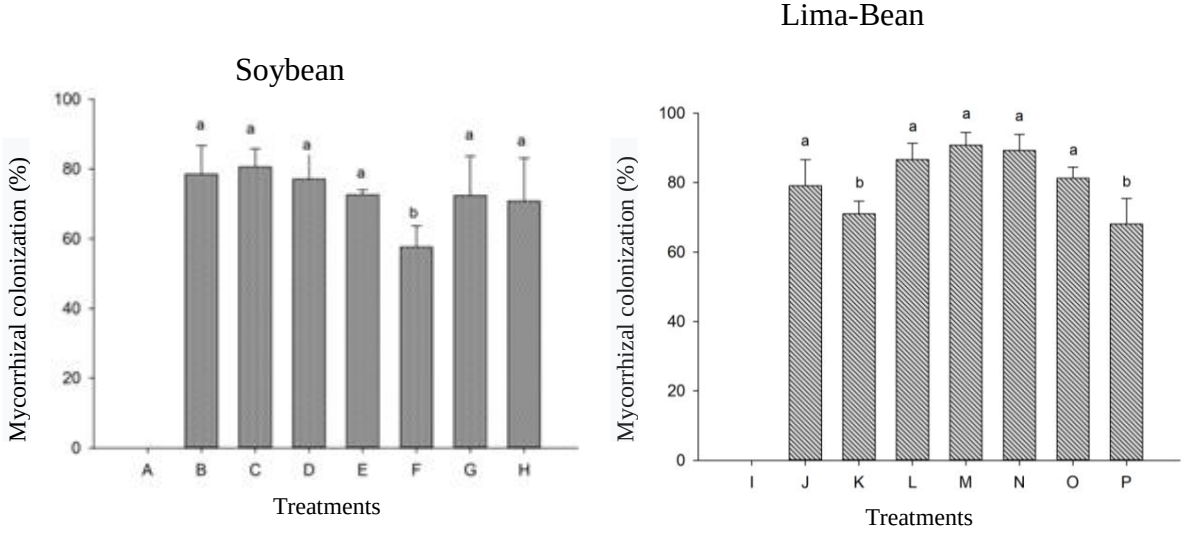


* Averages followed by the same letter do not differ by the F test at 5%.

Figure 2. Root dry mass of Soybean and Lima-bean to the following treatments inoculation: A- Without Mycorrhizal fungi (control); B- *Claroideoglomus etunicatum*, C- *Dentiscutata heterogama*, D- *Acaulospora morrowiae*, E- *Claroideoglomus etunicatum* + *Dentiscutata heterogama*, F- *Claroideoglomus etunicatum* + *Acaulospora morrowiae*, G- *Dentiscutata heterogama* + *Acaulospora morrowiae*, H-

Claroideoglomus etunicatum + Dentiscutata heterogama + Acaulospora morrowiae, I- Without Mycorrhizal fungi (control), J- *Claroideoglomus etunicatum*, K- *Dentiscutata heterogama*, L- *Acaulospora morrowiae*, M- *Claroideoglomus etunicatum + Dentiscutata heterogama*, N- *Claroideoglomus etunicatum + Acaulospora morrowiae*, O- *Dentiscutata heterogama + Acaulospora morrowiae* and P- *Claroideoglomus etunicatum + Dentiscutata heterogama + Acaulospora morrowiae*.

The results obtained do not show colonization for the non-inoculated plants, indicating no experiment contamination. All evaluated treatments roots were highly colonized with averages greater than 60% to both plant species, also in soybean, there was antagonism between *C. etunicatum* and *A.morrowiae* in consortium, which was superseded when there was a triple inoculation between *C. etunicatum* + *A.morrowiae* and *D. heterogama*. In beans, the same was observed for *D. heterogama* + *A. morrowiae* with the difference that the addition of *C.etunicatum* to this triple association (*C. etunicatum* + *D. heterogama* + *A. morrowiae*) was not able to reverse the antagonism (Figure 3). AMF have different strategies for root colonization, which is ordained by hyphae morphology. How thin the hyphae can be, higher infective they are and quickly roots plant are colonized, despite their low ability to explore the soil (LALIBERTÉ, 2017; NOVAIS DE et al., 2014) All fungal species tested here have fine and delicate hyphae and consequently high colonization capacity. Also rhizobia inoculation can improve significantly AMF colonization, given the synergism of tripartite symbiosis (OMIROU et al., 2016; OSUNDE et al., 2003; SAKAMOTO; OGIWARA; KAJI, 2014; SANGINGA; THOTTAPPILLY; DASHIELL, 2000) although, due the self-regulation of plant host or environmental factor, others researchers noticed the opposite (CATFORD, 2003; MEIXNER et al., 2005).



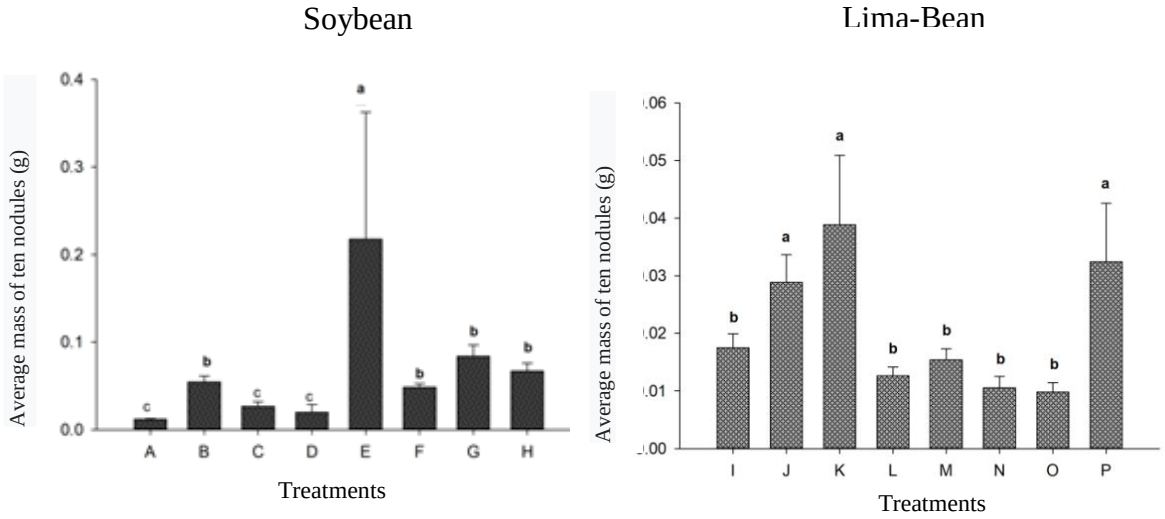
* Averages followed by the same letter do not differ by the F test at 5%.

Figure 3. Mycorrhizal colonization in Soybean and Lima-bean to the following treatments inoculation: A- Without Mycorrhizal fungi (control); B- *Claroideoglomus etunicatum*, C- *Dentiscutata heterogama*, D- *Acaulospora morrowiae*, E- *Claroideoglomus etunicatum* + *Dentiscutata heterogama*, F- *Claroideoglomus etunicatum* + *Acaulospora morrowiae*, G- *Dentiscutata heterogama* + *Acaulospora morrowiae*, H- *Claroideoglomus etunicatum* + *Dentiscutata heterogama* + *Acaulospora morrowiae*, I- Without Mycorrhizal fungi (control), J- *Claroideoglomus etunicatum*, K- *Dentiscutata heterogama*, L- *Acaulospora morrowiae*, M- *Claroideoglomus etunicatum* + *Dentiscutata heterogama*, N- *Claroideoglomus etunicatum* + *Acaulospora morrowiae*, O- *Dentiscutata heterogama* + *Acaulospora morrowiae* and P- *Claroideoglomus etunicatum* + *Dentiscutata heterogama* + *Acaulospora morrowiae*.

The nodules weight evaluated indicated in soybean plants only one superior treatment, the *G.etunicatum* + *D.heterogama,* reaching 0.2 g and differing significantly from the other treatments (Figure 4). The Lima-bean nodules weight obtained in the treatments *C.etunicatum, D.heterogama* and in the mix the highest averages (Figure 4). Soybean plants, usually presents greater N_2 fixation, total amount of nodules as well as its dry weight mass in AMF presence (ANTUNES; RAJCAN; GOSS, 2006; GOSS; DE VARENNES, 2002). Mortimer et al (2008) also find similar Lima bean results of nodules dry weight when inoculated *G. etunicatum* and *Rhizobium leguminosarum* in *Phaseolus vulgaris* (L.). The dry weight of nodules does not necessarily mean a larger volume for N_2 fixation. The tripartite symbiosis can work effectively when the three symbionts are benefited, on the other hand several studies has shown the legume nodule as a P sink. This situation can happen when the nodule is structurally and physically well-developed but doesn't have N_2 fixing efficiency, for example senescent nodules are inactive and demanded high P amount to be structured or when N or P content in soil is too high or too low for the demand of plant development and structuring of the nodule, and AMF can't help to provide this macronutrient to them (ALMEIDA et al., 2000; MORTIMER; PÉREZ-FERNÁNDEZ; VALENTINE, 2008; VADEZ et al., 1997).

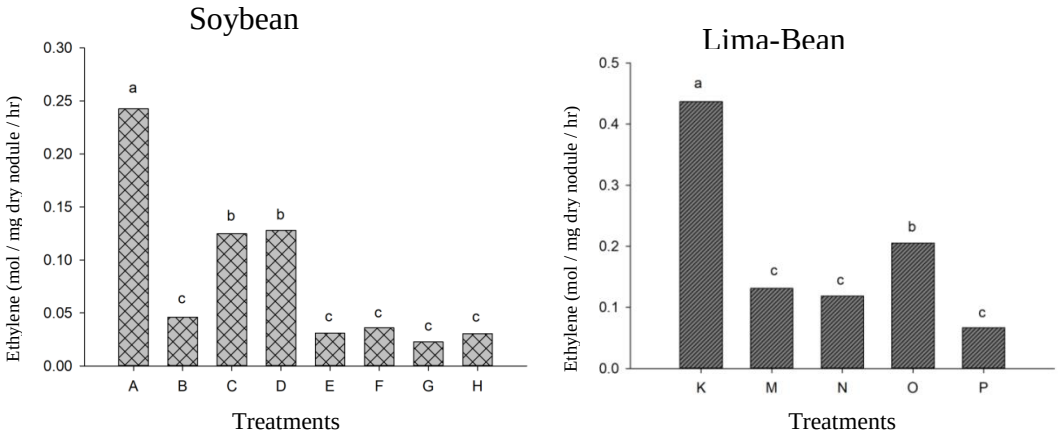
Almost all the nodules submitted to nitrogenase enzyme analysis showed ethylene measurements, indicating the acetylene reduction and consequently the nitrogenase activity (**Figure 5**). The Lima-bean nodules were very small and could not reach ethylene measurements to all treatments (**Figure 4 and 5**). The uninoculated soybean nodules (-AMF) treatment presented the highest nitrogenase activity followed by *D.heterogama* and *A. morrowiae* treatments, and for Lima-bean nodules, *D.heterogama* had the greatest values followed by *D.heterogama* + *A. morrowiae* treatments. Tripartite symbiosis was not so efficient for soybean plants, since the highest apport of N_2 fixation came from uninoculated

treatments with AMF. Those AMF treatments which increased nitrogenase activity may have help the NFB to provide better efficiency, as explain Kiers et al., (2016) at the biological market theory, expecting that individuals have a preference for interacting with more beneficial partners.



* Averages followed by the same letter do not differ by the F test at 5%.

Figure 4. Nodules weight Dry mass of Soybean and Lima-bean to the following treatments inoculation: A- Without Mycorrhizal fungi (control); B- *Claroideoglomus etunicatum*, C- *Dentiscutata heterogama*, D- *Acaulospora morrowiae*, E- *Claroideoglomus etunicatum* + *Dentiscutata heterogama*, F- Claroideoglomus etunicatum + *Acaulospora morrowiae*, G- *Dentiscutata heterogama* + *Acaulospora morrowiae*, H- *Claroideoglomus etunicatum* + *Dentiscutata heterogama* + *Acaulospora morrowiae*, I- Without Mycorrhizal fungi (control), J- *Claroideoglomus etunicatum*, K- *Dentiscutata heterogama*, L- *Acaulospora morrowiae*, M- *Claroideoglomus etunicatum* + *Dentiscutata heterogama*, N- *Claroideoglomus etunicatum* + *Acaulospora morrowiae*, O- *Dentiscutata heterogama* + *Acaulospora morrowiae* and P- *Claroideoglomus etunicatum* + *Dentiscutata heterogama* + *Acaulospora morrowiae*.



* Averages followed by the same letter do not differ by the F test at 5%.

Figure 5. Nitrogenase Activity in Soybean and Lima-bean to the following treatments inoculation A- Without Mycorrhizal fungi (control); B- *Claroideoglomus etunicatum*, C- *Dentiscutata heterogama*, D- *Acaulospora morrowiae*, E- *Claroideoglomus etunicatum* + *Dentiscutata heterogama*, F- *Claroideoglomus etunicatum* + *Acaulospora morrowiae*, G- *Dentiscutata heterogama* + *Acaulospora morrowiae*, H- *Claroideoglomus etunicatum* + *Dentiscutata heterogama* + *Acaulospora morrowiae*, K- *Dentiscutata heterogama*, M- *Claroideoglomus etunicatum* + *Dentiscutata heterogama*, N- *Claroideoglomus etunicatum* + *Acaulospora morrowiae*, O- *Dentiscutata heterogama* + *Acaulospora morrowiae* and P- *Claroideoglomus etunicatum* + *Dentiscutata heterogama* + *Acaulospora morrowiae*.

Despite tripartite symbioses showed less acetylene reduction than the uninoculated AMF soybean treatments, theses nodules were not senescent, since they have nitrogenase activity and was structurally intact, been possible observed the bacteroids inside then (Figure 6 and figure 7).

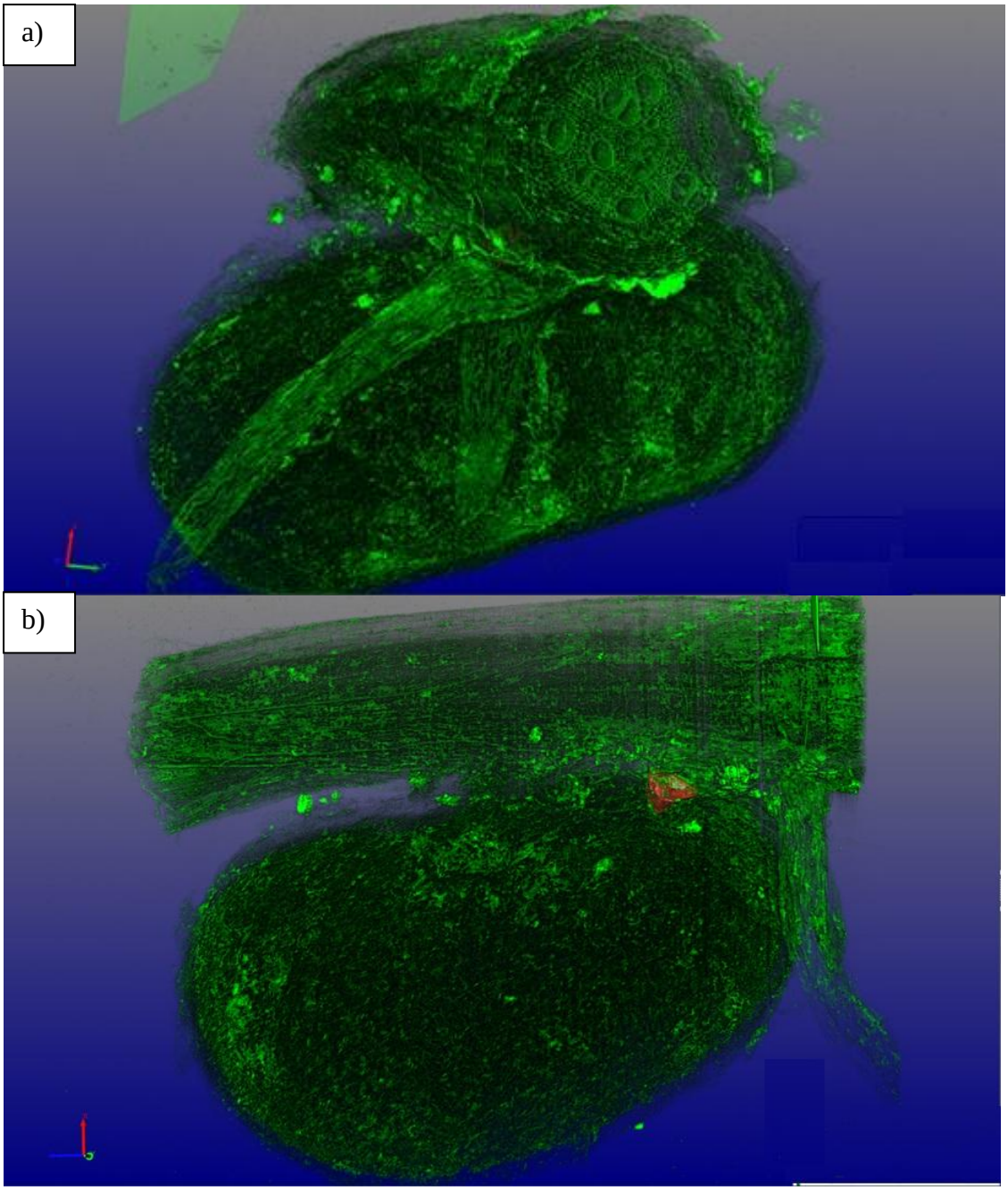


Figure 6 – Root fragment of the soybean plant coupled to the nitrogen fixing bacteria (*Bradyrhizobium japonicum*) nodule in three dimensions visualization from a) Front root view and b) Side root view obtained through X-ray microtomography in synchrotron analysis and processed by PARAVIEW software.

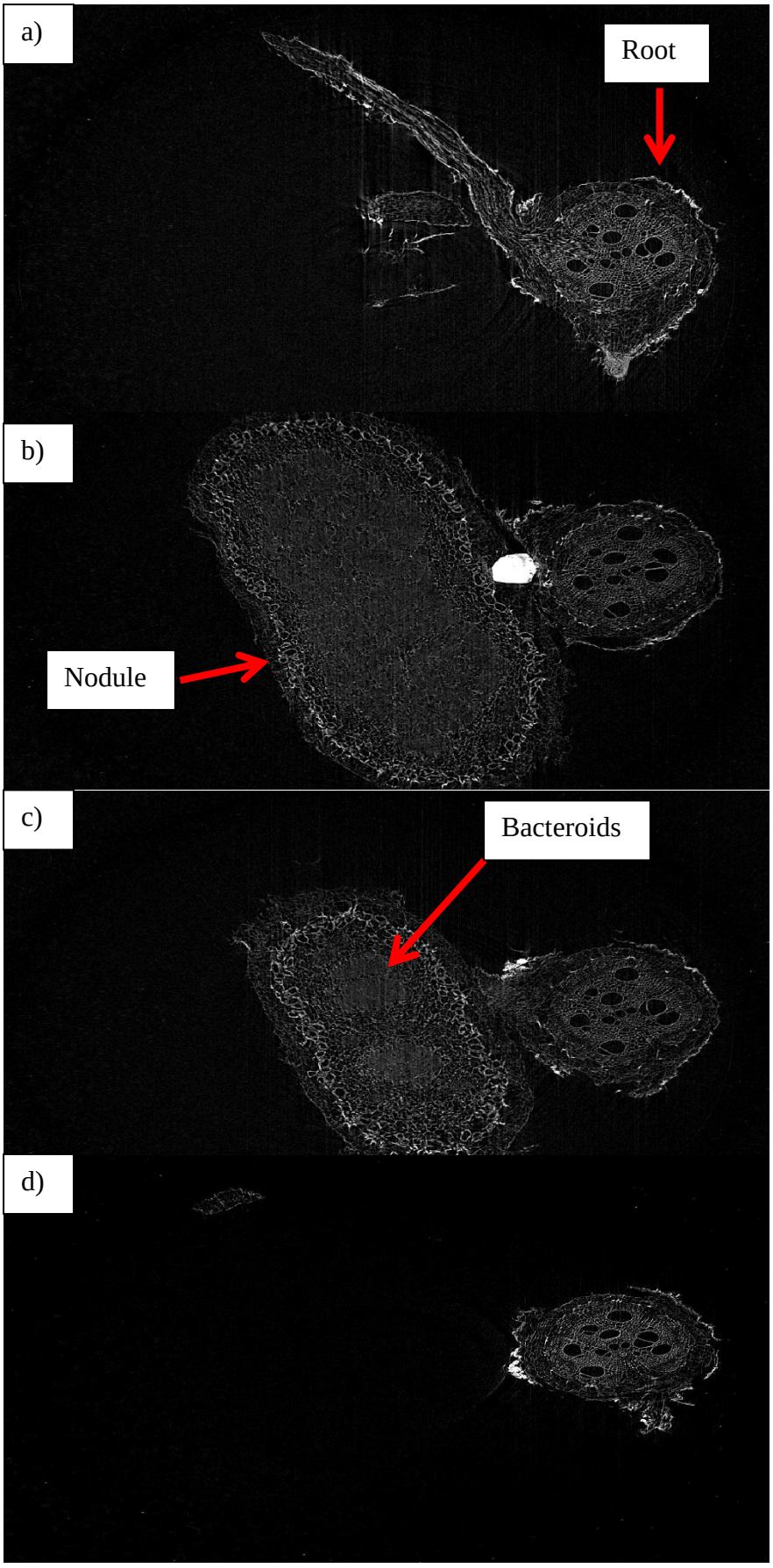

a)
Root
b)
Nodule
c)
Bacteroids
d)

Figure 7 – Root fragment of the soybean plant coupled to the nitrogen fixing bacteria (*Bradyrhizobium japonicum*) nodule in two dimensions visualization from a) Front root view; b) middle root view and c) bacteroids inside nodule view and d) end of root view obtained through X-ray microtomography in synchrotron analysis and processed by AVIZO software.

The nodules analyzed at synchrotron laboratory do not show hypha infection on soybean and Lima-bean nodules corroborating with Baird and Caruso (1994), Duhoux et al., (2001a) and Vidal-Dominguez et al., (1994). We also verify all tissues of nodules without fragment its structure been the first time that this technoly is applied in soybean and Lima bean nodules.

5. CONCLUSIONS

The IMX beamline proved to be satisfactory for morphological identification of structures on legume nodules; There is not mycorrhizal colonization in an active soybean nodule; Mycorrhizal colonization in soybean plants reduces the efficiency of biological N_2 fixation under nutritional stress of P and N.

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