UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIA DO SOLO

SEED INOCULATION, BIOLOGICAL NITROGEN FIXATION, AND RESPONSE TO NITROGEN FERTILIZATION IN SOYBEAN GROWN IN CENTER-SOUTH REGION OF PARANÁ STATE

INOCULAÇÃO DE SEMENTES, FIXAÇÃO BIOLÓGICA DE NITROGÊNIO E RESPOSTA À ADUBAÇÃO NITROGENADA EM SOJA NA REGIÃO CENTRO-SUL DO PARANÁ

Vítor Gabriel Ambrosini
(Thesis)

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I dedicate it to my grandmother Judit (*in memoriam*),
who was one of the best people I have met.
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SEED INOCULATION, BIOLOGICAL NITROGEN FIXATION, AND RESPONSE TO NITROGEN FERTILIZATION IN SOYBEAN GROWN IN CENTER-SOUTH REGION OF PARANÁ STATE

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Abstract

The main agricultural challenge for upcoming decades is to feed the growing world population as sustainable as possible. As soybean [*Glycine max* (L.) Merril] is the most important agricultural legume around the world, grown worldwide as source of protein and oil, increasing its productivity by reducing possible yield gaps instead of increasing the acreage by opening new arable lands should be the focus of researchers and producers. Therefore, three studies were performed in the Center-south region of Pananá, Southern Brazil, aiming to evaluate soybean response to seed inoculation in areas with a history of this practice; determining the nitrogen (N) derived from the air (Ndfa) performance and its relationship with environmental variables, plant, and BNF traits to find driving variables of the crop yield performance; accessing possible N limitations to soybeans through full-N fertilization; and evaluating the crop yield response to the starter N. Findings of this thesis showed that soybean response to inoculation was inconsistent on a regional-scale, regarding the type of inoculant. The Ndfa averaged 61%, similar to the worldwide average and lower than the previous estimate for Brazil. Mean air temperature, total soil N, available phosphorus and exchangeable calcium were the most significant variables related to Ndfa performance. Nitrogen limitation was higher in low yield environments likely due to issues with N supply (through N_2 fixation and/or soil). Furthermore, high seed yield was related to greater values of Ndfa and contents of soil organic matter (SOM). Hence, improving soil fertility to promote crop growth and BNF process and adopting conservation management practices to increase SOM should be the focus of farmers to reduce N limitation and increase soybean seed yield. Research efforts should be applied to quantify rhizobia persistence in the soil and its efficacy at N fixation after continuous cropping without inoculation, as well determining N balance for the region. Furthermore, future studies should be given to find sustainable ways to reduce soybean yield gap by N limitation.

INOCULAÇÃO DE SEMENTES, FIXAÇÃO BIOLÓGICA DE NITROGÊNIO E RESPOSTA À ADUBAÇÃO NITROGENADA EM SOJA NA REGIÃO CENTRO-SUL DO PARANÁ

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Resumo

O grande desafio da agricultura para as próximas décadas é alimentar a população mundial crescente da maneira mais sustentável possível. A soja [*Glycine max* (L.) Merril] é a leguminosa mais importante mundialmente em termos agrícolas por ser fonte de proteínas e óleo para a alimentação. Portanto, incrementar sua produtividade por meio da redução de possíveis *yield gaps* ao invés de abrir novas áreas agricultáveis deveria ser o foco de pesquisadores e produtores. Assim, três estudos foram conduzidos na região Centro-Sul do Paraná, Sul do Brasil, com o objetivo de avaliar a resposta da soja a inoculação de sementes em áreas com histórico desta prática; determinar a contribuição do nitrogênio (N) derivado do ar (Ndfa) e a sua relação com variáveis ambientais, de planta e componentes da fixação biológica de N (FBN) para verificar quais as variáveis determinantes do rendimento da soja; acessar possíveis limitações de N para soja por meio da dose cheia de N (full-N); e avaliar a resposta da soja ao nitrogênio de arranque. Os resultados desta tese mostraram que a resposta da soja à inoculação foi inconsistente em escala regional, independente do tipo de inoculante. A média de Ndfa foi 61%, similar à média mundial e menor do que estimativas prévias para o Brasil. Temperatura média do ar, fósforo disponível e cálcio trocável foram as variáveis mais significativas em relação ao desempenho do Ndfa. A limitação nitrogenada foi mais alta em ambientes de baixo rendimento devido a limitações no suprimento de N (através da FBN e/ou do solo). Além disso, alta produtividade de soja foi relacionada a maiores valores de Ndfa e de matéria orgânica do solo (MOS). Sendo assim, incrementar a fertilidade do solo, a fim de promover o crescimento de plantas e os processos da FBN, e adotar práticas de manejo conservacionistas para aumentar o teor de MOS deveriam ser o foco de produtores para reduzir possíveis limitações de N e incrementar o rendimento da soja. Esforços de pesquisa devem ser aplicados para quantificar a persistência de rizóbios no solo e sua eficácia na fixação de N após o cultivo contínuo sem inoculação, bem como determinar o balanço de N para a região. Ainda, futuros estudos devem ser feitos para encontrar formas sustentáveis de reduzir o déficit de produtividade da soja por limitação de nitrogênio.

SUMMARY

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1 – General Introduction

Soybean [*Glycine max* (L.) Merril] is the most important agricultural legume around the world, grown worldwide as a source of protein and oil. Brazil accounts for one-third (119 million metric tons in 2017/2018) of global soybean production (341 million metric tons in 2017/2018), making it the world's largest producer alongside US (USDA, 2019). The success of soybean production in Brazil is, in part, due to efforts that breeding programs have been doing to release more productive cultivars, as well the advances in techniques of phytosanitary control and soil management. No less important than this, the focus of breeding programs on isolating well-adapted rhizobium strains to the Brazilian conditions was also essential for the expansion and success of soybean in the country (ALVES; BODDEY; URQUIAGA, 2003). Favoring biological nitrogen fixation (BNF) process through seed inoculation with rhizobia strains instead of using nitrogen (N) fertilization, saving billions of dollars every year (HUNGRIA; MENDES, 2015).

The N required by soybean crops might be obtained from two main sources: BNF and mineral N from soil, and the proportion of each source depends on many factors, including the effective association between plant and bacteria (SINCLAIR; NOGUEIRA, 2018). Protecting the environment by increasing agricultural production as sustainably as possible is a concern. Biological N fixation by legumes supports sustainability of food production by meeting the high demand of N by these crops and reducing the need for nitrogen fertilizers (HUNGRIA; MENDES, 2015). Therefore, strategies to increase soybean yields and production in a more sustainable way most focus on adjusting agricultural management practices to enhance BNF.

Achieving efficient symbiotic activity in nodules is a primary condition for the plant to have uniform access to a source of N during the growing season, then seed inoculation with *Bradyrhizobium* bacteria strains is the most recognized strategy to promote BNF in soybeans (HUNGRIA; MENDES, 2015). Nevertheless, inoculation does not guarantee high BNF in soybeans, because many environmental factors might affect N_2 fixation and plant growth, limiting the amount of N supplied by BNF (LIE, 1971). However, there are only a few studies about regional characterizations including analysis of factors related to climate, soil, and plant that can help understand the complexity of environmental conditions affecting N_2 fixation, contributing to implementation strategies to increase contribution of BNF on N supply to soybeans.

Due to the high seed protein concentration, soybeans require 80 kg N ha^{-1} per 1000 kg of seed produced (HUNGRIA; MENDES, 2015; HUNGRIA; NOGUEIRA; ARAUJO, 2015; SALVAGIOTTI et al., 2008). This great requirement associated to the rising N demand by soybeans (BALBOA; SADRAS; CIAMPITTI, 2018) led researchers to question whether BNF and soil could provide enough amounts of N to sustain high seed yields (SALVAGIOTTI et al., 2008, 2009). La Menza et al. (2017) and Ortez et al. (2018) found a N limitation to soybeans attaining its maximum yield potential, especially in high yield environments, confirming the yield gap hypothesis due to N. Also, many studies were carried out applying small N rates to soybeans, aiming to avoid limitations to BNF and reducing a possible yield gap due to lack of N on the first days of a soybean cycle – while nodules are not completely formed and N_2 fixation is not active (GAI; ZHANG; LI, 2017). However, results of those studies are contradictory (SALVAGIOTTI et al., 2008), and soybean yield response to small N rates is expected only in N-deficient soils (DADSON; ACQUAAH, 1984).

The Center-south region of Paraná State, Southern Brazil, is highlighted in the national scenario of crop production. The regional soybean seed yield average is higher than 4000 kg ha^{-1}, and more than 6000 kg ha^{-1} are frequently obtained in many farms linked to the Cooperativa Agrária Industrial. This cooperative has its own research foundation (Fundação Agrária de Pesquisa Agropecuária – FAPA). Crop management studies (plant breeding, phytopatology, entomology, and soil fertility) on a regional scale are performed by FAPA season by season aiming to increase crop yields as sustainably as possible. The high soybean seed yield in these cooperative sites are attained without N fertilization, probably due to high N supply capacity of the soils in this region as evidenced by Fontoura and Bayer (2009). However, as farmers were targeting to further increase seed yield, this thesis was proposed (in a partnership between UFRGS and FAPA) aiming to provide answers to questions related to N supply for soybeans.

For the soil and environmental conditions of the Center-south region of Paraná, where there are usually no stresses related to water deficiency and/or heat and the predominant soils have more than 40 g kg^{-1} of organic matter and good capacity of N supply, the general hypothesis of this thesis are: i) inoculation does not increase soybean seed yield in fields with inoculants applying history; ii) the contribution of N derived from air (Ndfa – the proportion of BNF on N supply) in the studied region is lower than the 80% established in previous studies for Brazil; iii) low, medium, and high Ndfa contribution groups to soybeans are determined by climatic and soil variables; iv) starter N fertilization (up to 40 kg ha^{-1}) does not increase soybean seed yield; v) even in soils with high soil organic matter content, there is a N limitation because of the high soybean N demand.

The general objectives of this thesis were: i) evaluating soybean yield response to seed inoculation with *Bradyrhizobium* in areas with a history of implementing this practice; ii) determining the Ndfa performance on the studied region by extensively characterizing climate, plant, and BNF traits to find discriminant variables that will help understand crop performance; iii) quantify soybean yield response to external N addition by evaluating application of lower fertilizer N rates as starter N fertilization and by providing full-N to the crop in order to understand if seed yields were limited by N when grown in Southern Brazil.

Aiming to answer the hypotheses and to meet the objectives of this thesis, the following studies were performed: Study 1 – Soybean yield response to *Bradyrhizobium* strains in fields with inoculation history in Southern Brazil; Study 2 – Environmental variables controlling biological nitrogen fixation soybeans in no-till fields in Southern Brazil; Study 3 – Assessing nitrogen limitation in inoculated soybeans grown in Southern Brazil.

CHAPTER 2 – Literature Review

1. Biological nitrogen fixation

1.1. Seed inoculation with Bradyrhizobium

Soybeans might obtain N from two main sources: BNF and mineral N from soil, and the proportion of each source depends of many factors, including the effective association between plant and bacteria (SINCLAIR; NOGUEIRA, 2018). For that, earlier soybean breeding approaches were based to identify genotypes able to restrict indigenous *Bradyrhizobium* serogroups with low efficiency to fix N (HUNGRIA; MENDES, 2015). Furthermore, rhizobia strains were also isolated aiming to improve the efficiency of the symbiosis. Brazilian breeding programs focusing on isolating rhizobia strains to improve N_2 fixation started in 1950s in Rio Grande do Sul (by professors João Ruy Jardim Freire and Caio Vidor, UFRGS) and later in Rio de Janeiro (by researchers José Roberto Peres and Johanna Döbereiner) (ALVES; BODDEY; URQUIAGA, 2003; FREIRE; VERNETTI, 1999; HUNGRIA; MENDES, 2015). Since then, researchers have been working to improve the BNF process through seed inoculation with rhizobia strains instead of using N fertilization, saving billions of dollars every year (HUNGRIA; MENDES, 2015).

Four inoculant strains are currently recommended for soybeans in Brazil: SEMIA 587 and SEMIA 5019 – *Bradyrhizobium elkanii strains*, SEMIA 5079 – *Bradyrhizobium japonicum*, and SEMIA 5080 – *Bradyrhizobium diazoefficiens* (FREIRE; VERNETTI, 1999; MENDES et al., 2014). Those plus the American (USDA 110) and the Argentinian (E109) strains (all of them identified and selected between 1950s and 1970s) make up around 90% of inoculants in the world, showing efficacy with the new released and high productive soybean cultivars (HUNGRIA; MENDES, 2015). A bacterium highly effective in fixing N might not be able to survive in the soil and establish a population for years. Soil is a very hostile and competitive environment, therefore, inoculation with exogenous and efficient strains will not necessarily promote increasing root nodulation, N_2 fixation rates and seed yield response (SINCLAIR; NOGUEIRA, 2018). The low competition capacity (with soil endogenous microorganisms) of some exogenous rhizobia strains led to the recommendation for annual inoculation practice (called reinoculation), aiming to improve BNF in soybean (CâMARA, 2014).

The results of soybean inoculation research are controversial regarding seed yield response. Brazilian studies, in Paraná and Mato Grosso do Sul States, showed increase in yield with inoculation in fields with a history of growing soybeans (BRANDÃO JUNIOR; HUNGRIA, 2000; MERCANTE et al., 2002, 2011). In the US, Schulz and Thelen (2008) in 14-site/years and Leggett et al. (2017) analyzed data from 187 trials and also observed response to inoculation and recommended application of this practice annually. On the other hand, no yield responses were reported in the same countries (CAMPOS; HUNGRIA; TEDESCO, 2001; CAMPOS, 1999; CAMPOS; GNATTA, 2006; DE BRUIN et al., 2010; NISHI; HUNGRIA, 1996). Positive soybean yield response to inoculation is attributed to: (a) more effective and efficient *Bradyrhizobium* strains than those living in the soil (HUNGRIA; MENDES, 2015), (b) sites with rhizobia densities below 10 cells g^{-1} of soil (THIES; SINGLETON; BOHLOOL, 1991), and (c) areas without previous legumes or inoculation history (SCHULZ; THELEN, 2008). However, soybeans grown in sites with a history of inoculation is not a guarantee of success on root nodulation, great BNF contribution, and high seed yield. For instance, Zilli et al. (2013) found up to 99% decreasing rhizobia population soon after the soybean harvest, especially in places with a prolonged dry season, where inoculation might provide great seed yield increases (ZILLI et al., 2008). While soil sampling for determining rhizobia population is not normal practice, farmers must consider seed inoculation year-by-year, once it is a low-cost agricultural practice.

1.2. Environmental factors affecting BNF

1.2.1. Soil N content

Biological N fixation by legumes might be affected by several environmental factors, including climate and soil variables. Once numerous studies about N fertilization in soybeans have been published (see item 2.2.1), it is well documented that an excess of mineral N (especially nitrate – NO_3^-) might prejudice the BNF process. The cost of N obtained from BNF (eight electrons and 16 ATP mol^{-1} N, or 6-7 g C g^{-1} N) is higher than the requirements for mineral N assimilation (12 ATP mol^{-1} N, or 4 g C g^{-1} N) by soybeans (CâMARA, 2014; KASCHUK et al., 2009). Therefore, there is a preference of obtaining N from the soil rather than from the air. Hence, increasing soil N uptake by plants gradually reduces the contribution of BNF to soybean nutrition (MAPOPE; DAKORA, 2016; SCHIPANSKI; DRINKWATER; RUSSELLE, 2010). However, N-deficient soils might not provide the amount of N required on the first days of a soybean cycle (DADSON; ACQUAAH, 1984). In those conditions, supplementing small N amounts through fertilization to soybeans grown in N-deficient soils might increase initial plant growth and also the contribution of BNF (COOPER; SCHERER, 2012). The influence of mineral N (soil mineralization + fertilization) is represented in the Fig. 1. With this scheme, Cooper and Scherer (2012) emphasized that increasing soil mineral N content is important up to a certain point, by ensuring enough N amount so the soybean plant can perform its physiological processes (such as photosynthesis) and to supply photoassimilates required for growth and activity of nodules. On the other hand, in soils with high mineral N content, there is a reduction on the contribution of BNF to soybean nutrition.

According to Streeter and Wong (1988), the BNF restriction by high NO_3^- supplies are related to three possible effects: prevention of root infection by rhizobium, reduction of nodule growth rates, and inhibition of the enzyme nitrogenase activity. The prevention of root infection might occur due to different events, which include restriction of root hair deformation, inhibition of signaling processes between soybean roots and rhizobia, and increasing the number of aborted root infections (STREETER; WONG, 1988). Success or failure on root infections could be measured by counting the number of nodules per plant. There are two main hypothesis regarding the reduction of nodule growth rates and inhibition of the enzyme nitrogenase: 1 – competition by carbohydrates between BNF process and NO_3^- assimilation, and 2 – nitrite accumulation (a byproduct of NO_3^- reduction) in the nodules, leading to an inhibition of nitrogenase and leghemoglobin activity (KANAYAMA; WATANABE; YAMAMOTO, 1990; STREETER; WONG, 1988). It is possible to identify inhibition of nodule growth through measurements of total and average nodule dry weight. Reduction of BNF contribution might be observed by analyzing total N in the plant shoot or, with high accuracy, using isotopic methods of ^{15}N (UNKOVICH et al., 2008).

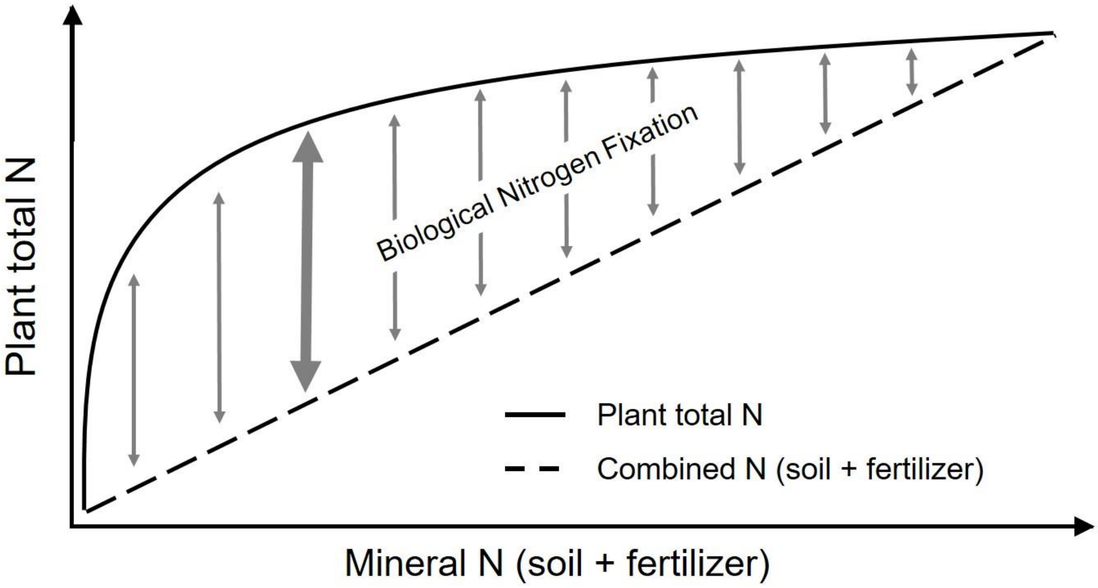

Figure 1. Representation of the proportional contribution of BNF for the total plant N as a function of N uptake (soil + fertilizer) by legumes. Solid and dashed lines represent the plant total N and the uptake mineral N from the soil, respectively. The space between these two lines represents the magnitude of the N obtained from BNF. The thickest vertical arrow represents the maximum BNF efficiency as a function of mineral N supply. Adapted from Cooper and Scherer (2012).

The hypothesis of carbohydrate deprivation for nodules by mineral N is based on the requirement of reducing energy for NO_3^- reduction and assimilation, which causes competition with the nodules for the available carbohydrates (STREETER; WONG, 1988). Nevertheless, in a preliminary study, Streeter (1981) concluded that inhibition of nodule growth and BNF was not caused by reduced carbohydrate accumulation in the nodules. On the other hand, this same author did not exclude the possibility of interference by NO_3^- on carbohydrates catabolism, reducing the capacity of nodules using these sugars – either by reducing carbohydrate transport to nodules or by their internal metabolism. Kanayama et al. (1990) found that carbohydrate deprivation was the reason for the reduction of nitrogenase enzyme activity when NO_3^- was supplied for a long time (three to seven days). They also observed that BNF was inhibited in a shorter time by decreasing the function of leghemoglobin by NO_3^-.

1.2.2. Other variables related to soil fertility

Nitrogen-fixing plants require photoassimilates as an energy source for growth and activity of nodules (MYLONA; PAWLOWSKI; BISSELING, 1995), having a high C cost (6-7 g C g^{-1} N) to sustain the BNF process (KASCHUK et al., 2009). Hence, limiting factors to the plant growth and photosynthesis are detrimental to the N_2 fixation process (TAMAGNO et al., 2018). For instance, it has been documented the role of high soil fertility on root nodulation and BNF process in different legume species (DIVITO; SADRAS, 2014; GATES; MÜLLER, 1979; GATES; WILSON, 1974; OLIVERA et al., 2004; TSAI et al., 1993). Gates and Müller (1979) reported that soybean root nodulation responded to phosphorus (P) and sulfur (S) supply until pod filling, and also that nodule developing continued throughout the whole soybean cycle, except under P deprivation. Tsai et al. (1993) found that rising levels of P, potassium (K) and S increased the contribution of BNF (from 52 to 65%, from low to high nutrient levels) on common bean nutrition. Divito and Sadras (2014) explained that limited P, K and S availability might decrease, directly or indirectly, nodule growth, leading to lower BNF contribution to legumes. The direct effect of P, K and S deprivation is due to the rapid reduction of these nutrients in the plant, while N concentration remains constantly increasing N:P, N:K and N:S ratios. These changes in nutrient stoichiometry have been proposed to activate an N-feedback signaling, regulating development and activity of nodules in the root. The indirect effect occurs by reducing the host plant’s growth (COOPER; SCHERER, 2012). Phosphorus also has an important role on the plant energy metabolism, then, P deprivation causes a negative impact on nodule’s energy status (OLIVERA et al., 2004). Sulfur deficiency might also reduce BNF by affecting activity of important enzymes, as nitrogenase (the main enzyme involved in BNF process), PEP-carboxylase, malate dehydrogenase and glutamate synthase (COOPER; SCHERER, 2012). Furthermore, it was also reported that high levels of P, K and

S reduced the deleterious effects of mineral N on the BNF process in soybeans (GATES; MÜLLER, 1979) and common beans (TSAI et al., 1993).

Other important soil variables to the BNF process are related to acidity and calcium (Ca) availability. Acidity is detrimental to plant growth, resulting in negative effects to nodulation and BNF, however, nodule formation is generally more sensitive to soil acidity than other aspects of plant growth (FERGUSON; LIN; GRESSHOFF, 2013). The direct effects of soil acidity on BNF are credited to high concentrations of H^+ and toxic metals, as Al^{3+} and Mn^{2+}, in acidic soils, affecting rhizobium growth and function (FERGUSON; GRESSHOFF, 2016). Low pH conditions prejudice signaling processes between plant and rhizobium by reducing flavonoid secretion and the expression of nodulation key genes, including *nod*A (FERGUSON; GRESSHOFF, 2016; FERGUSON; LIN; GRESSHOFF, 2013). Acidic conditions is also detrimental to root hair formation and curling, impairing nodule formation (MIRANSARI et al., 2006). Indirectly related to soil acidity, calcium is an essential component of the initial process of BNF. The role of Ca on BNF is related to the event called Ca spiking, a *nod* gene-dependent host response that triggers a signaling cascade leading to nodule development (EHRHARDT; WAIS; LONG, 1996; LÉVY et al., 2004; WAIS; KEATING; LONG, 2002).

1.2.3. Environmental factors

Environmental factors play a determinant role on plant growth, development and metabolism, and unfavorable changes in environmental conditions (water availability, temperature, light, salinity, soil fertility) are detrimental to vegetal metabolism and development (MOSA; ISMAIL; HELMY, 2017). All the stages of symbiosis (including pre-infection phase) between rhizobium and a host legume are also affected by environmental factors, and the symbiotic system is primarily affected by stressful conditions (LIE, 1971). For instance, N_2 fixation of legumes, like soybeans and cowpeas (*Vigna unguiculata*), are highly sensitive to drought stress (SINCLAIR et al., 2015; SINCLAIR; SERRAJ, 1995). The detrimental effect of water-deficit conditions to the BNF process is related to decreased phloem flow from the host plant to nodules, leading to a limitation of N product removal from nodules and aninhibition of N_2 fixation (SINCLAIR; NOGUEIRA, 2018). On the other hand, excess water (flooding) is also unfavorable to the host plant due to low-oxygen supply (hypoxia) to the root system, impairing the aerobic respiration and reducing plant energy status (BAILEY-SERRES; VOESENEK, 2010), which reduces the BNF process (SÁNCHEZ et al., 2011). Likewise, N_2 fixation is limited under hypoxia due to the reduction of oxygen supply to nodules (JAMES; CRAWFORD, 1998).

Another important environmental variable to BNF is air temperature. For instance, George et al. (1988) found that low mean air temperature reduced soybean photosynthesis, decreasing N_2 fixation indirectly due to low plant energy status. However, the most common stress related to the temperature in legumes is due to heat conditions (KEERIO; WILSON, 1998; ZAHRAN, 1999). Heat stress leads to a decreasing nitrogenase activity and accelerates the nodule senescence process, reducing the N_2 fixation (HUNGRIA; FRANCO, 1993). Collino et al. (2015), studying both soil and weather factors in Argentina, reported that air temperature can explain more about BNF when seed yield is below 3700 kg ha^{-1}, while soil variables are most important when seed yield is above this threshold.

1.3. Contribution of BNF to soybean

BNF can supply circa 60% of the total N required for soybean production around the globe (CIAMPITTI; SALVAGIOTTI, 2018; SALVAGIOTTI et al., 2008). Herridge et al. (2008) estimated the amount of N fixed annually by soybeans in different countries and concluded that the contribution of BNF to soybean nutrition is lower in China (50%) and the US (60%) than in Argentina (80%) and Brazil (80%). The main reasons for the differences between these countries are soil conditions and management practices. While the areas used for soybean production in the US are generally able to provide great amounts of N to plants, less N amount is available in Argentina and Brazil. Besides, Argentinian and Brazilian farmers commonly use seed inoculation, no-tillage system and avoid application of N to soybeans, which can contribute to greater N derived from the air (Ndfa – the proportional contribution of BNF) in these countries (HERRIDGE; PEOPLES; BODDEY, 2008; HUNGRIA et al., 2005).

Regional studies developed in Brazil confirm the high contribution of BNF (75-92%) to N supply for soybeans (ALVES et al., 2006; HUNGRIA et al., 2006). The proportional contribution of BNF (in percentage) varies accordingly to many environmental conditions (as reported above), and soil mineral N supply is one of the most important factors affecting BNF (MAPOPE; DAKORA, 2016). For the present study, performed in the Center-south region of Paraná State, Southern Brazil, it is expected that there is a lower contribution of BNF to soybeans than the 80% reported in the literature, because soils from this region generally have a great potential to supply N for crops (FONTOURA; BAYER, 2009).

2. Nitrogen fertilization in soybeans

2.1. Starter nitrogen fertilization

The main reason for starter N fertilization in soybeans is to supply N in the initial period of the crop cycle (14 to 20 days after sowing), while the nodules are still in formation and BNF has not yet occured. Aiming evaluate the benefits of initial nitrogen fertilization, Gai et al. (2017) applied N rates at soybean sowing and observed increasing root activity, plant N content, leaf chlorophyll content and photosynthetic rates with starter N up to 50 kg N ha^{-1}, which lead to gains in yield components. However, it is important to note that seed inoculation was not performed in this study and no information was provided on the number of rhizobia cells in the soil or even on soybean cultivation history in the area.

For the Center-south region of Paraná (Southern Brazil) – where soybean average soybean yields are high, reaching a more than 6000 kg ha^{-1} in some areas, the proposal for supplemental N application is supported by the estimates of Salvagiotti et al. (2008; 2009), which indicated the need of 450 kg N ha^{-1} to attain seed yields of 5400 kg ha^{-1}. Of these, 250-300 kg N ha^{-1} should be provided by BNF, requiring 150-200 kg N ha^{-1} from the soil. These authors also assume the soil N uptake by soybeans is around 100 kg N ha^{-1}, and is essential to the complementation of 50-100 kg N ha^{-1} through fertilizers to reach the maximum productive potential. Aiming to supply N to increase soybean yields but avoiding inhibiting BNF processes, studies applying small N rates at sowing have been developing. Some of these studies found seed yield increasing due to small (up to 40 kg ha^{-1} – BOROOMANDAN et al., 2009; OSBORNE; RIEDELL, 2006) or even to high starter N rates (120-160 kg ha^{-1} de N – CALISKAN et al., 2008; DADSON; ACQUAAH, 1984). On the other hand, many others showed lack of response to starter N to soybeans in Brazil (ARATANI et al., 2008; BALBINOT

JUNIOR et al., 2016; HUNGRIA et al., 2006; JENDIROBA; CâMARA, 1994; MENDES; HUNGRIA; VARGAS, 2003) and other countries around the world (HERRIDGE; BROCKWELL, 1988; JANAGARD; EBADI-SEGHERLOO, 2015; JOSIPOVIĆ et al., 2011; KAMARA et al., 2012; MRKOVAČKI; MARINKOVIĆ; AĆIMOVIĆ, 2008).

Some studies that reported a lack of soybean response to N fertilization also found impairing effects over root nodulation caused by N (lower nodule number and dry weight), reducing BNF contribution to soybean nutrition (HERRIDGE; BROCKWELL, 1988; HUNGRIA et al., 2006; MENDES; HUNGRIA; VARGAS, 2003). Furthermore, among the studies showing increases in seed yield due to N fertilization, there is not a consensus about the viability of starter N to soybeans. For example, Dadson and Acquaah (1984) reported that BNF is probably the most economically advantageous option to supply N to soybeans. Nevertheless, the same authors stated that in soils with low N supply capacity, N rates up to 40 kg N ha^{-1} might stimulate nodule formation and initial plant growth. This was corroborated by Osborne and Riedell (2006), who also attributed seed yield increases to higher initial soybean growth promoted by N fertilization at sowing. Other studies demonstrated that increasing yields by starter N fertilization might occur in water-deficient cropping seasons, a stressing condition to plants that impair the BNF process (KUBOTA; HOSHIBA; BORDON, 2008; PURCELL; KING, 1996). Therefore, according to these studies, starter N fertilization would only be feasible under the following conditions: (a) BNF is impaired by some limiting factor, or (b) in soils that could not supply the small N amounts needed by soybeans in early development stages.

Although the results of research carried out in Brazil indicate that nitrogen fertilization in soybeans is not feasible (ARATANI et al., 2008; BALBINOT JUNIOR et al., 2016; HUNGRIA et al., 2006; JENDIROBA; CâMARA, 1994; MENDES; HUNGRIA; VARGAS, 2003), it has been considered as an agronomic practice by many soybean farmers in the country. This has been occurring due to misused extrapolating results of trials carried out in specific sites as a basis for recommending fertilization in sites with different characteristics. This also happens either by pressure from the fertilizer industry in order to increase sales of nitrogenous fertilizers, or by the N effects (in this case, topdressing fertilization) on the visual aspect of the crop (such as the dark green coloration of the plants), but it is not usually reflected in increased productivity. Therefore, more research is important, especially in high yielding environments (more than 4500 kg ha^{-1}), where research on this subject is scarce and in which contribution of BNF to supply the entire soybean demand for N is uncertain (SALVAGIOTTI et al., 2008).

2.2. Nitrogen as a seed yield limiting factor to soybeans

Seed yield, plant biomass and harvest index have been increasing from the 1920s to 2015, leading to a positive time trend for nutrient uptake (BALBOA; SADRAS; CIAMPITTI, 2018). During this period, the same author reported that N uptake increased at a rate of 1.57 kg N year^{-1}. Some authors reported that soybeans require 80 kg N ha^{-1} per 1000 kg of seed produced (HUNGRIA et al., 2005; HUNGRIA; MENDES, 2015; SALVAGIOTTI et al., 2008). The rising N demand of soybeans led researchers to question whether BNF and soil could provide enough N to sustain high seed yields (SALVAGIOTTI et al., 2008; 2009). Therefore, it is possible that N supply might be a limiting factor for soybeans to attain high yield potential, causing a yield gap (CAFARO LA MENZA et al., 2017; ORTEZ et al., 2018). Yield potential is attained when a well-adapted cultivar is grown under ideal conditions, without limitation of water and nutrients, and in the absence of abiotic (light, salinity, drought) or biotic (diseases, insects, weeds) stresses (EVANS, 1993). Aiming to explore possible N limitation to soybeans, few studies were carried out using large amounts of N ($\geq$ 300 kg N ha^{-1}) to supply all the requirements of soybean production (CAFARO LA MENZA et al., 2017; HERRIDGE; BROCKWELL, 1988; ORTEZ et al., 2018; RAY; HEATHERLY; FRITSCHI, 2006; WILSON et al., 2014).

Seed yield gap caused by N was reported in some of these studies. Ortez et al. (2018) and Wilson et al. (2014) reported yield responses to high N amounts in modern cultivars (more productive). Their results agree with Cafaro La Menza et al. (2017), who associated higher seed yield increments to high yield environments, which is a consequence of high N demand of more productive cultivars and/or environments. On the other hand, Ray et al. (2006) did not find a relationship between yield gaps and yield environments. Therefore, it would be interesting to study yield gaps in different yield environment levels, and learn which leads to increasing N demands by soybeans.

CHAPTER 3 – Soybean yield response to *Bradyrhizobium* strains in fields with inoculation history in Southern Brazil

1. Introduction

Soybeans [*Glycine max* (L.) Merr.] are grown worldwide as a source of protein and oil. Due to the high seed protein concentration, crop nitrogen (N) requirements usually exceed the amount that soil can provide (Salvagiotti et al., 2008). Soybeans have developed the ability to fix atmospheric-N through a symbiotic relationship with soil rhizobia (*Bradyrhizobium* spp.) in order to fulfill plant N demand. Biological N fixation (BNF) supply averages 50-60% of the plant N demand for soybean production around the globe (SALVAGIOTTI et al., 2008). Nitrogen provided via BNF is linked to yield increases, thus further improvements on N fixation would benefit high-N demanding and high-yielding soybean systems (CIAMPITTI; SALVAGIOTTI, 2018).

Inoculation is recognized as a crucial management practice to enhance bacterial infection early in the season and future nodulation activity (CHIBEBA et al., 2015). Under no occurrence of abiotic/biotic stresses (HUNGRIA; MENDES, 2015), the effect of management on soybeans such as tillage, liming (FERGUSON; GRESSHOFF, 2016), and nutrient availability (DIVITO; SADRAS, 2014) have been reported to impact the overall nodulation efficiency.

Positive soybean yield responses have been commonly reported in areas without previous legumes or inoculation history (SCHULZ; THELEN, 2008) and in sites with low rhizobia densities in the soil (THIES; SINGLETON;

BOHLOOL, 1991). However, soybean yield response to seed inoculation is not consistent for areas with a history of utilizing this practice (inoculation). For instance, increases of up to 31% in soybean yield have been reported from comprehensive studies in Brazil (BRANDÃO JUNIOR; HUNGRIA, 2000; MERCANTE et al., 2002, 2011) and up to 30% in the US (LEGGETT et al., 2017; SCHULZ; THELEN, 2008), whereas no yield responses were reported in the same countries (CAMPOS; HUNGRIA; TEDESCO, 2001; CAMPOS, 1999; CAMPOS; GNATTA, 2006; DE BRUIN et al., 2010; NISHI; HUNGRIA, 1996). Positive soybean yield response to inoculation is mainly attributed to more effective and efficient *Bradyrhizobium* strains than those living in the soil (HUNGRIA; MENDES, 2015), while negligible or no yield response is expected in areas with a history of planting soybeans and when introduced bacteria are successfully established (THIES; SINGLETON; BOHLOOL, 1991).

Thirty-one field trials were conducted, from 1999 to 2017, aiming to evaluate soybean yield response to seed inoculation with *Bradyrhizobium* in areas with a history of implementing this practice in Southern Brazil (Set of trials 1: 21 trials conducted from 1999/2000 to 2014/2015). Moreover, we aimed to explore and identify plant traits and environmental factors responsible for changes in key bacteria-plant symbiosis (e.g., nodule number and weight, N content) explaining yield response to inoculation (Set of trials II: 10 trials conducted in 2015/2016 and 2016/2017).

2. Material and methods

2.1. Site description

Field trials were carried out in the Center-south region of Paraná State, southern region of Brazil. The regional climate is a humid temperate climate with a moderately hot summer (Cfb), according to the Köppen classification (APARECIDO et al., 2016), without a dry season. Annual precipitation ranges from 1,550 to 1,800 mm, with an occurrence of weekly precipitation during spring/summer, and an annual mean temperature ranging from 16.5 to 18.5 °C, a 25-year long-term average (APARECIDO et al., 2016). At all locations, soils were classified as Hapludox (SOIL SURVEY STAFF, 2014). Soybeans are planted in a no-till system, and inoculation is a common farming practice on this region, with several years of inoculated field grown soybeans.

2.2. Set of trials I: treatment descriptions and experiments conducted

Twenty-one field trials were conducted from 1999/2000 to 2014/2015 in areas with a history of soybean growth (Table 1). Two or three treatments were imposed in each trial: (i) control, without seed inoculation; and (ii-iii) inoculated, with solid and/or liquid inoculant (Table 1). Both inoculant formats were applied in seven field trials, while solid (nine trials) or liquid inoculant options (five trials) were all tested relative to a control (without inoculation) (Table 1). Commercial inoculants containing *Bradyrhizobium* strains were applied on seeds according to a Brazilian recommendation (250 g solid inoculant or 100 mL liquid inoculant per 50 kg of seeds). All field trials were arranged according to a randomized complete block design, with three to six replicates.

Table 1. Growing season, soybean cultivar, fertilizer rate, maturity group, and inoculant type (solid, S, and liquid, L) used in 21 field trials conducted in Guarapuava (Center-south of Paraná), from 1999/2000 to 2014/2015 growing seasons, in Southern Brazil.

Field trial number	Growing season	Cultivar	Fertilizer rate[1] (kg ha^{-1})	Maturity group	Inoculant
1	1999/2000	Embrapa 59	200	7.1	S
2	2000/2001	Embrapa 59	100	7.1	S / L
3	2000/2001	Embrapa 59	100	7.1	S / L
4	2001/2002	BRS 154	120	7.2	S / L
5	2001/2002	BRS 154	120	7.2	S / L
6	2001/2002	BRS 154	120	7.2	S
7	2001/2002	Embrapa 59	120	7.1	S
8	2002/2003	BRS 154	100	7.2	S / L
9	2002/2003	BRS 154	100	7.2	S / L
10	2002/2003	BRS 154	100	7.2	S
11	2003/2004	BRS 154	120	7.2	S / L
12	2003/2004	BRS 154	120	7.2	S
13	2005/2006	BRS Torena	150	7.0	S
14	2005/2006	CD 215	150	5.9	S
15	2006/2007	BRS Torena	150	7.0	S
16	2006/2007	CD 215	150	5.9	S
17	2011/2012	AFS 110	250	6.3	L
18	2013/2014	BMX Ativa	200	5.6	L
19	2013/2014	AFS 110	200	6.3	L
20	2014/2015	BMX Ativa	200	5.6	L
21	2014/2015	BMX Ativa	200	5.6	L

S – only solid inoculant; L – only liquid inoculant; S / L – solid and liquid inoculant. 1 – Fertilizer 0-20-20 was applied in trial #1, while 0-25-25 was applied in all other trials.

Plots had eight rows spaced 40 cm apart and 5 m long. Soybeans were sowed between the second half of November and the first half of December, as recommended for the region, with a target plant density ranging from 30 to 35 plants m^{-2}. Fertilization (Table 1), except for N, and phytosanitary control were applied according to regional recommendations to control pests and disease. A combined harvest was made in 6.4 m^2 of the middle rows in each plot between the second half of April and the first half of May. Seed yield was adjusted to 130 g kg^{-1} moisture content.

2.3. Set of trials II: Treatment descriptions and experiments conducted

Ten trials were conducted in five different sites (Campina do Simão, Taguá, Pinhão, Candói and Guarapuava), in 2015/2016 and 2016/2017 growing seasons. The trials were conducted on the same farm in both growing seasons, but not in the same field. Thus, each trial was considered an independent site. Soil samples from 0-20 cm soil depth were collected before the trial began (Table 2). Precipitation and temperature data for the evaluated seasons at each location is shown in Appendix 1.

Two treatments were imposed: (i) control, without seed inoculation, and (ii) inoculated, with a liquid inoculant, containing *Bradyrhizobium elkanii* (SEMIA 5019) plus *Bradyrhizobium japonicum* (SEMIA 5079), applied at rate of 100 mL per 50 kg seeds. Plots had eight rows spaced 40 cm apart and 5 m long.

Soybean 'BMX Apolo RR' (Don Mario 5.8i) variety, undetermined growth habit, was sown at 30 seeds m^{-2}. Seeds received the same fungicide and insecticide treatments before planting time, occurring between the end of October and the first half of November in both growing seasons. Fertilization was applied as 250 kg ha^{-1} of 0-25-25 (N-P_2O_5-K_2O). Phytosanitary treatments were applied according to regional recommendations.

Table 2. Characterization of 0-20 cm soil layer from field conducted in 2015/2016 and 2016/2017 growing seasons in Southern Brazil.

Site	Mn	S	P	K	Ca	Mg	Al	H + Al	$CEC_{pH\ 7,0}$	Clay	SOM	V	pH H_2O
	mg dm^{-3}				$cmol_c$ dm^{-3}					g kg^{-1}		%	
2015/2016 Growing Season													
C. Simão 1	32	85	1.5	47	4.5	3.8	0.0	4.4	12.8	470	41	66	5.6
Taguá 1	6	21	5.3	66	5.1	2.8	0.1	3.9	11.9	400	51	68	5.4
Pinhão 1	3	10	25.0	60	7.1	4.4	0.0	2.8	14.4	340	47	81	6.2
Candói 1	4	16	8.5	97	6.3	2.6	0.0	4.9	14.0	280	60	65	5.5
Guarapuava 1	4	12	6.5	194	6.6	2.8	0.0	3.1	13.0	340	46	76	5.8
2016/2017 Growing Season													
C. Simão 2	20	31	2.6	114	6.1	4.2	0.0	5.5	16.1	470	47	66	5.6
Taguá 2	7	13	2.1	98	7.1	4.1	0.0	5.5	16.9	400	53	68	5.7
Pinhão 2	4	16	4.0	288	8.3	5.3	0.0	4.9	19.2	340	52	75	5.8
Candói 2	3	13	8.4	147	8.1	3.6	0.0	5.5	17.5	280	57	69	5.7
Guarapuava 2	9	15	7.3	231	6.5	2.8	0.1	8.7	18.6	340	50	53	5.4

Mn: extracted by HCl 0.1 mol L^{-1}; S: extracted by $Ca(H_2PO_4)_2$ containing 500 mg P L^{-1}; P, and K: extracted by Mehlich-1; Ca, Mg, and Al: extracted by KCl 1 mol L^{-1}; clay content: determined by the pipette method; SOM – soil organic matter: determined by wet oxidation-redox titration (Walkley-Black) method; CEC – cation exchange capacity; V – base saturation.

At the phenological stage R1 (flowering; FEHR; CAVINESS, 1977), five plants per plot were collected and fractioned in root, shoot (aboveground plant – stem + leaves), and nodules. Samples were dried at 65 ºC until constant weight was achieved and dry weight was obtained for all plant fractions. In the first year, nodule number and dry weight were analyzed in all roots, while in the second year those variables were obtained only from crown root to enable the measurements. Based on nodules on the crown root, data of nodule number and dry weight from the whole root in the second year were estimated according to equations from Cardoso et al. (2009). Total N content in the shoot was calculated by multiplying the dry weight and its N concentration determined by the Thermo Fisher Scientific CN Analyzer (Flash 200 model). Seed yield was adjusted to 130 g kg^{-1} moisture content.

2.4. Statistical analysis

All 31 field trials followed a randomized complete block design with three to six replicates, depending on the location. For Set of trials I analysis, data was divided into two groups. The first group aimed to evaluate if seed yield responded differently to solid and liquid inoculants and contained information for both inoculants applied within the same trial. Each trial data from group 1 was compared by t-test. Data of solid and liquid inoculants applied in the same trial were also grouped using the average of both inoculation treatments. This data was used as a single inoculation treatment and were included in group 2. This group compared a control treatment (without seed inoculation) and a treatment with seed inoculation (with solid or liquid inoculant). Data from group 2 was compared by t-test within each trial and an average of the 21 trials. Aiming to assess if response to inoculation was related to the local yield potential, environmental indexes were established. Each index was calculated as the average yield of both treatments from each of the 21 field trials.

For the Set of trials II, the data of seed yield, dry weight, C and N content, and nodulation variables was submitted to analysis of variance (ANOVA). Inoculation, trials and interaction within trials were considered as fixed effects, and blocks were considered as random effects. Means were compared with Tukey HSD using the lsmeans function (lsmeans R package; LENTH, 2016) at the 0.05 confidence level. Stepwise multiple regression analysis was performed including all soil variables to identify which were responsible for the large variability due to environmental effects on nodule number and nodule dry weight. Variables used in the model were chosen based on the p-values.

3. Results and discussion

3.1. Set of trials I: Soybean yield response to seed inoculation at regional-scale

Solid and liquid inoculants were compared in seven out of the 21 trials of the first dataset. Seed yield only presented statistically significant differences between inoculation sources in one out of seven trials, with liquid inoculant out yielding the solid format by 9% (Table 3). Across all field trials, seed yield did not statistically differ between inoculant formats: 2919 (solid) and 2883 (liquid) kg ha^{-1} (Table 3). Thus, from this point onwards averages of both inoculation treatments were used as a single treatment (with inoculation) for the rest of the analysis for this dataset.

Table 3. Seed yield of soybeans inoculated with solid and liquid inoculants in seven field trials, in Southern Brazil.

Field trial	Seed yield		Δ Yield		p-value (3)
	Solid inoculant	Liquid inoculant			
	kg ha^{-1}		kg ha^{-1} (1)	% (2)	
2	2802	2513	-288	-10	0.051^{ns}
3	3199	3483	284	9	0.037*
4	2761	2894	133	5	0.630^{ns}
5	2579	2227	-352	-14	0.130^{ns}
8	2798	2829	31	1	0.733^{ns}
9	2821	2702	-119	-4	0.384^{ns}
11	3475	3535	60	2	0.527^{ns}
Average	2919	2883	-36	-1	0.753^{ns}

(1) Difference of seed yield between treatments with soybeans inoculated with solid and liquid inoculant.
(2) Relative difference between soybeans inoculated with solid and liquid inoculant, [(seed yield with solid inoculant – seed yield with liquid inoculant)/seed yield with solid inoculant] x 100
(3) *p*-value referent to t-test.
Levels of significance: *p < 0.05; ns: not significant.

Soybean seed yield ranged from 1853 to 5352 kg ha^{-1} (Table 4) and the overall mean was 3292 kg ha^{-1}. Seed yield did not respond to inoculation in a regional-scale ($p>0.05$): control 3298 kg ha^{-1} and inoculated 3286 kg ha^{-1}.

Differences per trial between control and inoculated ranged from -323 to 736 kg ha^{-1} in absolute terms and from -8.6 to 24.7% in relative terms (Table 4). Differences were statistically significant in only 3 out of 21 trials, but only in one favoring the inoculation effect on yields (Table 4). Seed yield histogram distribution for the difference between inoculated and control is portrayed in Fig. 2.

Table 4. Comparison of seed yield of inoculated vs. non-inoculated soybeans with *Bradyrhizobium* strains in 21 field trials, conducted from 1999/2000 to 2014/2015, in Southern Brazil.

Field Trial	Seed Yield		Δ Yield		p-value^2
	Inoculated	Non-inoculated			
	kg ha^{-1}		kg ha^{-1}	%^{1}	
1	3841	3105	736	24.7	0.032*
2	2658	2912	-255	-8.6	0.020*
3	3341	3429	-88	-2.4	0.406^{ns}
4	2827	2816	11	0.3	0.956^{ns}
5	2403	2313	90	9.5	0.793^{ns}
6	2994	2737	256	9.2	0.301^{ns}
7	1853	1964	-111	-3.6	0.500^{ns}
8	2814	2755	59	2.6	0.672^{ns}
9	2761	2840	-78	-2.4	0.554^{ns}
10	2616	2602	13	0.9	0.919^{ns}
11	3505	3500	5	0.2	0.934^{ns}
12	2270	2180	90	8.0	0.788^{ns}
13	2847	3055	-208	-5.3	0.359^{ns}
14	2935	2866	69	7.4	0.791^{ns}
15	3110	3371	-262	-7.1	0.217^{ns}
16	3000	3127	-128	-3.8	0.312^{ns}
17	5352	5302	50	1.0	0.798^{ns}
18	4465	4379	87	2.1	0.463^{ns}
19	4065	4253	-187	-4.2	0.242^{ns}
20	4608	4931	-323	-6.6	0.042*
21	4745	4813	-68	-1.3	0.801^{ns}
Average	3286	3298	-12	-0.3	0.269^{ns}

[1] [(inoculated soybean yield – non-inoculated soybean yield)/non-inoculated soybean yield] x 100
[2] *p*-value referent to t-test.

Results reported in this study showed no differences in inoculation at varying yield levels or environments (Fig. 3). In agreement with our findings, previous studies in Brazil and the US have reported a lack of seed yield response to inoculation in areas where soybeans have been previously grown (CAMPOS;

HUNGRIA; TEDESCO, 2001; CAMPOS, 1999; CAMPOS; GNATTA, 2006; DE BRUIN et al., 2010). Among these studies, Campos et al. (2001) evaluated nodule occupation by established rhizobia population in the soil, concluding that these strains were able to infect the root and fix atmospheric N. Using a similar method, Mendes et al. (2000) reported that the introduced strains were able to compete with the naturalized strains and establish a great nodule occupation (40% or more) in soybean roots up to three years after the inoculation practice. Therefore, the overall neutral yield response to the inoculation practice might be interpreted as if the rhizobia population established in the soil (naturalized strains) were efficient on infecting roots from soybean plants and fixing atmospheric N_2. Seed yield response to inoculation might be expected when indigenous rhizobia populations are below 10 cells g^{-1} of soil (THIES; SINGLETON; BOHLOOL, 1991).

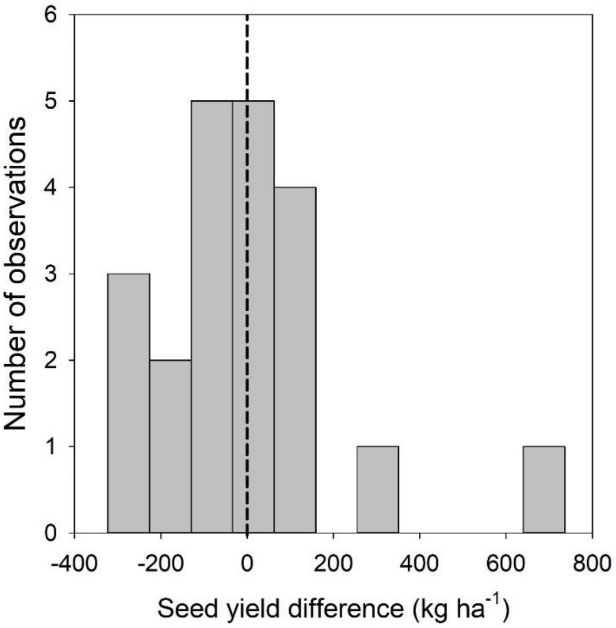

Figure 2. Cumulative frequency for seed yield difference between inoculated and non-inoculated seed soybean with *Bradyrhizobium* strains for 21 field trials, conducted from 1999/2000 to 2014/2015 growing seasons, in Southern Brazil. Dotted line represents neutral (around zero) seed yield response.

A main weakness of this regional-scale characterization is related to the lack of estimation of the number of rhizobia cells per gram of soil, and overall N fixation process (including nodulation, direct BNF measurements, etc.), impairing the ability to identify the key factors from the bacteria-plant viewpoint affecting seed yield response to the inoculation practice. To overcome this limitation, ten trials were performed (Set of Trials II) to evaluate variables related to root nodulation, plant dry weight, C and N content besides seed yield.

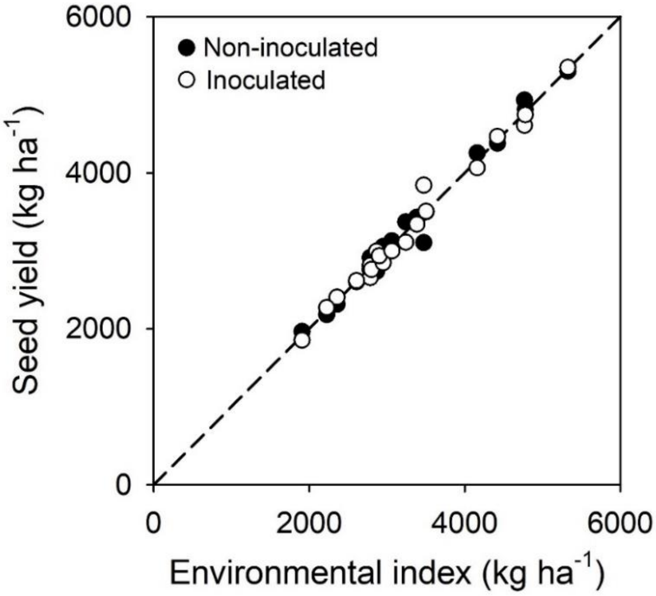

Figure 3. Relationship between seed yield of inoculated (opened circles) and non-inoculated (closed circles) soybean and environmental index in a total of 21 field trials conducted from 1999/2000 to 2014/2015 growing seasons in Southern Brazil. Dashed line is the 1:1 relation. Each data point represents a mean calculated from three to six replicates. The environmental index was calculated as the average yield of both treatments from each trial (Exp. 1).

3.2. Set of trials II: Plant growth and N components underpinning yield formation

From all ten trials evaluated, overall inoculation did not influence the variables analyzed (Table 5). Seed yield response to inoculation presented a similar pattern as encountered in the regional-scale analysis: lack of a consistent yield response ($p>0.05$). Overall, the Campina do Simão site, where soybeans were preceded by *Pinus* spp., presented a lower seed yield, averaging 3,628 kg ha^{-1} in 2015/16 and 2016/17 seasons; while the other four trials, with soybeans grown in no-tillage systems for more than ten years, yields were above 5,300 kg ha^{-1} (Table 5). Yield components followed the same pattern as seed yield, with lower seed number and seed weight in Campina do Simão relative to the rest of the trials, and with an overall lack of response to inoculation ($p>0.05$; Table 5). Thus, averaging 2015/16 and 2016/17 seasons, inoculation did not present a statistical yield benefit relative to the control across sites.

Table 5. Seed yield and components, nodule number and weight, shoot C and N content, and shoot and root dry weight of soybeans non-inoculated and inoculated with *Bradyrhizobium* strains in 10 field trials conducted in 2015/2016 and 2016/2017 growing seasons.

	Seed			Nodule		Shoot			Root
	Yield	Number	Dry weight	Number	Dry weight	C content	N content	Dry weight	Dry weight
	kg ha^{-1}	seeds m^{-2}	mg seed^{-1}	nodules plant^{-1}	mg plant^{-1}	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}	kg ha^{-1}
C. Simão 1	3278 *f*	1810 *e*	181 *bcd*	65 *bcd*	149 *de*	529 *c*	49 *d*	1230 *c*	270 *c*
C. Simão 2	3978 *e*	2366 *d*	168 *e*	27 *e*	140 *e*	762 *bc*	68 *cd*	1830 *bc*	390 *abc*
Taguá 1	5336 *cd*	2936 *bc*	182 *bc*	95 *a*	246 *abc*	1105 *ab*	129 *a*	2460 *ab*	390 *abc*
Taguá 2	5426 *c*	3097 *ab*	175 *d*	32 *e*	174 *cde*	901 *abc*	85 *abcd*	2340 *ab*	450 *ab*
Pinhão 1	4952 *d*	2713 *c*	183 *bc*	98 *a*	333 *a*	1034 *ab*	100 *abc*	2430 *ab*	330 *bc*
Pinhão 2	5901 *ab*	3201 *a*	184 *bc*	49 *cde*	195 *bcde*	854 *abc*	80 *bcd*	2160 *bc*	510 *a*
Candói 1	5491 *bc*	3073 *ab*	179 *cd*	68 *bc*	232 *bcd*	971 *ab*	97 *abc*	2340 *ab*	270 *c*
Candói 2	6045 *a*	3237 *a*	187 *ab*	33 *e*	148 *de*	1100 *ab*	111 *abc*	2760 *ab*	360 *abc*
Guarapuava 1	5289 *cd*	2861 *bc*	185 *b*	85 *ab*	266 *ab*	1056 *ab*	120 *ab*	2490 *ab*	360 *abc*
Guarapuava 2	6272 *a*	3277 *a*	192 *a*	38 *de*	192 *bcde*	1186 *a*	109 *abc*	3150 *a*	420 *abc*
Control	5219	2869	182	64	222	975	99	2370	390
Inoculation	5183	2848	182	59	208	938	93	2280	360
Inoculation (I)	ns	ns	ns	ns	ns	ns	ns	ns	ns
Trial (T)	***	***	***	***	***	***	***	***	***
I × T	ns	ns	ns	ns	ns	ns	ns	ns	ns

Trials succeed by number 1 and 2 were conducted in 2015/2016 and 2016/2017 growing seasons, respectively.
Means with different letters within columns differ by the Tukey's test at $p \leq 0.05$.
Levels of significance: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns: not significant.

Brazilian studies, in Paraná and Mato Grosso do Sul States, showed increases in yield with inoculation in fields with a history of soybeans cultivation (BRANDÃO JUNIOR; HUNGRIA, 2000; MERCANTE et al., 2002, 2011). In the US, Schulz and Thelen (2008) in 14-site/years and Leggett et al. (2017) analyzed data from 187 trials and also observed response to inoculation and recommended application of this practice annually. However, economic success of inoculant applied to soybeans is linked to a response to inoculation. Consequently, a high economic return is expected to inoculation when soil rhizobia population is low, on the other hand when rhizobia population is high, a low or no economic return is expected (THIES; SINGLETON; BOHLOOL, 1991). Because of this, De Bruin et al. (2010) performed 73 trials in a US Midwest region between 2000 and 2008 to evaluate soybean yield response to inoculants and concluded the probability of economic return with inoculation was less than 50% for that region, and did not warrant annual inoculation. Nonetheless, research efforts should continue to investigate the rhizobia residence time and population after several years of continuous cropping without inoculation, mainly in no-till systems that promote less abiotic stresses to rhizobia in soil due to more suitable moisture and temperature conditions (HUNGRIA; VARGAS, 2000).

Lack of yield response to inoculation could be potentially linked to a high number and more efficient naturalized rhizobia strains, lack of severe stress conditions (e.g., drought, heat, low pH, and unbalanced nutrition) that could affect response to inoculation (HUNGRIA; MENDES, 2015), and high soil capacity to provide N (MAPOPE; DAKORA, 2016). As related to the last potential cause, high soil N supply could inhibit BNF, as an impediment of root infection by rhizobium, lower nodule growth and inhibition of nitrogenase activity (STREETER; WONG, 1988).

Variation in nodule number and dry weight were observed among the ten sites ($p<0.001$; Table 5). Stepwise multiple regression analysis for nodule number explained 89% ($p<0.001$) of the variation on this trait related to changes in soil cation exchange capacity (CEC), phosphorous (P) and potassium (K) supply, while the model adjusted for nodule dry weight explained 34% ($p<0.01$) of the variability due to soil P supply (Table 6). Among these variables, CEC negatively influenced nodule number, but this should be carefully examined due to the narrow variation observed in CEC, 11.9 to 19.2 $\text{cmol}_c\ \text{dm}^{-3}$ (Table 2).

Overall, with broader ranges of CEC (<10 to >40 $cmol_c\ dm^{-3}$) in soils, lower inhibition might be expected in soils with high CEC due to the higher potential in ammonium adsorption by the soil particles (NOMMIK; VAHTRAS, 1982), slowly releasing N and diminishing the negative effect of inorganic N on nodulation and BNF processes (SATO et al., 2011).

Table 6. Linear regression model for number and dry weight of nodules as affected by soil variables.

Explanatory variable	Regression coefficient	Standard error	*p*-value	R^2 (model)
Nodule number (nodules $plant^{-1}$)				0.89
Intercept	192.54	18.69	<0.001	
CEC	-11.04	1.43	<0.001	
P	2.07	0.40	<0.001	
K	0.17	0.05	0.003	
Nodule dry weight (mg $plant^{-1}$)				0.34
Intercept	160.38	24.14	<0.001	
P	7.68	2.52	0.007	

The positive effect of P on nodule number and dry weight and K on nodule number can be explained by the studies from Divito and Sadras (2014) on P, K, and sulfur starvation on root nodulation and BNF. Authors suggested that P and K deprivation reduced both nutrient concentrations in plants, while N concentration remains constant increasing N:P and N:K ratios. These changes in nutrient stoichiometry have been proposed to activate an N-feedback signaling, regulating nodule development and activity in the root. Thus, this process results in less nodulation with lower soil P and K concentrations.

Despite the differences in nodulation among the environments, good root nodulation was detected in both treatments at all locations, without presenting significant differences in nodule number per plant, nodule dry weight and N content between control and inoculated treatments ($p>0.05$; Table 5). Therefore, the hypothesis that root nodulation and nodule growth reduction or BNF inhibition by high N availability or by abiotic stresses can be rejected in this study. The main hypothesis to explain these results is still based on the fact that indigenous rhizobia strains were able to infect soybean roots and fix atmospheric N (CAMPOS; HUNGRIA; TEDESCO, 2001; MENDES; VARGAS; HUNGRIA, 2000), reducing the probability of soybean seed yield response to inoculation (DE BRUIN et al., 2010).

The next challenges to researchers are to establish more consistent relations between BNF and soil and meteorological variables. Likewise, the determination of the residence time without losing the efficiency in native rhizobia strains after years of no soybeans grown and/or inoculation will help to identify areas with a history of soybean cultivation where this practice will be more effective. Lastly, but not least, a better understanding of the *Bradyrhizobium* strains should be pursued, using the same strains Guimarães et al. (2008) used when they found differences in their ability to fix N when investigated using the same soybean variety (cv. Celeste). Likewise, Pauferro et al. (2010) found that the “B value” for soybeans was primarily governed by the rhizobium strain more than the effect of the variety. The B value is defined as the difference between the ^{15}N natural abundance of the legume plant grown entirely on BNF (OKITO et al., 2004). Future studies should consider studying the differential ability of rhizobium strains to fix N to find the specific strain-variety combination to optimize BNF process and attain maximum soybean seed yields.

4. Conclusion

Soybean seed yield response to inoculation was inconsistent in Oxisols under no-till in 21 field trials at the South-central region of Paraná State (Brazil), regardless of the type of seed inoculant tested (solid or liquid). In addition, lack of differences in plant growth, nodulation, and N parameters were all documented from the second set of 10 field trials. In this study, P was beneficial for nodule number and dry weight, while K was positive to nodule number. The high number and dry weight of nodules observed even under the control treatment indicates that successfully established rhizobia strains population in the soil were as efficient as the ones introduced via the inoculation practice. Future research should focus on identifying the persistence of rhizobia in the soil and its efficacy at N fixation after continuous cropping without inoculation.

CHAPTER 4 – Environmental variables controlling biological nitrogen fixation soybeans in no-till fields in Southern Brazil

1. Introduction

The increasing global demand for food and concerns about environmental protection have been pushing forstrategies that increase agricultural production as sustainably as possible. Biological nitrogen fixation (BNF) by legumes supports sustainability of food production by meeting the high demand of N by these crops and reducing the need for nitrogen fertilizers (HUNGRIA; MENDES, 2015). Therefore, strategies to raise high soybean [*Glycine max* (L.) Merril] yields and production in a more sustainable way must focus in adjusting agricultural management practices to enhance BNF. Nitrogen derived from the air (Ndfa, which signifies the proportion of N supplied through BNF) represents ca. 60% of the total N required for soybean production (CIAMPITTI; SALVAGIOTTI, 2018; SALVAGIOTTI et al., 2008), differing among top producing countries (Herridge et al., 2008) mostly due to soil conditions and management practices. The soils of soybeans growing areas in US are generally able to provide a great amount of N to plants, reducing contribution of Ndfa (ca. 50%) on supply N (HERRIDGE; PEOPLES; BODDEY, 2008). On the other hand, less N amount is usually available in Brazilian soils of productive areas, where farmers commonly use seed inoculation, no-tillage systems, and avoid appling N fertilizer to soybeans, increasing contribution of Ndfa (ca. 80%) on soybean N nutrition (HERRIDGE; PEOPLES; BODDEY, 2008; HUNGRIA et al., 2005).

Regional studies developed in Brazil confirm the high contribution of Ndfa (69-94%) to supply N for soybeans (ALVES et al., 2006; HUNGRIA et al., 2005, 2006). However, most soils of those regions usually have a low capacity to release mineral N to crops as in other regions with higher soil N or organic matter content where Ndfa decreases significantly its importance as a source of N to the crop (MAPOPE; DAKORA, 2016). The proportion of N obtained from N_2 fixation or soil depends on many factors including soil, weather, and their interaction. For instance, soil acidity can reduce soil rhizobia population by aluminum toxicity (Ferguson and Gresshoff 2016) and also low pH conditions might disrupt the signal between plant and rhizobia (FERGUSON; LIN; GRESSHOFF, 2013). Limited phosphorus (P), potassium (K) and sulfur (S) availability decreases, directly or indirectly, nodule growth (DIVITO; SADRAS, 2014) and high mineral N availability reduces Ndfa by inhibiting root infection by rhizobia, reducing nodule growth and nitrogenase activity (KANAYAMA; WATANABE; YAMAMOTO, 1990; STREETER; WONG, 1988). Other climate factors such as soil water-deficit conditions (SINCLAIR; NOGUEIRA, 2018) or lower mean air temperature can reduce photosynthesis levels, indirectly decreasing Ndfa (GEORGE; SINGLETON; BEN BOHLOOL, 1988).

Climate change is pushing and changing features of the agricultural landscape in many parts of the world and, thus, crop adaptability. Moreover, the genetic interplay between the bacteria strain, host, and their interaction mediated by the environment makes it challenging to find more effective strategies for plant breeding, which has given minor consideration to BNF-associated traits in soybeans (SINCLAIR; NOGUEIRA, 2018). A possible breeding strategy would be exploit the interactions of BNF and plant traits with the environment by clustering geographical regions with similar responses to BNF and potential for future crop adaptation. Likewise, regions can be classified in the way it influences crop responses to the amount of N fixed. Variations in soil and weather conditions, even in the same region, should also be considered.

The complex interaction between BNF and multiple environmental factors is scarce in literature with a small number of studies focused on this approach. The goal of this study was to delimitate an important geographical region for soybean production in Brazil based on Ndfa performance by extensively characterizing climate, plant, and BNF traits to find discriminant variables that would help to understand crop performance. Results of this study will contribute to implementing strategies to increase contribution of BNF on N supply to soybeans and could even encourage studies of plant breeding based on environmental factors as selection strategies.

2. Material and Methods

2.1. Sites description

Twenty-four sites were selected to determine Ndfa and N-fixed (representing the proportion and the amount, in kg ha^{-1}, of N supplied through the BNF, respectively) in the Center-south region of Paraná State, Southern Brazil (Fig. 4). Regional climate is a humid temperate climate with a moderately hot summer (Cfb), according to the Köppen classification (APARECIDO et al., 2016), without a dry season. Cumulative rainfall, mean relative humidity, mean air temperature and thermal time varied from 628 to 1711 mm, 79 to 88%, 19.2 to 22.7 °C and 1616 to 2248 °Cd during the soybean growth cycle, in 2017/2018, respectively (Table 7). Sites altitude ranged from 499 and 1120 m above sea level (Table 8). Soils in all fields were classified as Hapludox (SOIL SURVEY STAFF, 2014) and were conducted in a no-till system. Soil fertilization, except for N, was made at soybean sowing (FONTOURA et al., 2015). Values of pH, clay content, soil organic carbon, soil total N, available P, exchangeable Ca, and available manganese (Mn) in the soil layer 0-20 cm determined at R5 development stage (beginning seed – FEHR; CAVINESS, 1977) are presented in Table 8. The entire set of variables is in Appendix 2.

Individual experimental plots at each site had six rows spaced at 0.40 m and 10 m long. Sowing dates ranged from September 26th to November 12th and plots were harvested from February 28th to April 15th to determine final seed yield (130 g kg^{-1} moisture content).

Soybean growth cycle varied from 131 to 177 days. Different soybean varieties and maturity groups were used among the sites (Table 8). Plant population (determined at R5 development stage) ranged from 23 to 41 plants m^{-2}. Each plot followed recommended phytosanitary treatments. Commercial inoculants containing *Bradyrhizobium elkanii* (SEMIA 5019) and *B. japonicum* (SEMIA 5079) strains were applied on seeds according to the Brazilian recommendation (100 mL liquid inoculant per 50 kg of seeds).

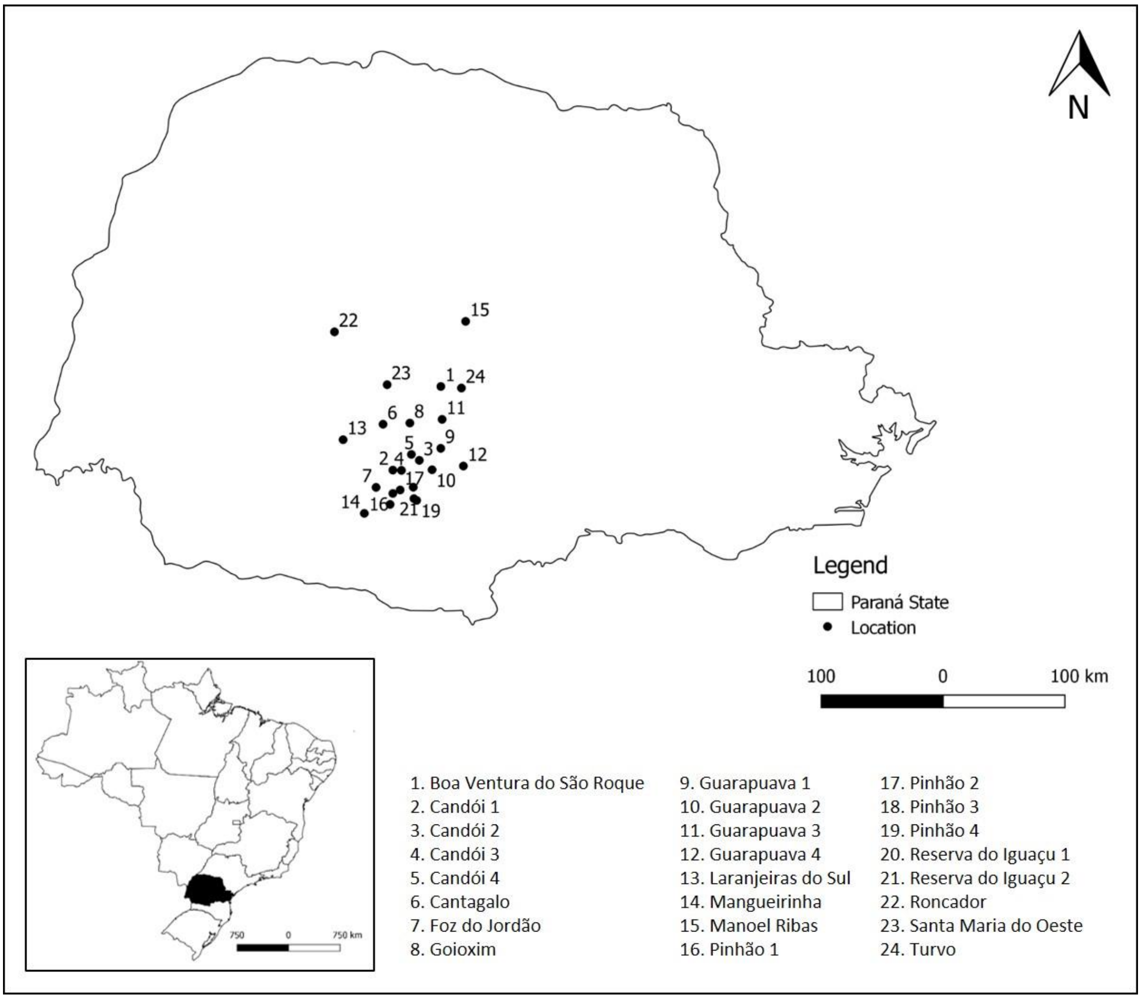

Figure 4. Position of Paraná State (in black) on Brazil map (inset), and distribution of the 24 sites used to determine biological nitrogen fixation in the Center-south region of Paraná, Southern Brazil (main figure).

Table 7. Meteorological variables during soybean growth cycle in the Center-south region of Paraná, Southern Brazil.

Variable	Period					
	S – R1	R1 – HV	S – R5	R5 – HV	R1 – R5	S – HV
Cumulative rainfall (mm)	585 (244–1227)	463 (202–776)	819 (395–1403)	229 (79–377)	234 (51–400)	1048 (628–1711)
Mean relative humidity (%)	79 (74–83)	86 (81–95)	81 (77–84)	86 (79–99)	88 (71–94)	83 (79–88)
Mean air temperature (°C)	20.1 (18.7–22.1)	20.9 (19.6–23.0)	20.3 (19.0–22.6)	20.8 (16.5–22.8)	20.6 (19.3–23.2)	20.5 (19.2–22.7)
Thermal time (°Cd)	827 (636–983)	1043 (804–1480)	1239 (1040–1440)	631 (355–938)	425 (178–664)	1858 (1616–2248)

Numbers outside and inside the parentheses correspond to average and minimum–maximum values of the variables in each period, respectively.
S: sowing; R1: beginning flowering; R5: beginning seed filling; HV: harvest.

Table 8. Soybean varieties and maturity groups, altitude, mean air temperature from R1 to R5, and soil variables in the upper 20 cm layer in the 24 sites of the Center-south region of Paraná, Southern Brazil.

Site	Soybean variety (maturity group)	Altitude	Mean air temperature (R1 – R5)	Soil						
				pH	Clay	Org. C	Total N	Available P	Exchangeable Ca	Available Mn
		m	°C		g kg^{-1}	g kg^{-1}		mg dm^{-3}	$cmol_c$ dm^{-3}	mg dm^{-3}
1	BMX Apolo RR (5.5)	1029	19.3	5.8	340	46.2	3.5	9.4	8.5	19
2	BMX Ativa RR (5.6)	944	20.7	5.9	340	38.4	2.5	7.6	7.5	4
3	K5616 (5.6)	958	20.5	5.7	340	43.0	2.6	5.0	7.2	5
4	M5917 IPRO (5.9)	917	20.2	6.8	280	41.1	2.4	7.2	10.8	1
5	BMX Apolo RR (5.5)	991	19.5	6.5	220	42.9	2.9	6.1	9.8	4
6	BMX Apolo RR (5.5)	889	21.1	5.8	540	32.5	2.3	3.3	8.1	8
7	BMX Elite IPRO (5.5)	833	20.0	5.2	340	50.2	3.3	2.5	4.5	17
8	BS 2606 IPRO (6.0)	961	20.8	5.7	280	43.6	2.8	11.0	7.4	9
9	BMX Apolo RR (5.5)	989	20.7	5.7	400	36.1	2.5	12.0	6.3	10
10	K5616 (5.6)	981	19.8	6.3	340	35.5	2.2	7.9	9.8	4
11	K6221 (6.2)	1069	20.4	5.6	280	43.1	2.9	13.0	6.6	16
12	BMX Apolo RR (5.5)	1120	19.5	6.1	340	37.3	2.5	11.0	7.9	4
13	K5616 (5.6)	827	21.0	5.6	540	36.1	2.7	6.7	6.8	19
14	DM 54i52 RSF IPRO (5.4)	813	21.5	6.1	470	29.7	2.3	6.6	8.5	8
15	BMX Vanguarda IPRO (6.0)	499	23.2	5.0	470	21.1	1.8	16.0	4.7	34
16	Roos Camino RR (5.3)	874	20.8	5.6	470	32.3	2.2	10.0	8.3	24
17	K5616 (5.6)	876	20.7	5.8	470	42.1	2.6	6.9	7.7	7
18	BMX Elite IPRO (5.5)	930	20.7	6.2	220	35.4	2.4	8.0	8.3	6
19	BMX Lança IPRO (5.8)	1045	19.9	6.1	470	37.5	2.7	16.0	8.7	8
20	BMX Apolo RR (5.5)	957	20.5	6.0	400	39.6	2.6	7.5	10.1	6
21	BMX Ativa RR (5.6)	1012	20.6	5.6	360	38.1	2.3	8.5	7.7	10
22	Roos Camino RR (5.3)	725	22.4	5.6	>600	33.6	2.5	6.1	6.9	40
23	BMX Ativa RR (5.6)	817	21.2	6.0	>600	35.1	2.8	10.0	7.9	46
24	K5616 (5.6)	1048	20.4	7.3	340	48.4	2.7	11.0	13.8	2

2.2. Determination of biological nitrogen fixation

At R5 growth stage, soybean plants of three subsamples measuring one linear meter each were cut at ground level (stem + leaves) in all the plots. At the same time, three different non-N_2-fixing species were sampled close to the plots to be used as reference plants to determine Ndfa and N-fixed (Table 9). Biomass samples were dried at 65 °C in a forced air oven until they reached a constant weight to determine shoot dry weight and N concentration in the shoot (Thermo Fisher Scientific CN Analyzer – Flash 200 model). Total N content in the shoot was calculated by multiplying the dry weight and N concentration.

Biological nitrogen fixation was determined by ^{15}N natural abundance method (SHEARER; KOHL, 1986). For this, soybean and reference plants samples were used in determining ^{15}N abundance using an automated continuous-flow isotope-ratio mass spectrometer (Delta V Advantage coupled to a Flash 2000 total C and N analyzer - Thermo Fisher Scientific, Waltham, USA). The percentage of N derived from the air (Ndfa) was calculated as:

$$Ndfa\ (\%) = [(\delta\ ^{15}N_{ref} - \delta\ ^{15}N_{soy}) / (\delta\ ^{15}N_{ref} - B\ value)] \times 100 \qquad Eq.\ (1)$$

where: $\delta\ ^{15}N_{ref}$ is the average shoot ^{15}N natural abundance of three non-N_2-fixing reference plants, $\delta\ ^{15}N_{soy}$ is the soybean shoot ^{15}N natural abundance at R5, and B value is the ^{15}N natural abundance in the soybean that relies only on BNF. $\delta\ ^{15}N_{reference}$ and $\delta\ ^{15}N_{soybean}$ are in Table 9. B value used was -2.62 ‰, the average of B values obtained by Guimarães et al. (2008) and Pauferro et al. (2010) for different Brazilian soybeans varieties and inoculants, composed of *Bradyrhizobium elkanii* and *B. japonicum* strains. Total aboveground N-fixed (kg ha^{-1}) was calculated by multiplying soybean shoot N content (kg ha^{-1}) and Ndfa (%)/100. The N derived from the soil was calculated as a difference between total shoot N and N-fixed.

Table 9. Species of reference plants and $\delta^{15}N$ values of reference and soybean plants used to determine biological nitrogen fixation in the 24 sites of the Center-south region of Paraná, Southern Brazil.

Site	Reference plants			$\delta\ ^{15}N$ values (‰)				
	Species 1	Species 2	Species 3	Ref. 1	Ref. 2	Ref. 3	Ref. average[(1)]	Soybean
1	*Eleusine indica* (L.) Gaertn	*Avena sativa* L.	*Bidens pilosa* L.	5.367	3.988	2.734	4.030	-0.063
2	*Eleusine indica* (L.) Gaertn	*Conyza bonariensis* (L.) Cronquist	*Euphorbia heterophylla* L.	8.284	3.398	3.154	4.945	0.605
3	*Eleusine indica* (L.) Gaertn	*Brachiaria plantaginea* (Link) Hitchc.	*Sonchus oleraceus* L.	8.103	8.560	6.977	7.880	-0.183
4	*Sida rhombifolia* L.	*Brachiaria plantaginea* (Link) Hitchc.	*Euphorbia heterophylla* L.	8.693	7.431	8.095	8.073	0.769
5	*Eleusine indica* (L.) Gaertn	*Ipomoea* sp.	-	6.742	3.601	-	5.172	0.439
6	*Eleusine indica* (L.) Gaertn	*Brachiaria plantaginea* (Link) Hitchc.	*Spermacoce latifolia* Aubl.	3.172	3.478	2.811	3.154	-0.022
7	*Eleusine indica* (L.) Gaertn	*Brachiaria plantaginea* (Link) Hitchc.	*Ipomoea* sp.	3.369	3.569	4.414	3.784	1.935
8	*Bidens pilosa* L.	*Brachiaria plantaginea* (Link) Hitchc.	*Euphorbia heterophylla* L.	3.607	3.450	2.975	3.344	1.080
9	*Eleusine indica* (L.) Gaertn	*Brachiaria plantaginea* (Link) Hitchc.	*Zea mays* L.	6.718	5.454	5.554	5.909	0.035
10	*Eleusine indica* (L.) Gaertn	*Brachiaria plantaginea* (Link) Hitchc.	*Euphorbia heterophylla* L.	14.132	16.599	12.471	14.401	-0.157
11	*Euphorbia heterophylla* L.	*Brachiaria plantaginea* (Link) Hitchc.	*Digitaria horizontalis* Willd.	5.348	6.614	6.614	6.192	0.268
12	*Eleusine indica* (L.) Gaertn	*Avena sativa* L.	*Spermacoce latifolia* Aubl.	3.923	2.991	2.896	3.270	-0.364
13	*Sida rhombifolia* L.	*Avena strigosa* Schreb.	*Digitaria horizontalis* Willd.	3.699	6.355	4.884	4.979	0.438
14	*Eleusine indica* (L.) Gaertn	*Brachiaria plantaginea* (Link) Hitchc.	*Commelina benghalensis* L.	2.209	4.398	3.336	3.314	0.663
15	*Eleusine indica* (L.) Gaertn	*Conyza bonariensis* (L.) Cronquist	*Digitaria horizontalis* Willd.	5.949	5.006	6.543	5.833	0.969
16	*Eleusine indica* (L.) Gaertn	*Bidens pilosa* L.	*Euphorbia heterophylla* L.	7.524	4.396	6.597	6.172	0.287
17	*Eleusine indica* (L.) Gaertn	*Brachiaria plantaginea* (Link) Hitchc.	*Euphorbia heterophylla* L.	5.515	4.324	4.409	4.749	0.709
18	*Eleusine indica* (L.) Gaertn	*Brachiaria plantaginea* (Link) Hitchc.	*Euphorbia heterophylla* L.	3.447	3.011	2.574	3.011	0.298
19	*Eleusine indica* (L.) Gaertn	*Avena strigosa* Schreb.	*Ipomoea* sp.	13.748	11.559	12.674	12.660	0.921
20	*Eleusine indica* (L.) Gaertn	*Brachiaria plantaginea* (Link) Hitchc.	*Bidens pilosa* L.	5.740	5.975	6.018	5.911	1.234
21	*Eleusine indica* (L.) Gaertn	*Conyza bonariensis* (L.) Cronquist	*Digitaria horizontalis* Willd.	5.105	2.829	4.481	4.138	0.257
22	*Eleusine indica* (L.) Gaertn	*Brachiaria plantaginea* (Link) Hitchc.	*Euphorbia heterophylla* L.	4.747	4.448	3.018	4.071	0.327
23	*Eleusine indica* (L.) Gaertn	*Conyza bonariensis* (L.) Cronquist	*Digitaria horizontalis* Willd.	6.359	3.913	5.788	5.353	0.564
24	*Eleusine indica* (L.) Gaertn	*Sida rhombifolia* L.	*Digitaria horizontalis* Willd.	6.803	7.147	7.491	7.147	0.014

[(1)] Average of the three non-N_2-fixing plant species.

2.3. Statistical analysis

Data from 24 plots were analyzed by descriptive statistics as each plot represented one repetition. Mean, standard deviation, amplitude (maximum and minimum values) and interquartile range (IQR; 25 – 75% percentiles) were used to summarize the following variables: seed yield, shoot dry weight, N content in shoot, Ndfa, N-fixed and N derived from soil. The 24 sites were categorized according to Ndfa contribution (Table 4), as low (<44%, three sites), medium (44 – 72%, 16 sites) and high (>72%, five sites) Ndfa, according to the classification proposed by Ciampitti and Salvagiotti (2018). Then, a discriminant multivariate analysis was performed to categorize groups of low, medium and high Ndfa according to environments. Similarly, a permutational analysis of variance (PERMANOVA) was performed aiming to validate the groups separation according to the variables related to soil and weather conditions.

Discriminant multivariate analysis (DA) was used in an attempt to summarize the environmental differentiation between pre-defined groups, while overlooking within-group variation. To avoid autocorrelation between variables we used a multivariate analysis that relies on data transformation using principal component analysis (PCA) as a prior step to DA. This procedure is known as discriminant analysis of the principal components (DAPC) and is recommended when we have more columns (variables) than rows (observations) on the data. Set (JOMBART; AHMED, 2011). The DAPC does not necessary imply loss of information and it was conducted with the R “ade4” package (CHESSEL; DUFOUR; THIOULOUSE, 2004). The high number of predictors presented on the model (20 variables) makes its interpretation harder and increase the likelihood of autocorrelation issues. For that reason we run the analysis in two steps. The first step was aimed to select the most important variables explaining differences of Ndfa. On this step, we include the entire set variables (Appendix 2) in the analysis. The contribution of each variable when running the analysis with all the 20 predictors is documented in Appendix 3. The second step was aimed to explore the relationship between the selected variables and the contribution of each one of them on separating the pre-defined Ndfa groups. For the second step we select the first ten variables (~45% of the variables) that contributed the most on the canonical variables.

3. Results

3.1. Contribution of N_2 fixation to supply soybean N demand

Soybean seed yields averaged 4014 kg ha^{-1} and ranged from 3597 to 4825 kg ha^{-1} (IQR50, 3828 to 4189 kg ha^{-1}) for the 24 sites (Table 10). Mean shoot dry weight averaged 8.5 Mg ha^{-1} (ranging from 6.1 to 10.8 Mg ha^{-1}; IQR50, 7.4 to 9.3 Mg ha^{-1}). Mean shoot N content was 272 kg ha^{-1} across sites, ranging from 211 to 359 kg ha^{-1} (IQR50, 255 to 282 kg ha^{-1}). Contribution of N-fixed (measured at R5 growth stage) averaged 167 kg ha^{-1} and ranged from 62 to 274 kg ha^{-1} (IQR50, 141 to 190 kg ha^{-1}), while mean contribution as a proportion of total plant N uptake (Ndfa) was 61% but ranged from 24 to 85%. Mean soil N contribution was 106 kg ha^{-1}, ranging from 41 to 244 kg ha^{-1} (Table 10).

The entire data set (24 sites) was classified in three groups according to their Ndfa values: low (<44%), medium (44–72%) and high (>72%). The low Ndfa group presented lower average seed yield (3784 kg ha^{-1}) and N-fixed (95 kg ha^{-1}) than the medium (seed yield = 3975 kg ha^{-1}; N-fixed = 164 kg ha^{-1}) and high (seed yield = 4273 kg ha^{-1}; N-fixed = 218 kg ha^{-1}) Ndfa groups (Table 10). Consequently, soil contribution on total shoot N content was greater for the low Ndfa group (200 kg ha^{-1}) relative to the medium (101 kg ha^{-1}) and high (65 kg ha^{-1}) groups. Shoot dry weight and N content did not follow any trends according to the Ndfa groups (Table 10).

3.2. Environmental variables contributing to Ndfa variation

Likewise the above classification originally proposed by Ciampitti and Salvagiotti (2018), similar trends were obtained (separation in low, medium and high Ndfa groups) by executing a linear discriminant analysis (LDA) utilizing ten variables (seed yield, shoot N content, altitude, mean air temperature from R1 to R5, clay content, exchangeable Ca, available Mn and P, soil organic C and soil total N) (Fig. 5A). Ellipses obtained within each group in the LDA represent confidence regions regarding the means of canonical scores at a 95% confidence level. Seed yield, temperature from R1 to R5, soil total N, clay content and available P loaded the most on first discriminant axis (data not shown) and related specially to discrimination of low to high Ndfa groups. Shoot N content, altitude and exchangeable Ca, loaded the most on second discriminant axis (data not shown), and associated particularly to the categorization of low to medium Ndfa groups (Fig. 5A). However, low and medium groups of Ndfa overlapped on the first discriminant axis, and high Ndfa group overlapped on the second discriminant axis, especially with the medium Ndfa group (Fig. 5A), making the separation in three groups not so clear. Therefore, performing a PERMANOVA, another approach to separate the groups based in the same ten variables, a clear separation was found between the high group of Ndfa and the other two (Table 11).

Table 10. Descriptive statistics related to seed yield, shoot dry weight, N content in shoot, amount of N obtained from BNF (N-fixed), N derived from the air (Ndfa) and from soil (Ndfs) measured at R5 in soybean grown in 24 sites of Southern Brazil.

Variable	Mean	Std. Dev.	Maximum	75%	25%	Minimum
All data						
Seed yield (kg ha^{-1})	4014	296	4825	4189	3828	3597
Shoot dry weight at R5 (Mg ha^{-1})	8.5	1.4	10.8	9.3	7.4	6.1
N content in shoot at R5 (kg ha^{-1})	272	35	359	282	255	211
N-fixed at R5 (kg ha^{-1})	167	43	274	190	141	62
Ndfa at R5 (%)	61	14	85	71	54	24
N derived from soil at R5 (kg ha^{-1})	106	46	244	123	78	41
Ndfs at R5 (%)	39	14	76	46	29	15
Low Ndfa (<44%)						
Seed yield (kg ha^{-1})	3784	218	4033	4033	3626	3626
Shoot dry weight at R5 (Mg ha^{-1})	9.9	0.8	10.8	10.8	9.1	9.1
N content in shoot at R5 (kg ha^{-1})	296	55	359	359	260	260
N-fixed at R5 (kg ha^{-1})	95	29	115	115	62	62
Ndfa at R5 (%)	32	8	41	41	24	24
N derived from soil at R5 (kg ha^{-1})	200	43	244	244	159	159
Ndfs at R5 (%)	68	8	76	76	59	59
Medium Ndfa (44-72%)						
Seed yield (kg ha^{-1})	3975	251	4491	4141	3825	3597
Shoot dry weight at R5 (Mg ha^{-1})	8.2	1.3	10.7	9.0	7.0	6.1
N content in shoot at R5 (kg ha^{-1})	265	27	329	282	252	211
N-fixed at R5 (kg ha^{-1})	164	20	199	178	153	127
Ndfa at R5 (%)	62	7	71	68	56	52
N derived from soil at R5 (kg ha^{-1})	101	24	158	117	81	71
Ndfs at R5 (%)	38	7	48	44	32	29
High Ndfa (>72%)						
Seed yield (kg ha^{-1})	4273	338	4825	4576	4005	3983
Shoot dry weight at R5 (Mg ha^{-1})	8.4	1.5	10.7	9.5	7.2	6.6
N content in shoot at R5 (kg ha^{-1})	283	43	350	316	251	228
N-fixed at R5 (kg ha^{-1})	218	41	274	257	183	163
Ndfa at R5 (%)	77	6	85	82	72	72
N derived from soil at R5 (kg ha^{-1})	65	15	79	77	52	41
Ndfs at R5 (%)	23	6	28	28	18	15

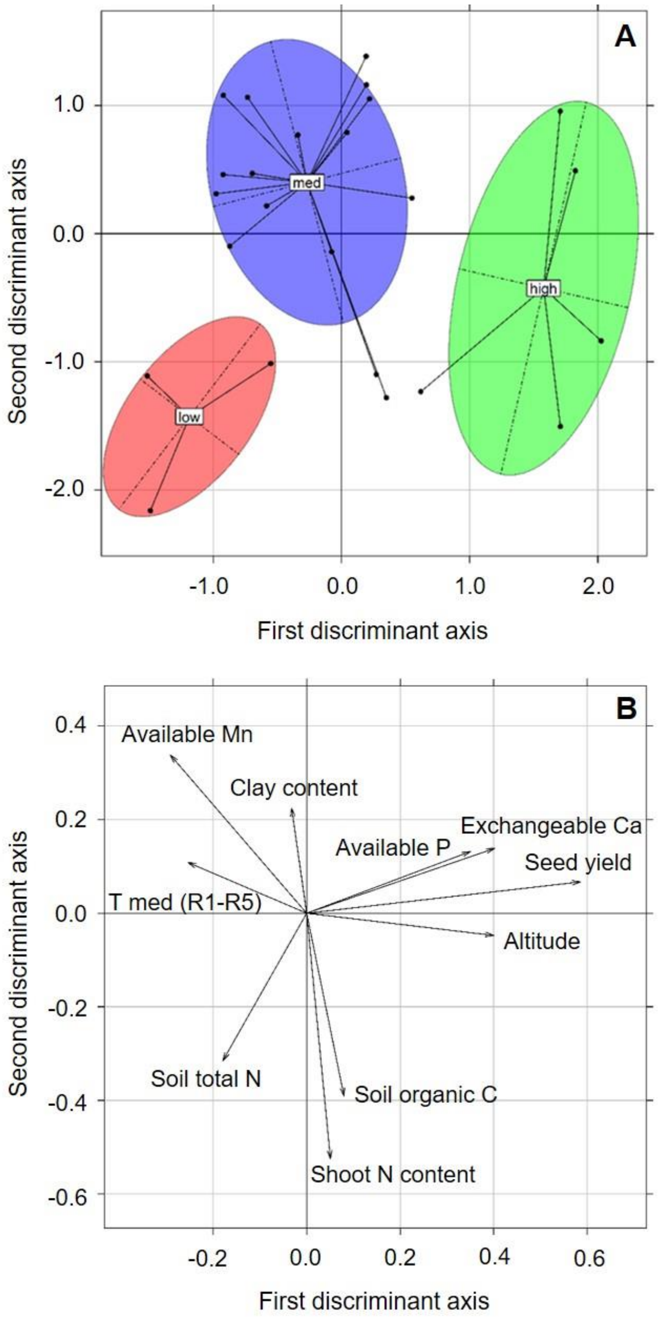

Figure 5. Canonical scores from linear discriminant analysis (LDA; A); and correlations between variables and canonical scores (B). Orange, blue and green ellipses represent low, medium and high Ndfa categories, respectively.

Table 11. Comparison between groups of nitrogen derived from the air (Ndfa) as affected by environmental variables using the PERMANOVA.

Comparison between groups of Ndfa	R^2	*p*-value
High vs. medium	0.45	0.048
High vs. low	0.18	0.049
Medium vs. low	0.07	0.306

The Ndfa groups were correlated to environmental variables presented in the canonical scores and discriminant analysis (Fig. 5B). The high Ndfa cluster was related in a similar direction to seed yield, altitude, exchangeable Ca, and available P; while the low Ndfa cluster was directly related to soil total N (Fig. 5). Less clear, but still important, for the medium Ndfa cluster, soil variables such as clay content and in a lower proportion available Mn, with a negative correlation for increases in soil total N – reducing contribution of the N fixation process as soil N supply increases. This analysis identified soil, plant and weather variables related to the Ndfa levels.

4. Discussion

4.1. Contribution of N_2 fixation to supply soybean N demand

Across all sites, average contribution of Ndfa to soybean N demand was 61%, similar to the mean value reported by Ciampitti and Salvagiotti (2018), but lower than the Ndfa value documented for Brazil, country-level (80%), by Herridge et al. (2008) and the Ndfa range recorded by researchers from 69 to 94% (ALVES et al., 2006; HUNGRIA et al., 2005, 2006). The reduced Ndfa proportion presented in this region is probably related to the high soil N availability documented by Fontoura and Bayer (2009). In addition, other factors such as no-till system (HUNGRIA; VARGAS, 2000), high soil fertility (DIVITO; SADRAS, 2014; FERGUSON; GRESSHOFF, 2016), absence of excess water (PURCELL; KING, 1996; SINCLAIR; SERRAJ, 1995) and/or heat stress (MUNÉVAR; WOLLUM II, 1981) may have contributed to an adequate growth and improve overall yields.

4.2. Environmental variables contributing to Ndfa variation

Environmental factors such as water supply, air temperature, light, salinity, and soil fertility are relevant for the symbiotic system in legumes (LIE, 1971). Impact of these factors (nutrient availability, soil acidity, water supply, and temperature) on N_2 fixation have been investigated as the individual effect (DIVITO; SADRAS, 2014; FERGUSON; GRESSHOFF, 2016; GATES; MÜLLER, 1979; MAPOPE; DAKORA, 2016; SCHIPANSKI; DRINKWATER; RUSSELLE, 2010; SINCLAIR et al., 2015; SINCLAIR; SERRAJ, 1995). Nonetheless, the analysis of the effect of multiple factors on N_2 fixation needs further investigation.

Thus, this research characterized 30 soil, plant, and weather variables (Appendix 2) in a regional approach (24 sites) for identifying factors related to Ndfa levels.

Among the environmental factors, soil total N was the most detrimental variable to Ndfa (Fig. 5B). High mineral N availability has a negative effect on the BNF process during nodule formation, by an impediment of root infection by rhizobium and by reducing nodule growth, and after nodule formation by inhibiting nitrogenase activity (KANAYAMA; WATANABE; YAMAMOTO, 1990; STREETER; WONG, 1988). Therefore, increasing N supply by soil reduces the contribution of N_2 fixation for legume nutrition (MAPOPE; DAKORA, 2016; SCHIPANSKI; DRINKWATER; RUSSELLE, 2010).

Detrimental effects to BNF are usually more pronounced in legumes under unbalanced nutritional conditions (DIVITO; SADRAS, 2014; GATES; MÜLLER, 1979; GATES; WILSON, 1974; LYND; ANSMAN, 1989), but increasing nutrient levels minimizes the negative impact of mineral N to N_2 fixation (TSAI et al., 1993). That might explain why exchangeable Ca and available P were related to the high Ndfa group and were opposed to the low group of Ndfa (Fig. 5B). Divito and Sadras (2014) observed that P deprivation reduced its concentrations in plants, while N concentration remains constant increasing N:P ratios. These changes in nutrient stoichiometry have been proposed to activate an N-feedback signaling, regulating nodule development and activity in the root. Thus, this process results in less nodulation with lower soil P concentrations. Phosphorus also has an important role on the plant energy metabolism, then, P deprivation has a negative impact on nodules energy status (OLIVERA et al., 2004). Regarding to Ca, its role in BNF is related the event called Ca spiking, a *nod* gene-dependent host response that triggers a signaling cascade leading to nodule development (EHRHARDT; WAIS; LONG, 1996; LÉVY et al., 2004; WAIS; KEATING; LONG, 2002). Thus, increasing the soil exchangeable Ca content (e.g. through liming) to the critical level is essential for obtaining a satisfactory root nodulation and high Ndfa contribution.

Altitude and mean air temperature from R1 to R5 were also related to the separation of Ndfa groups (Fig. 5). As altitude and air temperature are variables inversely proportional, higher altitudes were related to the high group of Ndfa and seed yield, mean air temperature was negatively correlated to those variables (Fig. 5B). Altitude ranged from 499 to 1120 m, leading to approximately

4 ºC of difference in mean air temperature from R1 to R5 between the coldest (19.3 ºC) and warmer (23.2 ºC) sites (Table 7 and 8). Therefore, it could be an unexpected relation between Ndfa and mean air temperature due to its small range. Small differences in mean air temperature (ca. 5 ºC) affecting Ndfa and seed yield were also reported by George et al. (1988), but with an opposite result to the found in this study. One may consider the increasing temperature effect on soil N mineralization (ELLERT; BETTANY, 1992; STANFORD; FRERE; SCHWANINGER, 1973), which could lead to a synergic effect with soil total N, increasing mineral N availability to soybean and reducing Ndfa. Nonetheless, the small range of mean air temperature from R1 to R5 (4 ºC) should not have great impact in the N mineralization. Therefore, it is more prudent to say that sites located in high altitudes also have low mean air temperature and high soil total N, which reduced Ndfa contribution.

As the soils of the studied region usually have a great capacity to supply N for crops due to predominance of soils with medium to high SOM content and soil total N (FONTOURA; BAYER, 2009), a low contribution of Ndfa and of N-fixed for soybean should be expected in this study (MAPOPE; DAKORA, 2016). However, the amount of N-fixed was higher in Center-south region of Paraná (167 kg ha^{-1} – Table 10) than the global average (136 kg ha^{-1}; CIAMPITTI; SALVAGIOTTI 2018). As worldwide N_2 fixation was determined in R6.5–R7, the difference between the results of this study and the worldwide average of N-fixed should be higher, considering we determined N-fixed in R5. Furthermore, high seed yields were related to high Ndfa (Fig. 5B; Appendix 4).

The main agricultural challenge for next decades is to feed the growing world population, hence, strategies are required to intensify crop production as sustainably as possible (FISCHER; CONNOR, 2018). These concerns about food security associated to the great correlation between Ndfa and seed yield found in this study (Fig. 5) and around the world (CIAMPITTI; SALVAGIOTTI, 2018) lead us to think that the right way to improve soybean seed yield as sustainably as possible is strategizing improvements to BNF.

5. Conclusion

The average proportion of N derived from the air (Ndfa) is 61% of the N supplied for soybean grown in Center-south region of Paraná State, Southern Brazil. This contribution is similar to the worldwide average and lower than the previous estimative for Brazil. In the studied region, among the set of 20 environmental variables, the variation in Ndfa is attributed mainly to the interaction of a few variables, especially the following related to soil fertility: soil total N (impacting negatively the Ndfa), and exchangeable Ca and available P (affecting Ndfa positively). Furthermore, seed yield and Ndfa are positively correlated, then, promoting BNF is a good strategy to improve soybean seed yield as sustainably as possible.

CHAPTER 5 – Assessing nitrogen limitation in inoculated soybeans grown in Southern Brazil

1. Introduction

Soybean [*Glycine max* (L.) Merril] is one of the most globally relevant field crop legume with a production of 341 million metric tons in 2017/2018 (USDA, 2019). Brazil accounts for one-third (119 million metric tons in 2017/2018) of the global soybean production, being the largest producer alongside of the United States (USDA, 2019). As a source of protein and oil for humans and animals, soybeans are a critical element for food security challenges. Increasing soybean seed yield within the existing acreage is a key to fill global food demands (FISCHER; CONNOR, 2018). Therefore, strategies to improve crop productivity at the farmer-scale should be further explored. Soybean yield potential is attained when a well-adapted variety is grown under ideal conditions, without water and nutrient limitations, and in absence of abiotic (light, salinity, heat, drought) or biotic (diseases, insects, weeds) stresses (EVANS, 1993).

Nitrogen (N) is one of the most important nutrients for soybeans, primarily acquired via two sources: biological nitrogen fixation (BNF) and mineral N derived from soil organic matter (SOM) mineralization. As the carbon requirements for mineral N assimilation (4 g C g^{-1} N) is lower than BNF (6-7 g C g^{-1} N) (KASCHUK et al., 2009), higher amounts of mineral N provided to soybeans decreases the BNF contribution (DADSON; ACQUAAH, 1984). Then, reducing a plausible yield-limitation caused by N in high yield environments (>6-7 Mg ha^{-1}) (SALVAGIOTTI et al., 2009) is a challenge, because increasing BNF might raise the energetic cost and could potentially penalize seed yield (TAMAGNO et al., 2018). On the other hand, applying N via fertilization might reduce soybean root nodulation and BNF process (KANAYAMA; WATANABE; YAMAMOTO, 1990; STREETER; WONG, 1988).

There is no consensus about N limitation in soybean and its relationship under different yield levels. For instance, Ray et al. (2006) found lack of yield response to the addition of external N at varying yield levels, whereas Cafaro La Menza et al. (2017) and Ortez et al. (2018) observed a response (but not consistent for the latter authors) to soybeans with an improved yield.. Lack of consistency on the yield response with the addition of external N to soybeans is clear from the recent investigations, and the evaluation of soil and N fixation parameters are critical not only to complement future research on this topic, but to better understand the factors affecting a potential yield response and exposing soybean to N limitations.

Connecting to N limitations, many studies investigated adding lower amounts of N fertilizer to soybeans early in the season via utilization of starter fertilizers (usually up to 40 kg N ha^{-1}). The rationale behind this practice is to supply low amounts of N early in the season – while nodules are not completely formed and N_2 fixation is not active (ABENDROTH; ELMORE; FERGUSON, 2006) and when N derived from mineralization can be scarce under low temperatures and/or low levels of soil organic matter (SOM) – N-deficient soils (DADSON; ACQUAAH, 1984). Results of those studies are contradictory, showing yield increases (e.g., OSBORNE; RIEDELL, 2006; BOROOMANDAN et al., 2009; GAI et al., 2017), and lack of yield response to N (e.g., HUNGRIA et al., 2006; MRKOVACKI et al., 2008; JOSIPOVIĆ et al., 2011; KAMARA et al., 2012; BALBINOT JUNIOR et al., 2016).

The objectives of this study were to quantify soybean yield response to external N addition by evaluating application of lower fertilizer N rates as starter N fertilization and by providing full-N to the crop in order to understand if seed yields were limited by N when grown in Southern Brazil.

2. Material and Methods

2.1. Field trials

Ten field trials were performed in five locations (Campina do Simão, Taguá, Pinhão, Candói and Guarapuava) in Center-south region of Paraná State, Brazil, during 2015/2016 and 2016/2017 cropping seasons (Table 12). All trials were conducted under no-till system, varying the time adoption from short-term (two years) in Campina do Simão site, to long-term, more than 10 years in Taguá and Pinhão sites, and 30 years in Candói and Guarapuava sites. All sites, with the exception of Campina do Simão, have a long history of soybean cultivation (more than 15 years). Regional climate is Cfb (humid temperate climate with moderately hot summer), according to Köppen classification, without a dry season (APARECIDO et al., 2016). Annual precipitation ranges from 1550 to 1800 mm, with occurrence of weekly rainfall during spring/summer, and annual mean temperature ranges from 16.5 to 18.5 °C, in average of 25 years (APARECIDO et al., 2016). Precipitation and temperature data for each site-year is shown in Appendix 1. Soils of the trials were classified as Hapludox (SOIL SURVEY STAFF, 2014). Across the years, the trials were conducted in the same farm at each site, but different locations within the farm; thus, each site-year were considered independent sites.

Six treatments were evaluated: a control (without N fertilization), four starter N fertilization rates (10, 20, 30, and 40 kg N ha^{-1} applied as urea (46% N) at sowing), and full-N fertilization (300 kg N ha^{-1} applied as urea in twice: 50% at sowing and 50% at R1 growth stage). Experiments were performed in completely randomized block design and factorial arrangement (N rate × location), with three or four repetitions. Plots consisted of eight planting rows spaced 40 cm apart and 5 m long.

Table 12. Geographical coordinates and characterization of 0-20 cm soil layer from field trials conducted in 2015/2016 and 2016/2017 growing seasons in Southern Brazil.

Sites	Latitude	Longitude	Mn	S	P	K	Ca	Mg	Al	H + Al	$CEC_{pH\ 7,0}$	Clay	SOM	V	pH H_2O
			mg dm^{-3}				$cmol_c\ dm^{-3}$					g kg^{-1}		%	
2015/2016 Growing Season															
C. Simão 1	25°03'57.83"S	51°50'01.98"W	32	85	1.5	47	4.5	3.8	0.0	4.4	12.8	470	41	66	5.6
Taguá 1	25°34'26.97"S	51°37'00.40"W	6	21	5.3	66	5.1	2.8	0.1	3.9	11.9	400	51	68	5.4
Pinhão 1	25°43'10.94"S	51°39'30.23"W	3	10	25.0	60	7.1	4.4	0.0	2.8	14.4	340	47	81	6.2
Candói 1	25°36'22.39"S	51°59'12.58"W	4	16	8.5	97	6.3	2.6	0.0	4.9	14.0	280	60	65	5.5
Guarapuava 1	25°32'51.07"S	51°29'50.83"W	4	12	6.5	194	6.6	2.8	0.0	3.1	13.0	340	46	76	5.8
2016/2017 Growing Season															
C. Simão 2	25°03'47.64"S	51°50'07.55"W	20	31	2.6	114	6.1	4.2	0.0	5.5	16.1	470	47	66	5.6
Taguá 2	25°34'17.16"S	51°37'05.82"W	7	13	2.1	98	7.1	4.1	0.0	5.5	16.9	400	53	68	5.7
Pinhão 2	25°39'59.69"S	51°42'51.09"W	4	16	4.0	288	8.3	5.3	0.0	4.9	19.2	340	52	75	5.8
Candói 2	25°26'38.70"S	51°54'32.81"W	3	13	8.4	147	8.1	3.6	0.0	5.5	17.5	280	57	69	5.7
Guarapuava 2	25°32'43.68"S	51°30'01.18"W	9	15	7.3	231	6.5	2.8	0.1	8.7	18.6	340	50	53	5.4

Mn: extracted by HCl 0.1 mol L^{-1}; S: extracted by $Ca(H_2PO_4)_2$ containing 500 mg P L^{-1}; P, and K: extracted by Mehlich-1; Ca, Mg, and Al: extracted by KCl 1 mol L^{-1}; clay content: determined by the pipette method; SOM – soil organic matter: determined by wet oxidation-redox titration (Walkley-Black) method; CEC – cation exchange capacity; V – base saturation.

For all trials, soybean ‘BMX Apolo RR’ (Don Mario 5.8i), indeterminant growth habit, was sown at 30 seeds m^{-2}. Liquid inoculant containing *Bradyrhizobium elkanii* (SEMIA 5019) + *B. japonicum* (SEMIA 5079) strains was applied at a rate of 100 mL per 50 kg seeds at all plots, except for the full-N treatment. Seeds received fungicide and insecticide treatments before sowing, which occurred between the end of October and mid-November. Soil samples were collected at sowing and their characterization (0-20 cm layer) is in Table 12. For all treatments, fertilization was managed as 250 kg ha^{-1} of 00-25-25 (N-P_2O_5-K_2O). Phytosanitary treatments were applied according to regional recommendations.

In the zero-N and starter N treatments, five plants per plot were collected at flowering (R1, FEHR; CAVINESS, 1977) growth stage and separated into root, shoot, and nodules. Roots were sampled using a shovel (inserted 15 cm from the main stem) to assess the 0-30 cm soil layer below the five plants collected. Samples were dried at 65 ºC until constant weight had been reached. In the first cropping season (2015/2016), nodule number and dry weight (DW) were analyzed in the entire root, while in second cropping season (2016/2017) those variables were obtained only from the crown root to facilitate the measurements. Nodule number and weight from the whole root in the second cropping season were estimated based on the data collected from the crown root according to equations fitted by Cardoso et al. (2009). Nitrogen concentration in the shoot was determined by the Thermo Fisher Scientific CN Analyzer (Flash 200 model), and N content in the shoot was calculated by considering the shoot DW. At harvesting, seed yield was determined and expressed as 130 g kg^{-1} moisture content.

2.2. Statistical analysis

Based on the objectives, data of each trial was divided in two data sets. On the first one, for starter N evaluation, the control treatment (zero-N) and the starter N rates treatments were analyzed. On the second, for N limitation study, zero-N and full-N were used. Data for both tests (starter N and N limitation) were submitted to analysis of variance. For starter N topic, blocks within site, and the interaction between site and treatment were considered as random factors. Means were compared by Tukey HSD using the lsmeans function (lsmeans R package; LENTH, 2016) at the 0.05 confidence level. For the N limitation test, a linear regression model was fitted between full-N and zero-N. In addition, the dataset was divided into terciles categorizing the sites in three yield levels according to the mean yield per site. Low (< 5000 kg ha^{-1}), medium (5000-6000 kg ha^{-1}), and high (> 6000 kg ha^{-1}) yield levels included three (Campina do Simão 1 and 2, and Pinhão 1), four (Taguá 1 and 2, Candói 1, and Guarapuava 1) and three (Pinhão 2, Candói 2, and Guarapuava 2) sites, respectively. Complementing the linear regression, the proportion of yield difference for full-N relative to zero-N was calculated for each yield level.

Regression models were developed between average yield (zero-N treatment) in each site and N derived from the air (Ndfa) and soil organic matter (SOM) aiming to understand if yield variations were related to the BNF and/or mineral N derived from SOM mineralization. The Ndfa measurements were obtained from studies conducted in the same sites during the 2017/18 season (Chapter 4). As soil type and weather characteristics were similar between years, and minor variation in Ndfa within a site across years is reported in the literature (ALVES et al., 2006), we think the Ndfa values can provide useful information in this analysis.

3. Results

3.1. Starter N fertilization

Soybean seed yields ranged from 3421 to 6137 kg ha^{-1} in average of all the ten sites, and with Guarapuava 2, Candói 2, and Pinhão 2 presented the highest seed yield and seed number (Table 13). Overall, Guarapuava 2 also had higher values of seed DW and shoot DW related to the other sites (Table 13). All the plant variables were generally lower in Campina do Simão 1 and 2 (Table 13). Regardless of potential trends in several factors, fertilizer N rates (10 to 40 kg N ha^{-1}) applied at sowing did not influence any of the variables analyzed ($p>0.05$; Table 13) relative to the control (no N added). Interaction effects (site × N rate) were observed for seed number, nodule number and shoot:root ratio (Table 13). However, the interaction effects were due to site differences, and not because of the N fertilization (treatment).

3.2. N limitation

Overall, full-N treatment increased seed yield in 236 kg ha^{-1} (from 5183 to 5419 kg ha^{-1}) related to zero-N ($p<0.0001$), which represents a yield increase of 4.6% across sites. Soybean yield for full-N versus zero-N relationships presented similar slopes ($p=0.12$) at varying yield levels (Fig. 6A), but differed only on the intercepts ($p=0.012$) of the adjusted model. When yields were evaluated in levels (low, medium, and high), a trend was observed for greater yield under full-N relative to the zero-N (Fig. 6B), with a larger separation on yield under low levels (7.2% yield difference for full-N vs. zero-N). Yield components were not affected by full-N fertilization in any of the yield environments ($p>0.05$; Table 14).

Seed yield in non-N-fertilized (zero-N) soybeans averaged 4068, 5384, and 6110 kg ha^{-1} in low, medium, and high yield levels. High yield levels were achieved through a combination of both high N fixation and a potential greater N contribution derived from N mineralization (Fig. 7). For N fixation, after 65% of Ndfa, seed yield tended to plateau, potentially emphasizing that N demand is not limited by this factor beyond that point (Fig. 7A). Above 5000 kg ha^{-1} for soybean seed yield, contribution of N derived from mineralization process seems to be a larger component of increasing yields and sustaining plant N demand (Fig. 7B).

In summary, starter N fertilization with small fertilizer N rates was not a useful practice aiming to increase soybean yields, potentially highlighting the absence of a N limitation early in the crop-growing season. For the full-N study, N limitation tended to be greater in low yields compared to medium-high yield levels, potentially connected with co-limitations on N demand coming from both N fixation and N mineralization processes.

Table 13. Seed yield and its components, root nodulation, N content, shoot and root growth at R1 stage of soybean fertilized with starter N in ten field trials conducted in 2015/2016 and 2016/2017 growing seasons, in Southern Brazil.

Treatment	Seed			Nodule		Shoot		Root	Shoot:root ratio
	Yield	Number	Dry weight	Number	Dry weight	N content	Dry weight	Dry weight	
	kg ha^{-1}	seeds m^{-2}	mg seed^{-1}	nodules plant^{-1}	mg plant^{-1}	kg ha^{-1}		kg ha^{-1}	
C. Simão 1	3421 *e*	1842 *h*	186 *b*	55 *b*	151 *de*	53 *d*	1200 *d*	270 *d*	4.8 *fg*
C. Simão 2	4004 *d*	2320 *g*	171 *d*	23 *d*	120 *e*	70 *cd*	1800 *c*	360 *cd*	5.3 *efg*
Taguá 1	5454 *b*	2939 *d*	186 *b*	81 *a*	206 *bc*	127 *a*	2520 *ab*	420 *bc*	6.2 *cde*
Taguá 2	5482 *b*	3106 *b*	176 *cd*	39 *c*	233 *b*	109 *ab*	3030 *a*	540 *a*	5.8 *def*
Pinhão 1	4853 *c*	2659 *f*	183 *b*	90 *a*	280 *a*	99 *abc*	2220 *bc*	330 *cd*	6.8 *bc*
Pinhão 2	5921 *a*	3201 *a*	185 *b*	44 *bc*	179 *cd*	76 *bcd*	2070 *bc*	450 *ab*	4.7 *g*
Candói 1	5491 *b*	3038 *c*	181 *bc*	79 *a*	227 *b*	101 *abc*	2280 *bc*	300 *d*	8.0 *a*
Candói 2	6044 *a*	3295 *a*	184 *b*	31 *cd*	115 *e*	109 *ab*	2610 *ab*	360 *cd*	7.5 *ab*
Guarapuava 1	5308 *b*	2876 *e*	185 *b*	88 *a*	239 *ab*	117 *a*	2520 *ab*	390 *bc*	6.4 *cd*
Guarapuava 2	6137 *a*	3182 *a*	193 *a*	30 *cd*	140 *de*	102 *abc*	3090 *a*	450 *ab*	6.8 *bc*
0 kg N ha^{-1}	5206	2846	181	57	202	93	2250	360	6.3
10 kg N ha^{-1}	5197	2822	184	53	188	86	2100	360	6.2
20 kg N ha^{-1}	5195	2821	184	56	191	98	2370	390	6.2
30 kg N ha^{-1}	5269	2876	183	58	191	104	2550	420	6.2
40 kg N ha^{-1}	5192	2863	182	56	173	101	2400	390	6.2
Site (S)	***	***	***	***	***	***	***	***	***
N rate (N)	ns	ns	ns	ns	ns	ns	ns	ns	ns
S × N	ns	**	ns	*	ns	ns	ns	ns	*

Means with different letters within columns differ by the Tukey's test at $p \leq 0.05$.
Levels of significance: ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; ns: not significant.

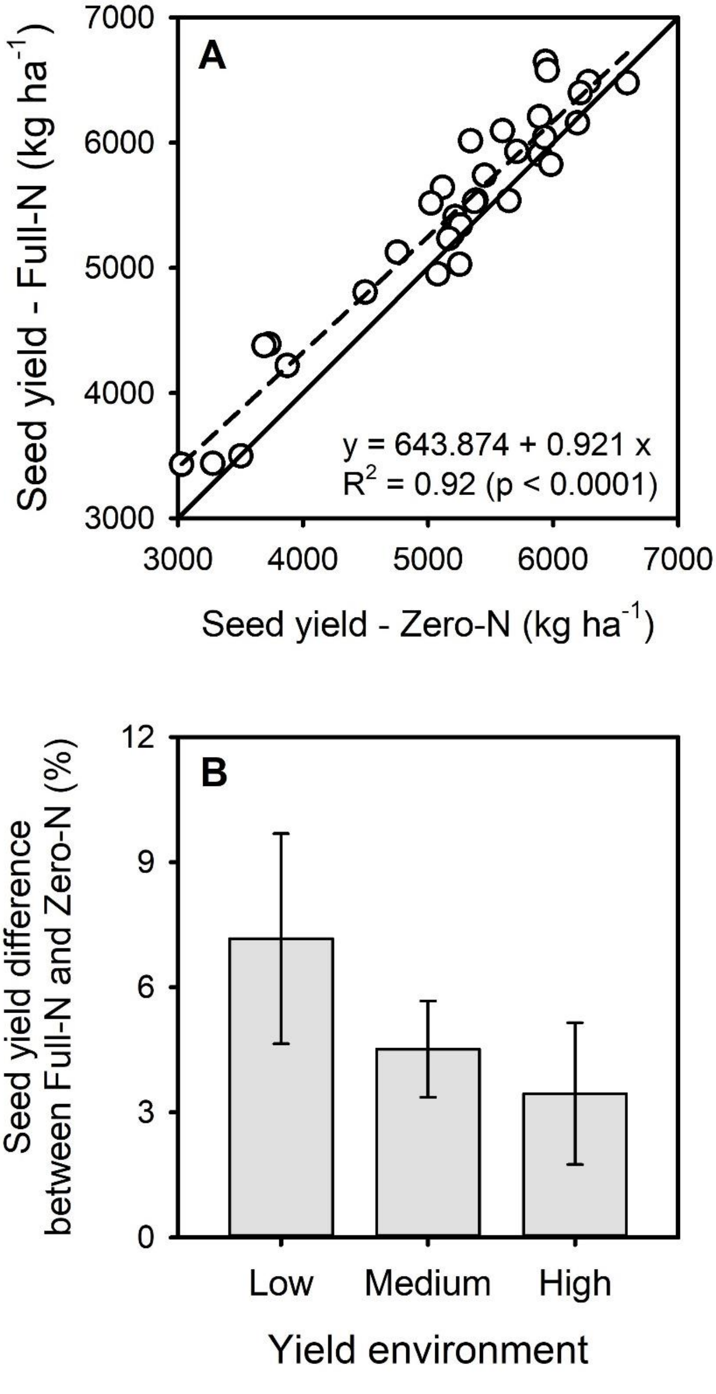

Figure 6. Seed yield in full-N vs. zero-N (A), and percentage of seed yield difference between both treatments for low (< 5000 kg ha^{-1}), medium (5000-6000 kg ha^{-1}), and high (> 6000 kg ha^{-1}) yield environments (B). In A, each data point represents a repetition of both treatments at all sites, and the diagonal solid line is a 1:1 line. In B, each bar represents the average of all sites for each yield environment. The absence of a letter means there was no significant difference by ANOVA ($p < 0.05$), and vertical lines are the standard error.

Table 14. Seed dry weight and number in full-N vs. zero-N for low, medium and high yield environment.

Yield environment	N rate	Seed number	Seed dry weight
		seeds m^{-2}	mg seed^{-1}
Low	Zero-N	2288 ns	178 ns
	Full-N	2394	182
Medium	Zero-N	2973 ns	181 ns
	Full-N	3097	182
High	Zero-N	3275 ns	187 ns
	Full-N	3370	187

ns: not significant by ANOVA ($p < 0.05$).

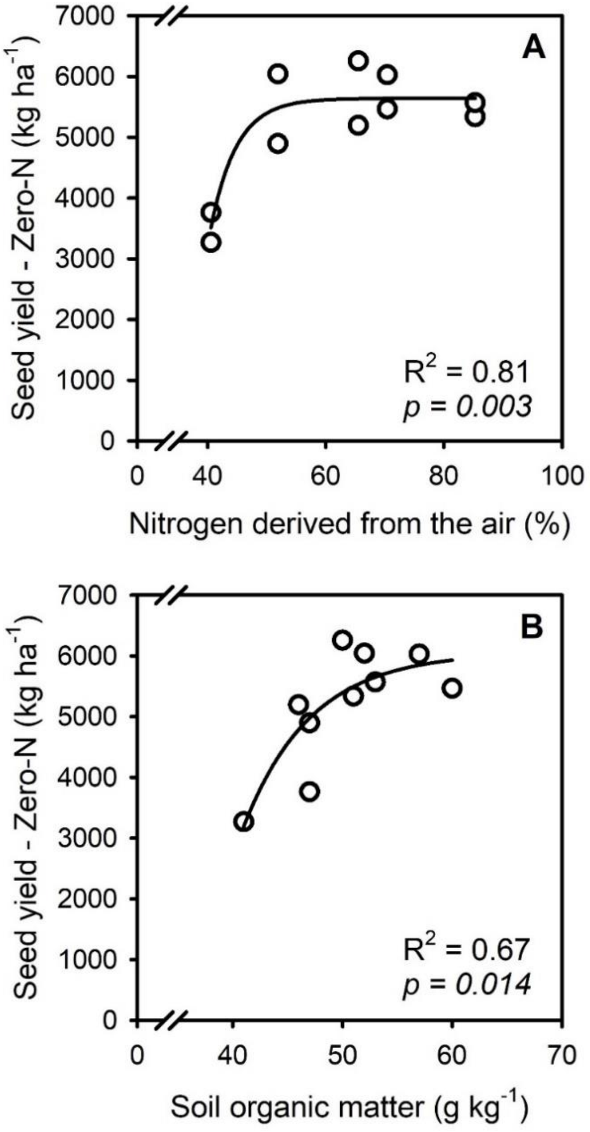

Figure 7. Relationship between seed yield in zero-N and nitrogen derived from the air (A) and soil organic matter (B). In both figures, each data point represents the average of every single site.

4. Discussion

4.1. Starter N fertilization

Soybean yield response to small N amounts should be expected only in N-deficient soils (DADSON; ACQUAAH, 1984), which is not the case of soils from the Center-south region of Paraná, Southern Brazil with high SOM (SOM > 41 g kg^{-1}; Table 12) (FONTOURA; BAYER, 2009). Lack of yield response to starter N is consistent with previous studies in Brazil (ARATANI et al., 2008; BALBINOT JUNIOR et al., 2016; HUNGRIA et al., 2006; JENDIROBA; CâMARA, 1994; MENDES; HUNGRIA; VARGAS, 2003) and around the world (HERRIDGE; BROCKWELL, 1988; JANAGARD; EBADI-SEGHERLOO, 2015; JOSIPOVIĆ et al., 2011; KAMARA et al., 2012; MRKOVAČKI; MARINKOVIĆ; AĆIMOVIĆ, 2008) with diverse soil and weather conditions.

4.2. N limitation

Interestingly, soybean yield response to full-N fertilization tended to be greater in low than medium-high yield environments (Fig. 6A, B), while based on previous studies, greater differences were expected in yield environments above 4500 kg ha^{-1} (SALVAGIOTTI et al., 2008). However, great N demand to sustain high seed yield is not the only issue driving N limitations in soybeans. For instance, our results showed that potential problems related to N supply via BNF (RAY; HEATHERLY; FRITSCHI, 2006) and/or soil mineral N availability (DADSON; ACQUAAH, 1984; SCHIPANSKI; DRINKWATER; RUSSELLE, 2010) are even more relevant and were not taken into consideration in previous investigations (CAFARO LA MENZA et al., 2017; ORTEZ et al., 2018).

As for the soybean yield limitations, the Center-south region of Paraná, Southern Brazil, usually does not have problems with water deficiencies and/or heat stresses (Appendix 1). Therefore, the main challenge for low-yielding soybean producers is adopting conservation management practices to increase SOM, such as no-till and crop rotation (BAYER et al., 2009; DIECKOW et al., 2005), providing adequate conditions for the BNF process (DIVITO; SADRAS, 2014; FERGUSON; GRESSHOFF, 2016). For high-yielding soybean producers, applying N fertilizers will impair BNF (KANAYAMA; WATANABE; YAMAMOTO, 1990; STREETER; WONG, 1988) and increasing N_2 fixation might come together with a rise on the energetic cost, which might penalize seed yield (TAMAGNO et al., 2018).

Feeding the growing world population is one of the greatest challenges for the next decades. Increasing crop productivity per unit area instead of opening new arable lands is one of agriculture's main challenges for the near future (FISCHER; CONNOR, 2018). However, applying high N amounts to attain the maximum yield potential is not environmentally profitable, hence, finding ways to increase BNF should be the main thought of soybean researchers in the next years. It is not our intention to recommend N fertilization to farmers.

5. Conclusion

The main key outcomes of this research were: i) starter N fertilization did not increase yields, potentially highlighting the absence of an early-season N limitation, and ii) N limitation tended to be greater in low-yield levels compared to medium-high yield levels, potentially connected with co-limitations on both N sources (N fixation and mineralization) to maintain soybean N demand. Producing soybeans in a sustainable manner will require focusing on production practices to conserve and, potentially, to increase in a long-term basis SOM and promote enhancing the BNF process for maintaining the large N demand required to achieve superior soybean yields. Future investigations should focus on obtaining a more complete characterization of soil, weather, and plant-related traits critical to improving the understanding of both N mineralization and N fixation processes.

CHAPTER 6 – Final discussion

The main agricultural challenge for the next decades is to feed the growing world population, and strategies are required to intensify crop production as sustainably as possible. Therefore, the target is raising crop yield by getting closer to the maximum yield potential instead of increasing acreage by opening new arable lands, but more than that it is desirable to use efficiently all the inputs in cropping to avoid environmental impacts (FISCHER; CONNOR, 2018). Thinking in this way, soybean has an advantage over other crops due to BNF, which allows its cultivation without applying N fertilizers. Despite that, there are still important questions about the nutritional N management of soybeans. For instance, although it is a low-cost agricultural practice, annual inoculation increases the operational time of soybean sowing management, leading many farmers to question whether this practice is even needed to ensure the establishment of root nodulation by rhizobium and, consequently, supplying the N required to obtain a satisfactory soybean seed yield. Results of Study 1 of this thesis showed inconsistent seed yield response to inoculation in 31 trials in the Center-south region of Paraná State (Brazil), regardless of the type of seed inoculant tested (solid or liquid). It was also found high number and dry weight of nodules even in non-inoculated soybeans, indicating that successfully established rhizobium strain populations in the soil were as efficient as the ones introduced via the inoculation practice. These results confirmed the hypothesis of lack of soybean response to inoculation in fields with inoculant application history.

Although re-inoculation did not increase soybean seed yield in the studied region, eventually stressing conditions (e.g., drought, hypoxia, heat) might reduce rhizobium population in the soil (HUNGRIA; MENDES, 2015; ZILLI et al., 2013), increasing the probability of soybean yield response to inoculation as a consequence of an insufficient symbiosis establishment (DE BRUIN et al., 2010; THIES; SINGLETON; BOHLOOL, 1991). However, the analysis of rhizobium population in the soil is not an agricultural practice; as well, it is unknown the rhizobium residence time in the soil after several years of continuous cropping without inoculation. Therefore, notwithstanding an inconsistent yield response to inoculation, as this practice does not represent a substantial increase in production costs, farmers should consider this input as an insurance, for potentially avoiding reduction of rhizobium soil population and thus ensuring adequate N supply for via BNF for attaining site-specific maximum soybean seed yields. Future research should consider studying the residence time without the efficiency loss in native rhizobium strains after years without soybeans grown and/or inoculated, helping to identify areas with a history of soybean cultivation in which this practice will be more effective. Likewise, looking at the differential ability of rhizobium strains to fix N and to find the specific strain-variety combination to optimize BNF process and attain maximum soybean seed yields would be helpful.

Furthermore, understanding how the complex interaction among environmental factors affect the contribution of N derived from the air (Ndfa – the proportion of N supplied through BNF) to soybean N nutrition might be helpful for the best use of resources in its cultivation. Factors related to climate conditions, such as water availability and temperature, are well documented in the literature as influencing BNF due to problems related to drought (PURCELL; KING, 1996; RAY; HEATHERLY; FRITSCHI, 2006; SINCLAIR; SERRAJ, 1995) and/or heat stresses (HUNGRIA; FRANCO, 1993; KEERIO; WILSON, 1998) in legumes grown in tropical and subtropical conditions. However, in the Center-south region of Paraná, Southern Brazil, that is not an issue due to mild temperatures and abundant rainfall frequently observed during the cropping season (Appendix 1 and 2). Consequently, findings from Study 2 showed that interaction among factors related to soil fertility were more important for Ndfa than variables related to climate conditions. For instance, soil total N was the most detrimental variable to Ndfa. However, increasing available P and exchangeable Ca minimized the negative impact of soil total N to N_2 fixation. Furthermore, seed yield was related to Ndfa. Therefore, improving soil fertility to benefit the BNF process is a key factor for increasing soybean yield in regions not affected by stresses related to climate conditions, like water deficiencies and high temperatures.

The average contribution of Ndfa to soybean N was 61%, lower than the previously country-level (80%) documented for Brazil (HERRIDGE; PEOPLES; BODDEY, 2008) and the Ndfa range recorded by researchers from 69 to 94% (ALVES et al., 2006; HUNGRIA et al., 2005, 2006), but similar to the worldwide mean value reported by Ciampitti and Salvagiotti (2018). The minor Ndfa found in this region compared to the whole country is probably related to the high soil N availability documented by Fontoura and Bayer (2009), while most soils cultivated with soybeans in Brazil have a low capacity to N supply for crops (HERRIDGE; PEOPLES; BODDEY, 2008). However, the contribution of N-fixed (measured at R5 growth stage) averaged 167 kg ha^{-1} and ranged from 62 to 274 kg ha^{-1} (IQR50, 141 to 190 kg ha^{-1}), which is higher than the worldwide average (CIAMPITTI; SALVAGIOTTI, 2018). Even the proportional contribution (Ndfa) of BNF in Center-south region of Paraná is lower than the Brazilian average; it was found that a great amount of N supplied (N-fixed, in kg ha^{-1}) to soybeans, allows this region to attain one of the highest average soybean seed yields of the country. The next step about this subject for the studied region is to determine the soybean N balance, calculated by the difference between the N amount exported by seeds and incorporated in the system through FBN. That will help us understand if soybeans grown in the edaphoclimatic conditions found in the Center-south of Paraná is depleting or incorporating N from the soil. Then, it will be possible to think about sustainable management strategies aimed at N reposition to the system in case of N depletion by soybeans.

Findings of Study 3 also showed an average yield gap due to N limitation of 4.6% for soybeans in Center-south of Paraná, which was lower for high yield environments (3.4%), with seed yield higher than 6000 kg ha^{-1}, and higher for low yield environments (7.2%), with seed yield lower than 5000 kg ha^{-1}. Small N amounts (up to 40 kg ha^{-1}) were also performed to evaluate if starter N fertilization could be a profitable way to improve soybean seed yield and reduce the yield gap. However, a lack of response was found and showed that starter N is not the preferential management practice to increase seed yield and reduce the yield by N limitation. This differs from previous reports that showed great yield gaps related to high yield environments (CAFARO LA MENZA et al., 2017; ORTEZ et al., 2018), our results showed higher yield gaps in low yield environments due to detriment over N supply (through N_2 fixation or soil). Furthermore, high seed yield was related to greater values of Ndfa and SOM. This result and others in this thesis led us to conclude that improving soil fertility to promote crop growth and BNF process and adopting conservation management practices to increase SOM should be the focus of farmers to reduce N limitation and increase soybean seed yield.

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APPENDICES

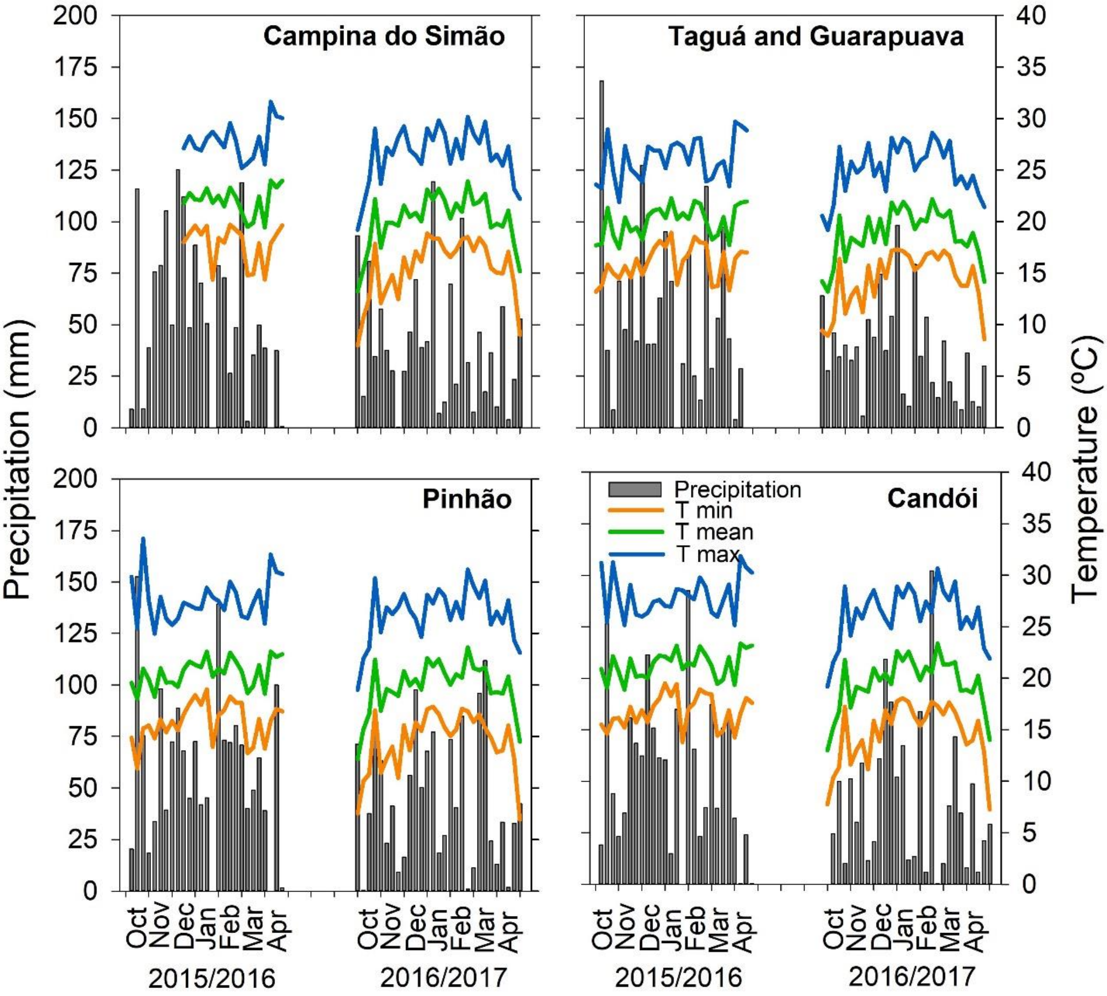

Figure 1. Precipitation and temperature (minimum, mean and maximum) of all the sites in 2015/2016 and 2016/2017 growing seasons.

Table 1. Entire set of variables determined in the 24 sites of Center-south region of Paraná, Southern Brazil.

Site	Soybean variety	Sowing	Population	Seed yield	Shoot dry weight	Shoot N content	N-fixed	Ndfa	Org. C	Total N	Soil pH	Clay
	(maturity group)	date	plants m^{-2}	kg ha^{-1}	Mg ha^{-1}	kg ha^{-1}	kg ha^{-1}	%	g kg^{-1}			%
1	BMX Apolo RR (5.5)	Oct. 18	33	3839	6.7	250	170	68	46.2	3.5	5.8	34
2	BMX Ativa RR (5.6)	Oct. 13	36	4491	6.9	238	168	70	38.4	2.5	5.9	34
3	K5616 (5.6)	Oct. 15	31	4202	8.4	273	211	77	43.0	2.6	5.7	34
4	M5917 IPRO (5.9)	Nov. 05	30	3825	9.4	258	181	70	41.1	2.4	6.8	28
5	BMX Apolo RR (5.5)	Nov. 11	31	3777	8.6	285	192	67	42.9	2.9	6.5	22
6	BMX Apolo RR (5.5)	Oct. 13	37	3877	8.8	290	160	55	32.5	2.3	5.8	54
7	BMX Elite IPRO (5.5)	Oct. 13	30	3626	9.9	260	62	24	50.2	3.3	5.2	34
8	BS 2606 IPRO (6.0)	Nov. 06	28	4033	9.1	268	109	41	43.6	2.8	5.7	28
9	BMX Apolo RR (5.5)	Oct. 18	37	4825	6.6	228	163	72	36.1	2.5	5.7	40
10	K5616 (5.6)	Oct. 16	32	4326	8.4	280	239	85	35.5	2.2	6.3	34
11	K6221 (6.2)	Nov. 11	36	3842	7.9	262	167	64	43.1	2.9	5.6	28
12	BMX Apolo RR (5.5)	Nov. 05	35	3881	9.1	282	185	66	37.3	2.5	6.1	34
13	K5616 (5.6)	Oct. 18	31	4149	7.6	258	133	52	36.1	2.7	5.6	54
14	DM 54i52 RSF IPRO (5.4)	Sep. 26	36	3694	10.8	359	115	32	29.7	2.3	6.1	47
15	BMX Vanguarda IPRO (6.0)	Oct. 10	33	4268	8.8	268	156	58	21.1	1.8	5.0	47
16	Roos Camino RR (5.3)	Oct. 15	34	3851	7.7	278	199	71	32.3	2.2	5.6	47
17	K5616 (5.6)	Oct. 16	35	3814	10.4	278	164	59	42.1	2.6	5.8	47
18	BMX Elite IPRO (5.5)	Oct. 23	23	4456	10.7	329	171	52	35.4	2.4	6.2	22
19	BMX Lança IPRO (5.8)	Oct. 23	31	4028	10.7	350	274	78	37.5	2.7	6.1	47
20	BMX Apolo RR (5.5)	Nov. 05	30	3597	7.3	255	137	54	39.6	2.6	6.0	40
21	BMX Ativa RR (5.6)	Oct. 16	37	3866	8.3	258	162	63	38.1	2.3	5.6	36
22	Roos Camino RR (5.3)	Oct. 12	36	4116	6.1	211	127	60	33.6	2.5	5.6	>60
23	BMX Ativa RR (5.6)	Nov. 12	41	3959	6.9	236	152	65	35.1	2.8	6.0	>60
24	K5616 (5.6)	Nov. 03	30	3983	7.9	282	203	72	48.4	2.7	7.3	34

Table 1. (continuation)

Site	Available P	Exchang. K	Exchang. Ca	Exchang. Mg	Available S	Base saturation	Available Mn	Altitude	Cum. rainfall		Mean air temperature	
	mg dm^{-3}		cmol$_c$ dm^{-3}		mg dm^{-3}	%	mg dm^{-3}	m	Until R1	R1 – R5	Until R1	R1 – R5
1	9.4	186	8.5	4.1	9.7	70	19	1029	734.6	96.6	20.0	19.3
2	7.6	131	7.5	3.8	13.0	71	4	944	463.2	351.2	19.7	20.7
3	5.0	155	7.2	2.0	8.3	64	5	958	652.4	180.8	19.5	20.5
4	7.2	83	10.8	7.3	8.8	92	1	917	447.4	161.0	20.2	20.2
5	6.1	196	9.8	3.6	8.1	82	4	991	498.8	247.8	20.3	19.5
6	3.3	58	8.1	3.5	6.4	73	8	889	615.8	199.4	20.6	21.1
7	2.5	210	4.5	1.7	16.0	41	17	833	528.8	399.6	21.2	20.0
8	11.0	241	7.4	2.7	10.0	63	9	961	571.6	308.8	20.6	20.8
9	12.0	213	6.3	2.6	8.7	66	10	989	630.8	167.4	19.4	20.7
10	7.9	173	9.8	4.7	19.0	83	4	981	1226.6	176.4	18.7	19.8
11	13.0	202	6.6	1.9	14.0	57	16	1069	453.0	256.8	20.3	20.4
12	11.0	101	7.9	4.3	9.5	76	4	1120	1010.4	202.2	19.4	19.5
13	6.7	215	6.8	3.4	9.6	66	19	827	740.8	110.4	20.1	21.0
14	6.6	115	8.5	3.1	13.0	79	8	813	660.8	364.8	19.0	21.5
15	16.0	106	4.7	1.3	8.8	45	34	499	243.6	313.6	22.1	23.2
16	10.0	72	8.3	2.9	8.4	65	24	874	483.4	364.2	19.6	20.8
17	6.9	204	7.7	2.1	12.0	65	7	876	538.4	343.8	19.7	20.7
18	8.0	203	8.3	4.6	8.5	81	6	930	689.4	219.8	19.9	20.7
19	16.0	214	8.7	4.3	8.9	78	8	1045	447.0	321.2	19.3	19.9
20	7.5	153	10.1	5.3	11.0	78	6	957	442.6	170.8	20.5	20.5
21	8.5	249	7.7	2.9	6.8	62	10	1012	766.4	164.0	19.6	20.6
22	6.1	154	6.9	3.8	9.9	69	40	725	377.0	344.8	21.4	22.4
23	10.0	255	7.9	3.8	22.0	78	46	817	298.0	96.6	21.6	21.2
24	11.0	154	13.8	7.8	8.6	95	2	1048	518.4	50.6	19.6	20.4

Table 2. Canonical weights for the variables of entire dataset.

Variable	Discriminant 1	Discriminant 2
Seed yield	0.86235782	-0.38233919
Shoot N content	0.22207919	-0.80690504
Plant population	-0.08478264	-0.27010763
Maturity group	0.07485637	0.28131840
Clay content	0.66146377	-0.10069244
Soil oganic C	0.92943322	-1.67839320
Soil total N	-0.95794375	-0.06617305
Soil pH	-0.10460094	0.30795677
Available P	0.13298249	-0.57793787
Available K	-0.14707610	0.09338417
Exchangeable Ca	0.66511387	-0.03124065
Exchangeable Mg	-0.42742497	-0.01483469
Available S	-0.10103043	-0.37172804
Available Mn	0.45707566	0.57920359
Base saturation	-0.10325702	-0.12106329
Altitude	-0.10302681	0.58000952
Cumulative rainfall (until R1)	-0.19758004	-0.56004031
Cumulative rainfall (R1 - R5)	-0.31747007	-0.15522101
Mean air temperature (until R1)	-0.01001821	-0.12536575
Mean air temperature (R1 - R5)	-1.01469196	-0.86951776

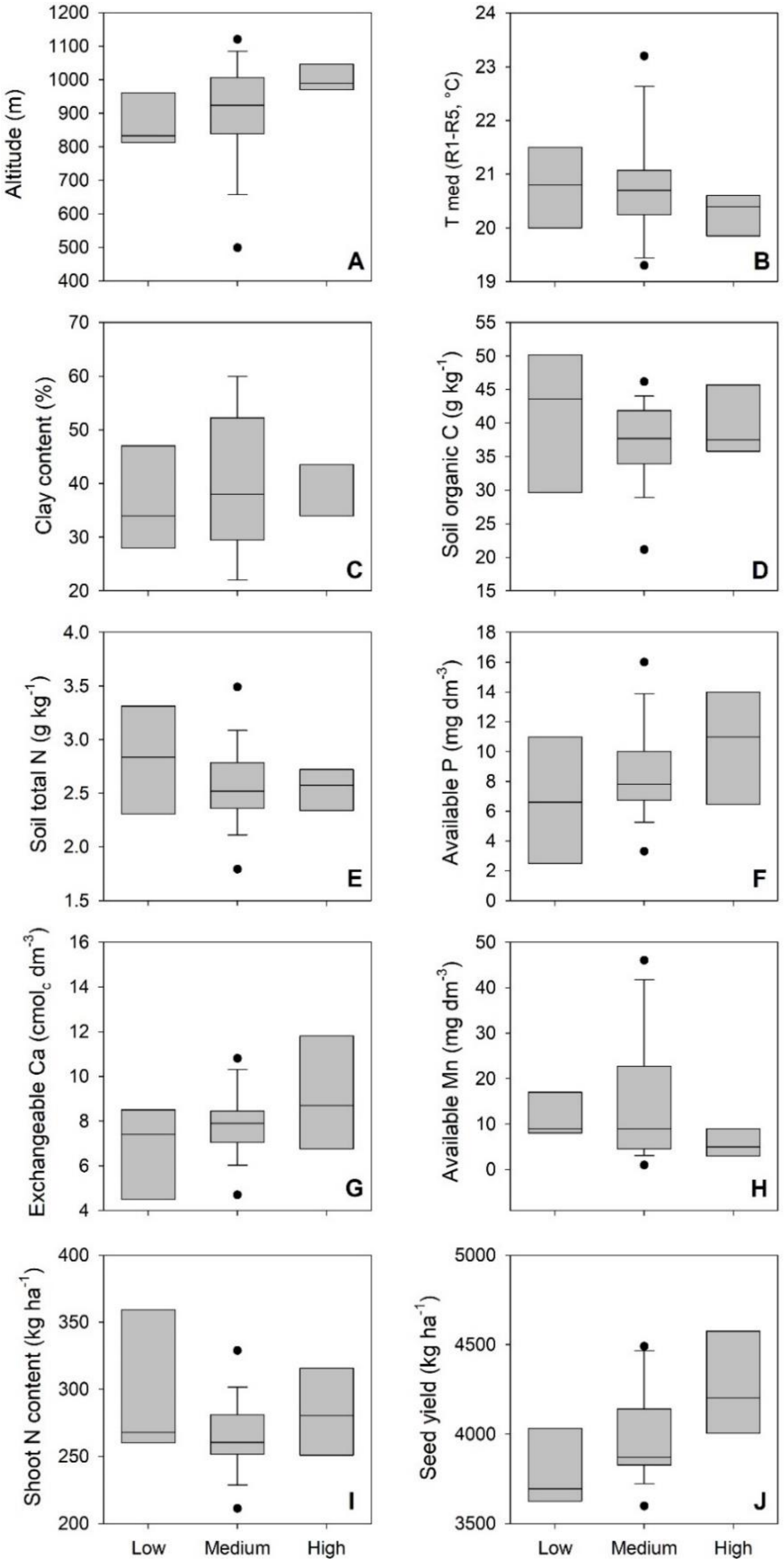

Figure 2. Boxplots comparison of altitude (A), air temperature (B), clay (C), organic carbon (D), total nitrogen (E), phosphorus (F), calcium (G), manganese (H), shoot N content (I) and seed yield (J) for low (<44%), medium (44-72%) and high (>72%) Ndfa groups.