University of São Paulo
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Changes in early soybean mycorrhization with the application of seaweed extracts to seeds

Marcos Altomani Neves Dias

Thesis presented to obtain the degree of Doctor in Science. Area: Crop Science

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SUMMARY

RESUMO

Alterações na micorrização precoce da soja com a aplicação de bioestimulantes nas sementes

Práticas agrícolas sustentáveis incluem uma melhor exploração dos recursos naturais do solo. Os fungos micorrízicos arbusculares (FMA) desempenham um papel importante neste contexto, com comunidades nativas nos solos que estabelecem uma associação mutualística com a maioria das espécies cultivadas e maximizam o acesso a nutrientes minerais, especialmente o fósforo. O tratamento de sementes pode consistir em uma alternativa interessante para estimular a colonização por FMA, desde estádios iniciais de crescimento da cultura, utilizando formulações de bioestimulantes aplicadas às sementes. Esta pesquisa teve como objetivo avaliar a micorrização precoce de plantas de soja em resposta a extratos de algas aplicados nas sementes. Os tratamentos consistiram de uma testemunha (CTR, água destilada), dois extratos de algas marinhas, das espécies *Ascophyllum nodosum* (ANE) e *Ecklonia maxima* (EME) e uma formulação sintética contendo formononetina (FOR), comercialmente utilizada como estimulante de micorrização. O primeiro experimento foi conduzido em casa de vegetação, com o objetivo de avaliar diferentes parâmetros de crescimento e desenvolvimento inicial das plantas de soja e a colonização de raízes por FMA, em resposta aos tratamentos de sementes; estas avaliações foram realizadas sob diferentes doses de fósforo adicionadas ao solo (0, 50, 100 e 200 $mg.dm^{-3}$). O segundo experimento foi conduzido em câmara de crescimento, com o objetivo de avaliar aspectos qualitativos e quantitativos da micorrização em resposta ao tratamento de sementes com bioestimulantes, nas fases iniciais de crescimento das plantas. Neste experimento, analisou-se a influência dos tratamentos na estrutura de comunidades de FMA, bactérias e fungos na rizosfera das plantas de soja, através de PCR/T-RFLP. As doses de fósforo aplicadas no solo influenciaram significativamente ($P < 0,10$) nos parâmetros biométricos e fisiológicos das plantas de soja, enquanto os tratamentos de sementes influenciaram significativamente apenas o teor de clorofila e o balanço de nitrogênio. A formononetina aplicada nas sementes de soja aumentou a colonização da raiz por FMA em relação ao controle, independentemente da dose de P aplicada ao solo, enquanto ANE e EME apresentaram valores intermediários para este parâmetro, não diferindo tanto da testemunha como da formononetina. Quando considerados alguns parâmetros qualitativos do estabelecimento de micorrizas, tanto os bioestimulantes sintéticos como os naturais apresentaram melhorias significativas. O estádio fenológico da soja influenciou significativamente as estruturas de comunidades de FMA, bactérias e fungos na rizosfera das plantas de soja, enquanto os tratamentos de sementes com bioestimulantes somente influenciaram significativamente a comunidade bacteriana. No entanto, uma variação relativamente grande nas estruturas da comunidade microbiana pôde ser atribuída à interação entre estádio fenológico das plantas e o tratamento de sementes com bioestimulantes.

Palavras-chave: *Glycine max* L.; Extratos de algas marinhas; Formononetina; Tratamento de sementes; Fungos micorrízicos arbusculares

ABSTRACT

Changes in early soybean mycorrhization with the application of seaweed extracts to seeds

Sustainable agriculture practices include a better exploitation of soil natural resources. Arbuscular mycorrhizal fungi (AMF) plays an important role in this context, with native communities in soils that establishes a mutualistic association with most plant species and maximizes their access to mineral nutrients, especially phosphorus. Seed treatment may consist in a good alternative to stimulate AMF root colonization, since early stages of crop growth, by using biostimulant formulations applied to seeds. This research had the objective to evaluate the early mycorrhization of soybean plants in response to seaweed extracts applied to seeds. The treatments consisted of a control (CTR, distilled water), two seaweed extracts, from *Ascophyllum nodosum* (ANE) and *Ecklonia maxima* (EME) species and a synthetic formulation containing formononetin (FOR), commercially used as mycorrhization stimulant. The first experiment was conducted in greenhouse, with the aim to evaluate different parameters of soybean early growth and development and AMF root colonization in response to the seed treatments, under different rates of phosphorus added to the soil (0, 50, 100 and 200 $mg.dm^{-3}$). The second experiment was conducted in growth chamber, with the objective to evaluate qualitative and quantitative aspects of mycorrhization in response to the seed treatments with biostimulants, at early plant growth stages. In this experiment, the influence of treatments on the structure of AMF, bacterial and fungal communities in the soybean rhizosphere were analyzed, through PCR/T-RFLP fingerprinting. Phosphorus rates applied to soil significantly influenced ($P<0.10$) biometric and physiological parameters of soybean plants, while the seed treatments only influenced leaf chlorophyll content and nitrogen balance at a significant level, despite some trends to increase leaf area, shoot dry mass and nodulation. Formononetin applied to soybean seeds increases AMF root colonization compared to control, independently of P rate applied to soil, while ANE and EME presented intermediate values for this parameter, not differing from both control and formononetin. When considered some qualitative parameters of mycorrhiza establishment, such as frequency and intensity of mycorrhization, both synthetic and natural biostimulants presented significant improvements, with EME and FOR providing higher values in general. The phenological stage of soybean significantly influenced AMF, bacterial and fungal community structures in rizosphere, while the biostimulant seed treatments only significantly influenced the bacterial community. However, a relatively large variation in the microbial community structures could be attributed to the interaction between phenological stage of plants and the seed treatment with biostimulants.

Keywords: *Glycine max* L.; Biostimulants; Formononetin; Seed treatment; Arbuscular mycorrhizal fungi

1. INTRODUCTION

Sustainability is a key driver for current and future generations in all economical activities, including agriculture. In crops such as soybean, which is a worldwide commodity traded between many countries, the crop production inputs and practices are always into the public opinion focus. Basically, every management decision in agriculture must consider the level of impact on the agroecosystem.

This research aimed to explore a possible interaction of three important and current subjects involving soybean crop production: 1) use of biostimulants to improve crop performance; 2) use of seed treatments; 3) soil biological fertility management.

Biostimulants from both natural and synthetic sources are currently available in the crop input market. Many of them have seaweed extracts on their composition, with *Ascophyllum nodosum* and *Ecklonia maxima* being among the most common species used for this purpose. Generally, seaweed extracts are applied to crops as foliar spray, but they also can be applied directly to soil or as seed treatment.

Seed treatment is a consolidated and very common practice in soybean crop production. This practice figures as a practical, precise and low-cost method that allows to deliver a large variety of inputs. Crop protection still the focus of most seed treatments today, although there is a large and growing interest for products such as microbial inoculants, fertilizers and biostimulants.

Another important approach to guarantee sustainability is to optimize the exploration of soil fertility. In this context, arbuscular mycorrhizal fungi are considered a very important soil resource, which can form a mutualist association with most crop species (including soybean) and increase nutrient and water uptake, despite several other benefits to the agroecosystem.

This research aimed to explore the interaction among these three parameters (biostimulants, seed treatment and mycorrhiza) that are constantly involved on soybean crop management. The main hypothesis to be tested was if *Ascophyllum nodosum* and, or, *Ecklonia maxima* extracts have the potential to affect soybean mycorrhization, due to their known stimulatory effects in plants. The use of natural and renewable sources of agricultural biostimulants, such as seaweed extracts, cooperates with the strong demand for more sustainable crops. This type of product must be used if the benefits and positive returns are proven.

2. LITERATURE REVIEW

2.1. Soybean crop overview: demand to increase yields with sustainable practices

Soybean is expected to be cultivated on approximately 130 million hectares worldwide in the 2018/2019 season (USDA, 2018). This number certainly follows an increasing demand for the grain, which is expected to continue rising for the next decades. Thus, as every other economic activity, there is a constant need to maintain or increase yields and profit margin, with sustainability.

As demonstrated in Table 1, Brazil is currently responsible for approximately 29% of the area cultivated with soybean, followed by the United States and Argentina as main producer countries. In terms of production, together, these three countries are expected to supply around 82% of world´s soybean grain in the 18/19 season, with 295.4 of 359.49 million metric tons (USDA, 2018). Only Brazil is expected to supply 33.5% (USDA, 2018).

Table 1. Soybean cultivated area in the world and main producer countries.

Country/Region	16/17	17/18	18/19 (Projected)
	Area (ha)		
World	**118.88**	**124.05**	**130.08**
Brazil	33.90	35.10	37.50
United States	33.47	36.23	35.96
Argentina	17.40	16.80	19.00
India	10.97	10.16	11.50
China	7.20	7.85	8.10
Paraguay	3.40	3.46	3.50
Russia	2.12	2.57	2.85

Source: USDA (2018)

Soybean is the fourth most cultivated crop in the world in terms of land use, following wheat, corn and rice, with 217.13, 183.95 and 161.60 million hectares, respectively (USDA, 2018). These large numbers highlight the importance of this crop in terms of land use and conservation.

Currently, consumers are highly aware of social and environmental impacts of the products they consume. Therewith, demands for organic and sustainable food is increasing (Lernoud and Willer, 2017). In the case of soybean, which is included in a large and complex food chain, this fact is very significant. Moreover, practices used for soybean cultivation certainly serve as reference for other crops with lower research investments.

As a major world supplier of soybean, Brazil have an important role as a reference in terms of productivity and adoption of sustainable practices in this crop. In the 17/18 season, approximately 62% of land cultivated with soybean in Brazil was in tropical regions, mostly located in the "Cerrado" or Brazilian Savannah region (CONAB: Companhia Nacional de Abastecimento, 2018). In general, these areas have soils with natural low chemical fertility, acidic, with high Al^{3+} levels and very low availability of mineral nutrients in general, especially phosphorus. Since the 1970´s, research efforts to adjust and improve soil fertility in these regions, associated to other areas of agronomic sciences, such as plant breeding, has allowed a successful exploration of Cerrado regions to produce soybean and other crops, such as corn, cotton and sugarcane. However, many challenges to manage crops in such conditions are frequent and still relevant (Sousa *et al.*, 2016).

In every case, a key aspect to sustain a long-term utilization of land to agriculture is soil health (Primavesi, 2008). In this context, a better use of soil resources and the microbial activity, such as from arbuscular mycorrhizal fungi, are crucial and must be taken into consideration for crop management practices.

2.2. Arbuscular mycorrhizal fungi (AMF) - main characteristics and importance in annual crops

Arbuscular mycorrhizal fungi (AMF) forms a mutualistic association (mycorrhiza) with most cultivated species (around 80%), which promotes a larger exploitation of soil resources by plants. The hyphal networks are capable to explore a much larger volume of soil than roots alone, thus enhancing the access to elements that present low mobility in soil or are present at very low levels (Bolan, 1991; Simard and Durall, 2004; Liu *et al.*, 2016).

Beyond the improvement on soil exploitation, these fungi present other important mechanisms that increase nutrients and water uptake, such as the release of organic acids, phosphatases, exchange of nutrients among plants and nutrient storage (Simard & Durall, 2004; Strack et al, 2003; Van Der Heijdn & Horton, 2009). Another important function of AMF in natural and agroecosystems is associated to the formation of a hyphal network and the production of glomalin, which improves soil structure and increase particles aggregation (Moreira and Siqueira, 2006; Dodd and Ruiz-Lozano, 2012).

In this mutualistic relationship, there is an exchange of carbon from plants to AMF, while P and other mineral nutrients are provided from the fungi to the host plant (Moreira and Siqueira, 2006). Use of carbon by AM fungi can be offset either by higher rates of

photosynthesis and, or, by saving in carbon costs for root production (Smith *et al.*, 2011). Moreover, another very important aspect related to mycorrhizal networks is the transfer of nutrients between plants, intra or interspecifically (Wahbi *et al.*, 2016).

Phosphorus availability to plants is very often associated to mycorrhiza. From one side, one of the main attributes given to this symbiosis is an improved access and uptake of P by plants, which is very important, as this element is one of the most difficult to acquire and, at the same time, is a macronutrient that represents approximately 0.2% to 0.5% of dry weight (Hawkesford *et al.*, 2011; Smith *et al.*, 2011). The very low solubility of phosphates of iron, aluminum and calcium results in low concentrations of P in soil solution, maintaining this element at critical low levels in many soils, which includes a large part of Brazilian cropland (Sfredo, 2008; Sousa *et al.*, 2016).

On the other hand, excessive use of P fertilizers is inversely related to mycorrhization, as plants do not favor the infection by the symbiont at high P rates, or even a possible increase of root growth associated to higher P supply can reduce the proportion of colonized roots (Miranda and Miranda, 2002; Nogueira and Cardoso, 2002; Smith *et al.*, 2011; Liu *et al.*, 2016; Motta *et al.*, 2016; Konvalinková *et al.*, 2017).

Excess or imbalanced use of mineral fertilizers, combined with crop protection practices, in general, cause negative effects on AMF communities in soils (Miranda and Miranda, 2002; Smith *et al.*, 2011). Mycorrhiza is not involved only in the P uptake and cycle, it is related to practically all mineral nutrients, playing a crucial role in their dynamic in agricultural and natural systems (Posta, Marschner and Römheld, 1994; Liu *et al.*, 2000; Cardoso, Navarro and Nogueira, 2003; Dodd and Ruiz-Lozano, 2012). As example, Storer *et al.* (2017) concluded that mycorrhiza also has a function to reduce N_2O emissions, with significant global implications to reduce the emission of this greenhouse gas.

Besides the negative environmental impact of indiscriminate or prophylactic use of crop inputs, such as fertilizers, crop protection formulations and others, recent studies have demonstrated that these “standard practices” are, in general, economically disadvantageous for soybean growers (Mourtzinis *et al.*, 2016; Orlowski *et al.*, 2016). As example with seed treatments, Jin, Germida and Walley (2013) verified a suppressive effect of different types of seed-applied fungicides on AMF colonization in pea and chickpea.

However, to a certain extent, it is possible to affirm that a trend to increase the concern with soil health parameters, which certainly includes AMF, is occurring (du Jardin, 2012, 2015). Recent advances in soil microbiology science are leading to new discoveries, highlighting the need for more attention to biological aspects of soil in the cropping

systems(Primavesi, 2008; Andreote, Gumiere and Durrer, 2014). Moreover, the need to increase nutrients use efficiency, especially phosphorus, is evident (Withers *et al.*, 2015, 2018; Rockström *et al.*, 2017).

The stimulation of AMF colonization is challenging, as many environmental and physiological factors are directly involved, such as soil pH, temperature, aeration, moisture, mineral composition, microbiota, root exudates of stimulatory or inhibitory compounds, plant species and the development of new and fine roots(Simard and Durall, 2004; Ceballos *et al.*, 2013; Liu *et al.*, 2016). Among the practices that must be adopted to favor this mutualistic association and promote a more resilient cropping system are: no-till, maintenance of crop residues covering soil surface, crop rotation (with mycotrophic species), rational use of inorganic fertilizers (especially P) and crop protection products (Primavesi, 2008; Hungria, Nogueira and Araujo, 2013; Cerezini *et al.*, 2016; Withers *et al.*, 2018). Additionally, the use of compounds that stimulates the establishment of mycorrhiza can be considered a complementary approach.

2.2.1. Arbuscular mycorrhizal fungi (AMF) management in annual crops

Basically, there are two ways to manage AMF in the field: application of AMF inoculum or work with indigenous AMF community (Plenchette *et al.*, 2005; Rodriguez and Sanders, 2015).

To date, the production of AMF inoculum is not considered as efficient as for other microorganisms (Rodriguez and Sanders, 2015). The obligatory symbiont characteristic limits inoculum production and their ability to adapt in different environments, competing with native communities. There are several commercial products in the market that offers AMF inoculants, however, there are few information and inconsistent results regarding their effectiveness when applied in large scale. Rodriguez and Sanders (2015) argue that the application of AMF in field conditions in too simplistic and ignores basic ecological principles.

The success of AMF inoculation in agricultural soils can be determined by many factors such as species compatibility, habitat niche availability for AMF and competition with native fungi (Verbruggen, 2017). These aspects need to be evaluated under local conditions for a more appropriate assessment of the viability of AMF inoculants. In the cases of seedling production in nurseries, for either horticulture or perennial crops, there is a higher chance of success using AMF inoculants, as the substrate and environmental factors can be better

controlled. Several researches to evaluate AMF inoculum are reported, for a large variety of crop species and with variable effects (Herrera-Peraza *et al.*, 2011; Koch *et al.*, 2011; Penton *et al.*, 2011; Ceballos *et al.*, 2013; Martin Alonso, Rivera Espinosa and Perez Diaz, 2013; Cely *et al.*, 2016).

Therewith, considering current practices used for soybean production and the large extensions of land occupied by this crop, the stimulation of indigenous AMF colonization can be considered a more appropriate approach (von Alten, Lindemann and Schönbeck, 1993; Plenchette *et al.*, 2005; Castillo *et al.*, 2014; Rodriguez and Sanders, 2015). Practices that promotes more root exudation of mycorrhiza stimulatory compounds can be a good alternative to increase mycorrhization levels. It is well known that root systems with fine, non-lignified and metabolic active parts also favor root colonization (Berbara, Souza and Fonseca, 2006).

Besides agronomical practices that may favor AMF colonization and maintenance in soil, some compounds have also been pointed as mycorrhization stimulants, such as the flavonoid formononetin (Silva-Júnior and Siqueira, 1997; Carneiro *et al.*, 1999; Siqueira *et al.*, 1999; Davies *et al.*, 2005; Catford *et al.*, 2006; de Novais and Siqueira, 2009; Ferreira, 2012; Peixe *et al.*, 2013; Castillo *et al.*, 2014; Santos, 2014; Cordeiro *et al.*, 2015; Ribeiro *et al.*, 2016; Savana da Silva *et al.*, 2017; Salgado Henrique Moreira, F., Maria de Sousa Moreira, F., Oswaldo Siqueira, J., Henrique Barbosa, R., Barbosa Paulino, H., & Aurélio Carbone Carneiro, 2017) and some natural products (von Alten, Lindemann and Schönbeck, 1993; Kuwada *et al.*, 1999, 2005, 2006; Sala, Freitas and Da Silveira, 2007; Paszt *et al.*, 2011, 2015). Both the synthetic and natural compounds are here classified as biostimulants, and more details are going to be discussed in the sequence.

As conclusion, the integration of different agronomic practices is crucial to maintain a rich, diversified and active community of AMF in cultivated soils, which is considered of high relevance to soil fertility and health.

2.3. Agricultural biostimulants

Khan et al. (2009), describe biostimulants as “materials, other than fertilizers, that promote plant growth when applied in small quantities”. Du Jardin (2015) describes biostimulants as “any substance or microorganism applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and, or, crop quality traits, regardless of its nutrients content. More recently, Van Oosten *et al.*(2017) defined agricultural biostimulants

as “a diverse classification of substances that can be added to the environment around a plant and have positive effects on plant growth, nutrition and stress tolerance (biotic and abiotic)”.

The use of biostimulants has become a common practice in agriculture (Craigie, 2011; Calvo, Nelson and Kloepper, 2014; Van Oosten *et al.*, 2017; Yakhin *et al.*, 2017). A better conscientization of consumers is increasing demand for sustainable produced or even organic products, which has a direct impact on the use of biostimulants in cropping systems. Biostimulants have multiple effects that may contribute to make agriculture more resilient and offer alternatives to synthetic products (Paszt *et al.*, 2015). Yakhin *et al.* (2017) reported that global biostimulants market is projected to reach 2.91 billion USD by 2021, with a CAGR (compound annual growth rate) of 10.4% from 2016 to 2021. Currently, Europe and North America are the main markets, corresponding to 41.7% and 21.5%, respectively. Asia and Latin America have 20% and 12.9%, respectively.

In a recent review about plant biostimulants, Calvo, Nelson and Kloepper (2014) divided them in five categories: 1) microbial inoculants; 2) humic acids; 3) fulvic acids; 4) protein hydrolysates and amino acids; 5) seaweed extracts. Among these types, seaweed extracts is the fastest growing market (Yakhin *et al.*, 2017).

2.3.1. Seaweed extracts

The objective of this part of the review is to provide an overview of characteristics and purposes of using seaweed extracts in agriculture, from a broader agronomic prospect. In recent years, very comprehensive reviews involving agricultural biostimulants and seaweed extracts were published (Khan et al. 2009;Craigie, 2011; du Jardin, 2012, 2015; Arioli, Mattner and Winberg, 2015; Battacharyya *et al.*, 2015; Van Oosten *et al.*, 2017; Yakhin *et al.*, 2017). Together, they have citations of several publications, with most of them associating the positive effects in plants to the use of seaweed extracts, or even a specific component present in the extracts. However, as these extracts contain a complex and diverse composition, many of the mechanisms of action still need better elucidation.

Seaweeds are considerably different from terrestrial plants in terms of biochemical and functional characteristics; they constitute a broad group with approximately nine thousand species, separated in three divisions: Phaeophyta, Rhodophyta and Chlorophyta, commonly named as brown, red and green, respectively. Among these, brown algae are more commonly used as agricultural biostimulants (Khan et al., 2009;du Jardin, 2012).

The utilization of seaweed biomass in agriculture occurs since the Roman Empire. Technologies to obtain the extracts from seaweeds were developed in the 1940´s, thus expanding the use of this type of product in agriculture, starting in the United States and Europe (Craigie, 2011; Arioli, Mattner and Winberg, 2015). More recently, it is estimated that the industry processes 10 to 12 million tons (frozen weight) of seaweed per year (Nayar and Bott, 2015), with diverse options for agricultural use, especially from brown seaweeds, which are commercialized as liquid or powder (Khan et al., 2009).

The extracts are obtained from renewable sources of strategically harvested seaweed biomass, with methods that involves acid or alkaline hydrolysis, cell rupture by pressure or even fermentation processes (Arioli, Mattner and Winberg, 2015). The extracts vary according to algae specie, presenting a wide range of mineral and organic components, including macro and micronutrients, aminoacids, vitamins, phenolic compounds, phytohormones (cytokinin, auxin, abscisic acid, gibberellin, brassinosteroids), complex polysaccharides, among others, with many of them absent in terrestrial plants (Khan et al., 2009; Stirk and Van Staden, 2014; Stirk *et al.*, 2014).

The most common classification applied to seaweed extracts is biostimulant (du Jardin, 2012; Calvo, Nelson and Kloepper, 2014). In general, many benefits from the application of these extracts are reported, either by directly promoting plant growth and stress resistance or through indirect pathways, by the interaction with microorganisms that constitutes the plant microbiome (Khan et al., 2009; Sangha *et al.*, 2014). In fact, the interaction with plant microbiome is inevitable, and a positive influence in it may improve plant health and performance in general (Andreote, Gumiere and Durrer, 2014).

A wide range of responses in plants are attributed to seaweed extracts, such as improved growth of roots and aerial organs, better nutrient uptake, higher flower and fruit set, senescence delay, improved resistance to biotic and abiotic stresses, among others (Arioli, Mattner and Winberg, 2015; Van Oosten *et al.*, 2017). These beneficial effects are mainly associated to signaling molecules present in the extracts (Craigie, 2011). Arioli, Mattner and Winberg (2015) considers algae extracts as potential inputs to fight against climatic stressors, such as drought, frost and heatwaves.

The complex composition of seaweed extracts causes multiple responses in plants (Figure 1), which varies according to environmental and genetic factors (Stirk and Van Staden, 2014). Therewith, a large part of mechanisms responsible for stimulatory effects in plants are not yet completely elucidated. Brassinosteroids were recently found in seaweed

extracts, while strigolactones may also be present, as pointed by Stirk and Van Staden (2014) and Arioli, Mattner and Winberg, (2015).

Currently, there are over 47 companies producing and marketing seaweed extracts for agricultural use, mainly from *Ascophyllum nodosum* (Calvo, Nelson and Kloepper, 2014; Van Oosten *et al.*, 2017). The extract of *Ecklonia maxima* is very commonly used as well. Both extracts are also present in several scientific publications (Kingman and Moore, 1982; Audibert *et al.*, 2010; Papenfus *et al.*, 2012; Jannin *et al.*, 2013; Khan *et al.*, 2013; Stirk *et al.*, 2014; Rengasamy *et al.*, 2014; Arioli, Mattner and Winberg, 2015; Goñi *et al.*, 2016; Martynenko *et al.*, 2016; Di Stasio *et al.*, 2017). In Brazil, both extracts are also among the most utilized in agriculture. In 2017, Brazil imported 709 tons of seaweed extracts as "fertilizer" (ABISOLO, 2018).

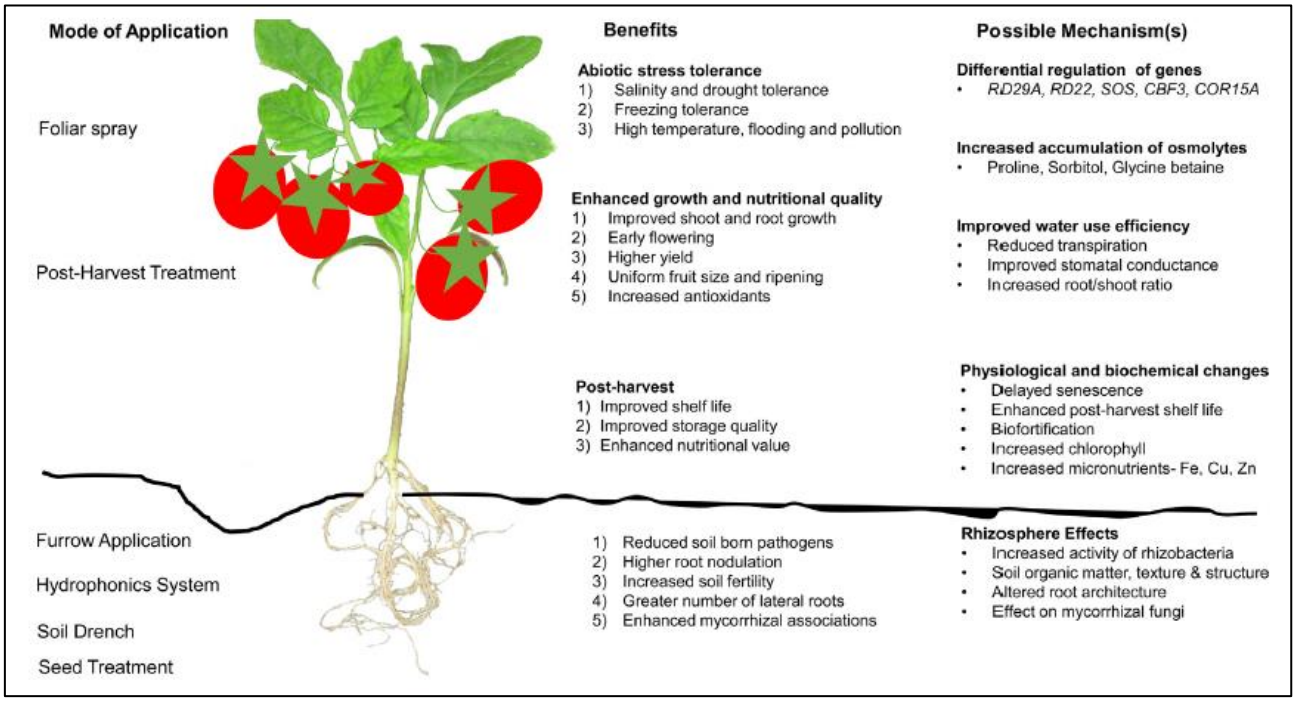

Figure 1. Schematic diagram containing methods of application of seaweed extracts and their possible effects on plants (source: Battacharyya *et al.*, 2015)

2.3.1.1. *Ascophyllum nodosum* **extract (ANE)**

The extract of *Ascophyllum nodosum* (L.) Le Jolie is one of the most frequently used as agricultural biostimulant (Yakhin *et al.*, 2017). Botanical aspects and geographical distribution of this algae are very well detailed in FAO (2015). This brown seaweed specie occurs in the North Atlantic shores of Canada and the estuarine rocky shores of northern Europe (Ugarte *et al.*, 2010).

The composition is very complex, with analogues of plant hormones (auxin, cytokinin, gibberellin, abscisic acid), phenolic compounds, betaines, polysaccharides, steroids and polyamines, mineral nutrients and others (Craigie, 2011).

The cytokinin-like effect of this extract is generally highlighted. Khan *et al.* (2011) reported that ANE compounds may elicit endogenous cytokinin-like activity, which may have a positive impact in modulating the growth and development of aerial plant organs.

Several responses in plants are attributed to the application of ANE. As examples, Fan *et al*. (2011) verified an stimulus to the production of phenolic compounds in spinach with the application of ANE. Jannin *et al.* (2013) observed increased concentration and lower degradation rates of chlorophyll in colza leaves with the application of ANE. In soybean, Martynenko *et al.* (2016) found better resistance to drought stress using ANE treated plants. Khan *et al.* (2013) reported that ANE improved nodulation parameters, root and shoot dry mass and shoot length in alfafa. Araujo (2016) verified positive responses of soybean plants with the application of ANE fractions in the seed treatment, resulting in improved earlier development.

It’s important to emphasize that, due to the large amount of ANE produced and their distinct sources, variations in terms of extracts composition may occur. Goñi *et al.* (2016) verified a very significant heterogeneity of responses among two ANE extracts by analyzing plant´s (Arabidopsis) transcriptome after their application. While one type of ANE dysregulated 4.47% of plants transcriptome, the other dysregulated only 0.87%. Despite the large difference in the transcriptome analysis, both extracts still presented positive results in terms of growth stimulus, increasing plant height and leaf number.

2.3.1.2. *Ecklonia maxima* **extract (EME)**

Ecklonia maxima (Osbeck) Papenfuss is a brown seaweed that naturally occurs in the west coast of South Africa, in low temperature and relatively nutrient rich waters (Stirk *et al.*, 2014). Since 1979, its biomass is harvested and commercialized for agricultural use (Stirk *et al.*, 2014).

Despite the decades of utilization, very recent studies are still demonstrating new characteristics and functionalities of EME. Ciepela et al. (2016) found that application of EME increased the contents of non-structural carbohydrates, improving the nutritional value of fodder plants. Di Stasio *et al.* (2017) found positive results with the application of EME in

mustard, in terms of biomass production, antioxidant activity, photosynthetic rate and chlorophyll content.

Rengasamy *et al.* (2014) studied a specific phlorotannin (Eckol) isolated from EME applied as seed treatment in maize. They verified a stimulus to maize root and shoot elongation and number of seminal roots; moreover, treated plants presented higher activity of α-amylase, which indicates a potential to positively influence the germination process.

In contrast to ANE, an auxin-like effect is frequently associated to EME (Rengasamy *et al.*, 2014; Stirk *et al.*, 2014). This indicates a stronger influence on root development, especially when applied as seed treatment. Stirk *et al*., (2014) recently identified other important groups of plant growth regulators in EME, abscisic acid, gibberellins and brassinosteroids. The authors infer that this cocktail of biological active compounds present in EME may act individually or in concert to contribute to the numerous favorable physiological responses elicited by its application in plants.

2.3.2. Formononetin

The isoflavonoid formononetin was identified as mycorrhization stimulant when compared to several other phenolic compounds, mainly by stimulating hyphal growth and differentiation into infection structures (Siqueira, Safir and Nair, 1991). Therefore, a patented method was developed to synthetically produce this molecule, and it is available for commercialization in some countries (Myconate®, Plant Health Care, Raleigh, United States).

In general, positive and significant results were found in terms of mycorrhization stimulus when this synthetic product was applied as seed treatment in different crops, including soybean, maize, wheat, cotton, potato and alfafa (Silva-Júnior and Siqueira, 1997; Siqueira *et al.*, 1999; Davies *et al.*, 2005; Catford *et al.*, 2006; de Novais and Siqueira, 2009; Castillo *et al.*, 2014, 2016; Cordeiro *et al.*, 2015; Ribeiro *et al.*, 2016; Salgado *et al.*, 2017).

Silva-Júnior and Siqueira (1997) reported an increase of 9.4% on soybean dry mass under field conditions, with the application of formononetin to soil. In terms of AMF colonization, the authors reported increases for both maize and soybean crops, with higher colonization percentage and higher density of vesicules and arbuscules. Siqueira *et al*. (1999) found an attenuation of heavy metals uptake by maize plants with the application of formononetin, probably as a consequence of increased AMF colonization.

Catford *et al*. (2006) identified a possible role of exogenous application of formononetin on plants nodulation (*Sinorhizobium meliloti*) in alfafa. In the same study, formononetin also promoted higher levels of mycorrhization in plants.

Castillo *et al*. (2014) and Castillo *et al*. (2016) reported significant increases in terms of wheat mycorrhization in response to application of formononetin to seeds. Savana da Silva *et al.*, (2017) verified a positive effect of formononetin applied as seed treatment in soybean, increasing the infection of root nodules by AMF, thus forming a tripartite symbiosis. Field trials also reported increased yields using this product (Myconate) as seed treatments (Peixe *et al.*, 2013; Santos, 2014; Cordeiro *et al.*, 2015; Ribeiro *et al.*, 2016; Salgado *et al.*, 2017). Ribeiro *et al*., (2016) also reported an increase in soybean yield, mycorrhization and nodulation with the use of formononetin as seed treatment, indicating a rate above 50 $g.ha^{-1}$ to be applied as seed treatment. Oppositely, Ferreira (2012) did not found a significant influence of formononetin on both AMF colonization and crop yield in field conditions, for corn and soybean.

2.3.3. Soybean seed treatment

Seed treatment is a common technique applied to soybean crop and can have a variety of agronomic purposes. The advantages of this technique compared to other delivery methods, such as soil or foliar applications, are mainly based on its lower operational costs, high uniformity of products distribution among plants in the field, early availability to plants (since germination starts), rational use of products and easy availability of modern equipment's for either "on-farm" or industrial scale operations (Taylor *et al.*, 1998; Dias *et al.*, 2018).

In Brazil, more than 90% of soybean seeds consumed contain a certain type of treatment (França-neto *et al.*, 2015), with the seed treatment operation being executed either in large (industrial) or small scale ("on-farm"). Currently, the components that are predominantly applied to seeds are: fungicides, insecticides, nematicides, micronutrients, microbial inoculants, biostimulants and polymers, with most of them applied in the liquid form.

Murillo-Williams and Pedersen (2008) evaluated the direct effect of seed treatment with three common fungicides on soybean mycorrhization. They found no negative correlation between the products and AMF root colonization, as they previously expected. The root colonization with fungicide-treated seeds even increased in some cases, as they might have reduced the abundance or eliminated the presence of AMF antagonists.

In general, there is a growing interest for the use of microbial inoculants and natural biostimulants in the seed treatment, with the aim to improve initial crop performance (Hungria, Nogueira and Araujo, 2013; Cerezini *et al.*, 2016; Wilson, Amirkhani and Taylor, 2018). The use of seed treatments to manage mycorrhiza establishment in annual crops was previously proposed in the 1990´s, mainly with studies involving synthetic formononetin. More recently, Castillo *et al.* (2014) also intended to evaluate a possible benefit of applying natural biostimulants and formononetin in the seed treatment of wheat on crop mycorrhization. This research had the aim to evaluate a possible effect of two commonly used crop biostimulants, the extracts of *A. nodosum* and *E.maxima*, on early soybean mycorrhization, comparing to synthetic formononetin and untreated seeds (distilled water).

3. MATERIAL AND METHODS

3.1. Seaweed extracts

3.1.1. *Ascophyllum nodosum* extract (ANE)

The *Ascophyllum nodosum* extract (ANE) was provided by Acadian Seaplants Ltd. (Dartmouth, Nova Scotia, Canada), in a liquid formulation containing 11.0% ($w.w^{-1}$) of the dry powder extract, with pH 5.80 and density 1.04 $g.cm^{-3}$.

3.1.2. *Ecklonia maxima* extract (EME)

The *Ecklonia maxima* extract (EME) was provided by Kelp Products (Pty) Ltd. (Simon´s Town, South Africa), in a liquid formulation containing 5.3% ($w.w^{-1}$) of the dry powder extract, with pH 4.30 and density 1.03 $g.cm^{-3}$.

3.1.3. Formononetin (FOR)

The source of formononetin was a commercial product registered as Myconate ® (Plant Health Care, Inc., United States), a powder formulation containing 95% of 4´-methoxy, 7´hydroxy isoflavone (formononetin), commercially recommended as seed treatment to stimulate AMF colonization.

Appendix A shows the visual aspects of ANE, EME and FOR.

3.2. Seed treatments and quality evaluation

For the seed quality evaluation and for the first experiment (greenhouse), treatments were applied to seeds using a stainless-steel pan coater (Appendix B) with a conical spreader in the center, equipped with a rotating motor (Leroy-Somer, model LS71, 0.75 KW, 3000 rpm). This equipment allows to simulate either on-farm or large-scale operations, with a uniform coverage of soybean seedcoat. For the second experiment (growth chamber), seeds were individually treated using a micropipette and analytical scale (for formononetin powder), aiming to provide a very precise amount of each formulation per seed. In both experiments,

seeds were treated within less than two hours and allowed to dry at room temperature before sowing.

Liquid treatments (distilled water, *Ascophyllum nodosum* and *Ecklonia maxima* extracts) were applied at a rate of 1 µL.seed^{-1}, while formononetin was applied at a rate of 0.33 µg.seed^{-1}. In the treatment with formononetin, seeds also received 1 µL.seed^{-1} of distilled water before the application of formononetin, aiming to maintain the same rate of liquid applied to seeds in all treatments. These rates were chosen based on average recommendation rates used for soybean crop, of 300 ml.ha^{-1} of seaweed extracts and 100 g.ha^{-1} of formononetin.

Preliminary tests of seed germination and vigor (seedling emergence) were conducted to evaluate the effect of treatments previously described on seed quality. The first test was conducted following procedures described on the Rules for Seed Testing (Brasil, 2009), with four replicates of 50 seeds per treatment and germination counting at the 5th and 8th days after sowing. The seedling emergence test was conducted in plastic trays, filled with 8 dm^3 of fine sand moistened at 60% of the water holding capacity, also with four replicates of 50 seeds per treatment; the final emergence counting occurred at the 9th day after sowing. These tests were conducted with two seed lots, from cultivars M6210IPRO and RK7214IPRO and results are presented on Table 5. As treatments did not affect seed quality, they were used to continue the study, as described on Table 1.

Table 2. Seed treatments evaluated in the first (greenhouse) and second (growth chamber) experiments.

ID	Rate (µL.seed^{-1})	Rate (µg.seed^{-1})	[1]Rate (ml.ha^{-1})	[1]Rate (g.ha^{-1})
CTR	1	-	300	-
ANE	1	-	300	-
EME	1	-	300	-
FOR	1	0.33	300	100

[1] Rates estimated with an average use of 50 kg of soybean seeds per hectare. CTR: distilled water; ANE: *Ascophyllum nodosum* extract; EME: *Ecklonia maxima* extract; FOR: formononetin (powder) and distilled water (liquid).

The cultivar M6210IPRO was selected to continue the study, as it is a modern cultivar (released in 2011) and recommended to use in a large part of Brazilian territory. It has an indeterminate growth habit.

3.3. Soil Substrate

The soil was collected in January 2016, on the surface layer (0-20 cm) of an uncultivated pasture field in Piracicaba, SP, Brazil, with coordinates 22°43'4.67"S, 47°37'1.98"W and elevation of 585 m. The area was predominantly covered by *Urochloa brizantha* (syn. *Brachiaria brizantha*). The soil is classified as dystrophic Red-Yellow Latosol (Oxisol). Firstly, it was sieved (2 mm mesh) to remove larger aggregates and organic residues and very well mixed to uniformize the sample. Afterwards, it was submitted to physical and chemical analysis, at the Laboratory of Soil Fertility (USP/ESALQ). Based on chemical results, the soil base saturation was adjusted to a target of 70% (from initial 48%), with the application of lime (calcium carbonate p.a., FMAIA, Belo Horizonte, MG, Brazil), which also elevated calcium levels and pH. After adjusted, the soil was reanalyzed, and the physical and chemical results are presented on Table 3.

Four rates of P in the substrate were used: a control treatment considered as "0" (no P added) and the application of 50, 100 and 200 $mg.dm^{-3}$ of P, as finely ground and sieved (mesh pore size: 0.59 mm) triple superphosphate (TSP). After the fertilizer was mixed, each soil sample were submitted to chemical analysis to determine P level (resin method), where they showed a very precise correlation with the rates applied, with results of 51, 100 and 199 $mg.dm^{-3}$.

The sieved soil was also characterized by AMF spore density prior to utilization in the experiment, using the wet sieving method (Gerdermann and Nicolson, 1963), with six replicates of 50 g. The samples were mixed in water and sieved (710 µm and 45 µm mesh pore sizes) with running water, followed by centrifugation (3 min.; 3500 rpm) in 70% sucrose solution. A spore density of 17 $spores.g^{-1}$ of soil was verified.

Soil indigenous AMF was evaluated by genus, with the collaboration of Denise L. C. Mescolotti (Laboratory of Soil Microbiology, USP/ESALQ), following information contained on the International Culture Collection of Vesicular Arbuscular Mycorrhizal Fungi (INVAM), available on http://invam.wvu.edu. Five genera were found and were constant in all samples: Scutellospora, Gigaspora, Ambispora, Glomus and Acaulospora. Appendix E contains a picture of a group of spores collected from the soil used in both experiments.

Table 3. Physical and chemical analysis of the soil used in the experiments, after sieving and liming.

Parameter	Analysis method	Unit	Result
Sand	n/a	$g.kg^{-1}$	800
Clay	n/a	$g.kg^{-1}$	176
Silt	n/a	$g.kg^{-1}$	24
pH	$CaCl_2$	n/a	5.7
Organic matter	Colorimetric	$g.dm^{-3}$	15
P	Resin	$mg.dm^{-3}$	8
S	Calcium phosphate	$mg.dm^{-3}$	8
K	Resin	$mmol_c.dm^{-3}$	1.6
Ca	Resin	$mmol_c.dm^{-3}$	28
Mg	Resin	$mmol_c.dm^{-3}$	7
Al	KCl	$mmol_c.dm^{-3}$	<1
H + Al	SMP	$mmol_c.dm^{-3}$	10
Sum of Bases	n/a	$mmol_c.dm^{-3}$	46.6
Cation exchange capacity (CEC)	n/a	$mmol_c.dm^{-3}$	34.5
Base saturation	n/a	%	72
Al^{3+} saturation	n/a	%	4
B	hot water	$mg.dm^{-3}$	<0.1
Cu	DTPA	$mg.dm^{-3}$	0.6
Fe	DTPA	$mg.dm^{-3}$	44
Mn	DTPA	$mg.dm^{-3}$	22.7
Zn	DTPA	$mg.dm^{-3}$	1.7

3.4. First experiment (greenhouse)

A greenhouse experiment was conducted with the main objective to evaluate the early colonization of soybean plants by arbuscular mycorrhizal fungi (AMF), in response to the seed treatments with *Ascophyllum nodosum* and *Ecklonia maxima* extracts, using distilled water and formononetin as control treatments.

These treatments were tested with increasing rates of P added to soil, with the intent to create different scenarios with this factor that directly influences mycorrhization. Evaluations of 1) leaf area, 2) leaf chlorophyll content, 3) leaf epidermal flavonoids, 4) leaf nitrogen balance, 5) root dry mass, 6) shoot dry mass, 7) chlorophyll fluorescence, 8) leaf transpiration, 9) internodes and total shoot length, 10) Leaf P concentration and accumulation and 11) nodulation parameters were conducted to better accompany plants growth and development and complement the data and discussion of results.

The experiment was arranged in a completely randomized design with four replicates, containing four plants each. A factorial design was established (4x4), with four rates of P added to soil (0, 50, 100 and 200 $mg.dm^{-3}$) and four types of seed treatments (CTR,

ANE, EME and FOR). Each plant was individually cultivated in propylene pots filled with 0.5 dm^3 of soil substrate. The phenological stages used as reference in this document are shown on Appendix D.

3.4.1. Cultivation details

The experiment was carried out in greenhouse with natural sun light, located at USP/ESALQ campus, Soil Science Department, Piracicaba, SP, Brazil. The sowing occurred on February 25th, 2016. Plants were maintained in greenhouse up to the V4 stage, with air temperature varying between 20 ^{o} C to 36 ^{o} C and soil temperature between 22 ^{o} C to 30 ^{o} C during the cultivation period. Soil moisture was kept uniform, between 45 to 65% of total water holding capacity, constantly monitored by weighing the pots. The plants were irrigated only with distilled water during their cycle.

A nutrient solution adapted by Sarruge (1975), without P, was applied at three phases of plants development, when plants reached V1, V2 and V3 phenological stages. In each occasion, 10 ml per plant was applied on soil surface.

3.4.2. Leaf area estimation

Leaf area was determined using a non-destructive method proposed by Richter *et al.* (2014), at the growth stages of V2, V3 and V4. The equation was firstly calibrated for the cultivar used in the study (M6210IPRO), by growing some plants from this cultivar to use as model. Therewith, the length (L, mm) and width (W, mm) of both unifoliolate leaves and of the central leaflet of each trifoliolate leaf were measured at the three growth stages during the experiment. The leaf area of unifoliolate (LA_u) and trifoliolate (LA_t) leaves were calculated according to Equations 1 and 2, respectively. Total plant leaf area was given by the sum of individual areas calculated in each equation.

$$LA_u = 0.7505.(L.W) \quad (1)$$

$$LA_t = 1.6919.(L.W) \quad (2)$$

Where:

LA_u : unifoliolate leaf area

LA_t : trifoliolate leaf area

L: maximum leaf length

W: maximum leaf width

3.4.3. Leaf chlorophyll, epidermal flavonoid and nitrogen balance index

Direct determinations of leaf chlorophyll, epidermal flavonoid and nitrogen balance index were conducted using the leaf-clip sensor Dualex 4 Scientific Dx4 (Force-A, Orsay, France), according to procedures described on Cerovic *et al.* (2012).

Measurements were performed at V2, V3 and V4 growth stages, in the central leaflet of the first, second and third trifoliolate leaf, respectively. Three spots per leaf were randomly chosen, avoiding leaf veins, to generate an average number per plant.

3.4.4. Chlorophyll fluorescence

The chlorophyll fluorescence was determined at the V3 stage of soybean plants, in the central leaflet of the second trifoliolate leaf, using a Junior-PAM fluorometer (Walz GmbH, Effeltrich, Germany). Only two rates of P added to soil were chosen for this evaluation (0 and 100 $mg.dm^{-3}$). First, leaves were covered for 30 minutes with aluminum foil for dark adaptation and interruption of photosynthetic activity, at ambient temperature. Afterwards, an actinic pulse of light of 1350 $\mu mol.m^{-2}.s^{-1}$ was instantly applied after removing the aluminum foil. The data provided by the equipment refers to maximum quantum efficiency of photosystem II (PSII).

3.4.5. Leaf transpiration

A steady-state porometer, model LI-1600 (Li-cor, Inc., Lincoln, NE, United States) was used to analyze leaf transpiration. Similar to chlorophyll fluorescence, only two rates of P added to soil were chosen for this evaluation (0 and 100 $mg.dm^{-3}$). The photosynthetic active radiation (PAR) during the measurements in the greenhouse ranged from 380 to 410 $\mu mol.m^{-2}.s^{-1}$, and values were recorded in an one-hour interval (8:30 am – 9:30 am). The average leaf temperature was 27.6 ^{o}C and air relative humidity of 66.2 %. The plants were analyzed at the V3 stage, on the central leaflet of the second trifoliolate leaf.

3.4.6. Shoot and internodes length

Total shoot and internode lengths were measured using a ruler (graduated in mm), after plants were collected and removed from soil (V4 stage).

3.4.7. Tissues dry mass and nodulation parameters

Shoots were separated from roots and both were rinsed in deionized water, to clean the surface and remove soil particles. Nodules were carefully separated from roots with forceps. All tissue materials were placed in paper bags and maintained in air-forced oven at 65 ^{o} C for 96 h. Afterwards, the dried samples were weighed in analytical scale (0.0001 g). Nodules count per plant was also determined.

3.4.8. Determination of AMF root colonization

Dried roots were cleared in 10% KOH solution for 40 minutes at 90 ^{o} C, followed by application of 10% H_2O_2 for 1 minute and staining with 5% acetic acid solution with 5% of Parker® ink (Newell Rubbermaid, Boulogne Billancourt, France). After stained, roots were maintained in lactoglycerol (lactic acid – glycerol – water, 1:1:1) until evaluation (Vierheilig *et al*., 1998).

The evaluation of AMF root colonization followed the gridline intersect method (Giovannetti and Mosse, 1980).

3.4.9. Phosphorus leaf concentration and content

Dried tissues of soybean leaf blades were ground in mortar to a very fine powder; afterwards, 15 mm-diameter pellets were produced from 200 mg of powder per replicate. The pellets were obtained in a stainless-steel matrix with a pressure of 15 tons.cm^{-2} during 120 s, using a hydraulic pellet press (model 36248 X-Press, Spex SampPrep, Metuchen, NJ, United States).

Fist, these pellets were used to obtain intensity measures (cps.µA^{-1}) of P in the soybean leaf tissues, using Energy Dispersive X-ray Fluorescence (EDXRF, equipment model EDX-720, Shimadzu, Kyoto, Japan). Afterwards, an equation to correlate cps.µA^{-1} to mg.kg^{-1} was obtained, by the comparison with materials of known concentration. The values were

then converted and presented as $mg.kg^{-1}$ of P. The P content (or accumulation) was calculated by the multiplication of P concentration and the dry mass of leaf blades. This analysis was conducted with the collaboration of Prof. Dr. Hudson W. P. Carvalho and Dr. Eduardo de Almeida, at the Laboratory of Nuclear Instrumentation (CENA/USP).

3.5. Second experiment (growth room)

A growth room experiment was conducted with two main objectives: 1) evaluate more detailed aspects of early soybean mycorrhization, at each growth stage (V0 to V3), in response to the same seed treatments applied on the first experiment; 2) evaluate the effects of seed treatments on AMF communities in soil, at each growth stage, using DNA based techniques. Other evaluations conducted at this part of the research are also important to enrich the discussion about seed treatments effects, such as their influence on bacterial and fungal communities in the soybean rhizosphere and the interaction with each phenological stage.

3.5.1. Cultivation details

The experiment was installed at USP/ESALQ, Crop Science Department, in Piracicaba, SP, Brazil. The sowing occurred in January 5th, 2017. Plants were cultivated in growth room, with artificial LED lighting (250 $\mu mol.m^{-2}.s^{-1}$ PAR intensity, 16h photoperiod), in 0.5-dm^3 propylene pots. Average air and soil temperatures were maintained constant at 25 ± 4 ºC and 23 ± 2 ºC, respectively.

The same soil substrate described on item 3.3 was used, with no P added (except for the evaluation of mycorrhization parameters at V2 stage, where two P rates were compared, 0 and 100 $mg.dm^{-3}$). Soil moisture was kept uniform, between 45 to 65% of total water holding capacity, constantly monitored by weighing the pots. The plants were irrigated with distilled water during all cycle. A nutrient solution adapted by Sarruge (1975), without P, was applied at two phases of plants development, when plants reached V1 and V2 phenological stages. In each occasion, 10 ml per plant was applied on soil surface.

Four treatments were evaluated: 1) 1 $\mu L.seed^{-1}$ of distilled water (CTR); 2) 1 $\mu L.seed^{-1}$ of ANE; 3) 1 $\mu L.seed^{-1}$ of EME; 4) 1 $\mu L.seed^{-1}$ of distilled water + 0.33 $\mu g.seed^{-1}$ of formononetin (FOR). Treatments were applied with a micropipette above seeds and allowed

to dry at room temperature before sowing. Six replicates per treatment were used, with two plants per replicate, arranged in a fully randomized.

Evaluations occurred at the growth stages V0, V1, V2 and V3. First, at each stage, rhizosphere samples (here considered as the soil fraction closely adhered to roots) were collected, by carefully removing with forceps, and stored in plastic microtubes (1.5 ml). Soil samples were maintained in freezer at -80 °C until DNA extraction.

After soil was collected, the plants were carefully removed from the soil with tap water and rinsed in deionized water. Roots and shoots were separated, placed in paper bags and dried in air-forced oven at 65 °C for 96 hours. Afterwards, shoot samples were weighed in analytical scale (0.0001 g). Roots were prepared to analyze the mycorrhizal parameters, as described in the sequence.

3.5.2. Mycorrhizal parameters

Dried roots were cleared in 10% KOH solution for 40 minutes at 90 ^{o}C, followed by application of 10% H_2O_2 for 1 minute and staining with 5% acetic acid solution with 5% of Parker® ink (Newell Rubbermaid, Boulogne Billancourt, France). After stained, roots were maintained in lactoglycerol (lactic acid – glycerol – water, 1:1:1) until evaluation (Vierheilig et al., 1998).

For each root system (replicate), 2 samples of fifteen 1-cm segments were mounted in microscope slides (75 x 26 mm). AMF root colonization was evaluated according to Trouvelot *et al*. (1986) and Faessel *et al.* (2010), with each root segment being observed under an optical microscope (100 x) and scored for: 1) total AMF colonization of each segment, scored from 0 to 5 according to Figure 1-A; 2) arbuscule presence in each root segment (Figure 1-B).

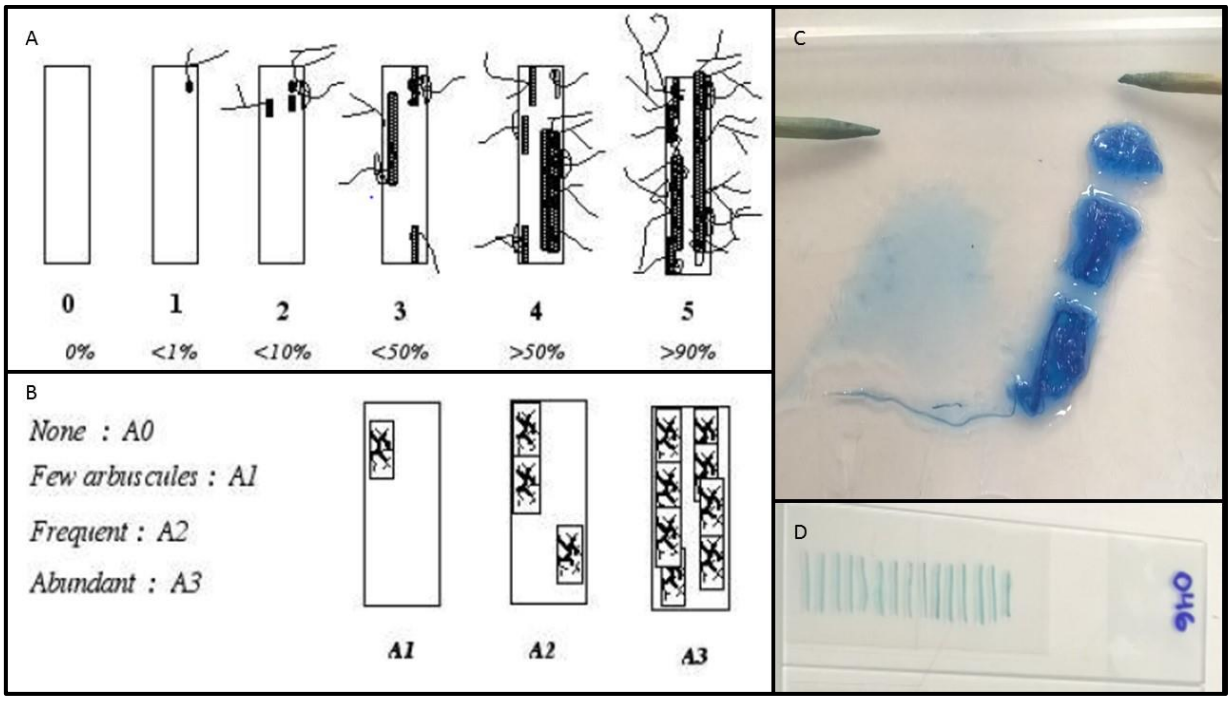

Figure 2. A: scores of mycorrhization in root fragments (adapted from Trouvelot et al., 1986); B: scores of arbuscule presence in root fragments (adapted from Trouvelot et al., 1986); C: root system of one plant being sampled; D: microscope slide mounted with 15 root fragments.

This range of classes allowed to estimate the level of mycorrhization in the plants, by calculating three parameters of AMF colonization, using the software Mycocalc (French National Institute for Agricultural Research, INRA, Dijon).

$$\boldsymbol{F\%} = \frac{\boldsymbol{CRS}}{\boldsymbol{TRS}} \times \mathbf{100}$$

(1)

Where:

F%: frequency of mycorrhiza in the root system

CRS: number of colonized root segments

TRS: total number of root segments

$$\boldsymbol{M\%} = \frac{(\mathbf{95}\times \boldsymbol{n5} + \mathbf{70}\times \boldsymbol{n4} + \mathbf{30}\times \boldsymbol{n3} + \mathbf{5}\times \boldsymbol{n2} + \boldsymbol{n1})}{\boldsymbol{TRS}}$$

(2)

Where:

M%: intensity of the mycorrhizal colonization in the root system

TRS: total number of root segments

$$\boldsymbol{A\%} = \boldsymbol{a} \times \frac{\boldsymbol{M}}{\boldsymbol{100}}$$

(3)

Where:

A%: arbuscule abundance in the root system

M: intensity of the mycorrhizal colonization in the root system (M%)

a%: arbuscule abundance in the mycorrhizal parts of root fragments (a%)

3.5.3. Rhizosphere analysis of microbial communities

Soybean rhizosphere samples, from each growth stage, were analyzed to verify the influence of seed treatments on AMF community structure, with PCR/T-RFLP. Additionally, total bacterial and fungal community structures were evaluated with the same technique.

3.5.3.1. **Soil DNA extraction**

Soil DNA was extracted from 400-mg samples of rhizosphere, using the Power Soil® DNA Isolation (MoBio, Carlsbad, United States), following the protocol established by the manufacturer. After extraction, a sample of 5 µL for each treatment was analyzed by agarose gel electrophoresis 1.5%, with TAE buffer 1x (400 mM Tris, 20 mM acetic acid, 1 mM EDTA), colored with GelRed™ (0.5 µg ml^{-1}) and visualized under UV light (DNR – Bio Imaging Systems/MiniBis Pro).

The extracted DNA were used in three analyses: 1) AMF community fingerprinting (PCR/T-RFLP); 2) Fungal community fingerprinting (PCR/T-RFLP); 3) Bacterial community fingerprinting (PCR/T-RFLP).

3.5.3.2. **Arbuscular mycorrhizal fungi, total bacterial and total fungal community structures analysis by PCR/T-RFLP**

Microbial communities' structure was analyzed in rhizosphere samples, with three replicates per treatment.

For AMF community analysis, ~380 bp length sections of the gene 28S rRNA was used (Antunes *et al.*, 2009; Barto *et al.*, 2011; Koch *et al.*, 2011). The extracted DNA was submitted to a nested-PCR protocol to amplify DNA from AMF. The first amplification used the primers LR1 (5'-GCATATCAA TAAGCGGAGGA-3') and FLR2 (5'-GTCGTTTAAAGCCATTACGTC-3') (van Tuinen *et al*., 1998; Trouvelot *et al*., 1999; Antunes *et al*., 2009; Koch *et al*., 2011). The first reaction was constituted of 2.5 µL of DNA, 2.0 µL of each primer at 10 mM, 6.25 µL of PCR buffer (10x), 2.5 mM of $MgCl_2$ 50 mM, 2.5 µL of dNTPs, 0.5 µL of Taq DNA polymerase, 10 µL of betaine solution and ultra-pure water, to complete 50 µL.

For the second reaction, it was used 2.5 µL of the product from first reaction, 2 µL of primers FLR3-FAM (5'-TTG AAA GGG AAA CGA TTG AAG T-3') and FLR4-VIC (5'-TAC GTC AAC ATC CTT AAC GAA-3') (Gollotte, Van Tuinen and Atkinson, 2004) at 10 mM, 6.25 µL of PCR buffer (10x), 2.5 mM of $MgCl_2$ 50 mM, 2.5 µL of dNTPs (2.5 mM), 0.5 µL of Taq DNA polymerase, 10 µL of betaine solution and ultra-pure water, to complete 50 µL. Both PCRs consists of an initial denaturation step at 93 ^oC for 3 min followed by 35 cycles (93 ^oC for 1 min, 58 ^oC for 1 min, 72 ^oC for 1 min) and a final extension step of 10 min at 72 ^oC in a thermocycler (Viriti®, Applied Biosystems).

For total fungal community analysis, the intergenic region of ribosomal DNA (ITS) was used, with primers ITS1F-FAM (5'-TCCGTAGGTGAACCTTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), following the procedure described by Avis et al.(2006). The reaction was constituted of 1 µL of DNA, 0.1 µL of each primer, 0.20 µL of Taq polymerase, 4.0 µL of dNTPs (2.5 mM), 6.0 µL of $MgCl_2$ 25 mM, 5.0 µL of PCR buffer (10x), completed with ultra-pure water to reach 50 µL. This amplification started at 94 °C for 1.5 min, 13 cycles at 94 °C for 35 s, 55 ° C for 55 s and 72 °C for 45 s; 13 cycles at 94 °C for 35 s, 55 °C for 2 min, 72 °C for 45 s, 9 cycles at 94 °C for 35 s, 55 °C for 3 min, 72 °C for 45 s and 10 min at 72 °C.

For bacterial community analysis, the universal primers to amplify the ribosomal region of 16S rRNA gene was used, FAM-8fm (5'- AGAGTTTGATCMTGGCTCAG-3') and 926R-Eub (5'-CCGTCAATTCCTTTRAGTTT-3') described by Heuer *et al*. (1997). The

reaction was constituted of 1 µL of DNA, 0.2 µL of each primer, 0.50 µL of Taq polymerase, 4.0 µL of dNTPs (2.5 mM), 6.0 µL of $MgCl_2$ 25 mM, 5.0 µL of PCR buffer (10x), completed with ultra-pure water to reach 50 µL PCR reaction for this amplification started at 95 °C for 4 min, 30 cycles at 95 °C for 30 s, 57 °C for 30 s, 72 °C for 45 s and 10 min at 72 °C. It resulted in a volume of 50 µL (with approximately 50 ng of DNA).

The products from PCR reactions were purified with QuiaQuick PCR purification kit (Quiagen) and quantified in agarose gel 2% with TAE buffer 1X, colored with Gel Red® and observed under UV light.

The amplified fragments of regions 28S rRNA, ITS and 16S rRNA were digested with the restriction enzymes *TaqI*, *HhaIII* and *HhaI*, respectively. For *TaqI* digestion, 10 µL of purified PCR product, 2 µL of enzyme buffer, 1.0 µL of the enzyme and 7.0 µL of ultra-pure water was used. For *HhaIII* digestion, 15 µL of purified PCR product, 2 µL of enzyme buffer, 0.5 µL of the enzyme and 2.5 µL of ultra-pure water. For *HhaI*, 10 µL of purified PCR product, 2 µL of enzyme buffer, 0.5 µL of the enzyme and 7.5 µL of ultra-pure water. Afterwards, samples were precipitated with the addition of 2.0 µL EDTA (125 mM), 2.0 µL of sodium acetate (3M) and 50 µL of ethyl alcohol, followed by centrifugation at 4000 rpm for 30 min at 4 °C. The precipitated fragmented DNA was washed with ethanol 70% and the samples were dried at room temperature; after, samples were resuspended in 9.5 µL of formamide Hi-D and 0.5 µL of Liz 1200 at the moment of reading. The solutions were read by an automatic sequencer ABI Prism 3500 (Applied Biosystems).

The occurrence and intensity of peaks with fragments with different sizes were compared with the software GeneMapper® v.4.1, which generated matrices with information of the peaks generated by the terminal restriction length fragments (TRFs). It was generated 119 peaks for 28S rRNA, 655 peaks for ITS region and 405 peaks for 16S rRNA.

3.6. Statistical analysis

A significance level of 10% was adopted for both experiments. This level was considered more appropriate to analyze the effects of natural biostimulants, as they are non-target specific and contain a complex composition; thus, effects are expected to present higher variability if compared to target-specific formulations.

Data was transformed when needed and submitted to analysis of variance, followed by regression analysis or mean comparison tests (Tukey´s). Non-parametric tests were used in

cases of data that did not fit normal distribution, using Wilcoxon´s test for pairwise comparison and Steel-Dwass test to compare between all pairs.

An analysis of variance was firstly performed to verify the effects of soybean phenological stages and seed treatments with biostimulants on the arbuscular mycorrhizal fungi community alpha-diversity (Shannon-index), with means compared by Tukey´s test in case of significance ($P<0.05$).

Afterwards, the variation of bacterial, fungal and arbuscular mycorrhizal fungi communities in response to the seed treatments with biostimulants were tested using non-metric multidimensional scaling (NMDS), using the Bray-Curtis similarity. A permutation MONOVA (PERMANOVA) was carried out to evaluate the correlation between phenological stages and seed treatments on the microbial community structures.

The statistical analyses were performed in R software, version 3.5.0 (R Core Team, 2018), using the packages ‘vegan’ (Oksanen *et al.*, 2016) and ‘agricolae’ (De Mendiburu, 2017).

4. RESULTS AND DISCUSSION

4.1. First experiment: rates of P added to soil associated to seed treatments with biostimulants (greenhouse)

Table 4 contains ANOVA results for the tests conducted in the first experiment (greenhouse), with four rates of P added to soil and four seed treatments. Significant results (*P<0.10*) occurred for almost all parameters in response to P levels in soil, except for leaf nitrogen balance (V3 stage), root dry mass, leaf transpiration and total shoot length. On the other hand, seed treatments with biostimulants only significantly (*P<0.10*) influenced three parameters: leaf chlorophyll content (V2 stage), leaf nitrogen balance (V2 stage) and arbuscular mycorrhizal fungi colonization. No significant difference was found by the interaction between both factors, P rate and seed treatment (P*ST).

These results indicate a strong effect of P supply from soil in many plant growth parameters. The rates of P used in this study ranges from normal P levels found in farmed soils to excessive levels (rates of 100 and 200 $mg.dm^{-3}$). However, it is important to consider that P levels in the planting furrow can very high concentrations, as indicated on Appendix E. The main intention of using this range was to evaluate the effect on mycorrhiza establishment. It is well known that high P levels in the soil negatively influences this mutualist association to occur (Moreira and Siqueira, 2006). Thus, the seed treatments evaluated in this study can have an influence on this process, maybe improving or stimulating AMF root colonization under unfavorable conditions. Oppositely, low levels of P generally stimulate AMF root colonization, and the effect of these biostimulant seed treatments under this condition also needed to be evaluated.

Table 4. Results of analysis of variance (ANOVA) for the tests conducted in the first experiment (greenhouse).

Evaluation	P	ST	P*ST
		Prob>F	
Leaf area V2	<0.0001*	0.5407	0.7031
Leaf area V3	<0.0001*	0.7157	0.8526
Leaf area V4	<0.0001*	0.7270	0.6281
Leaf chlorophyll content V2	0.0004*	0.0413*	0.5558
Leaf chlorophyll content V3	0.0097*	0.2353	0.3675
Leaf chlorophyll content V4	0.0636*	0.2865	0.3969
Leaf Epidermal flavonoids V2	0.0008*	0.5669	0.4122
Leaf Epidermal flavonoids V3	<0.0001*	0.6750	0.1931
Leaf Epidermal flavonoids V4	<0.0001*	0.6454	0.9636
Leaf nitrogen balance V2	0.0227*	0.0095*	0.2483
Leaf nitrogen balance V3	0.5985	0.1944	0.1416
Leaf nitrogen balance V4	<0.0001*	0.7815	0.7695
Root dry mass	0.2859	0.2890	0.3073
Shoot dry mass	<0.0001*	0.6852	0.4193
Chlorophyll fluorescence	0.0145*	0.4473	0.5631
Leaf transpiration	0.2578	0.9488	0.8862
Total shoot length	0.2764	0.8829	0.3525
Nodules dry mass	<0.0001*	0.6473	0.9595
Nodules count	<0.0001*	0.7386	0.9971
Arbuscular mycorrhizal fungi colonization	<0.0001*	0.0614*	0.2801

P: P rates added to the soil (0, 50, 100 and 200 mg dm^{-3})
ST: seed treatments (Distilled water, *Ascophyllum nodosum* extract, *Ecklonia maxima* extract or formononetin)
* Significant ($P<0.10$)

4.1.1. Soybean growth and development parameters in response to different P rates added to soil

4.1.1.1. **Results**

Soybean leaf area in response to increasing P rates added to soil was analyzed at V2, V3 and V4 growth stages (Figure 3). Higher P levels in soil promoted leaf growth at each growth stage (*P<0.10*), with maximum leaf area achieved at the rates of 143.3, 136.1, and 137.5 mg dm^{-3} of P, for V2, V3 and V4, respectively.

As plants were analyzed *in vivo*, the same groups of plants from each P fertilizer rate were analyzed at all stages. It is interesting to note that maximum leaf area was obtained with almost the same rate (approximately 140 mg dm^{-3}) at all ages analyzed, which can be indicated as an optimum value for the growth conditions that the soybean was cultivated, at least for leaf area increase.

Results of leaf chlorophyll content analyzed at V2, V3 and V4 are shown in Figure 4. In a similar way to leaf area, there were significant (*P<0.10*) responses for P rates in the soil, at all growth stages. However, at V2 and V3, leaf chlorophyll decreased as P rate increased, while at V4 the opposite occurred. Minimum leaf chlorophyll concentrations at V2 and V3 were found at the P rates of 173.1 and 203.1 mg dm^{-3}, respectively, while a maximum peak of leaf chlorophyll concentration at V4 was found at the P rate of 148.6 mg dm^{-3}.

Leaf epidermal flavonoids data is shown in Figure 5. The values of epidermal flavonoids decreased (*P<0.10*) as P rates added to soil increased, at all growth stages analyzed. Minimum flavonoid index values occurred at the P rates of 200, 150 and 150 mg dm^{-3} for V2, V3 and V4, respectively. The mean values of leaf flavonoid index increased around 25% from V2 to V3, and 38% from V3 to V4.

Leaf nitrogen balance results are presented on Figure 6. Significant (*P<0.10*) responses to P rates in the soil were found only at V2 and V4 stages. In the first, leaf nitrogen index decreased as P rate increased, while in the second the opposite occurred. At V3 stage, P rates did not influence the nitrogen balance index, maintain an average value of 26.6. The index values decreased almost by half (around 48.5%) from V2 to V4 growth stage.

Data of soybean root and shoot dry mass is presented on Figure 7. A significant response (*P<0.10*) to P rates added to soil could be observed only for shoot dry mass, with maximum value achieved at 127.8 mg dm^{-3} of P, 35% higher than the treatment with no P added. On the other hand, root dry mass was not directly affected by the increasing rates of P added to soil, keeping an average value of 587.3 mg per plant.

Results of shoot and internodes length are presented in Figure 8. Total shoot length was not significantly (*P<0.10*) influenced by P rates added to soil, with maximum mean value (141.8 mm) obtained at 100 mg dm^{-3} P rate. Internodes length were influenced (*P<0.10*) by the availability of P, except the first internode (int1), which maintained an average length of 44 mm. At internodes 2, 3 and 4, the length slightly decreased (*P<0.10*) as P rates increased in soil, while internode 5 the opposite occurred, increasing the length as P rates increased. As the internode lengths slightly changed until the last internode (int5), it's possible to verify that this part was determinant to total shoot length. At this part, a maximum value was reached a 131.3 mg dm^{-3}.

Figure 9 (A and B) contains results of P leaf concentration and accumulation in soybean leaves, in response to phosphorus supply to the soil. As expected, as P rates added to soil increased, higher amounts of P could be taken up by plants and directed to leaf tissues. Thus, a significant increase (*P<0.10*) of approximately five-fold on both P concentration and

content was verified on soybean leaf blades, comparing the lowest to the highest P rate added to soil (0 and 200 mg dm^{-3} of P added).

Both parameters are important to measure, as P concentration gives a relative value, while P content or accumulation gives an absolute value. The last one also considers the plant biomass accumulation, as sometimes is possible to achieve higher concentration only with reduced plant growth. In this case, as P content increased proportionally to P concentration in leaves, it is possible to conclude that plants also presented an increase on biomass and could utilize the source of P for nutrition and improved growth.

Figure 10 contains data of nodulation parameters. Both nodules count (NC) and dry mass (NDM) increased (*P<0.10*) as P levels in soil also increased, with highest values obtained at 146 and 137.8 mg dm^{-3} of P added to the soil, respectively. Again, this range between 130 and 140 mg dm^{-3} of P provided the maximum value in the regression analysis, thus indicating this rate as the most appropriate for optimum growth of soybean plants under the conditions that plants were cultivated.

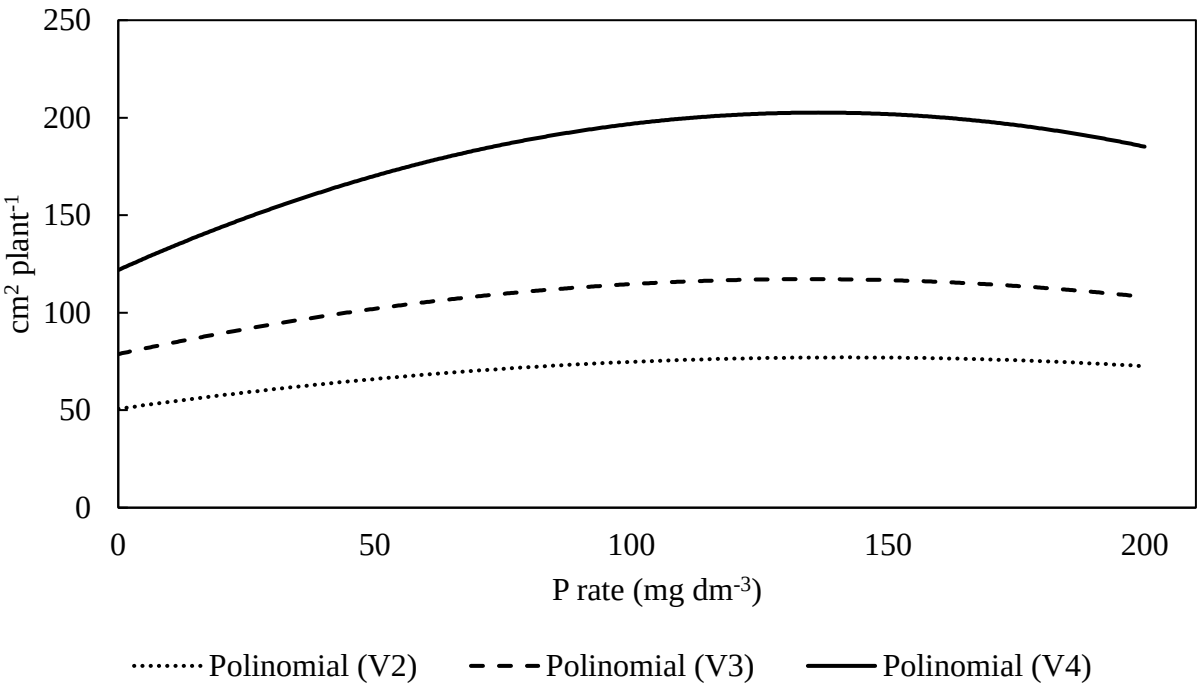

Figure 3. Soybean leaf area at three growth stages (V2, V3 and V4), in response to four rates of P added to the soil: 0, 50, 100 and 200 mg dm^{-3}.
V2: $y= -0.0013x^2 + 0.3727x + 50.7$ (R^2=0.53, CV= 9.7%, *P>F=<0.0001*)
V3: $y= -0.0021x^2 + 0.5716x + 78.8$ (R^2=0.63, CV= 7.0%, *P>F=<0.0001*)
V4: $y= -0.0043x^2 + 1.1824x + 121.9$ (R^2=0.78, CV= 9.2%, *P>F=<0.0001*)

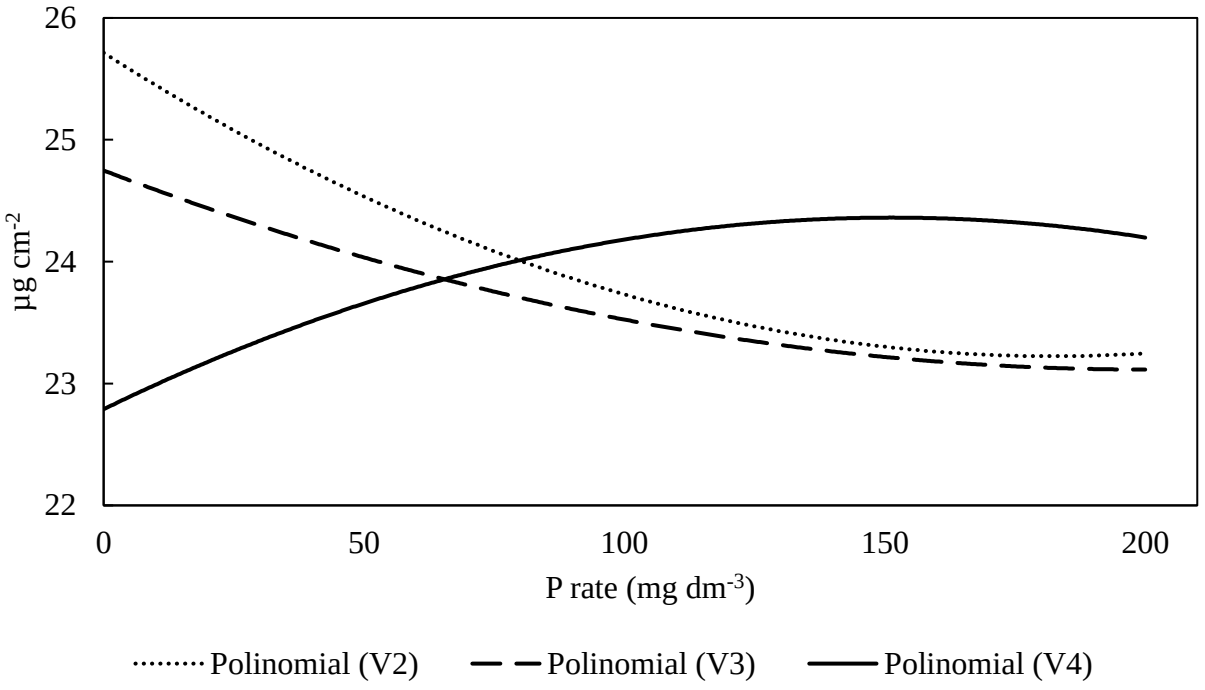

Figure 4. Soybean leaf chlorophyll content at three growth stages (V2, V3 and V4), in response to four rates of P added to the soil: 0, 50, 100 and 200 mg dm^{-3}.
V2: y= 8E-05x^2 - 0.0277x + 25.7 (R^2=0.20, CV= 8.7%, *P>F=0.0004*)
V3: y= 4E-05x^2 - 0.0163x + 24.7 (R^2=0.11, CV= 7.7%, *P>F=0.0097*)
V4: y= -7E-05x^2 + 0.0208x + 22.8 (R^2=0.07, CV= 8.9%, *P>F=0.0636*)

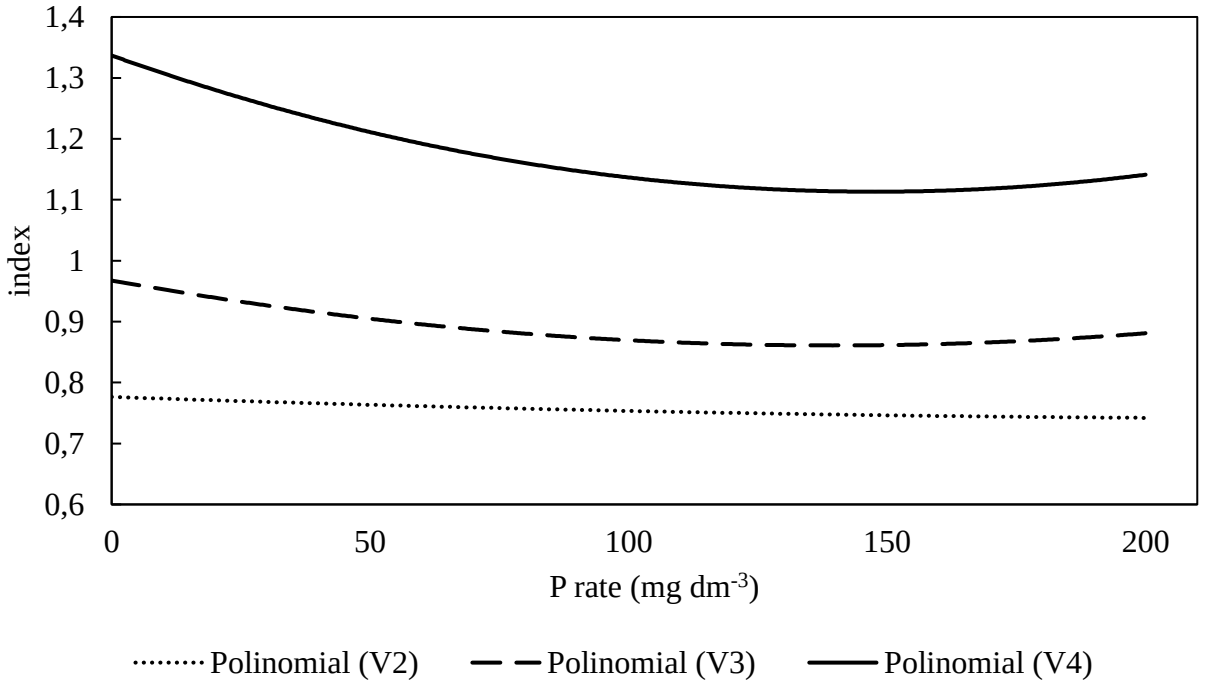

Figure 5. Soybean leaf epidermal flavonoids at three growth stages (V2, V3 and V4), in response to four rates of P added to the soil: 0, 50, 100 and 200 mg dm^{-3}.
V2: y= 6E-07x^2 - 0.0003x + 0.7765 (R^2=0.18, CV= 3.9%, *P>F=0.0008*)
V3: y= 5E-06x^2 - 0.0015x + 0.9675 (R^2=0.37, CV= 6.9%, *P>F=<0.0001*)
V4: y= -1E-05x^2 + 0.0030x + 1.3367 (R^2=0.56, CV= 9.0%, *P>F=<0.0001*)

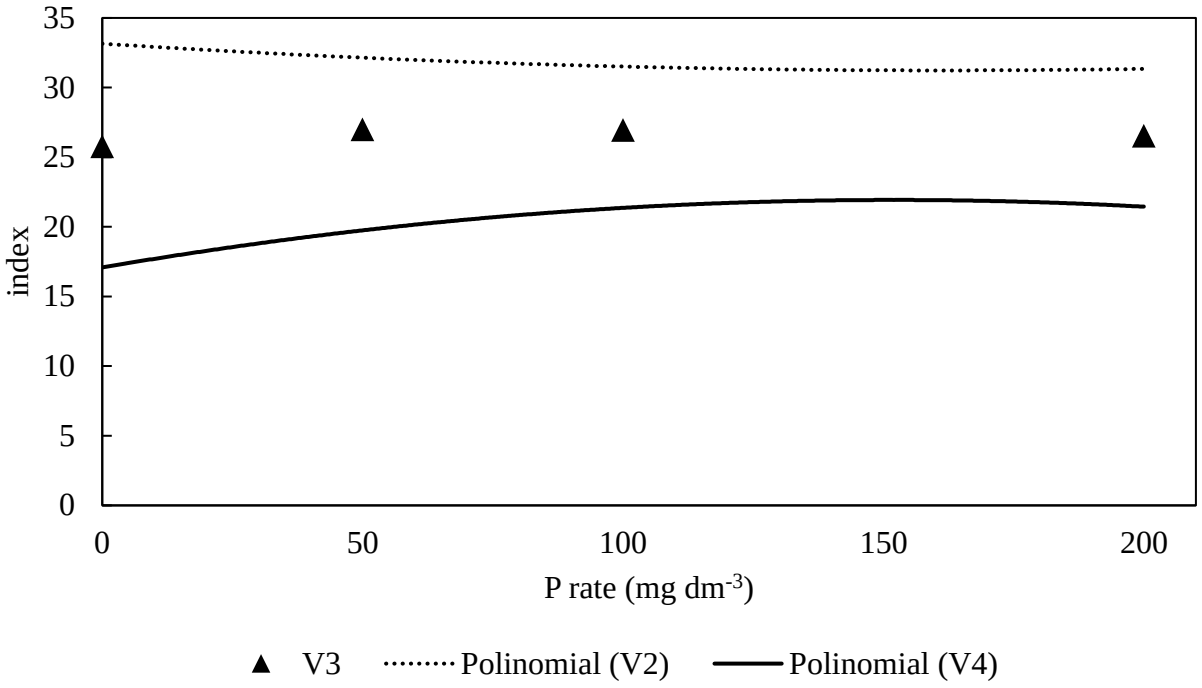

Figure 6. Soybean leaf nitrogen balance index at three growth stages (V2, V3 and V4), in response to four rates of P added to the soil: 0, 50, 100 and 200 mg dm^{-3}.
V2: y= 7E-05x^2 - 0.0240x + 33.2 (R^2=0.08, CV= 7.5%, *P>F=0.0227*)
V3: y= 26.6 (CV= 10.6%, *P>F=0.5985*)
V4: y= -0.0002x^2 + 0.0637x + 17.1 (R^2=0.41, CV= 14.0%, *P>F=<0.0001*)

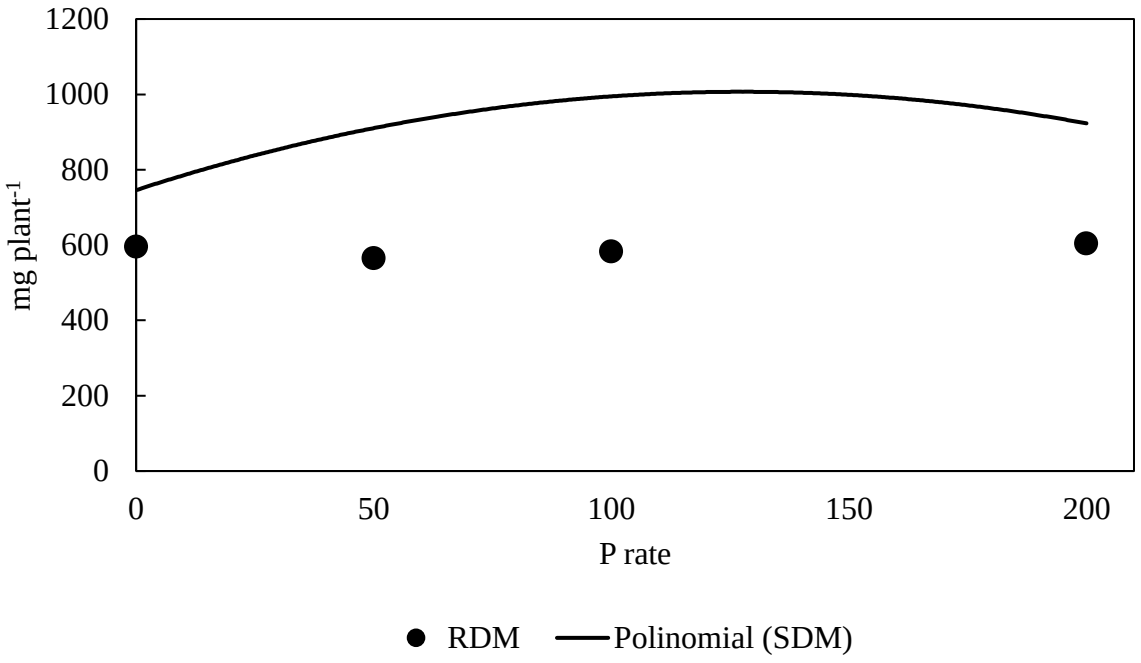

Figure 7. Soybean root (RDM) and shoot (SDM) dry mass at the fourth fully expanded leaf stage (V4), in response to four rates of P added to the soil: 0, 50, 100 and 200 mg dm^{-3}.
RDM: y= 587.3 (CV= 13.0 %, *P>F=*<0.0001)
SDM: y= -0.016x^2 + 4.0898x + 745.9 (R^2=0.62, CV= 8.9%, *P>F=*0.2859)

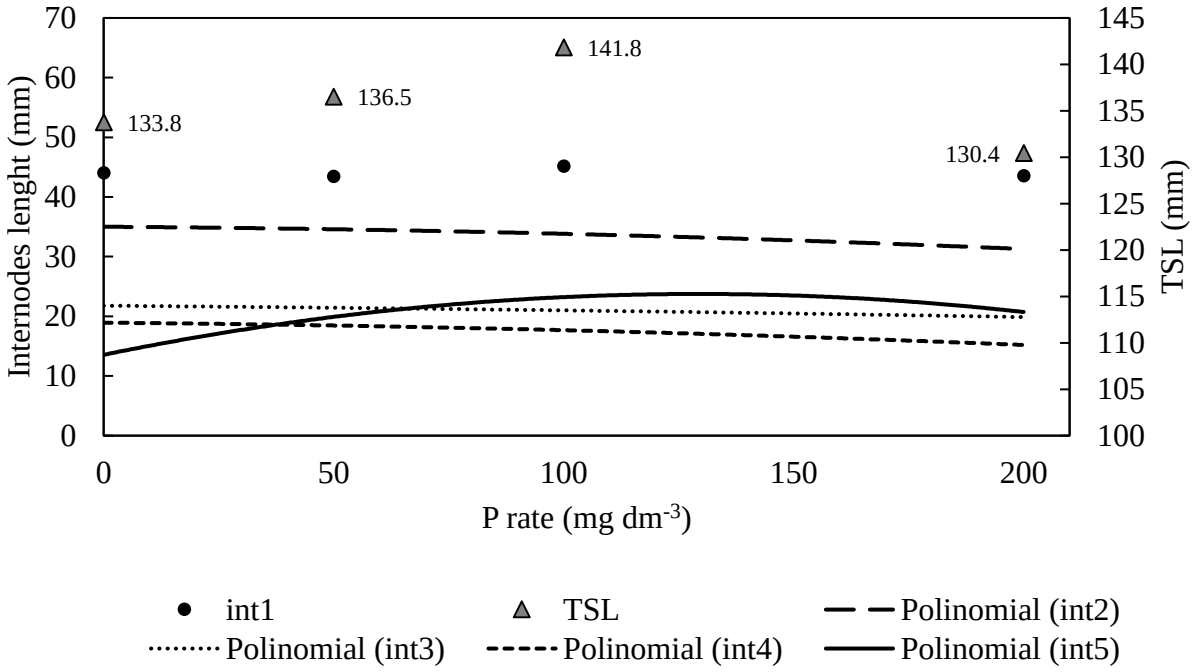

Figure 8. Internodes and total shoot length of soybean plants at the fourth fully expanded leaf stage (V4), in response to four rates of P added to the soil: 0, 50, 100 and 200 mg dm^{-3}.

TSL: y = 135.6 (CV= 6.7%, *P>F=0.2764*)
Int1: y= 44.0 (CV= 10.8%, *P>F=0.9377*)
Int2: y= $-7E-05x^2$ - 0.0049x + 35.6 (R^2=0.08, CV= 14.6%, *P>F=0.0214*)
Int3: y= $-2E-05x^2$ - 0.0061x + 21.7 (R^2=0.14, CV= 9.1%, *P>F=0.0007*)
Int4: y= $-6E-05x^2$ - 0.0064x + 18.9 (R^2=0.15, CV= 21.2%, *P>F=0.0022*)
Int5: y= $-0.0006x^2$ + 0.1576x + 13.5 (R^2=0.34, CV= 31.9%, *P>F=0.0022*)

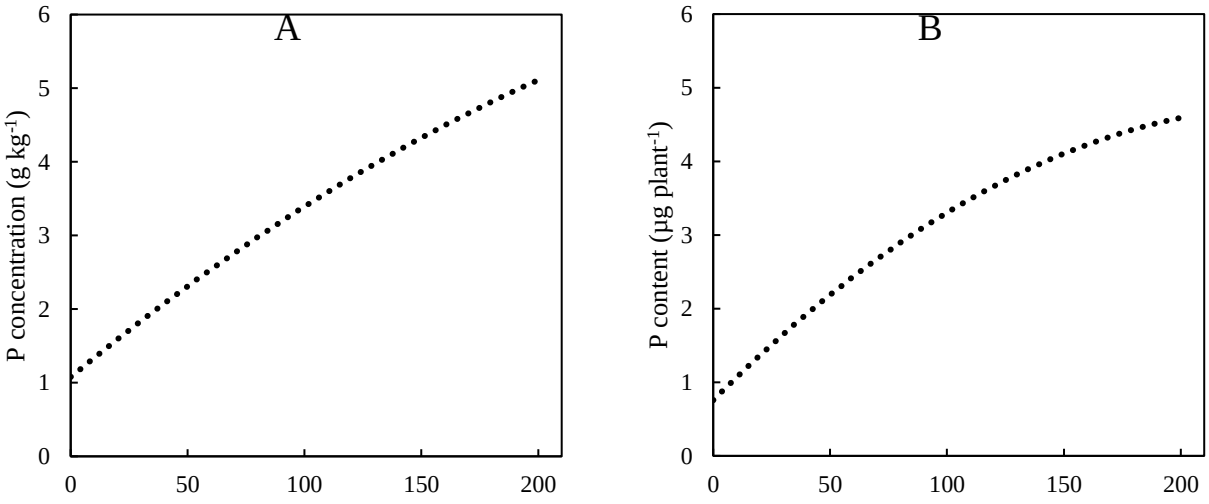

Figure 9. Phosphorus concentration (A) and content (B) in soybean leaf blades at the fourth fully expanded leaf stage (V4), in response to four rates of P added to the soil (0, 50, 100 and 200 mg dm^{-3})

P concentration: y= $-3E-05x^2$ + 0.0261x + 1.0782 (R^2=0.98, CV= 20.8 %, *P>F=<0.0001*)
P content: y= $-6E-05x^2$ + 0.0318x + 0.7555 (R^2=0.96, CV= 23.4 %, *P>F=<0.0001*)

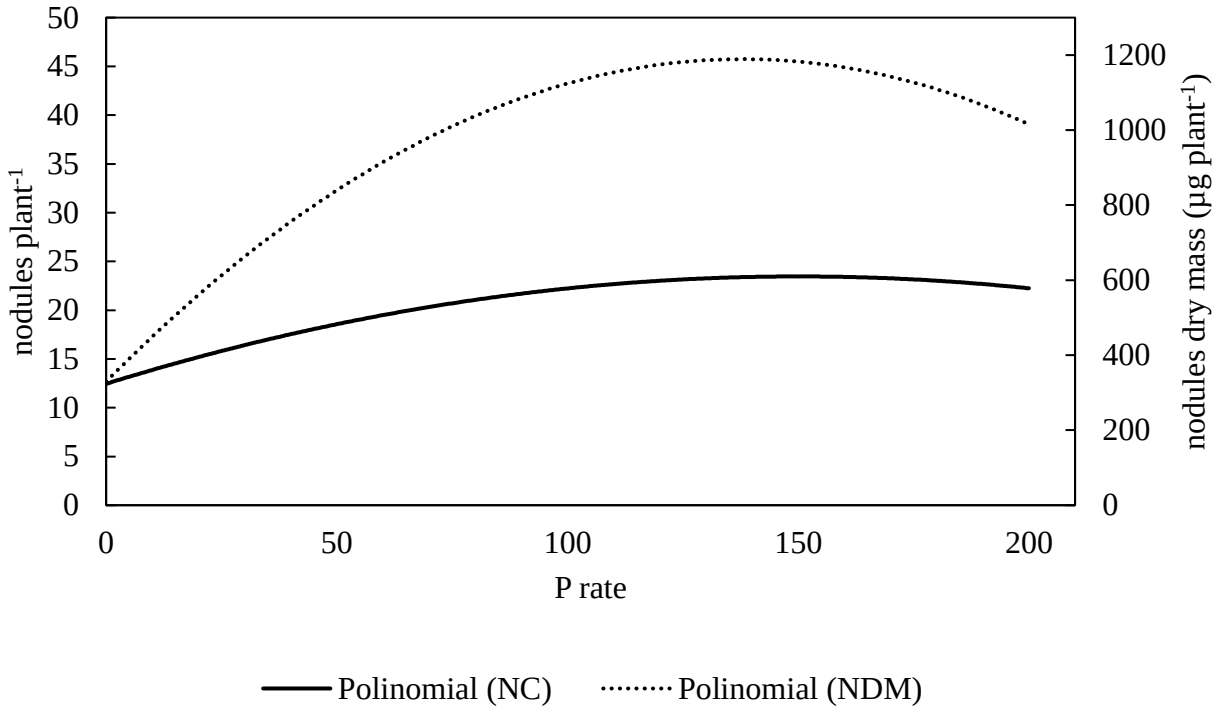

Figure 10. Soybean root nodules count (NC) and dry mass (NDM) in response to four rates of P added to the soil: 0, 50, 100 and 200 mg dm^{-3}.
NC: $y= -0.0005x^2 + 0.146x + 12.5$ (R^2=0.84, CV= 39.5%, *P>F=<0.0001*)
NDM: $y= -0.0451x^2 + 12.434x + 331.0$ (R^2=0.39, CV= 33.3%, *P>F=<0.0001*)

4.1.1.2. **Discussion**

The different rates of P applied to soil in this study had the main objective to provide different conditions to understand the dynamics of AMF root colonization and the effect of seed treatment under these different conditions, which is explored on item 4.1.3. A similar approach using different levels of P in the soil to study mycorrhiza symbiosis were used by several authors (Bolan, 1991; Carneiro *et al.*, 1999; Nogueira and Cardoso, 2002; Peixe *et al.*, 2013; Motta *et al.*, 2016; Konvalinková *et al.*, 2017).

In this experiment, it was possible to observe some direct effects of phosphorus supply, applied as finely ground triple superphosphate, on soybean early growth and development. As mentioned before, almost the exact rates of P applied to soil were verified in the soil test, indicating the availability of this element to plants.

In general, a higher supply of P favored an increase of leaf area, leaf chlorophyll concentration (at V4 stage), nitrogen balance index (at V4 stage), shoot dry mass, P concentration and accumulation of leaves and nodulation (nodules count and dry mass). P concentration in leaf tissues ranged from 1.1 g kg^{-1} to 5.1 g kg^{-1}, which certainly established

very distinct nutritional conditions to evaluate the effect of seed treatments and mycorrhization, as wished. The optimal rate o P that maximized most growth parameters ranged from 130 to 140 mg dm^{-3}. At the rate of 140 mg dm^{-3}, P leaf concentration was 4.14 g kg^{-1}.

According to Malavolta (2006), the best range of P concentration on soybean leaves is between 4 and 5 g kg^{-1}, despite some controversy of using nutrient leaf concentration as a proper parameter to measure plant nutritional status (Urano *et al.*, 2007; Parent *et al.*, 2013).

An adequate supply of P is a crucial aspect to achieve higher yields in soybean (Sousa *et al.*, 2016). In recent studies involving modern soybean cultivars and production systems, both in the United States and Brazil, Bender, Haegele and Below (2015) and Barth *et al.* (2018) reported phosphorus harvest indexes of 81% and 84%, respectively.

The inverse correlation found between P rate added to soil and leaf chlorophyll concentration was probably due to a dilution effect; as leaf area increased, lower amounts of chlorophyll pigment was present per square centimeter. On the other hand, the positive correlation found between P rate and leaf chlorophyll found at V4 indicates that plants were already prepared to uptake P from soil and maintain vigorous growth, producing enough chlorophyll and, consequently, greener leaves.

Flavonoids are phenolic compounds that have the main function of preventing plant cells from oxidative stresses and are mainly concentrated in epidermal cells (Agati *et al.*, 2009; Cerovic *et al.*, 2012). The results indicate a clear role of P supply in reducing the stressful conditions in plants, with a stronger influence on the results observed during V4 stage, where the mean values of flavonoid index ranged from 1.34 (no P added) to 0,66 (150 mg dm^{-3} of P added), thus with higher rates of P resulting in lower flavonoid production by plants. The importance of P on the energy supply and several enzyme reactions probably are related to this result observed. Also, plants lacking P may also have suffered some stressful conditions, leading to the production of flavonoids

The nitrogen balance index followed the same pattern as the chlorophyll concentration in leaves. Higher index values were found at early stages, V2 and V3, respectively. To a certain extent, the same trend verified for chlorophyll concentration can be verified in the nitrogen index results, with a tendency to decrease as P rate increase during early stages (V2) and the opposite during later stages of growth (V4).

Bressan *et al.* (2001) found a linear and positive correlation between plant tissue dry mass and rates of P added to soil, on both soybean and sorghum. This result might be explained by the resource allocation strategy of plants with higher P supply. As examples, a

higher P supply led to increases on both leaf area and shoot biomass, while plant height was not significantly affected

In terms of nodulation, a similar response to P rates was found by different authors (Bressan *et al.*, 2001; Soares *et al.*, 2016), indicating that phosphorus has an important role in this type of symbiosis, mainly as a key component of energy sources, such as ATP, highly required during nodulation processes and normal nodule functioning. Moreover, an inadequate supply of P limits the translocation of sugars, crucial for this symbiosis (Hawkesford *et al.*, 2011).

4.1.2. Soybean growth and development parameters in response to seed treatments with biostimulants

4.1.2.1. **Results**

The seed treatments evaluated in this study did not affect soybean seed physiological quality (Table 5), evaluated on two cultivars, M6210IPRO and RK7214IPRO. The amount of liquid and powder (in the case of FOR) was enough to uniformly cover the seedcoat and were based on average recommended rates for these formulations. The seeds can be considered of good quality, with germination and seedling emergence equal or higher than 96% and 95%, respectively, considering all treatments.

The biostimulants applied through seed treatment did not influence ($P<0.10$) soybean leaf area (Table 6), through all growth stages; ANE presented the highest mean value at V2 (9% higher than CTR), while FOR presented the highest mean values at both V3 and V4 stages (5 and 6% higher than CTR, respectively). In average, leaf area increased 155% from V2 to V4. Except for EME treatment at V3 stage, all mean values of leaf area were higher for biostimulant-treated plants when compared to CTR. This indicates a possible trend of the formulations to promote leaf area increase, despite not yet with a significant difference.

Leaf chlorophyll analysis only presented a significant ($P<0.10$) response to seed treatments at the V2 stage, when all treatments differed from the control and showed lower chlorophyll concentration values. At V3 and V4, all treatments presented similar values (Table 7). Again, despite not statistically significant, lower chlorophyll concentration per square centimeter possibly indicates a larger leaf expansion given by the biostimulant effect, as occurred for the increasing P rates presented on Figure 4.

Table 8 contains results of leaf epidermal flavonoids in response to seed treatments with biostimulants. In general, no effect of treatments on this parameter could be observed. In average, a 59% increase on flavonoid index was observed from V2 to V4 stage.

Nitrogen balance index results are shown in Table 9. The response of this parameter to the biostimulants applied to seeds was significant (*P<0.10*) only at the V2 growth stage, when FOR presented a lower value than CTR, with 30.9 and 33.6, respectively. ANE and EME did not differ from both CTR and FOR, with intermediate mean values. In average, nitrogen balance index decreased around 40% from V2 to V4.

The seed treatments did not influence root and shoot dry mass (*P<0.10*), with no difference compared to the control (Table 10). Meanwhile, all treatments with biostimulants presented higher mean values of shoot dry mass compared to CTR, with 3.2%, 3.9% and 4.9% increases verified with the application of ANE, EME and FOR, respectively. This also suggests a potential of biostimulants to improve plant growth, even though not statistically significant.

Table 11 contains results of chlorophyll fluorescence (CF) and leaf transpiration (LT), in response to both P supply in the soil and seed treatment with biostimulants. CF was significantly influenced by P level in the soil, with higher P supply resulting in increased CF value, while the seed treatments did not affect (*P<0.10*) this parameter. However, EME and FOR showed higher mean values of chlorophyll fluorescence, with increases around 3% and 5%, respectively. For LT, neither P level nor seed treatments influenced the results (*P<0.10*), with higher mean values for LT occurring at a lower level of P in the soil and ANE seed treatment. This last one showed around 7% increase in leaf transpiration mean value compared to CTR.

Internodes and total shoot length showed no response (*P<0.10*) to the seed treatments with biostimulants, except for internode 3, where all biostimulants shortened the space between the second and third nodes (Table 12). To a certain extent, it´s possible to verify an influence of seed treatments in the last internode measured (int5), the same that increased with the increased supply of P in the soil. FOR showed a 20.1% increase compared to CTR, while ANE and EME showed 5 to 6% increase, respectively.

Table 13 contains values of P concentration and content in response to seed treatment with the biostimulants. Values ranged from a minimum of 2.62 to 3.02 mg kg^{-1} and no direct effect of treatment on this parameter could be verified. In terms of accumulation, which involves both concentration and plant biomass, EME treatment showed the highest mean value.

Results of soybean nodulation in response to the seed treatments are presented on Table 14. In general, treatments did not significantly (*P<0.10*) differ from the control treatment for the parameters tested, nodules count and nodules dry mass. On the other hand, analyzing the data of nodules dry mass (NDM), a clear trend to increase mean values in treatments containing biostimulants can be observed, with FOR providing the highest average value (approximately 14% higher than CTR).

Table 5. Germination (G) and seedling emergence (SE) of soybean seed lots from cultivars M6210IPRO and RK7214IPRO, in response to different seed treatments: distilled water (CTR), *Ascophyllum nodosum* extract (ANE), *Ecklonia maxima* extract (EME) and formononetin (FOR).

	M6210IPRO		**RK7214IPRO**	
Treatment	G (%)	SE (%)	G (%)	SE (%)
CTR	$97^{ns} \pm 1.8$	$98^{ns} \pm 0.9$	$98^{ns} \pm 0.7$	$95^{ns} \pm 1.7$
ANE	98 ± 0.7	97 ± 1.3	96 ± 1.3	96 ± 1.7
EME	96 ± 1.3	96 ± 1.1	96 ± 1.7	97 ± 1.2
FOR	98 ± 0.9	98 ± 0.5	97 ± 1.1	97 ± 0.8
CV(%)	2.3	2.7	2.4	2.5
ANOVA (*P*>F)	0.1962	0.7808	0.1565	0.9189

[ns] Non-significant (*P*<0.10)

* Numbers following the signal ± corresponds to Standard Error of the Mean (SEM)

** Distilled water was applied at 1 µL.seed^{-1} on both CTR and FOR treatments; seaweed extracts were applied at 1 µL.seed^{-1} on both ANE and EME treatments; FOR was applied at 0.33 µg.seed^{-1}.

Table 6. Soybean leaf area at three growth stages (V2, V3 and V4), in response to four seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

Treatment	V2	V3	V4
		cm^2 plant^{-1}	
CTR	63.4^{ns}	99.3^{ns}	163.1^{ns}
ANE	69.0	101.3	167.9
EME	66.3	98.7	170.1
FOR	65.4	104.2	173.0
CV (%)	9.7	7.0	9.2
Prob>F	0.5407	0.7157	0.7270

[ns] Non-significant (*P*<0.10)

Table 7. Soybean leaf chlorophyll content at three growth stages (V2, V3 and V4), in response to four seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

Treatment	V2	V3	V4
		µg cm^{-2}	
CTR	25.5 a	23.3^{ns}	23.8^{ns}
ANE	24.0 b	23.7	23.5
EME	24.0 b	24.6	23.0
FOR	23.7 b	23.8	24.4
CV (%)	8.7	7.7	8.9
Prob>F	0.0413	0.2353	0.3675

^{ns} Non-significant (P<0.10)

* Means followed by the same letter in the column do not differ by Tukey´s test (P<0.10)

Table 8. Soybean leaf epidermal flavonoids at three growth stages (V2, V3 and V4), in response to four seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

Treatment	V2	V3	V4
		Index	
CTR	0.7588^{ns}	0.9105^{ns}	1.1941^{ns}
ANE	0.7590	0.9131	1.2059
EME	0.7520	0.8911	1.1953
FOR	0.7666	0.9078	1.2300
CV (%)	3.9	6.9	9.0
Prob>F	0.5669	0.6750	0.6454

^{ns} Non-significant (P<0.10)

Table 9. Soybean leaf nitrogen balance index at three growth stages (V2, V3 and V4), in response to four seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

Treatment	V2	V3	V4
		Index	
CTR	33.6 a	25.7^{ns}	20.3^{ns}
ANE	31.7 ab	26.2	19.7
EME	31.9 ab	27.8	19.5
FOR	30.9 b	26.5	20.2
CV (%)	7.5	10.6	14.0
Prob>F	0.0095	0.1944	0.7815

^{ns} Non-significant (P<0.10)

* Means followed by the same letter in the column do not differ by Tukey´s test (P<0.10)

Table 10. Soybean root (RDM) and shoot (SDM) dry mass in response to four seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

Treatment	RDM	SDM
	mg plant^{-1}	
CTR	585.4^{ns}	867.5^{ns}
ANE	598.5	895.4
EME	568.6	901.5
FOR	596.5	909.6
CV (%)	8.9	13.0
Prob>F	0.2890	0.6852

^{ns} Non-significant (P<0.10)

Table 11. Chlorophyll fluorescence (CF) and leaf transpiration (LT) evaluated in soybean plants at the third fully expanded leaf stage, in response to two rates of P added to the soil (0 and 100 mg dm^{-3}, low and high, respectively) and four seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

Treatment	CF	LT
	Maximum quantum yield of PSII (Fv/Fm)	µg H_2O cm^{-2} s^{-1}
P		
Low	0.6580	4.47^{ns}
High	0.7354*	3.98
ST		
CTR	0.6911^{ns}	4.14^{ns}
ANE	0.6613	4.44
EME	0.7095	4.14
FOR	0.7248	4.20
CV (%)	11.0	21.6
ANOVA (*Prob*>F)		
P	0.0145	0.2578
ST	0.4473	0.9488
P*ST	0.5631	0.8862

^{ns} Non-significant (P< 0.10)

* Means differ by Student´s t-test (P<0.10)

Table 12. Total shoot and internode lengths of soybean plants, at the fourth fully expanded leaf stage (V4), in response to four seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

Treatment	Int1	Int2	Int3	Int4	Int5	TSL
	----------mm----------					
ST						
CTR	42.5^{ns}	34.0^{ns}	22.4 a[1]	18.8^{ns}	17.9^{ns}	135.5^{ns}
ANE	44.8	34.5	21.3 ab	17.6	18.9	137.1
EME	45.8	33.8	19.9 b	16.8	19.0	135.3
FOR	43.0	32.4	20.4 b	17.1	21.5	134.6
CV (%)	10.8	14.6	9.1	21.2	31.9	6.7
Prob > F	0.2107	0.6573	0.0003	0.4323	0.3502	0.8829

^{ns} Difference between means are non-significant at a 0.10 level

[1] Means followed by the same letter in the column do not differ by Tukey´s test (P<0.10)

Table 13. Concentration and accumulation of P in soybean leaf blades at the fourth fully expanded leaf stage (v4), in response to four seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

Treatment	P concentration (mg kg^{-1})	P accumulation (µg plant^{-1})
CTR	3.02^{ns}	2.66^{ns}
ANE	2.93	2.62
EME	2.97	2.81
FOR	2.96	2.73
CV (%)	20.8	23.4
Prob>F	0.8521	0.6953

^{ns} Non-significant (P<0.10)

Table 14. Soybean root nodules count (NC) and dry mass (NDM) in response to four seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

Treatment	NC (nodules plant^{-1})	NDM (µg plant^{-1})
CTR	19.2^{ns}	775.3^{ns}
ANE	17.8	823.0
EME	18.8	860.2
FOR	20.0	882.1
CV (%)	39.5	33.3
Prob>F	0.7386	0.6473

^{ns} Non-significant (P<0.10)

4.1.2.2. **Discussion**

The use of agricultural biostimulants are generally associated with improvements on crop growth and development, leading to higher performance and better results in terms of yield or quality of harvested materials (Yakhin *et al.*, 2017). The use of biostimulants in the

seed treatment have interesting results and consists in a growing trend (Araújo, 2016; Wilson, Amirkhani and Taylor, 2018), as crop early growth and establishment in the field are a crucial steps towards the rise of yield potentials.

Firstly, a crucial parameter to determine the feasibility of using a given formulation as seed treatment is its safeness to seed physiological quality. By the results presented on Table 5, it is possible to affirm that the rates applied does not negatively influence soybean seeds germination and seedling emergence parameters, thus indicating that treatments do not harm soybean seeds.

By the results found in this experiment, in general, biostimulants alone presented non-significant (*P<0.10*) responses for most parameters, except leaf chlorophyll concentration (V2), nitrogen balance index (V2) and arbuscular mycorrhizal fungi colonization. Similarly, the interaction between biostimulants and P rates applied to soil did not result in any significant difference for the tests conducted.

On the other hand, for some parameters such as leaf area, shoot dry mass and nodulation, biostimulant-treated plants provided higher mean values compared to CTR treatment if compared in terms of percentual differences. In some cases, these average (but random) increases might be economically significant and may justify the commercial use of these biostimulant formulations through seed treatment.

The three biostimulants tested in this experiment have their effects very well documented in the scientific literature (Massa, 2010; Craigie, 2011; Calvo, Nelson and Kloepper, 2014; Arioli, Mattner and Winberg, 2015; Goñi *et al.*, 2016; Van Oosten *et al.*, 2017; Yakhin *et al.*, 2017). In many cases, positive physiological effects in plants are directly attributed to their use. Different from formononetin, which is a synthetic and target-specific formulation, the seaweed extracts have a much more complex composition and a broader range of effects expected in plants, which might be more dependent of environmental factor to favor their optimum effect (Yakhin *et al.*, 2017).

Comparing the extracts of both seaweeds according to their composition and effects on plants, it is expected to have a stronger effect of ANE on shoots (or aerial plant organs), while EME would primarily act on roots. The first is known to promote a cytokinin-like effect on plants, while the second promotes an auxin-like effect (Kingman and Moore, 1982; Khan *et al.*, 2011; Papenfus *et al.*, 2012; Rengasamy *et al.*, 2014; Stirk and Van Staden, 2014; Stirk *et al.*, 2014). However, non-significant influence was found on both parameters in terms of dry mass accumulation, although the mean values of shoot dry mass were 3.2% and 3.9% higher for ANE and EME, respectively.

Despite non-significant, mean values of leaf area presented on Table 6 were generally higher for treatments containing biostimulants. This might explain the results found on Table 7, where CTR treatment showed higher chlorophyll concentration in leaves at the V2 stage, which probably occurred due to a dilution effect caused by higher leaf area of biostimulant-treated plants.

An effect of biostimulants on the biosynthesis of phenolic compounds in plants is expected (Calvo, Nelson and Kloepper, 2014). In this case, no correlation was found between the seed treatment with biostimulants with the quantity of epidermal flavonoids in leaves.

The accumulation of dry mass is a very common parameter to measure biostimulants effects on plants. Higher values of dry mass accumulation from a given treatment frequently indicate a better photosynthetic capacity and conversion into biomass (Carvalho, 2013; Sangha *et al.*, 2014; Di Stasio *et al.*, 2017). Another approach of biostimulants may be related to improvements on resource allocation in plants, which can direct their photoassimilates to strategic organs in order to maximize growth or yield (Di Stasio *et al.*, 2017).

Savana da Silva *et al.* (2017) reported a positive and synergistic effect of formononetin on soybean root nodules colonization by AMF. Possibly, the interaction between rhizobia, AMF and soybean plants are synergistic, and led to higher nodulation. Similar results of formononetin positively affecting nodulation was found by Catford *et al.* (2006) in alfafa. Oppositely, Ribeiro *et al.* (2016) found no correlation between formononetin rates and nodulation parameters in soybean, at field level. By the results found in this experiment, despite non-significant ($P<0.10$), it is possible to verify a clear trend of all three biostimulants positively affecting the nodules dry mass accumulation, while the values for nodules count were very similar to CTR. Formononetin promoted the higher accumulation, followed by EME. Moreover, it is also important to emphasize that no negative effects on nodulation was observed with the application of biostimulants to seeds, which is a very important parameter to consider when choosing the products for soybean seed treatment, due to the crucial importance of biological nitrogen fixation to crop performance, yield and sustainability (Cerezini *et al.*, 2016).

4.1.3. Soybean mycorrhization parameters in response to P rates added to soil and seed treatment with biostimulants

4.1.3.1. Results

The effect of increasing rates of P added to soil on soybean mycorrhization is shown on Figure 11. As expected, soybean root colonization by AMF was inversely proportional to P rates, with minimum levels of P resulting in higher percentages of mycorrhiza establishment in soybean plants. At the lowest P level in soil, the AMF colonization was approximately 60.5%, while with the P rates of 100 mg dm^{-3} and 200 mg dm^{-3}, the percentages of AMF-colonized roots dropped to 48.1% and 27.7%, respectively, indicating a strong and negative effect of P availability in soil with mycorrhiza formation.

The differences of AMF root colonization induced by different levels of P availability provided an interesting condition to evaluate how the seed treatments perform under each situation, being one of the objectives of this study.

Figure 12 contains the results of AMF colonization in response to the biostimulant seed treatments. Only the treatment with formononetin significantly ($P<0.10$) differ from the control, with a mean value 20.6% higher. ANE and EME treatments did not differ from both CTR and FOR treatments, despite presenting 13.9% and 14.4% more roots colonized than CTR, respectively.

Figure 13 contains the same data of Figures 11 and 12 but combining both P rate and seed treatment factors for an exploratory analysis. Except for treatment EME at P0 rate, all treatments containing biostimulants presented higher mean values of AMF root colonization, under all P levels, thus, again confirming an indication of a positive effect of both seaweed extracts on soybean mycorrhization.

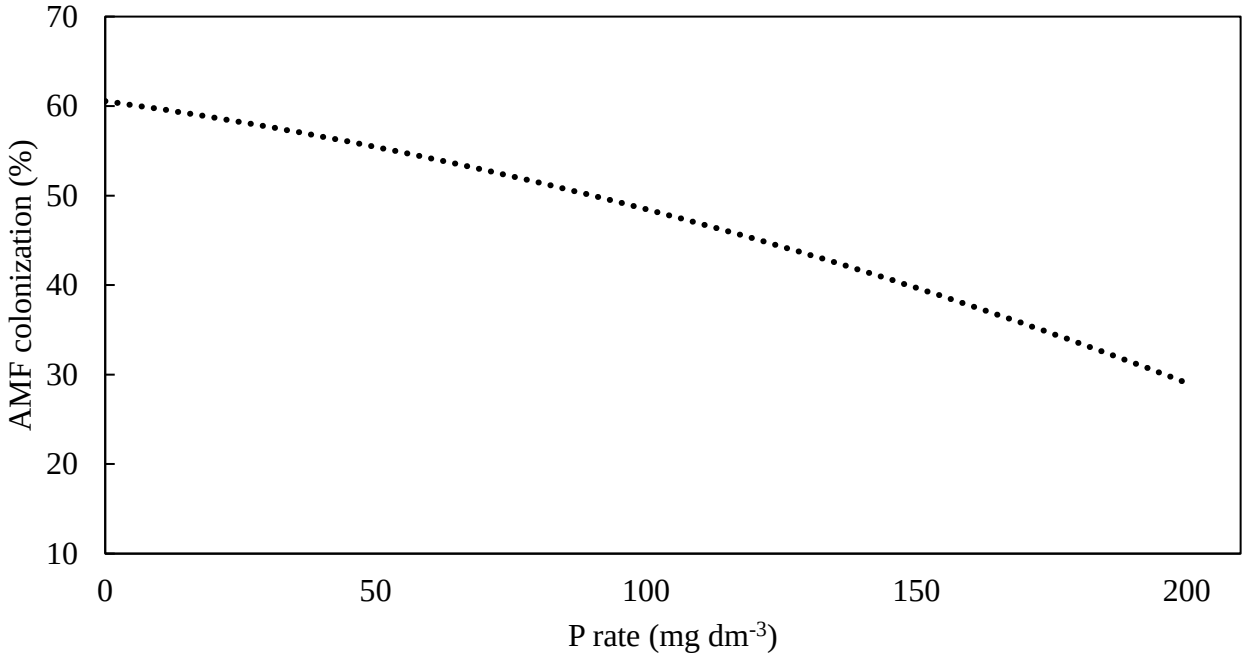

Figure 11. Arbuscular mycorrhizal fungi (AMF) colonization in soybean in response to four rates of P added to the soil: 0, 50, 100 and 200 mg dm^{-3}.
$y= -0.0004x^2 - 0.0841x + 60.5$ (R^2=0.60, CV= 32.0%, *P>F=<0.0001*)

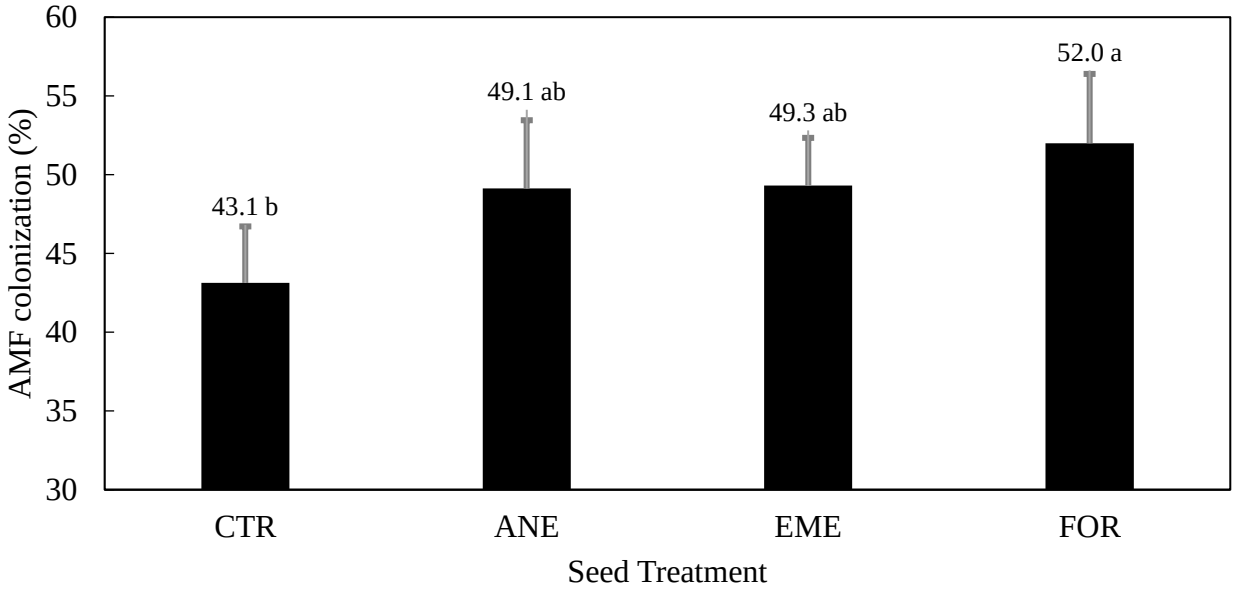

Figure 12. Arbuscular mycorrhizal fungi (AMF) colonization in soybean in response to four seed treatments: distilled water (CTR, 1 µl.$seed^{-1}$), *Ascophyllum nodosum* extract (ANE, 1 µl.$seed^{-1}$), *Ecklonia maxima* extract (EME, 1 µl.$seed^{-1}$) and formononetin (FOR, 0.33 µg.$seed^{-1}$).

ns Non-significant (*P*<0.10)
* Means followed by the same letter above bars do not differ by Tukey´s test (*P*<0.10)

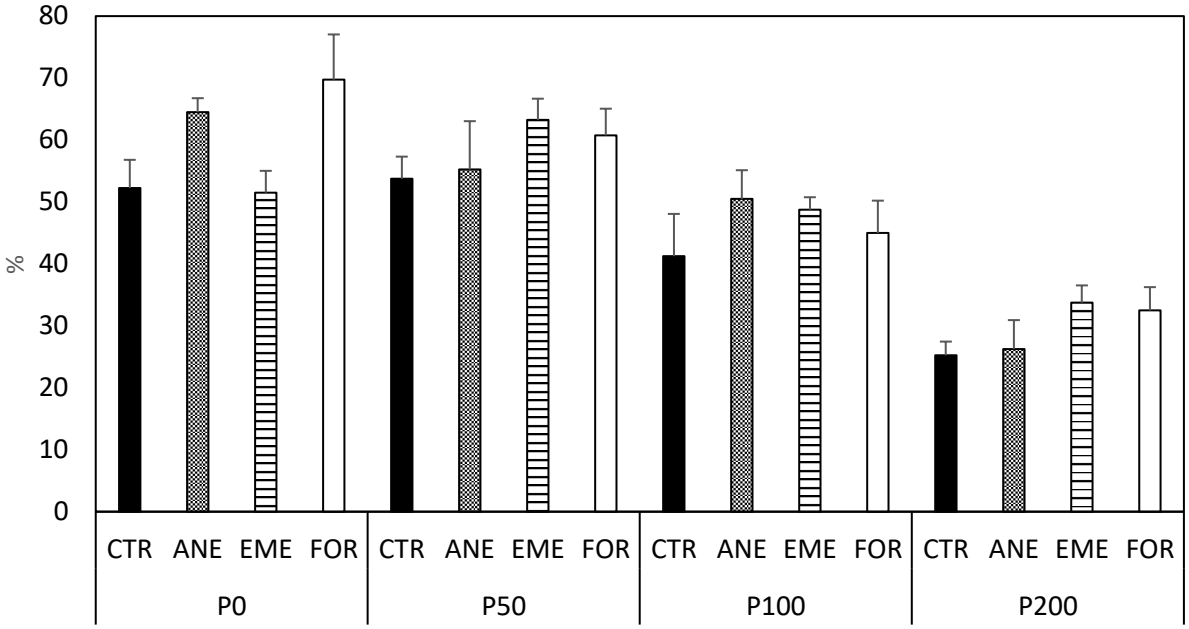

Figure 13. Root colonization by arbuscular mycorrhizal fungi in soybean plants at the fourth fully expanded leaf stage (V4), in response to four rates of P added to the soil (P0: 0 mg dm^{-3}; P50: 50 mg dm^{-3}; P100:100 mg dm^{-3}; P200: 200 mg dm^{-3}) and four seed treatments: distilled water (CTR, 1 µl.$seed^{-1}$), *Ascophyllum nodosum* extract (ANE, 1 µl.$seed^{-1}$), *Ecklonia maxima* extract (EME, 1 µl.$seed^{-1}$) and formononetin (FOR, 0.33 µg.$seed^{-1}$).

4.1.3.2. **Discussion**

The level of root colonization found in this experiment can be considered adequate or comparable to field level results. As examples of AMF root colonization levels in soybean, Ferreira (2012) found a range between 63.3% and 69.5%; Cordeiro *et al.* (2015) between 39% and 54%; Ribeiro *et al.* (2016) and Savana da Silva *et al.* (2017) between 55% and 66%.

The relationship between mineral P supply and mycorrhiza is extensively studied and frequently used as a classical example of antagonism (Bolan, 1991; Moreira and Siqueira, 2006; Smith *et al.*, 2011; Liu *et al.*, 2016; Motta *et al.*, 2016; Savana da Silva *et al.*, 2017). In this experiment, the inverse correlation between AMF root colonization to increased rates of P in the soil occurred as expected. Roots colonized by AMF reduced more than 50% from the lowest to the highest P level in soil.

This is an important parameter to take into consideration under field conditions for soybean cropping systems, where P fertilizers are generally placed in-furrow, next to the seeds and, consequently, to the early developing root system (Sousa *et al.*, 2016). The amount of available P in this zone can be very high (see Appendix E) and may inhibit mycorrhiza

establishment since early stages of growth, which may negatively the crop sustainability in the mid to long term or even the maintenance of native AMF in the area.

The positive effects of the isoflavonoid formononetin on AMF colonization agrees with several studies that confirmed its potential to stimulate mycorrhization (Silva-Júnior and Siqueira, 1997; Siqueira *et al.*, 1999; Soares and Martins, 2000; Catford *et al.*, 2006; Cordeiro *et al.*, 2015; Castillo *et al*., 2014; Ribeiro *et al*., 2016; Salgado *et al*, 2017; Savana da Silva *et al.*, 2017). Some phenolic compounds are known to have an effect on different types of symbiosis in plants, such as formononetin of mycorrhiza (Siqueira, Safir and Nair, 1991). However, some authors reported no significant effect of this synthetic biostimulant on crop mycorrhization (Ferreira, 2012)

In the same way, despite not statistically significant, the treatments containing seaweed extracts also promoted an increase (in percentual value) of AMF root colonization compared to the control with distilled water (CTR). This result corroborates with the ones reported by Kuwada *et al.*(1999, 2005, 2006), which also evaluated the direct responses of AMF colonization to seaweed extracts. These authors concluded that seaweed extracts and some of its isolated compounds were able to positively influence AMF root colonization and hyphal growth.

Paszt *et al.* (2015) also evaluated the effect of some natural biostimulants on strawberry mycorrhization, finding positive results in terms of AMF abundance and diversity for some types of product, especially humic substances.

Castillo et al 2014 evaluated the effect of seed treatments with both formononetin and natural biostimulants on wheat mycorrhization. The authors concluded that formononetin accelerated mycorrhiza formation, while some natural biostimulants also presented intermediate or no effect on mycorrhization.

4.2. Second experiment: soybean mycorrhization and soil rhizosphere parameters associated to the seed treatments with biostimulants (growth chamber)

4.2.1. Mycorrhization parameters

4.2.1.1. **Results**

Figure 14 contains results of shoot dry mass, which gives an idea of how plants developed during cultivation in the growth chamber. It is possible to verify more expressive

growth rates from V1 to V2 and V2 to V3, with plants presenting average increases of 152% and 132% in biomass production, respectively in each phase. Comparing the mean values among biostimulants in each growth stage, it is possible to verify that at V2 they showed superior mean values compared to CTR, with EME treatment presenting the highest value (167.7 mg $plant^{-1}$), 18.8% superior than CTR. At V3 stage, they also showed superior mean values compared to CTR, with ANE treatment showing the highest value (375.5 mg $plant^{-1}$), 4.2% superior than CTR.

An overview of soybean mycorrhization process during early growth stages (V0 to V3) is presented on Figure 15. It is possible to observe that AMF root colonization starts very early and can reach most of the root system already at V1 stage, with a frequency of mycorrhiza in the root system being higher than 50%. An increase of more than seven-fold could be observed from V0 to V3 for the Frequency (F%). Also, the M% and A% parameters give an idea of quality or functionality of mycorrhiza, indicating that this mutualistic association can start and function very early in the plants cycle, contributing to the host plant since this phase. This fact reinforces the idea to utilize seed treatments to influence on mycorrhization.

Table 15 gives different parameters of soybean mycorrhization, according to each growth stage (V0, V1, V2 and V3) and seed treatment with biostimulants. FOR treatment presented the highest frequency (F%) on both V0 and V1 stages, while EME presented the highest value at V2 and ANE at V3. EME did not differ from FOR at any stage tested in terms of frequency (F%). At V3, no significant difference ($P<0.10$) of frequency (F%) was found among treatments.

Similarly, as also shown on Table 15, the mycorrhizal colonization in the root system (M%) started very early in the cycle, with FOR treatment presenting higher values at V0 and V1 and being surpassed by EME at V2 and ANE in V3. For the arbuscule abundance parameter, significant differences ($P<0.10$) occurred only during V1 and V2 growth stages, with EME treatment presenting the higher values.

Table 16 contains results of the same AMF colonization parameters (F%, M% and A%) during V2 stage, under two contrasting levels of P in the soil, 0 and 100 mg dm^{-3} of P added. As result, the highest level of P available in the soil significantly ($P<0.10$) reduced all three mycorrhization parameters. The seed treatments also significantly affected all three parameters, except ANE treatment for frequency (F%). EME showed the highest value for all three parameters tested, increasing the frequency (F%) by 46.7% compared to the CTR treatment, as an example.

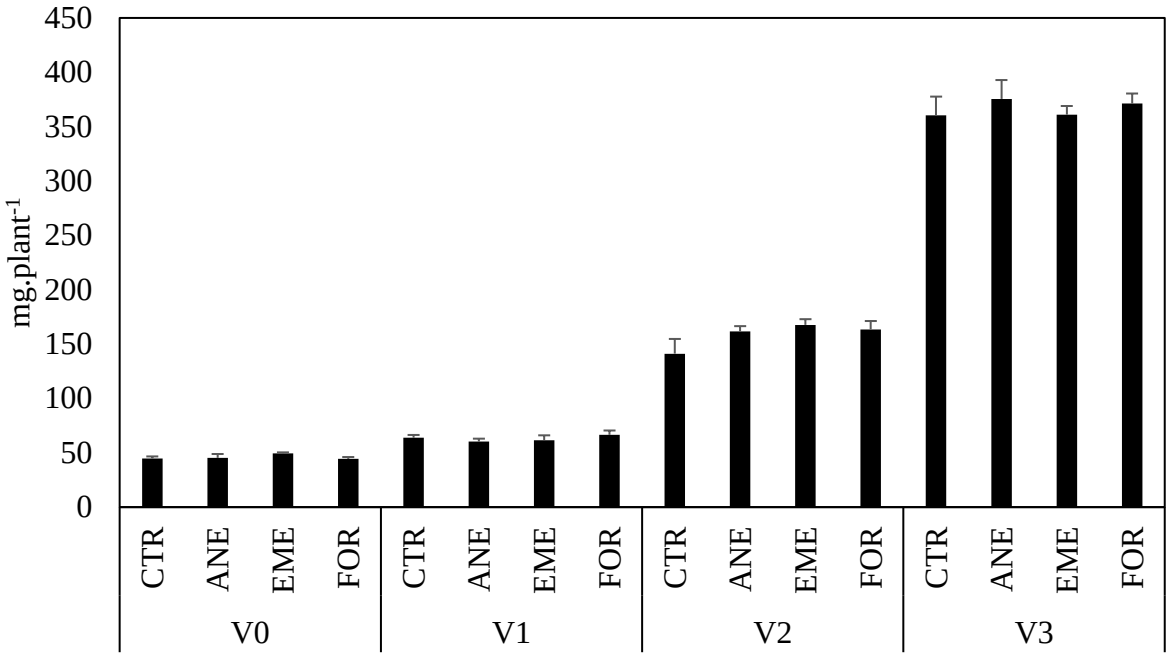

Figure 14. Shoot dry mass of soybean plants cultivated in growth room (second experiment), at each growth stage (V0, V1, V2 and V3), in response to different seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

* Bars above columns represent the Standard Error of the Mean (SEM). Difference between means in each growth stage are non-significant (P<0.10).

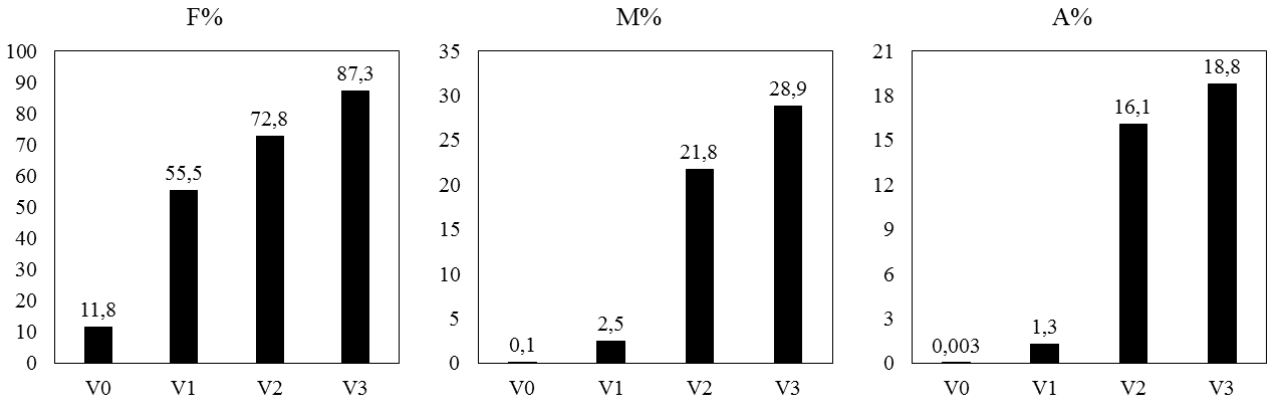

Figure 15. Frequency of mycorrhiza in the root system (F%), intensity of the mycorrhizal colonization in the root system (M%) and arbuscule abundance in the root system (A%) evaluated in soybean plants at different growth stages (V0, V1, V2 and V3).

Table 15. Frequency of mycorrhiza in the root system (F%), intensity of mycorrhizal colonization in the root system (M%) and arbuscule abundance in the root system (A%) in soybean plants at different growth stages (V0, V1, V2 and V3) and in response to different seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

	F%	M%	A%
V0			
CTR	4.0 b[1]	0.04 b	0.00^{ns}
ANE	15.4 ab	0.15 a	0.00
EME	10.0 ab	0.10 ab	0.00
FOR	26.7 a	0.31 a	0.00
V1			
CTR	53.6 b	0.8 b	1.00 b
ANE	51.8 b	2.2 ab	1.53 b
EME	70.1 ab	4.4 a	1.73 a
FOR	76.5 a	2.9 a	1.30 b
V2			
CTR	63.8 b	8.9 c	4.90 b
ANE	68.4 b	22.2 b	15.7 a
EME	87.4 a	38.8 a	32.0 a
FOR	76.2 ab	27.4 ab	21.1 a
V3			
CTR	84.7 ^{ns}	28.6 ^{ns}	17.3 ^{ns}
ANE	94.1	36.4	24.0
EME	91.2	23.4	14.5
FOR	86.1	28.9	20.8

[2] Means followed by the same letter in the column, within each growth stage, do not differ by the Steel-Dwass test (P<0.10)

Table 16. Frequency of mycorrhiza in the root system (F%), intensity of mycorrhizal colonization in the root system (M%) and arbuscule abundance in the root system (A%), in soybean plants cultivated with two P rates added to the soil (0 and 100 mg dm^{-3}), evaluated at the two fully expanded leaf stage (V2) and in response to different seed treatments: distilled water (CTR, 1 µl.seed^{-1}), *Ascophyllum nodosum* extract (ANE, 1 and 10 µl.seed^{-1}), *Ecklonia maxima* extract (EME, 1 and 10 µl.seed^{-1}) and formononetin (FOR, 0.33 µg.seed^{-1}).

	F%	M%	A%
P rate			
Low	74.4 a[1]	23.4 a	17.3 a
High	61.4 b	11.2 b	7.3 b
ST			
CTR	54.6 b[2]	5.7 b	2.9 b
ANE	66.7 b	14.7 a	9.3 a
EME	80.6 a	26.0 a	19.1 a
FOR	69.6 b	22.8 a	18.0 a

[1] Mean values followed by the same letter in the column do not differ by Wilcoxon´s test (P<0.10). [2] Mean values followed by the same letter in the column do not differ by Steel-Dwass test (P<0.10)

4.2.1.2. **Discussion**

Data of soybean shoot dry mass shows a very high growth rate of plants at this initial development period, which gives an indication of intense metabolic activity. It is important to consider that seed treatments have their effect mainly concentrated at the initial stages of plants development, during this phase of fast growth.

According to data shown on Figure 15, mycorrhiza starts to establish very early in soybean plants. At V0, plants were still finishing their germination process (see Appendix C). This indicates that soybean plants are receptive to AMF infection since the very early stages of growth, or since the germination phase.

The intensity and arbuscule abundance of mycorrhizal colonization, presented on Figure 15, also suggests that AMF not only are able to colonize roots very early in the cycle, as they are also capable of start a functional symbiosis, indicated by the presence of arbuscules.

To a certain extent, the biostimulants influenced mycorrhization until V2 stage. At V3, no difference between the three parameters could be verified. However, in the case of frequency (F%) at V3, despite statistically non-significant, the biostimulants still promoted higher mean values.

Castillo *et al.* (2014) verified significant increases of frequency and intensity of mycorrhization in wheat in response to seed treatments with formononetin and some natural biostimulants, including a seaweed extract with *Ascophyllum nodosum* in the composition.

Table 16 reinforces the negative effect of high P availability in soil to root colonization by AMF. All three mycorrhization parameters analyzed had their values reduced at the higher rate of P applied to soil. It is possible to verify that occurred a 17.5% reduction in the frequency (F%), while there was a reduction of more than 50% for both intensity and arbuscular abundance parameters, thus indicating a detrimental effect of excess P on more qualitative aspects of mycorrhization.

Despite the large number of technical and scientific publications that confirms the benefits of mycorrhiza in agricultural systems, there is still little concern from farmers, consultants, salesforce or other decision makers in the production process regarding the maintenance and better use of this important soil resource, especially when compared to other crop management practices, such as machinery, cultivar selection or crop protection. Plenchette *et al.* (2005) raised the same concern; these authors pointed for the need to improve and facilitate the access to analytical methods that analyze, for example, relative

mycorrhizal dependency, soil mycorrhizal infectivity and receptivity. This would provide indicators that could be used as routine for crop management decisions.

4.2.2. Rhizosphere microbial communities' structure

4.2.2.1. **Results**

An analysis of variance (alpha-diversity) was conducted for AMF community, in response to the seed treatments with biostimulants, soybean phenological stages and the interaction between these factors. A significant response ($P<0.10$) occurred for both phenological stage and the interaction, while the biostimulants alone did not influence (p=0.2403) AMF community structure. Figure 16 contains a graphical representation of ANOVA and mean comparisons among growth stages (from V0 to V3). V2 stage differed from the others, while V0 did not differ from V1 and V1 did not differ from V3.

The variation of AMF community according to seed treatments was then analyzed within each growth stage, with results presented on Figure 17. The AMF community profile was obtained from a total of 119 distinct groups (peaks). A larger variation among treatments can be observed both at V0 and V2 stages. Treatment FOR does not overlap with any treatment at V2 and V3 stages, while treatment EME does not overlap with any treatment at V1 stage, indicating some changes on AMF community structure attributed to these treatments. At V3 stage, EME and FOR are closer and more distant from CTR than ANE. To a certain extent, EME showed fewer overlap with CTR compared to ANE, at V1, V2 and V3 growth stages. This might indicate that EME have higher potential to modify or interfere on AMF community diversity compared to ANE, reinforcing the results presented on Tables 15 and 16.

Bacterial community profile was analyzed from a total of 404 distinct groups (peaks). In general, they showed large overlap between treatments, except with FOR at V0 and V1 and EME at V1 and V3. Again, both treatments EME and FOR showed higher potential to alter microbial community structure of rhizosphere soil. To a certain extent, at V2 and V3 stages, the treatments can be considered very close in terms of bacterial community structure. CTR and ANE presented more overlap, indicating a weak effect of ANE in changing bacterial community in rhizosphere soil.

Fungal community structure was analyzed from a total of 655 distinct groups (peaks) and is presented in Figure 19. In this group, fewer overlaps can be observed compared to

AMF and bacteria. ANE did not show overlap with CTR at V0, V2 and V3 stages; EME did not overlap with CTR only at V0 and V3; FOR did not overlap with CTR at V0, V1 and V2.

Figure 20 contains the results of PERMANOVA, where the influence of phenological stage, seed treatment and the interaction among them were analyzed on the AMF, bacterial and fungal community structures. Soybean phenological stage significantly influenced (*P<0.10*) all communities analyzed, while seed treatments only influenced (*P<0.10*) bacterial community, with p=0.1197 and p=0.3195 for fungi and AMF, respectively. The interaction among both factors significantly (*P<0.10*) influenced the three types of microbial communities. They showed a relatively high influence in determining the structure of the communities, explaining 38.55%, 25.61% and 24.73% of results for bacteria, fungi and AMF, respectively. Despite non-significant, seed treatments explained 5.0% and 6.7% of the variation on AMF and fungal communities, respectively, while the same treatments explained 10.2% of variation on bacterial community.

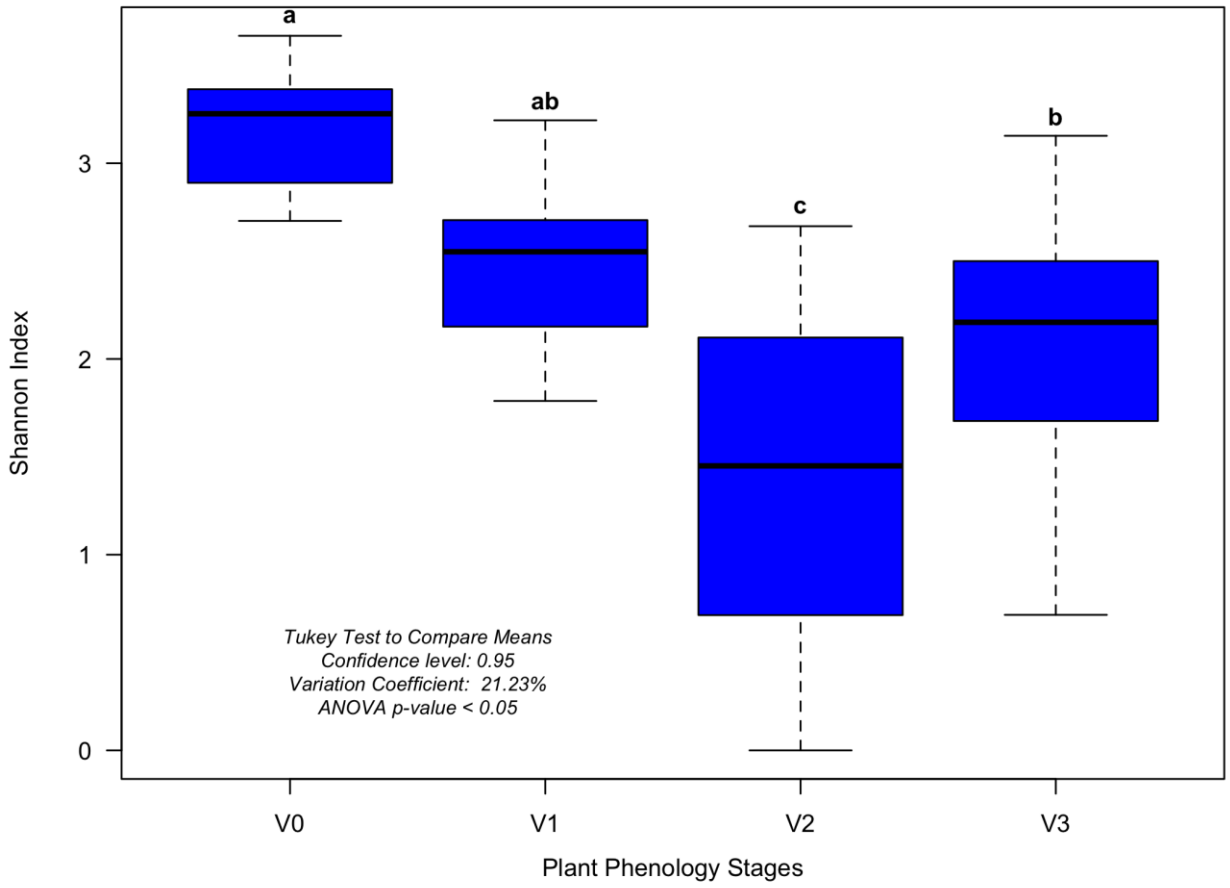

Figure 16. Analysis of variance of arbuscular mycorrhizal fungi community structure (alpha-diversity) according to each soybean phenological stage (V0, V1, V2 and V3).

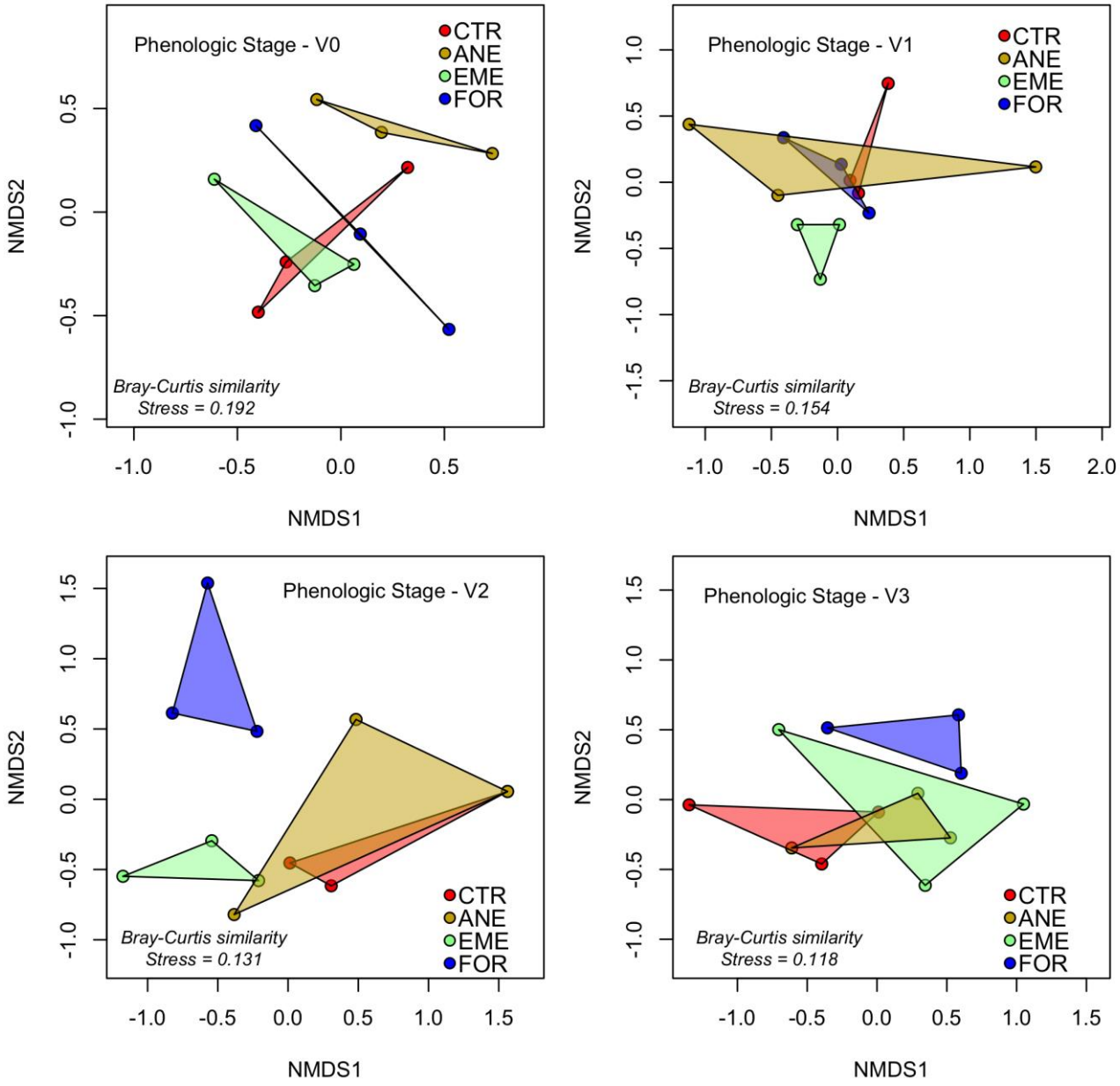

Figure 17. Arbuscular mycorrhizal fungi community structure in soybean rhizosphere analyzed with PCR/TRFLP fingerprinting, at different phenological stages (V0, V1, V2, V3) and in response to four types of seed treatment: distilled water (1 µL.seed^{-1}, CTR), *Ascophyllum nodosum* extract (1 µL.seed^{-1}, ANE), *Ecklonia maxima* extract (1 µL.seed^{-1}, EME) and formononetin (0.33 µg.seed^{-1}, FOR).

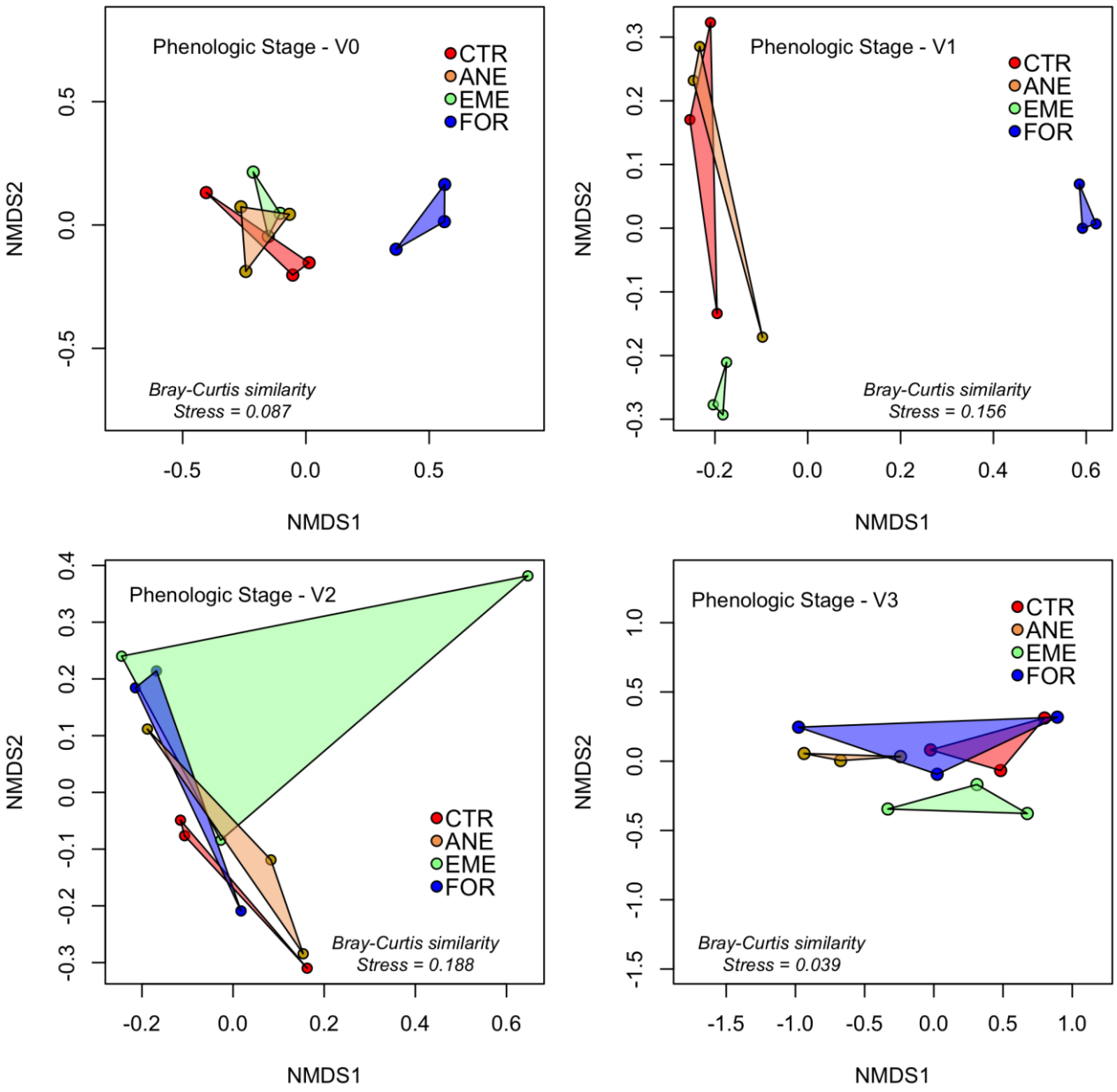

Figure 18. Bacterial community structure in soybean rhizosphere analyzed with PCR/TRFLP fingerprinting, at different phenological stages (V0, V1, V2, V3) and in response to four types of seed treatment: distilled water (1 µL.seed^{-1}, CTR), *Ascophyllum nodosum* extract (1 µL.seed^{-1}, ANE), *Ecklonia maxima* extract (1 µL.seed^{-1}, EME) and formononetin (0.33 µg.seed^{-1}, FOR).

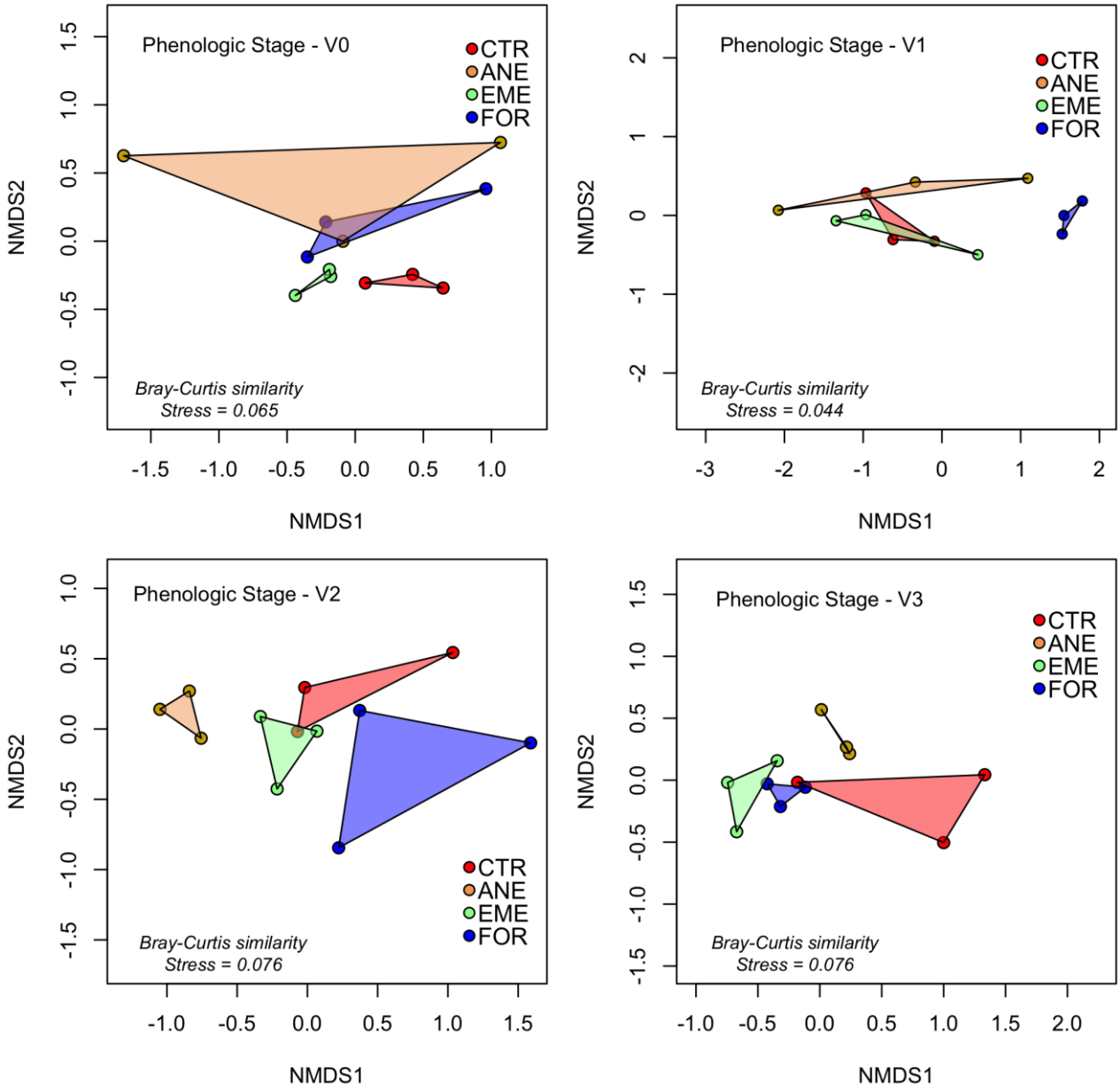

Figure 19. Fungal community structure in soybean rhizosphere analyzed with PCR/TRFLP fingerprinting, at different phenological stages (V0, V1, V2, V3) and in response to four types of seed treatment: distilled water (1 µL.seed^{-1}, CTR), *Ascophyllum nodosum* extract (1 µL.seed^{-1}, ANE), *Ecklonia maxima* extract (1 µL.seed^{-1}, EME) and formononetin (0.33 µg.seed^{-1}, FOR).

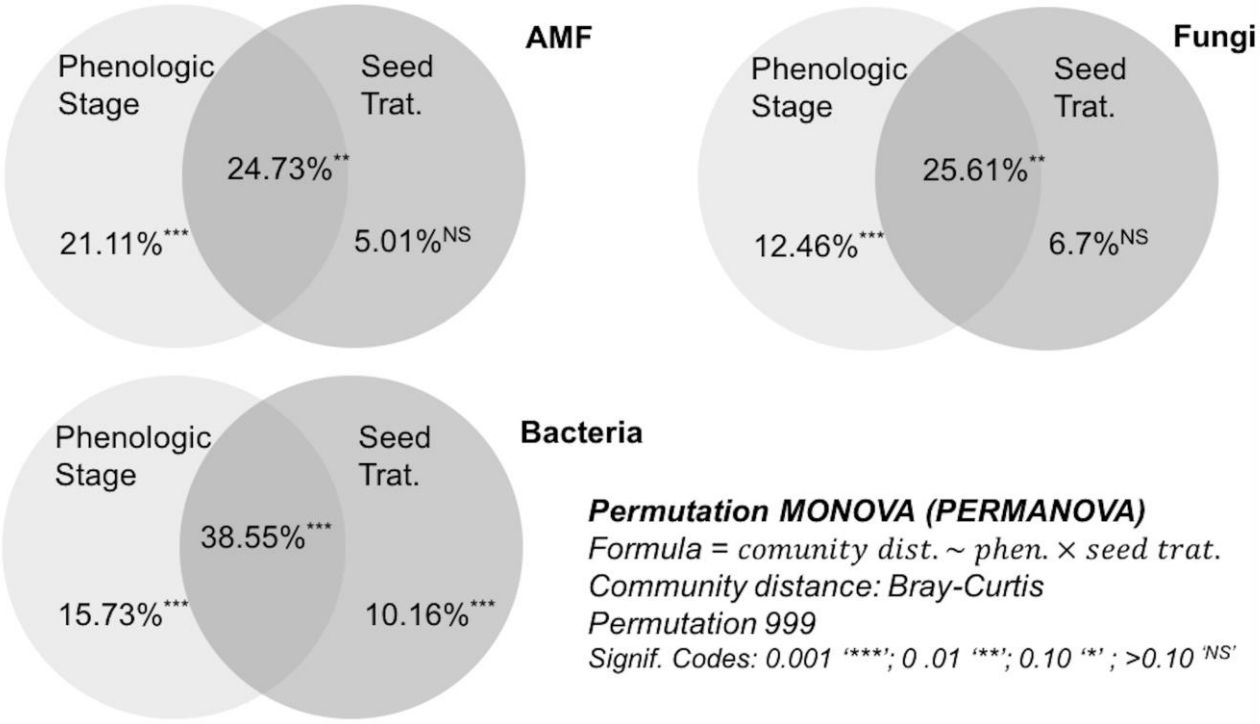

Figure 20. PERMANOVA results of arbuscular mycorrhizal fungi (AMF), total fungal and bacterial communities in soybean rhizosphere, analyzed with PCR/TRFLP fingerprinting, in response to different phenological stages (V0, V1, V2, V3) and in four types of seed treatment: distilled water (1 µL.seed^{-1}, CTR), *Ascophyllum nodosum* extract (1 µL.seed^{-1}, ANE), *Ecklonia maxima* extract (1 µL.seed^{-1}, EME) and formononetin (0.33 µg.seed^{-1}, FOR).

4.2.2.2. **Discussion**

Biotic and abiotic factors can play important roles on the composition of soil microbial communities, such as plant species, plant age, distance from plant root systems, soil mineral composition, agronomic practices, among others (Moreira and Siqueira, 2006; Antunes *et al.*, 2009; Barto *et al.*, 2011). Andreote, Gumiere and Durrer (2014) pointed for the importance of plant genotype, agricultural practices and phenological stage of plants in determining the composition of microbiomes. The effect of plant growth stages on the rizosphere microbial communities was studied by several authors (Houlden *et al.*, 2008; Cavaglieri, Orlando and Etcheverry, 2009; Hussain *et al.*, 2012; Chaparro, Badri and Vivanco, 2013; Liang *et al.*, 2015; Breidenbach *et al.*, 2016).

Liang *et al.* (2015) studied the influence of different soybean varieties on AMF community diversity. These authors found no genotype influence of AMF community, while

phenological stage of plants significantly influenced their diversity. The same occurred in this experiment, for AMF community, with a significant influence of soybean growth stages and non-significant effect of seed treatments with biostimulants. Barto et al (2011), analyzing AMF community structure through PCR/TRFLP, verified that the invasive species *Alliaria petiolata* reduced AMF colonization and promoted significant changes in the community composition, evaluated on sugar maple (*Acer saccharum*) from natural forest sites.

It was expected a stronger influence of the seed treatments with biostimulants on the AMF community, for both the natural and synthetic products, as they were able to alter the colonization percentages. Instead, only 5% of community structure was influenced by these treatments, which can also be seen as a good result if we consider that they are able to increase root colonization without impacting on community structure when compared to the control. Similar results were found by Antunes et al. (2009), where even the introduction of AMF inoculum did not alter the community structure of indigenous AMF, on both disturbed and undisturbed soils. In the same way, Koch *et al.*, (2011) found that the introduction of the invasive and non-mycorrhizal specie *Alliaria petiolate* did not negatively influenced AMF community as they expected.

In this experiment, a significant modification of community structure attributed to the seed treatments occurred only for bacteria, explaining 10.2% of variation. This reinforces the importance of agronomic practices, even seed treatments, in determining the microbial community structure. For bacteria, both EME and FOR treatments showed fewer overlap with the control, indicating stronger effect on bacterial community structure.

No reference was found in the literature that correlates the impact of formononetin on AMF community structure, despite the considerable number of researches published with this synthetic formulation. They generally refer only to its effect on root colonization parameters. In this work, the results indicate a possible selection process promoted by formononetin on AMF species that were present in the soybean rhizosphere.

5. CONCLUSION

Phosphorus rates applied to soil significantly influenced ($P<0.10$) biometric and physiological parameters of soybean plants, while the seed treatments only influenced leaf chlorophyll content and nitrogen balance at a significant level. Formononetin applied to soybean seeds increases AMF root colonization compared to control, independently of P rate applied to soil, while ANE and EME presented intermediate values for this parameter, not differing from both control and formononetin. When considered some qualitative parameters of mycorrhiza establishment, such as frequency and intensity of mycorrhization, both synthetic and natural biostimulants presented significant improvements, with EME and FOR providing higher values in general. The phenological stage of soybean significantly influenced AMF, bacterial and fungal community structures in rizosphere, while the biostimulant seed treatments only significantly influenced the bacterial community. However, a relatively large variation in the microbial community structures could be attributed to the interaction between phenological stage of plants and the seed treatment with biostimulants.

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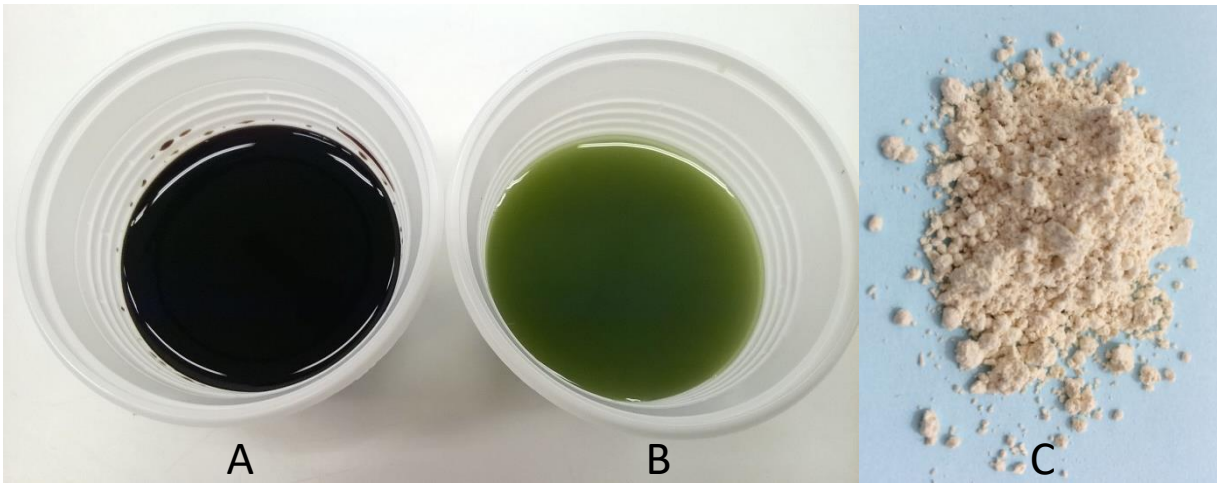
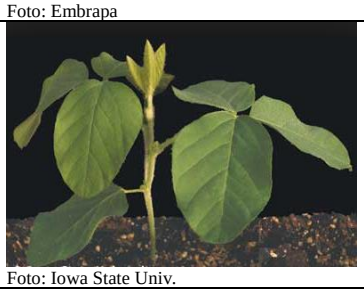
APPENDICES

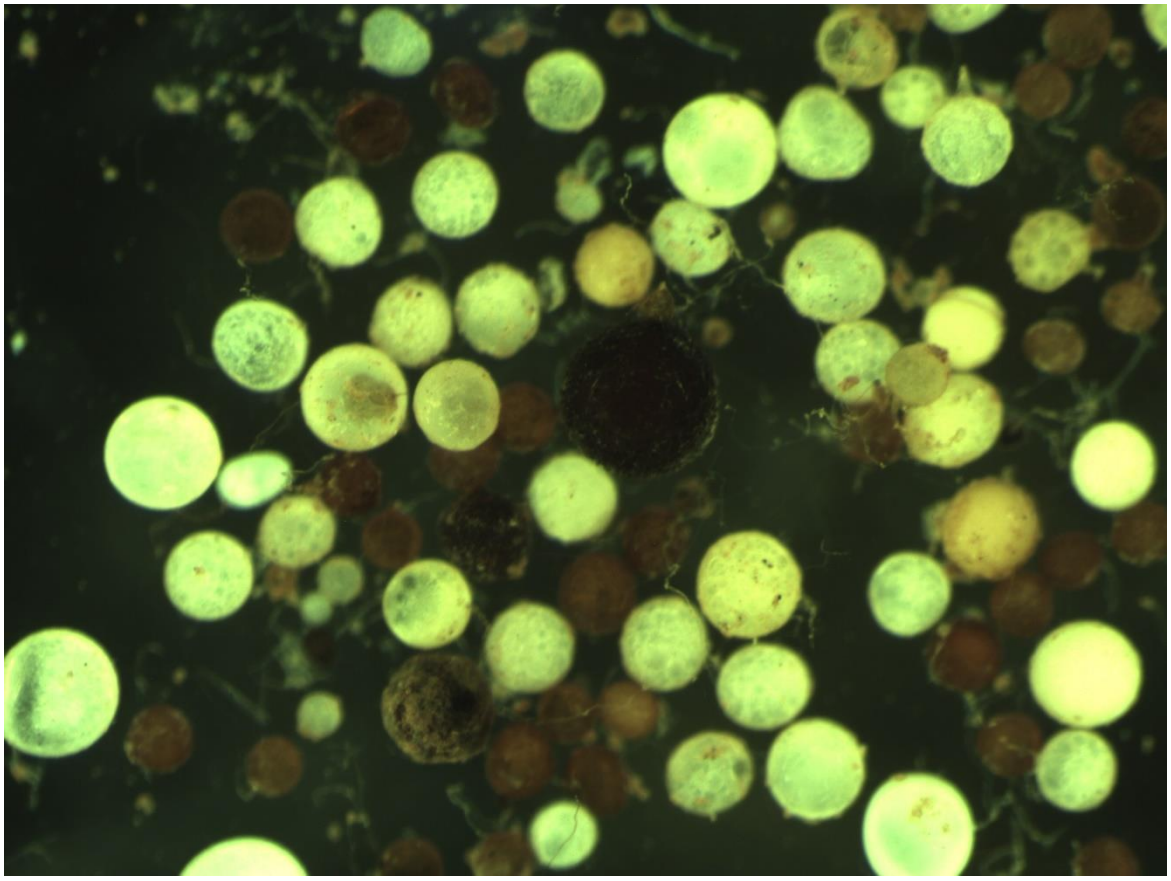


APPENDIX A. Visual aspects of the biostimulants used in the experiment. A:*Ascophyllum nodosum* extract (ANE); B: *Ecklonia maxima*.extract (EME); C: formononetin (FOR).

APPENDIX B. Pan coater used for seed treatment, in the Laboratory of Seed Analysis, University of São Paulo, "Luiz de Queiroz" College of Agriculture.

Identification	Characteristic	Image
V0	Cotiledon leaves separated	 Foto: Embrapa
V1	Folioles of first trifoliate leaf separated	 Foto: Purdue Univ.
V2	Folioles of second trifoliate leaf separated	 Foto: Embrapa
V3	Folioles of third trifoliate leaf separated	 Foto: Iowa State Univ.
V4	Folioles of fourth trifoliate leaf separated	 Foto: soilcropandmore.info

APPENDIX C. Soybean phenological stages considered in this document.



APPENDIX D. Arbuscular mycorrhizal spores present in one of the soil samples analyzed for genus identification. The same soil was used in the first and second experiments. Image from microscope (40x)

VOLUME OF SOIL PER HECTARE (0-20 cm SURFACE LAYER)

$1ha = 10{,}000\ m^2 \times 0.2\ m = 2{,}000\ m^3$ (or 2,000,000 dm^3)

ESTIMATED VOLUME OF SOIL IN SOYBEAN PLANTING FURROW* (0-20 cm SURFACE LAYER; 0.5 m BETWEEN ROWS)

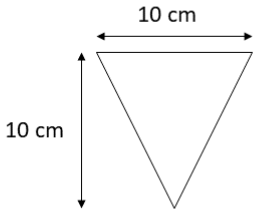
$$1\ ha = \frac{10000\ m^2 \times 0.1\ m \times 0.1m}{0.5\ m} = 200\mathrm{m}^3$$

CONSIDERING AN AVERAGE APPLICATION OF 100 Kg ha^{-1} OF P_2O_5 (OR 44 Kg ha^{-1} OF P):

44 kg in 2,000,000 dm^3 (total area, 1 ha) = 22 mg dm^{-3} of P

44 kg in 200,000 dm^3 (only in furrow*, 1 ha) = 220 mg dm^{-3} of P

** Considering a planting furrow of 10 cm x 10 cm*



APPENDIX E. Estimated values of phosphorus concentration in soybean planting furrow, considering it as a "closed system", with the aim simulate the amount of P that soybean roots at early stages of growth may find available in soil, at early stages of growth.