UNIVERSIDADE ESTADUAL PAULISTA – UNESP

CÂMPUS DE JABOTICABAL

CONCENTRATION EFFECT ON ENDOPHYTIC ESTABLISHMENT OF PLANT GROWTH-PROMOTING MICROORGANISMS

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Paola Andrea Escobar Diaz - Born on March 10th, 1985, in the city of Bogotá D.C., Colombia, daughter of Roberto Escobar Dueñas and Maria Margarita Diaz Diaz. She joined to the Undergraduate Program in Bacteriology and Clinical Laboratorist at Universidad Colegio Mayor de Cundinamarca de Bogotá (UCMC) in March 2005. In December 2009, she received the title of Bacteriologist and Clinical Laboratory specialist after presenting her research work entitled “Microbiology Analysis of potable water in Hospitals of Bogotá” under the advice of Prof. Ana Praxedis Gonzales, PhD. In March 2016, she started the MSc studies at the Graduate Program in Agricultural Microbiology under the advice of Prof. Everlon Cid Rigobelo, PhD, at the Faculty of Agricultural and Veterinary Sciences, Campus of Jaboticabal-FCAV/Unesp. In March 2018, she defended her master’s thesis entitled “*Bacillus* spp. as growth promoters in cotton crop”. In August 2018, she started the PhD studies at the Graduate Program in Agricultural Microbiology at the same institution. During her PhD studies, she co-supervised scientific initiation students and undergraduate conclusion works, in addition to the publication of scientific articles in international journals arising from her PhD work and partnerships.

“Thus, the task is not so much to see what no one yet has seen, but to think what nobody yet has thought about that which everybody sees.”

Arthur Schopenhauer

To God, for the infinite possibilities to start again because He is my strength, and I know that I will always have someone to guide me during this journey.

To my brothers and my boyfriend!

I dedicate

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SUMMARY

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DOSE EFFECT FOR RHIZOSPHERIC AND ENDOPHYTIC ESTABLISHMENT OF PLANT GROWTH PROMOTING MICROORGANISMS

ABSTRACT - Agriculture plays an important role in the Brazilian economy. In this context, mineral fertilizers and pesticides are applied on a large scale to increase productivity to meet the demand of the world population. One of the negative aspects associated with these activities is to lose the soil quality and physical and chemical properties. This issue has become an environmental concern due to changing climatic conditions and public health risks that affect food security and sustainability of agriculture. This situation required the development of sustainable and ecologically correct alternatives to agriculture. Therefore, the study of soil microorganisms associated with plants has great potential as they are directly or indirectly involved in promoting growth and increasing plant productivity through the production of phytohormones, availability of nutrients and production of volatile organic and antimicrobial compounds (antibiotics, lytic enzymes and siderophores). Microorganisms interact in different ways with plants, benefiting them and guaranteeing resources for themselves in return. Several species of fungi and bacteria are reported as plant growth promoters in several cultures and have been used as biological inoculants in agriculture to increase crop productivity. Thus, the present study aimed to determine the effect of concentration for the rhizospheric and endophytic establishment of three strains of *Aspergillus* and three strains of *Bacillus* selected for their potential as plant growth promoters. The plant growth promotion and colonization in cotton plants were evaluated in greenhouse. The parameters dry mass, phosphorus and nitrogen contents in the plants and the number of colony forming units in the plants and in the soil were evaluated. Subsequently, two strains of fungi and bacteria were selected and tested at two concentrations in the field to determine the effect of strains and concentrations on cotton yield. It was verified that the colonization of cotton plants and crop productivity was variable and not associated with concentration. Finally, further studies are needed to determine the plant growth promoting ability of *Bacillus* and *Aspergillus* in other crops and their impact on productivity and environment.

Keywords: cotton, concentration, Inoculants, plant growth promotion, sustainability

CHAPTER 1 - General Considerations

1. INTRODUCTION

Agriculture provides products for many purposes (e.g., fiber, energy, fuel, food, among others). These products, as well as the production methods used, need to keep up with the demand generated by the world population that has been growing along with the availability of land used for agriculture (Sazvar et al.,2018).

On the other hand, biotic and abiotic factors such as soil quality, moisture, climatic conditions, water availability can be affected by androgenic actions such as soil contamination and saturation due to the excessive use of fertilizers and pesticides (Chandra and Sobti, 2019). The use of these chemicals provides an average increase in productivity by providing some nutrients and control of pests and diseases; however, they affect interactions between plants and microorganisms in the rhizosphere, altering the soil characteristics and its balance over time. In addition, prolonged use of mineral fertilizers can harm ecosystem and agricultural systems (Meena et al., 2020; Willett et al., 2019; Arif et al.,2020).

According to Shrestha et al. (2020), initiatives in sustainable agriculture aim at the conservation of natural resources, helping to mitigate the adverse effects caused by the excessive use of mineral fertilizers. These initiatives take advantage of the biological potential of roots and rhizosphere, which together with natural microbiota, favor nutrient uptake, allowing better soil use and exploitation, in addition to increasing tolerance to biotic and abiotic stress (Meena et al., 2017; Bertola et al., 2021).

In general, there are microorganisms that live in the soil and interact with plants. Soil microorganisms are part of a fundamental system and are an important soil component, as they become an integral part of the crop production system. Microorganisms form symbiotic associations on the surface of plants or endophytic interactions in roots, stems, or leaves. Studies have shown that plant growth promoting microorganisms (PGPM), in addition to participating in biochemical processes, interact

with plants, inducing systemic responses that improve their metabolic capacity (de la Fuente Cantó et al., 2020; Lopes et al., 2021).

Based on plant-microorganism interactions, there are several bacteria and fungi species that have plant growth-promoting characteristics with enormous potential. The use of PGPM is an alternative to face the future challenges aimed at sustainable agriculture for crop yields (Aloo et al., 2019). The plant-bacteria interactions promote growth and increase in productivity by mechanisms such as the production of phytohormones and availability of nutrients (phosphorus, nitrogen, and iron) through the solubilization of phosphates, biological nitrogen fixation (BNF) and production of siderophores. Fungi are currently recognized as an important source of organic and bioactive compounds. They produce secondary metabolites, including phytohormones such as auxins, gibberellins, cytokinins, and abscisic acid (Adeleke and Babalola, 2021).

Declining soil fertility, environmental conditions, as well as biological soil health are other important criteria to be considered in the search for nutrient management strategies to improve crop yields and restore degraded soils. Given this situation, the development of sustainable technologies based on microorganisms emerged as an innovative and ecologically correct proposal to improve soil fertility and plant growth (Fasusi et al., 2021). The application of PGPM as biofertilizers in sustainable agricultural practices has shown reduction in the use of mineral fertilizers in the field. However, more information is needed in the incessant search for a better understanding of the complex relationships between plant, soil, and microorganisms.

In this context, the present study aimed to determine microorganisms capable of promoting the growth of cotton plants and to determine the effect of the concentration of microorganisms on cotton yield under greenhouse and field conditions.

2. LITERATURE REVIEW

2.1. Environmental and economic impacts of the use of agricultural inputs

In recent decades, Brazil has become one of the largest agricultural producers in the world. Over the 2020-2021 period, some agribusiness activities were affected by the COVID-19 pandemic; however, despite the enormous problems caused by the pandemic, excellent results were obtained in terms of agricultural production (CONAB 2021).

Most of the agribusiness growth was due to increased productivity. Brazil is one of the countries whose productivity has grown, on average, 86.0%. This is due to the increased use of inputs, cultivable land, labor, energy, and mineral fertilizers. Agricultural productivity growth has been the safest way to meet growing food needs (Liszbinski et al., 2020).

During recent high-consumption agricultural systems and technologies, mineral fertilizers (N, P or K) have been excessively applied to provide the nutrients needed to increase agricultural productivity worldwide (Omara et al., 2019). In Brazil, the domestic production of mineral fertilizers has been insufficient to supply the demand of agricultural producers, causing the country to import about 70% of nitrogen (N), 50% of phosphorus (P_2O_5) and more than 90% of potassium (K_2O) of the total consumed [National Association for National Fertilizer Diffusion (ANDA), 2020]. Therefore, Brazilian agricultural production depends on the import of mineral fertilizers, and this makes the country vulnerable to exchange and price fluctuations and, consequently, to the risk of shortages of basic inputs (Farias et al., 2020).

The advantage of fertilizers is that they increase productivity in view of the limitation of cultivable area; however, only a limited amount (30-40%) of these nutrients is absorbed by plants due to the low efficiency of fertilizer use and the rest is lost in the soil, causing environmental pollution. The high consumption of fertilizers can have negative environmental impacts as excess can pollute groundwater. In addition, natural pollinators, birds, and beneficial microorganisms present in the soil can also be affected, altering the balance in ecosystems (Ning et al., 2017; MAPA, 2022).

Savci (2012) describes fertilizers as sources of Hg, Cd, As, Pb, Ni and Cu, heavy metals present in mineral fertilizers, which are difficult to degrade, making them persistent pollutants. Today, the use of fertilizers is seen as a necessity to improve productivity and to replace soil nutrients, but it has also brought serious consequences due to their persistence in nature, accumulating in the soil, plants and, therefore, being consumed and causing harmful long-term effects on human health (Kulkarni and Goswami, 2019).

2.2. The cotton crop and its position in the context of the use of agricultural inputs

Cotton (*Gossypium hirsutum*) is one of the main crops in Brazil and its fiber has been used by humans for centuries (Shahrajabian et al., 2020). The main cotton product is the fruit composed of the seed (58%), which contains 15% oil, 3% fiber, 40% protein, and 42% bagasse. The fiber, which is composed of cellulose layers, is the main economic cotton product (Siakeng et al., 2019).

Cotton is the natural fiber most widely used in the world and has been the preferred choice of the textile industry and consumers since the beginning of the industrial revolution. From then on, cotton production increased considerably and represents about half of fibers used for clothing and textile products (Zhang et al., 2015; Wang and Memon, 2020). Despite the increase in the use of synthetic fibers, cotton is still the most important natural fiber in the world. Currently, more than 100 countries invest in cotton cultivation, with China, India, the United States, Pakistan, and Brazil being the largest world`s producers (Tausif et al., 2018; Alves et al., 2021).

Commercial cotton production began in the Northeastern region of Brazil, with the state of Maranhão being the first major producer and exporter of the product to Europe. Due to the development and economic importance of the crop, the state of São Paulo began the planting of shorter-fiber herbaceous cotton, but due to the high costs of land and competition from other crops, such as sugarcane, corn and soybeans, cotton was planted in new areas such as Mato Grosso and Goiás. Currently,

the crop is planted mainly in three macro-regions, North-Northeastern region (Tocantins, Maranhão, Piauí, Ceará, Rio Grande do Norte, Paraíba, Pernambuco, Alagoas and Bahia), Mid-western region (Mato Grosso, Mato Grosso do and Goiás) and the South-Southeastern region (São Paulo, Paraná and Minas Gerais) with Brazil being the fourth largest world`s producer, accounting for approximately 57% of cotton (plume+seed) exporting 1.37 million tons, surpassing 1.18 million tons of the last harvest (CONAB, 2021).

Brazil is one of the few countries that can produce cotton in humid tropical conditions. Excessive rainfall during the plant's cycle favors the occurrence of pests and diseases that harm the crop, requiring the use of pesticides (Furtado et al., 2016). This situation has favored the development of several research programs involving breeding aimed at insect resistance and herbicide tolerance (Qaim, 2020). Among the main advances for the control of cotton pests, cotton varieties genetically modified with genes of the bacterium *Bacillus thuringiensis* (Bt) stand out. The varieties currently available in Brazil produce the Cry1Ac protein. The expression of this protein in cotton plants gives the plant resistance to caterpillars that feed on cotton bolls (Santos and Torres, 2010).

According to report on the global situation of transgenic crops (ISAAA, 2018), part of cotton production in Brazil and in the world is made from transgenic seeds. The demand for pesticides by cotton is very large and when observing data on the use of pesticides in production, concern for the environment and public health is inevitable. The great demand for cotton by Asian consumers and the lower production in the USA allowed a 13.4% increase in the planted area in Brazil in the 2021/22 harvest (CONAB, 2021).

Cotton farming consumes most of the fertilizers produced and imported by Brazil and, due to nutritional requirements, this crop is demanding and, despite being tolerant to water and saline stresses, its maximum productivity is reached when there is good water availability. Brazilian cotton farming uses an average of 28.6 L of pesticides per hectare. The use of mineral fertilizers is justified by the conditions required by the soil in the cerrado regions, where the largest cotton production is concentrated and the culture requires fertilization to provide plants with essential elements and overcome the nutritional crop deficiencies (Pignati et al. 2017; ANDA, 2020).

The use of large amounts of fertilizers is a common practice in agriculture. Fertilizers are widely consumed in the world and influence the production cost. Conventional farming practices often use large amounts of mineral fertilizers and pesticides that can have adverse effects on humans, animals, and environment. This situation is harmful to the environment because it brings imbalance, compaction, and soil degradation (Ronquim, 2010; Rahman and Zhang, 2018). As with other crops, cotton growth and production are directly impacted by the supply of nutrients that are absorbed by the cotton plant practically throughout the entire cycle from the beginning of flowering to fruiting, decreasing as the bolls ripen (Rehman and Farooq, 2019). Knowing the metabolic effect caused by excess or deficiency of nutrients on cotton growth, development and production components is important to reduce productivity losses caused by nutritional stress. The availability of nutrients in the soil provided by the application of fertilizers has long-term consequences such as decrease in microbial diversity and imbalance in soil microbial biomass (Sun et al., 2015).

2.3. Alternatives to the application of agricultural inputs and the use of microorganisms in agricultural production

Given the negative consequences of the excessive use of agricultural inputs, interest in the development of technologies and research as alternatives that contribute to increase yields and crop protection has intensified, generating balance between production and other aspects (social, economic, political, and cultural) and that are sustainable in the long-term (Singh et al., 2017).

Given the need to reduce the use of agricultural pesticides and mineral fertilizers, management practices such as crop rotation, improvement of crops with agronomic and physiological characteristics of interest, have been established for some decades; however, these efforts are not being enough to mitigate the negative impacts of soil degradation (Lopes and Albuquerque, 2018; Lopes et al., 2018).

For Ahmad et al. (2018), these already implemented practices should be complemented with safe alternatives, such as the use of microorganisms that promote

plant growth and that can improve soil quality through symbioses with the plant. According to Xiong et al. (2021), all terrestrial plants are colonized by diverse, complex, and interactive communities of microorganisms. The study of microbial communities that inhabit different habitats and their contribution to the development and protection of plant growth has received great interest in the last two decades (Glick and Gamalero, 2021). Plant growth-promoting microorganisms (PGPM) have demonstrated ability to exert effects on the plant through direct (biological nitrogen fixation, phosphate solubilization, production of phytohormones) or indirect modes of action (production of siderophores and biofilm), in addition to establishing beneficial soil microbiota (Chitnis et al., 2020; Khan et al., 2021).

According to Gavilanes et al. (2021), the plant can efficiently take advantage of 10-25% of the phosphate provided by mineral fertilization, while insoluble phosphorus (inorganic and organic) in the soil can be made available by PGPM found in the soil. Phosphorus can be made available through the release of hydrogen ions, organic acids, or production of enzymes (phosphatases and phytases) capable of hydrolyzing organic or inorganic phosphorus, making them ready for absorption by roots.

The utilization of PGPM has several benefits, such as production of phytohormones, contribution to the mitigation of abiotic and biotic stresses, increase grain yield, increase in seed emergence rates, plant biomass and resistance to agricultural pests and diseases (Sehrawat and Sindhu, 2019; Zhang et al., 2021). Microorganisms can be considered technological inputs, as they reduce the use of mineral fertilizers by assisting in the assimilation of nutrients via processes such as nitrogen fixation and solubilization of phosphorus, potassium, and zinc in the field (Chebotar et al., 2015; Itelima et al., 2018; Mącik et al., 2020).

Currently, the use of biological products, also called biofertilizers, composed of live or latent microorganisms, which are applied to the soil, seeds, or seedlings, has emerged as an economically and ecologically viable alternative to mineral fertilizers. According to Chagas et al. (2017), the manufacture of biological products for pest and disease control grew more than 70% in the last year in Brazil, moving approximately USD 48.4 million, which demonstrates the economic potential and sustainable application for agricultural production.

Many studies with microorganisms are still in laboratory and greenhouse stages, which makes necessary to prove not only the interaction between microorganisms but also the antagonism between them; further studies need to be carried out under field conditions, because few are the microbial species that can be effectively transformed into commercial products (Steffen et al., 2019). Studies on the culture, environment, soil conditions, microorganism characteristics and behavior are important to assess the colonization potential and the behavior of PGPM in the field, as these factors are directly related to plant development.

2.4. Microorganisms to stimulate plant production

Each part of the plant carries out, to a greater or lesser degree, its own selection of microorganisms, comprising representatives of all three primary life domains - Bacteria, Archaea and Eucarya. The root system of plants secretes exudates such as sugars, polysaccharides, amino acids, aromatic acids, aliphatic acids, fatty acids, sterols, phenolic compounds, enzymes, proteins, plant growth regulators and secondary metabolites that are involved in attracting beneficial organisms, forming microbiomes associated with the plant including nitrogen fixers, phosphate-solubilizing bacteria, mycorrhizal fungi, endophytic fungi, biocontrol agents, bioremediation agents, plant growth-promoting rhizobacteria and pathogenic microorganisms (Bhattacharyya et al., 2016; Sehrawat and Sindhu, 2019; Singh et al., 2021).

These associations are dynamic and can be influenced by soil resident organisms, local edaphic factors, environmental conditions, crop quality, plant biotic and abiotic stress (Toju et al., 2018).

According to their location, microorganisms can be classified as rhizospheric, epiphytic and endophytic (Rossmann et al., 2017). Epiphytic microorganisms are defined as living on the surface of plant organs and tissues; eventually, they can enter a plant and remain for a certain period. Due to physicochemical differences in the shoots of plants, the leaf microbiome differs substantially from that of roots, which contributes to microbiological diversity. Several factors such as soil pH, rapid

fluctuations in temperature, relative humidity, ultraviolet radiation, and nutrient availability can influence the colonization, proliferation, diversity, and distribution of beneficial and pathogenic microorganisms (Alsanius and Wohanka, 2019; Islam et al., 2020).

Several studies have provided information that demonstrate the composition of the microbiome found in the soil rhizosphere. Rhizospheric microorganisms are selected through a wide diversity of compounds (exudates) secreted by plant roots, forming the rhizodeposition that modifies the soil chemical and physical properties and, therefore, selecting a differentiated microbial community near the root surface (Walker et al., 2003; Iannucci et al., 2021). Likewise, microorganisms including fungi, actinomycetes, algae, protozoa and nematodes are stimulated in the rhizosphere. According to Kumar (2016), some of the exudates act as repellents that inhibit the growth of pathogenic microorganisms and contribute to the growth of rhizobacteria that promote plant growth through phytohormones considered phytostimulators, or rhizobacteria that contribute to the degradation of organic pollutants and reduction of heavy metals in soils, called rhizoremediators (Chaudhary and Shukla, 2019) (Fig. 1).

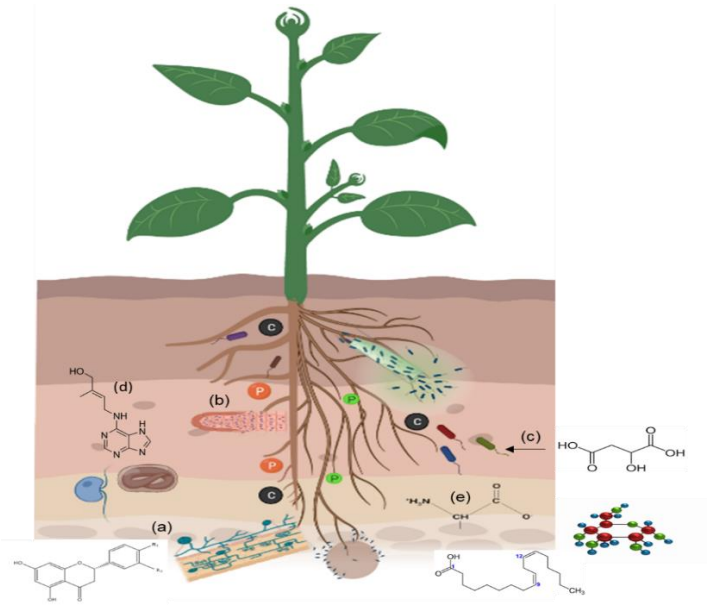

Figura 1. Chemical communication between plant roots and other organisms in the rhizosphere. Roots secrete a wide range of compounds, including sugars and amino acids that are involved in attracting microorganisms (chemotaxis). Among the main compounds are (a) flavonoids (signaling molecules) that interact with mycorrhizae, (b) rhizobia, (c) aliphatic acids (malic acid) involved in the recruitment of plant growth-promoting

> rhizobacteria such as *Bacillus subtilis*, (d) growth regulators (cytokinins) involved in cell division and differentiation, which confer resistance against nematodes, (e) organic acids, amino acids and sugars involved in bacterial attraction and bacterial *quorum sensing* (Badri et al. 2009).

Endophytic microorganisms can colonize the internal tissues of plants in at least part of their life cycle, they live intra or intercellularly without developing disease symptoms in plants (Orozco-Mosqueda et al., 2021). According to their life strategies, endophytic microorganisms can be classified as "mandatory" or "facultative". Mandatory endophytes are strictly dependent on the host plant for their growth and survival and their transmission to other plants can be vertically or through vectors. Facultative endophytes have a stage in their life cycle in the host plant, but they can survive outside the plant and their colonization is variable and essentially depending on bacterial species, genotype, host developmental stage and environmental conditions (Alves et al., 2014).

Endophytic microorganisms, generally found in the soil, infect the host plant, initially colonizing the cracks formed at root junctions and spreading to intercellular spaces in the root (Slaughter, 2021). To become established in a plant, endophytic microorganisms need to be able to gain rapid and widespread entry into young plants, find environment within the plant that provides fixed carbon, optimal pH, oxygen tension, and moisture.

According to Bashir et al. (2022), the stomata found in leaf tissues, wounds caused by microbial phytopathogens, or nematodes can be considered entry points for the colonization of endophytes in the plant. Endophytic microorganisms can promote plant growth through mechanisms such as release of phytohormones (Rana et al., 2020), nitrogen fixation and availability of mineral nutrients (Prasad et al., 2019). The availability of mineral nutrients by bacteria, in addition to improving plant development, provides tolerance to biotic and abiotic stress (Kumar et al., 2022; Fasusi et al., 2021). To promote growth, the microorganism needs to colonize the plant's endosphere after colonizing the rhizosphere. Colonization is a process that uses several characteristics involving motility, fixation, plant-polymer degradation, and evasion of plant defenses (Afzal et al., 2019).

2.5. Mechanisms of action used by plant growth promoters

2.5.1. Direct Mechanisms

Plant growth promoting microorganisms (PGPM) are phylogenetically diverse and when associated with the plant, interact in different ways (symbiosis, parasitism, commensalism, amensalism and neutralism), promoting plant growth among other benefits (Dubey et al., 2016; Glick and Gamalaro, 2021). The direct action of PGPM involves interaction between microorganisms and plants in the rhizosphere, improving the uptake of essential nutrients such as nitrogen, phosphorus, potassium, and iron (Kumar and Verma, 2018; Naik et al., 2019; Hakim et al., 2021).

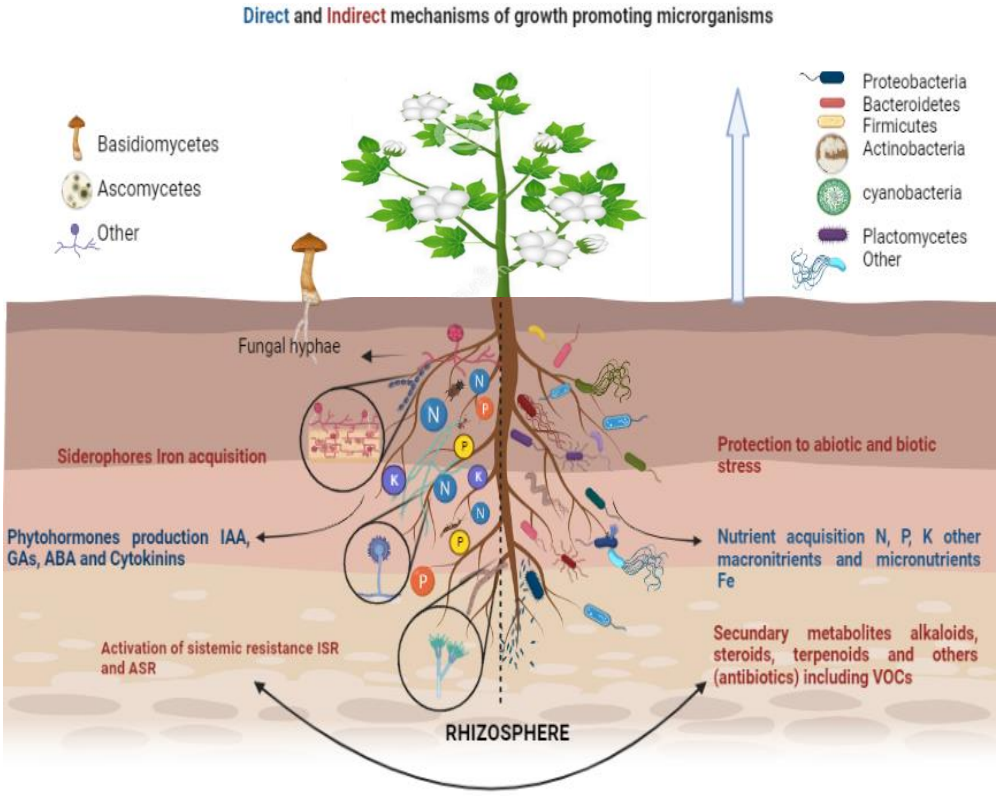

Figure 2. Examples of phyla in microbiome composition. a) bacteria (right) and fungi (left) present in the rhizosphere and the direct and indirect mechanisms in which they are involved.

2.5.2. Biological nitrogen fixation

Nitrogen is a fundamental nutrient for the synthesis of biomolecules such as proteins, nucleic acids, and numerous primary and secondary metabolites essential for the proper functioning of plants (Chang et al., 2015). In most soils, nitrogen that can be assimilated by plants in the form of nitrite, nitrate or ammonia is scarce. Nitrogen availability is affected by different parameters such as soil type, climate, crop type, and nitrogen deficiency can lead to unbalanced plant growth (Vicente and Dean, 2017; Schulze et al., 2019).

The application of nitrogen fertilizer has been the method most widely used since the 1960s to supply nitrogen to plants. However, with the discovery of diazotrophic bacteria and plant growth-promoting rhizobacteria, it was possible to discover the advantages of the use of atmospheric nitrogen, since these microorganisms carry out biological nitrogen fixation (BNF) converting N_2 into its ammoniacal form (NH_3) through the action of the nitrogenase enzyme composed of proteins encoded by the group of nif “operons”, together with other structural genes that activate the Fe protein, electron donation, biosynthesis of Fe Mo cofactor and many other regulatory genes obligatory for the synthesis and enzymatic activity that participate on the basis of the biological nitrogen fixation process (Zgadzaj et al., 2016; Puri et al., 2018; Angel et al., 2018).

According to Lindström and Mousavi (2020), BNF is considered, after photosynthesis, the most important biological process of plants. There is a diversity of nitrogen fixing bacteria known to establish a symbiotic association with legumes through the formation of nodules, mainly those of the genera *Rhizobium* and *Bradyrhizobium*. These bacteria convert N_2 into ammonia for plants to use it as a nitrogen source (Aasfar et al., 2021). Likewise, some species of free-living plant growth-promoting rhizobacteria or grass-associated endophytic bacteria such as *Azospirillum* can also promote biological nitrogen fixation, but in less efficient ways (Chang et al., 2015).

However, free-living bacteria of the genera *Azotobacter, Azospirillum, Herbaspirillum, Burkholderia, Bacillus* and *Paenibacillus* close to roots manage to establish themselves in the soil through exudates provided by the plant (amino acids,

peptides, proteins, enzymes, vitamins, and hormones) and in exchange, the nitrogen fixed by bacteria can be absorbed by the plant. Soil microorganisms maintain the optimal concentration of soil nutrients, providing better plant growth and crop yields. (Goswami et al., 2016; Mahanty et al., 2017; Rana et al., 2020; Subrahmanyam et al., 2020; Babalola et al., 2021).

Nitrogen fixation for plants has been concentrated on bacteria; however, symbiotic relationships between plant and soil fungi have demonstrated the ability to transfer nitrogen to plants. Studies such as that carried out by Behie and Bidochka (2014) determined five fungi species including *Metarhizium* spp., *Beauveria bassiana* and *Lecanicillium lecanii* that, in addition to killing target insects and endophytically colonizing the plant, were able to transfer nitrogen from insects to soyben, bean and wheat plants.

According to Barra-Bucarei et al. (2021), in their study with bean and grass plants, showed that fungi of the species *Metarhizium robertsii* could be a channel through which plants could obtain nitrogen from insects. It is possible that the endophytic capacity and pathogenicity of *Metarhizium* can establish a method of transferring nitrogen to host plants through fungal mycelia. This information helps understanding the role that fungi play in nitrogen cycling.

Studies on fungal nutrient transfer to plants have been mainly focused on mycorrhizal fungi. Certain endophytic fungi such as *Metarhizium* and *Beauveria* are also capable of transferring nitrogen to host plants. Genes and their transporters involved in the movement of nitrogen and phosphorus were identified. These advances may further elucidate the role of fungi in these nutrient exchanges (Behie and Bidochka 2014). However, further studies are still needed in relation to nitrogen transfer to plants through fungi in different cultures.

In general, microorganisms can be considered a sustainable alternative as their use can considerably reduce the amount of nitrogen fertilizers used in agriculture (Steffen et al., 2019; Elnahal et al., 2022).

2.5.3. Nutrient uptake

The direct action of plant growth-promoting microorganisms involves an increase in nutrient uptake and production of phytohormones, which is directly related to increase in biomass, expansion of the root system, plant height and productivity (Bamisile et al., 2018). The availability of nutrients such as phosphorus, nitrogen, potassium, magnesium, zinc, iron, and copper are part of the strategy of microorganisms to provide nutrients to the host plant (Rana et al., 2019).

2.5.4. Phosphorus solubilization

Phosphorus is a limiting nutrient for plant growth and development. It is also a structural component of many enzymes, coenzymes, phosphoproteins, phospholipids, and nucleic acids. It participates in important physiological and metabolic processes such as photosynthesis, respiration and membrane formation, energy transfer, macromolecular biosynthesis, and signal transduction (Tian et al., 2021).

Plants absorb phosphorus in the form of monobasic ($H_2PO_4^-$) and dibasic (HPO_4^{-2}) ions, although phosphorus found in soil is mainly in the insoluble form, which cannot be absorbed by plants (Admassie et al., 2020).

In soil, microorganisms play a fundamental role in the transformation of inorganic phosphorus, which is carried out through solubilization and mineralization mechanisms by bacteria and some fungi (Mehta et al., 2019). Regardless of being in interaction with the plant, microorganisms can solubilize inorganic phosphorus through the secretion of organic acids (gluconic, acetic, lactic, malic, succinic, tartaric, 2-ketogluconic, oxalic and citric acids) through the metabolism of sugar resulting from root exudates.

These acids released by microorganisms act as cation chelators that at the same time allow the release of insoluble phosphate compounds and promote acidification of the microbial cell and the environment around them (Awais et al., 2017;

Patel et al., 2015; Fabianska et al., 2019). In addition to organic acids, H+ protons and inorganic acids are also released as alternative mechanisms for the solubilization of inorganic phosphates (Saber et al., 2009; Gupta et al., 2015).

On the other hand, solubilization of organic phosphates or mineralization of organic phosphorus is necessary for the cycling of phosphorus from plant and animal remains that contain high levels of phosphate compounds (Ahemad and Kibret, 2014; Goswami et al., 2014). This mineralization is promoted by enzymes such as phytases, phosphatases, phosphohydrolases or phosphonatases and C-P lyases, classified as acidic or alkaline according to their optimal activity pH.

These enzymes can be secreted outside the plasma membrane or remain trapped in the membrane as soluble proteins (Teymouri et al., 2016). According to Kafle et al. (2019), the main phosphorus-supplying molecules after mineralization are nucleic acids, phospholipids and phosphate sugars that are easily degraded, whereas phytic acids, polyphosphates and phosphonates are slowly mineralized.

Various genera like *Agrobacterium, Arthrobacter, Azotobacter, Bacillus, Beijerinckia, Burkholderia, Enterobacter, Erwinia, Flavobacterium, Microbacterium, Mesorhizobium, Pseudomonas, Rhizobium, Rhodococcus* and *Serratiae* are described in literature as phosphorus solubilizers (Bhattacharyya and Jha, 2012; Oteino et al., 2015). In this sense, microorganisms capable of solubilizing and mineralizing phosphates can promote the use of natural soil phosphates, benefiting crops, reducing production costs, and increasing fertilization efficiency (Fabiańska et al., 2019).

In the case of fungi, the metabolic pathways and molecules involved have not yet been well described. According to Hiruma et al. (2018), phosphorus transport between plants colonized by arbuscular mycorrhizal fungi could be associated with several genes related to phosphorus transport such as PHT1; 2 ePHT1; PHT3; and it is unclear whether transporters accumulate on the biotrophic surface and whether they are necessary in the growth promotion process mediated by endophytic colonization.

Ortega-Garcia et al. (2015) demonstrated that *Trichoderma asperellum* inoculation significantly reduced the use of phosphorus fertilization in onions. Likewise, Baron et al. (2018) demonstrated the accumulation of significant amounts of

phosphorus in corn inoculated with *Aspergillus sydowii*, even receiving lower fertilizer doses. Finally, further studies aimed at clarifying the mechanisms of plant growth promotion using fungi need to be carried out.

2.5.5. Production of phytohormones

Plant hormones or phytohormones are natural organic molecules that play an important role in plant development. Phytohormones are chemical messengers that influence the plant's ability to react to the environment and tolerate stressful conditions (Enders and Strader, 2015; Khan et al., 2020). Phytohormones are sintered in certain parts of the plant and transported to other locations at very low concentrations. Thus, phytohormones influence physiological processes such as differentiation, development, growth, and stomatal movement (Sureshbabu et al., 2016).

Some rhizobacteria and fungi can produce different types of phytohormones, including auxins, cytokinins, gibberellins, ethylene, and abscisic acid, and more recently strigolactones and brassinosteroids. Phytohormones can influence their own physiological processes as well as the physiological processes of plants. The potential for phytohormone production by fungi has been little explored under different environmental conditions despite their potential in agriculture (Sureshbabu et al., 2016; Singh et al., 2017; Khanna et al., 2021).

Auxins are responsible for the formation of root primordia and plant development (Taiz and Zeiger, 2006; Lin and Sauter, 2019). One of the most well-known auxins is indoleacetic acid (IAA). In plants, IAA participates in cell division, elongation, fruit development and senescence, in addition to stimulating the development of roots, leaves and flowers (Phillips et al., 2011). In dicots, IAA specifically induces the formation of lateral roots while in monocots, IAA induces the formation of adventitious roots (Lakehal and Bellini, 2019).

IAA can affect the plant in a positive way (shoot and root growth) or negatively (root growth inhibition) (Duca et al., 2014). IAA production is part of the signaling and

communication system between plants and microorganisms present in the rhizosphere (Spaepen et al., 2007; Waqas et al., 2012).

The fungus *Paecilomyces formosus* LHL10 strain isolated from cucumber plants produce IAA and gibberellins (Khan et al., 2012). Furthermore, *Trichoderma* strains isolated from soil showed ability to produce IAA, in addition to significantly increasing phosphorus solubilization, plant height, shoot and root dry mass and chlorophyll content in tomato leaves (Bader et al., 2020). Likewise, Baron et al. (2020) demonstrated the ability of fungi *Purpureocillium lilacinum, Purpureocillium lavendulum* and *Metarhizium marquandii* to produce IAA and solubilize phosphorus from fluorapatite, promoting increase in shoot and root dry mass and the availability of nutrients such as phosphorus and nitrogen in soybean, corn, and bean.

On the other hand, seedlings treated with IAA-producing rhizobacteria had significant effect on height, shoot dry mass and root system (Saghafi et al., 2018). Other examples of bacteria capable of synthesizing IAA are *Aeromonas punctata* (Iqbal and Hasnain, 2013), *Azospirillum brasilense* (Pham et al., 2022), *Bacillus subtilis* (Diaz et al., 2019) and *Burkholderia* (Laird et al., 2020).

According to Keswani et al. (2020), the main precursor for the synthesis of IAA is tryptophan (encoded by *trp* genes). Tryptophan participates in the indole-3-acetamide, indole-3-pyruvate, tryptamine, and indole-3-acetonitrile pathways. However, only a small set of genes and enzymes involved in these pathways have been characterized (Spaepen et al., 2007; Spaepen and Vanderleyden, 2011).

Other class of phytohormones are gibberellins responsible for different developmental processes such as seed germination, stem elongation, flowering, and fruiting in higher plants (Saleem et al., 2015). Gibberellins have positive effect on leaf size (which facilitates sunlight absorption) and on root meristem size (Martinez et al., 2016).

Studies have shown that with the presence of gibberellin-producing microorganisms in their rhizospheres, plants have better growth rates (Vacheron et al., 2013). Some gibberellin-producing bacteria species include *Bacillus amyloliquefaciens* (Shahzad et al., 2016) and *Bacillus pumilus* (Joo et al., 2004). Khan et al. (2008)

demonstrated the production of gibberellin by fungus *Penicillium citrinum* IR-3-3, which was able to promote growth by increasing the height of dwarf mutant rice, which due to its genetic modifications was deficient in producing gibberellin naturally.

According to Glick (2020), ethylene participates in leaf and fruit maturation, seed germination, leaf senescence, flower wilting, root initiation, elongation and branching, nodule formation and leaf abscission. Excess ethylene is also harmful to plants causing defoliation, root growth inhibition and early-stage senescence. Some bacteria species such as *Bacillus* spp. (Santoyo et al., 2019) and *Pseudomonas* spp. (Sandhya et al., 2010; Kamran et al., 2016) are known for producing ACC-deaminase (ACCD), an enzyme produced to reduce high ethylene levels, helping to regulate its adverse effects on plants.

Abscisic acid (ABA) is a phytohormone that participates in mediating stomatal closure (Kumar et al., 2019). This phytohormone is produced by plants, algae, bacteria, and fungi (Seo et al., 2006). In water stress situations, ABA levels increase, and stomata are partially closed as an adaptive response to prevent water loss (Frankenberger and Arshad, 2020).

In general, a microorganism that can produce or change the concentration of plant growth regulators such as IAA, gibberellins, cytokinins and ethylene is called phytostimulator (Poveda and González-Andrés, 2021). In this sense, phytohormone-producing microorganisms can be used to improve crop physiology, biomass, and yield (Enders and Strader, 2015; Kang et al., 2019).

2.6. Indirect Mechanisms

2.6.1. Systemic resistance activation

Plants have developed mechanisms to defend themselves against infection by pathogens and pest infestation. Plants are associated with bacteria and fungi that play the role of biological agents, inducing systemic resistance through receptors that activate effective defense responses after detection of pathogen-associated molecules

(PAMPs) (Pinho et al., 2020). In this way, the plant reacts to signals caused by infestation by herbivores, nematodes, or infection by pathogenic microorganisms. In association with certain microorganisms, the plant responds against pathogens and pests by increasing its innate immunity (Tabassum et al., 2017).

According to Coll et al. (2011) the recognition of PAMPs triggers signaling events, including ion fluxes, production of reactive oxygen species (ROS), production of phytohormones such as salicylic acid, jasmonic acid and ethylene, which actively participate in plant resistance systems as they act as flags of these responses. Phytoalexins (phenolic compounds and proteins related to pathogenesis) lead the plant to a type of programmed cell death such as hypersensitivity that occurs at the site where the pathogen attempts to invade (Chadha et al., 2015).

In response to a variety of pathogens, the plant has two pathways, systemic acquired resistance (SAR) and systemic induced resistance (SIR).

Systemic acquired resistance (SAR) causes necrotic lesions resulting from the accumulation of hydrogen peroxide and production of pathogen-related proteins (PR-Proteins) (Van Loon et al., 2006; Yan et al., 2019). Bari and Jones (2009) observed that after activating this response, some plants release methyl salicylate, a volatile derivative from the salicylic acid that can be perceived by neighboring plants of the same species, inducing them to activate their defense mechanisms. After activation, it leads to the production of compounds such as β-glucan present in the cell wall or lytic enzymes such as glucanases and xylanases, which are recognized by receptors in plants (Latz et al., 2018; Yan et al., 2019; Poveda et al., 2020).

Systemic induced resistance (SIR) is associated with the production of jasmonate and ethylene, without involving the expression of PR-proteins. Exposure to non-pathogenic microorganisms can increase resistance to future pathogen attack. Non-pathogenic microorganisms, such as rhizobacteria, activate signaling pathways, involving jasmonic acid and ethylene, which trigger systemic resistance induced throughout the plant. Both responses give the plant the ability to delay or prevent the entry of pathogens, in addition to promoting plant growth (Glazebrook, 2005; Busby et al., 2016).

2.6.2. Production of antibiotics and secondary metabolites by plant growth-promoting fungi and bacteria

Antibiotics are natural or synthetic substances, organic compounds of low molecular weight produced by microorganisms in low concentration and act by inhibiting or causing the death of disease-causing pathogens including bacteria, fungi, viruses, and protozoa that affect humans, plants, and animals (Tan et al. Zoe, 2001; Elnahal et al., 2022).

More than 230 metabolites such as alkaloids, steroids, terpenoids, peptides, polyketones, flavonoids, quinols, phenols, chlorinated compounds, and volatile organic compounds (VOCs) are produced by plant-associated microbial strains, many of them endophytic fungi (Gunatilaka, 2006; Lugtenberg et al., 2016; Latz et al., 2018; Kaddes et al., 2019).

Some bacteria can produce a single antibiotic, while others can produce several substances (Reimer and Bode, 2014). In literature, *Bacillus subtilis* and *B. amyloliquefaciens* species are described as producing subtilin and bacilisin, respectively (Leclère et al., 2005, Yu et al., 2002).

Fungi are a large reservoir of volatile organic molecules (VOCs). The production of several of these bioactive compounds by fungi can facilitate the dominance of their biological niche and offer protection to the plant against harmful invaders. Most studies on fungi are focused on the role of pest biocontrol through the production of enzymes, such as proteases and chitinases, and antibiotics involved in mycoparasitism processes (Viterbo et al., 2002).

According to Pena et al. (2019), endophytic fungus *Muscodor brasiliensis* obtained from *Cinnamomum zeylanicum* (cinnamon) can inhibit and kill some other fungi and bacteria through the production of volatile compounds, among which isoamyl acetate is the most active. Other secondary metabolites produced by endophytic fungi are alkaloids, which accumulate in plants and are toxic to various pest species and even vertebrates (Faeth, 2002; Gimenez et al., 2007; Johnson et al., 2013; Lugtenberg et al, 2016).

Ecological applications and benefits of metabolites produced by fungi are promising, as the constant use of synthetic chemicals in agriculture is harmful to humans, animals, and environment (Lugtenberg et al., 2016). Factors such as season, age, environment, site, and temperature can influence the production of antibiotics and volatiles by fungi (Dastogeer et al., 2020). However, environmental degradation, loss of biodiversity, soil deterioration and water scarcity exacerbate the problem of antibiotic resistance by bacteria, emergence of new viruses, increase in the incidence of fungal infections and resurgence of previously controlled pests, which leads to the urgency for the search for new useful compounds to assist plants (Kaddes et al., 2019).

Consequently, in Brazilian biodiversity, the chance of finding endophytic microorganisms of plants in different environments and ecosystems is great. In this sense, research is trying to discover new effective natural compounds for the treatment of diseases, also promoting the potential of biological products as an alternative for sustainable agriculture.

2.6.3. Production of siderophores by bacteria and fungi

Iron is a micronutrient found in soil often unavailable for assimilation by plants and microorganisms. Iron is an essential element for all living cells and is involved in fundamental processes of electron transfer in photosynthesis and respiration and acts as a catalyst in the synthesis of chlorophyll (Hu et al., 2017).

Generally, iron is in the soil in the unavailable form (insoluble oxy-hydroxides) for assimilation by plants and microorganisms (Rajkumar et al., 2010). Thus, plants have developed different strategies such as the release of protons and organic acids by roots to decrease the soil pH and increase the iron availability or the release of low molecular weight molecules called siderophores that bind to iron and, then they are absorbed by root cells (Johnstone and Nolan, 2015; Chowdappa et al., 2020; Ibiang et al., 2020).

Siderophore-producing microorganisms can stimulate plant growth, directly, by improving iron nutrition and indirectly, by competitively acquiring Fe^{3+}, thus inhibiting the growth of pathogens in the rhizosphere (Ma et al., 2011). Likewise, rhizobacteria and fungi can produce a wide range of siderophores that act outside the cell membrane as agents that chelate iron from organic mineral compounds, capturing iron molecules and binding to complex receptors located in the membrane where they are absorbed, thus making iron available to the plant (Huo et al., 2021).

Suebrasri et al. (2020) demonstrated the production of siderophores by *Trichoderma koningii* ST-KKU 1, *Macrophomina phaseolina* SS 1 L 10 and *M. phaseolina* SS 1 R 10 endophytic strains and suggested that the production of siderophores by fungi was important in promoting bean growth. Similarly, Eslahi et al. (2020) demonstrated the production of siderophores by recombinant endophytic *Trichoderma harzianum* strains in bean plants. In addition, the production of siderophores by endophytic fungi is poorly known and characterized (Card et al., 2016).

In the case of bacteria, Johnstone and Nolan (2015) suggest that certain *Pseudomonas* species produce siderophores that chelate iron in the rhizosphere, thus inhibiting the development of some microorganisms. Murali et al. (2021) verified that the production of siderophores by *Pseudomonas* sp. and *Bacillus megaterium*, together with the production of phytohormones and the action of ACC-deaminase, contributed to the increase in productivity in brown mustard plants (*Brasica juncea*). Finally, other genera involved in the production of siderophores correspond to *Azotobacter, Azospirillum, Bacillus, Dickeya, Klebsiella, Nocardia, Pantoea, Paenibacillus* and *Streptomyces* (Gáll et al., 2016; Goudjal et al., 2016; Romero-Perdomo et al., 2017; Banik et al., 2016; Kesaulya et al., 2018; Pourbabaee et al., 2018).

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CHAPTER 2 - *Aspergillus* spp. and *Bacillus* spp. as growth promoters in cotton plants under greenhouse conditions[1]

ABSTRACT - This study aimed to verify the potential of three *Aspergillus* and *Bacillus* species as growth promoters in cotton plants under greenhouse conditions. The experiment was conducted with a completely randomized design with seven treatments (six microorganisms plus one control) and five replicates until the flowering stage at 70 days after emergence. The inoculation of cotton plants with *Bacillus velezensis* (Bv188) and *Bacillus subtilis* (Bs248 and Bs290) had a positive effect on total nitrogen extraction (899.31, 962.18, and 755.41 mg N/kg dry matter, respectively) compared to the control (459.31 mg N/kg dry weight), total phosphorus extraction (121.94, 124.31, and 99.27 mg P/kg dry matter, respectively) compared to the control (65.10 mg P/kg dry matter), and total dry matter (41.08, 43.59, and 49.86 g/plant, respectively) compared to the control (26.70 g/plant), as well as biomass carbon (72.26, 35.18, and 14.7 mg/kg soil, respectively). Cotton plants inoculated with *Aspergillus brasiliensis* (F111), *Aspergillus sydowii* (F112), and *Aspergillus* sp. (*versicolor* section) (F113) had higher total nitrogen extraction (953.33, 812.59, and 891.62 mg N/kg dry matter, respectively) compared to the control (459.31 mg N/kg dry matter), a higher total phosphorus (122.30, 104.86, and 118.45 mg P/kg dry matter, respectively) compared to the control (65.10 mg P/kg dry matter), a higher total dry matter (37.52, 37.41, and 53.02 g/plant) compared to the control (26.70 g/plant), and greater respiratory activity (14.98, 10.43, and 7.11 mg CO_2/100 g soil, respectively) compared to the control (3.5 mg CO_2/100 g soil). The fungi *A. brasiliensis* (F111) and *A. sydowii* (F112) promoted higher phosphorus absorption by cotton plants, which was reflected by the lower amount of nutrients in the soil (7.10 and 16.96 g P/dm^3 soil) than in the control (26.91 g P/dm^3 soil). The results suggest that *B. subtilis* 248 promoted an increase in phosphorus extracted from the roots and total and phosphorous compounds from the root dry matter and increased the value of soil respiratory activity, and this bacterium could be used as an inoculant in cotton crops.

Keywords: phosphorus solubilization, nitrogen extraction, dry matter, *Aspergillus brasiliensis*, *Bacillus velezensis*

[1] This chapter corresponds to the scientific paper published in the journal Frontiers in Sustainable Food Systems 5: 709267, 2021.

INTRODUCTION

Cotton (*Gossypium* spp.) is a crop of economic importance for Brazil because the country reached production of 2.49 million tons in the 2020/2021 harvest (CONAB, 2021). The uses of cotton extend far beyond fiber, although cotton fiber is the main source for the textile industry. When the boll is opened, the seed of cotton can be used as raw material in the oil industry for human consumption or animal feed (Constable and Bange, 2015; Lima et al., 2016).

Cotton crops have high production costs due to the high demand for mineral fertilizers and pesticides (Khan et al., 2017). Nitrogen is needed in greater quantities than other nutrients in cotton production systems (Hou et al., 2007), and its excessive use causes economic losses and environmental risks. Global estimates indicate that between 85 and 90 million metric tons of nitrogen fertilizer are applied to soil every year, and 40–70% is lost by leaching, with only a small part used by plants; thus, additional nitrogen fertilizer is applied to the soil (Good et al., 2004; Wu and Liu, 2008). Another nutrient that plays an important role in plants is phosphorus, which forms biological molecules; however, its low availability and high fixation in the soil are problems that lead to deficiencies; thus, phosphorus is a limiting factor for plant growth (Anand et al., 2016). In view of the above, the nutritional requirements for cotton production can be addressed by using plant growth-promoting microorganisms (Diaz et al., 2019).

Various microorganisms, such as bacteria, actinobacteria, and fungi, can promote plant growth by fixing atmospheric nitrogen, solubilizing nutrients (not available in special soil types), and increasing nutrient absorption (Spaink, 2000; Harrison, 2005). These microorganisms have the ability to synthesize hormones, including indole acetic acid (IAA), cytokinins, auxins, and gibberellins, which are essential to promote plant growth (van Loon, 2007; Contreras-Cornejo et al., 2009). In addition, they can increase plant growth through the synthesis of secondary metabolites, volatile compounds, and enzymes and increase photosynthesis (Zhang et al., 2008; Vacheron et al., 2013). These microorganisms can also improve plant health and trigger resistance to pathogens and herbivorous insects, thereby inducing

systemic defense responses (Van Wees et al., 2008; Segarra et al., 2009; Hossain et al., 2016).

Studies have shown that the use of plant growth-promoting microorganisms as inoculants is a viable strategy in current agricultural production systems (Romero-Perdomo et al., 2017; Numan et al., 2018; Diaz et al., 2019); therefore, the search for new microorganisms must be constant to face current and future problems in cotton crops.

In this context, the aim of this study was to verify whether the microorganisms *Bacillus velezensis*, *Bacillus subtilis*, *Aspergillus brasiliensis*, *Aspergillus sydowii*, and *Aspergillus* sp. (versicolor section) have the ability to promote growth in cotton plants under greenhouse conditions.

MATERIALS AND METHODS

Study Location

The experiment was carried out in a greenhouse in the Horticulture Sector of the “Júlio de Mesquita Filho” São Paulo State University (UNESP), Campus of Jaboticabal, São Paulo, Brazil (21°14′28″S, 48°17′23″W). According to the Köppen and Geiger classification, the local climate corresponds to a tropical climate with a dry winter season (Peel et al., 2007), an average temperature of 22°C and an average annual rainfall of approximately 1,340 mm. The predominant soil at the study site is classified as eutrophic red latosol with a clayey texture (52% clay, 23% silt, and 24% total sand) (EMBRAPA Solos, 2018).

Experimental Design and Experiment Management

The experiment was carried out in a completely randomized design with seven treatments (six microorganisms and one control without inoculation) and five replicates. The experiment was carried out until the flowering stage at 70 days after

emergence (DAE). Pots with a capacity of 5 L were filled with sieved soil (particles smaller than 1 cm in diameter). Fertilization was carried out according to a soil analysis and according to nutritional recommendations for pot experiments proposed by Malavolta et al. (1989) for cotton crops. The amounts of nutrients used in the soil were as follows: nitrogen (N: 3.33 g urea/pot), phosphorus (P: 5.5 g P_2O_5/pot), potassium (K: 1.66 g KCl/pot), calcium (Ca: 6.25 g of Super simple SS/pot), magnesium (Mg: 0.5 g MgO/pot), sulfur (S: 3.125 g of SS/pot), zinc (Zn: 0.125 g $ZnSO_4$/pot), boron (B: 0.025 g H_3BO_3/pot), molybdenum (Mo: 0.002 g molybdate/pot), copper (Cu: 0.03 g $CuSO_4$/pot), and manganese (Mn: 0.08 g $MnSO_4$/pot). All nutrients were mixed with sieved soil 1 week before sowing. The moisture content of the pots was maintained at approximately 70% of the field capacity with daily irrigation. Four cotton seeds (*Gossypium hirsutum*-IMA7501 WS) were sown per pot, and thinning was carried out 15 days after seedling emergence, maintaining one plant per pot.

Inoculum Preparation

The microorganisms used in the study belong to the collection of the Laboratory of Soil Microbiology, UNESP, Campus of Jaboticabal. These microorganisms (bacteria and fungi), *B. subtilis* strain Bs248 (Access Number MZ133755), *B. subtilis* strain Bs290 (Access Number MZ133476), *B. velezensis* strain Bv188 (Access Number MZ133757), *A. brasiliensis* strain F111 (Access Number MZ133758), *A. sydowii* strain F112 (Access Number MZ133759), and *Aspergillus* sp. versicolor section strain F113 (Access Number MZ133456), were selected because they have growth promotion characteristics, such as phosphorus solubilization, biological nitrogen fixation, and IAA production (Baron et al., 2018; Diaz et al., 2019; Milani et al., 2019).

Each bacterial isolate was multiplied in an Erlenmeyer flask containing 90 ml of sterile nutrient broth and incubated for 24 h at 28°C in a bacteriological incubator. After 24 h of incubation, absorbance readings of each isolate were performed in a spectrophotometer at 630 nm, and in parallel, 100 µl of each isolate was sown in Petri dishes (Kloepper et al., 1989) containing nutrient agar to determine the concentration and adjustment for 10^8 colony-forming units (CFU/ml).

For fungi, a suspension of conidia was prepared by scraping Petri dishes containing fungi grown on potato dextrose agar for 7–10 days at 25°C. Fungi were scraped with 0.1% Tween 80 in sterile distilled water. Fungi suspensions were filtered to remove excess mycelium. The concentration of each fungus was determined according to the conidia count in a Neubauer chamber and adjusted to 10^8 spores/ml.

Cotton seeds were individually inoculated with microorganisms (bacteria or fungi) by immersion for 8 h at 25°C (Jaber and Enkerli, 2016). Immersion was carried out in the dark under agitation on a shaking platform at 130 rpm. After the immersion period, cotton seeds were sown in pots containing sieved and fertilized soil as previously described. Cotton seedlings were inoculated every 15 days with 10 ml of suspension containing the respective microorganism at a concentration of 10^8 CFU or spores/ml. Inoculations were carried out by applying the inoculum to the plant base and stem using a graduated micropipette (Kasvi single-channel premium black k1–1000 PB). Treatments were as follows: Bv188 = *B. velezensis* strain Bv188, Bs248 = *B. subtilis* strain Bs248, Bs290 = *B. subtilis* strain Bs290, F111 = *A. brasiliensis* strain F111, F112 = *A. sydowii* strain F112, F113 = *Aspergillus* sp. versicolor section strain F113, and Control = No inoculation.

Biometric Plant Parameters

Shoot and Root Dry Matter

At 70 DAE, the experiment was disassembled, and the roots of the cotton plants were washed with running water to remove excess soil. After washing, the plants were separated into roots and shoots and placed in paper bags for drying in an oven with air circulation at 65°C until reaching constant weight. The weight of the root and shoot dry matter was determined using an analytical scale (Mettler Toledo model AB204).

Preparation of Soil Samples

Thirty-five soil samples were separated into two subsamples of approximately 100 g each. One subsample was sieved and dried at room temperature for chemical analysis, and the other was kept in a refrigerator for microbiological analysis. After harvest, the presence of the inoculated microorganisms was checked from each soil sample.

Phosphorus Determination

Soluble phosphorus in soil was determined using the method proposed by Watanabe and Olsen (1965). For the determination of phosphorus in plants, the phosphorus concentrations in roots and shoots were determined according to the methodology proposed by Sarruge and Haag (1974) and modified by Bezerra Neto and Barreto (2011). A sample of 0.5 g of plant tissue from the digester tube was added to 5 ml of concentrated nitric acid and 1 ml of concentrated perchloric acid. The mixture was left to rest for 1 day. Then, complete digestion was performed using a block digester. The material was washed with distilled water to obtain 50 ml of extract. A reading was performed in a spectrophotometer at 470 nm using 5 ml of extract plus 1 ml of specific reagent composed of a mixture of 5% ammonium molybdate and 0.25% ammonium vanadate.

Total Nitrogen Concentration in Plant and Soil

The nitrogen concentration in the shoots and roots was determined according to Sarruge and Haag (1974) with sulfuric digestion of the plant material to measure the nitrogen concentration associated with obtaining 90% dry matter production. The total nitrogen in the soil was determined according to the methodology proposed by Bremner (1996) and modified by Wilke (2005).

Microbial Respiratory Activity

Respiratory activity was determined by the method of quantifying the released CO_2 according to Jenkinson and Powlson (1976) using wide-mouthed glass flasks, to which 100 g of soil (dry or wet) was added. Inside the flasks, two beakers were used (one containing 20 ml of NaOH and the other 20 ml of distilled water). Beakers were placed inside glass flasks that were sealed with plastic film and incubated in the dark for 7 days. The microbial respiratory activity was measured based on the amount of CO_2 released from the soil samples. After incubation, the remaining NaOH was quantified by titration with HCl.

Microbial Biomass Carbon

Microbial biomass carbon was determined by the irradiation-extraction method (Islam and Weil, 1998; Mendonça and Matos, 2017) using a microwave oven with a power of 900 W (Panasonic model NN-ST 252 WRUN) and frequency of 2,450 MHz. After irradiation, samples were subjected to a 0.5-mol/L potassium sulfate extractor, and microbial biomass carbon was determined by oxidation with 0.066 mol/L potassium dichromate and titration with 0.033 mol/L ferrous ammonium sulfate (Brookes et al., 1982).

Nitrogen and Phosphorus Extraction

Nitrogen extraction from the roots was calculated by multiplying the nitrogen content in the root and root dry matter for each replicate. Nitrogen extraction from the shoots was calculated by multiplying the nitrogen content in the shoot and shoot dry matter for each replicate. Total nitrogen extraction was calculated by multiplying the sum of the nitrogen content of the roots and shoots and the sum of the root and shoot dry matter for each replicate. For the phosphorus extraction calculation, the procedure was the same as that used for nitrogen.

Presence of Microorganisms Previously Inoculated

To verify the presence of the microorganisms previously inoculated in the soil sample, 10 g of rhizospheric soil was added to 95 ml of 0.1% pyrophosphate solution (w/v) and subjected to serial dilution (Wollum, 1982) up to a concentration of 10^{-4}. A 0.1-ml aliquot from the dilution (10^{-4}) was transferred to Petri plates containing Bunt and Rovira medium (Bunt and Rovira, 1955), pH 7.4, for bacteria and maltose agar for fungi. Then, plates containing the bacterial and fungal inoculum were kept at 30°C for 72 h and 7 days, respectively. After this period, the colonies were visualized with the aid of a magnifying glass at 6 × magnification, and a Gram stain test was performed.

Statistical Analysis

Data were log (x + 1) transformed to avoid violating the assumption of the ANOVA. Data normality and homogeneity of variance were assessed by the Shapiro–Wilk and Levene tests ($\alpha = 0.05$), respectively. The parameters under study were analyzed by the ANOVA *F* test ($\alpha = 0.05$) to identify differences. Subsequently, multiple comparisons of averages were performed using Duncan's test ($\alpha = 0.05$) to identify significant differences between treatments. Statistical analyses were performed using R software for Windows (R Core Team, 2020).

RESULTS

Inoculations with Bv188, Bs248, Bs290, F111, F112, and F113 increased the amount of nitrogen extracted from the roots (177.14, 173.48, 166.73, 197.09, 158.71, and 193.50 mg N/kg dry matter, respectively) compared to the control (66.31 mg N/kg dry matter), from the shoots (722.27, 704.68, 588.68, 756.24, 653.88, and 698.12 mg N/kg dry matter, respectively) compared to the control (393.00 mg N/kg dry matter), and from the total (899.31, 962.18, 755.41, 953.33, 812.59, and 891.62 mg N/kg dry

matter, respectively) compared to the control (459.31 mg N/kg dry matter) (Figure 1); moreover, they increased the amount of phosphorus extracted from the roots (47.17, 46.75, 40.68, 40.61, 42.22, and 42.31 mg P/kg dry matter, respectively) compared to the control (21.87 mg P/kg dry matter), from the shoots (74.78, 77.56, 58.59, 81.69, 62.64, and 76.15 mg P/kg dry matter, respectively) compared to the control (43.23 mg P/kg dry matter), and from the total (121.94, 124.31, 99.27, 122.30, 104.86, and 118.45 mg P/kg dry matter, respectively) compared to the control (65.10 mg P/kg dry matter) in cotton plants (Figure 2).

Cotton plants inoculated with bacteria Bv188, Bs248, and Bs290 showed higher weight of the root dry matter (12.37, 15.03, and 20.60 g/plant, respectively) compared to the control (7.05 g/plant), of the shoot dry matter (28.71, 28.56, and 29.27 g/plant, respectively) compared to the control (19.65 g/plant), and of the total dry matter (41.08, 43.59, and 49.86 g/plant, respectively) compared to the control (26.70 g/plant) (Figure 3). In the case of fungi, inoculation with F111, F112, and F113 presented greater weights of root dry matter (13.17, 11.87, and 21.28 g/plant, respectively) and total dry matter (37.52, 37.41, and 53.02 g/plant, respectively).

The nitrogen content in the shoot dry matter (10.16 g N/kg dry matter) (Figure 4A) and phosphorus content in the root dry matter (4.28 g P/kg dry matter) were higher in cotton plants inoculated with Bv188 (Figure 4B) and Bs248 (Figure 5A) compared to the control (20.02 N/kg dry matter). There were no differences in the nitrogen content in the root dry matter ($p = 0.175$), and the phosphorus content in the shoot dry matter was not different from that in the control except for F112, which was lower ($p < 0.003$) (Figure 5B).

The nitrogen percentage was lower in soils inoculated with Bv188, Bs248, Bs290, F111, F112, and F113 (5.12, 5.23, 8.07, 5.23, 5.23, and 6.02%, respectively) than in the control (8.77%) (Figure 6A). The soluble phosphorus content was lower only in soils inoculated with fungi F111 and F112 (7.10 soil and 16.97 mg/dm3 soil, respectively) compared to the control (26.91 mg/dm3 soil) (Figure 6B). The biomass carbon results indicated that inoculation with Bv188, Bs248, Bs290, F111, and F113 (72.26, 35.18, 32.54, 38.29, and 45.16 mg C/kg soil, respectively) positively affected this variable compared to the control (6.41 mg C/kg soil) (Figure 6C). In the case of respiratory activity, a positive effect was only observed for inoculation with

microorganisms Bv188, Bs248, F11, F112, and F113 (16.41, 23.21, 14.98, 10.43, and 7.11 mg CO2/100 g soil, respectively) compared to the control (3.50 mg CO2/100 g soil) (Figure 6D).

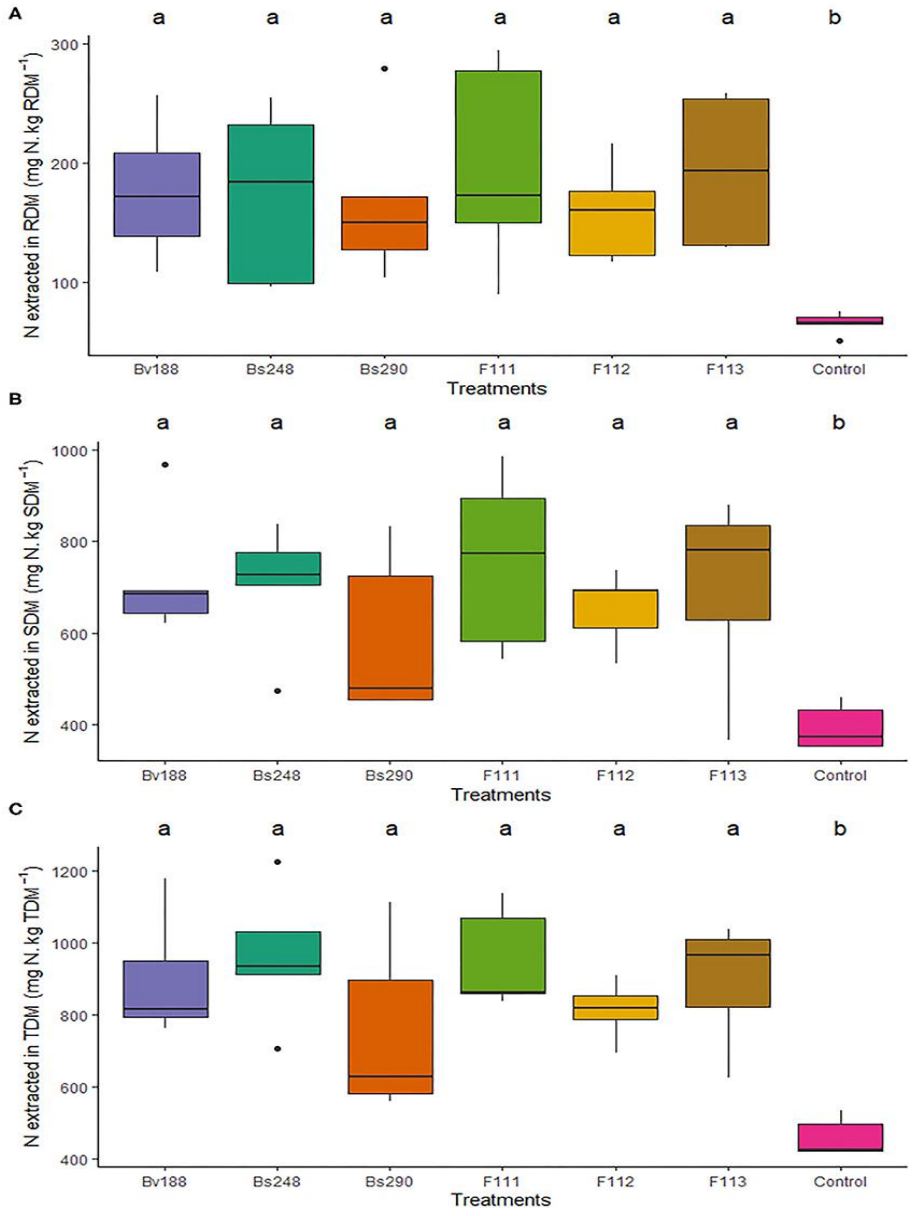

FIGURE 1 | Boxplots (median and quartiles) of N extracted from the roots (A), shoots (B), and total dry matter (C) of cotton plants inoculated with the treatments. Different letters in the rows indicate significant differences between means (Duncan's, $p < 0.05$). RDM, root dry matter; SDM, shoot dry matter; TDM, total dry matter; Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; F111, *Aspergillus brasiliensis*; F112, *Aspergillus sydowii*; F113, *Aspergillus* sp. *versicolor* section; Control, no inoculation.

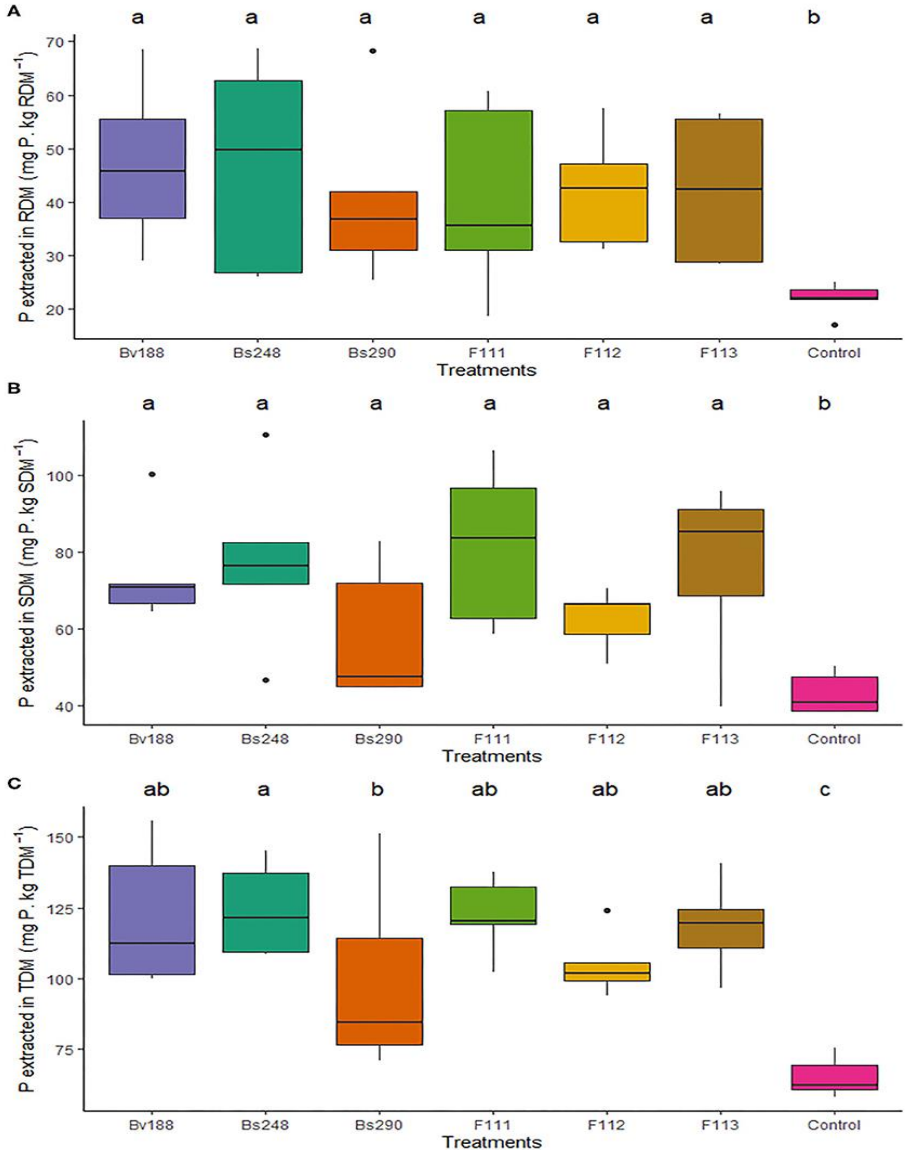

FIGURE 2 | Boxplots (median and quartiles) of P extracted from the roots **(A)**, shoots **(B)**, and total dry matter **(C)** of cotton plants inoculated with the treatments. Different letters in the rows indicate significant differences between means (Duncan's, $p < 0.05$). RDM, root dry matter; SDM, shoot dry matter; TDM, total dry matter; Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; F111, *Aspergillus brasiliensis*; F112, *Aspergillus sydowii*; F113, *Aspergillus* sp. *versicolor* section; Control, no inoculation.

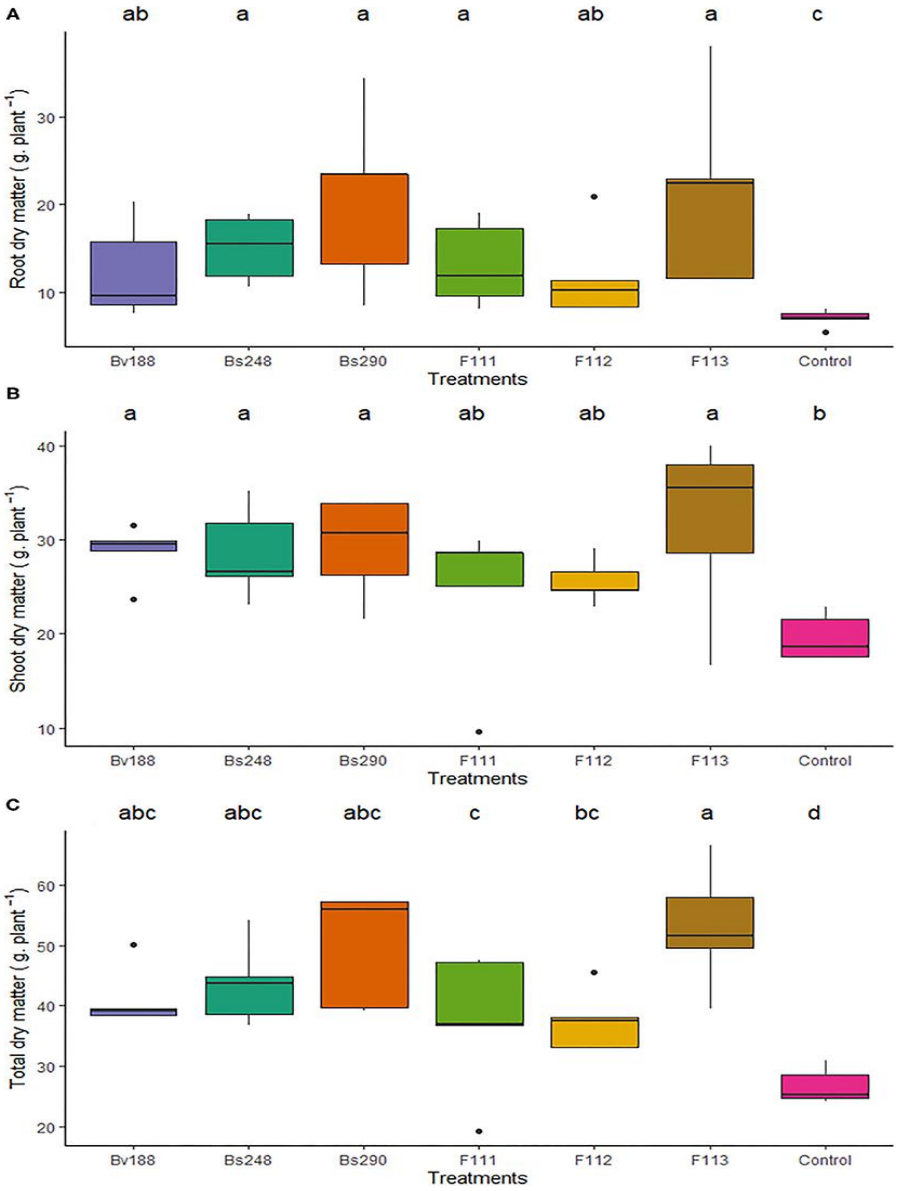

FIGURE 3 | Boxplots (median and quartiles) of the root **(A)**, shoot **(B)**, and total dry matter **(C)** of cotton plants inoculated with the treatments. Different letters in the rows indicate significant differences between means (Duncan's, $p < 0.05$). Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; F111, *Aspergillus brasiliensis*; F112, *Aspergillus sydowii*; F113, *Aspergillus* sp. *versicolor* section; Control, no inoculation.

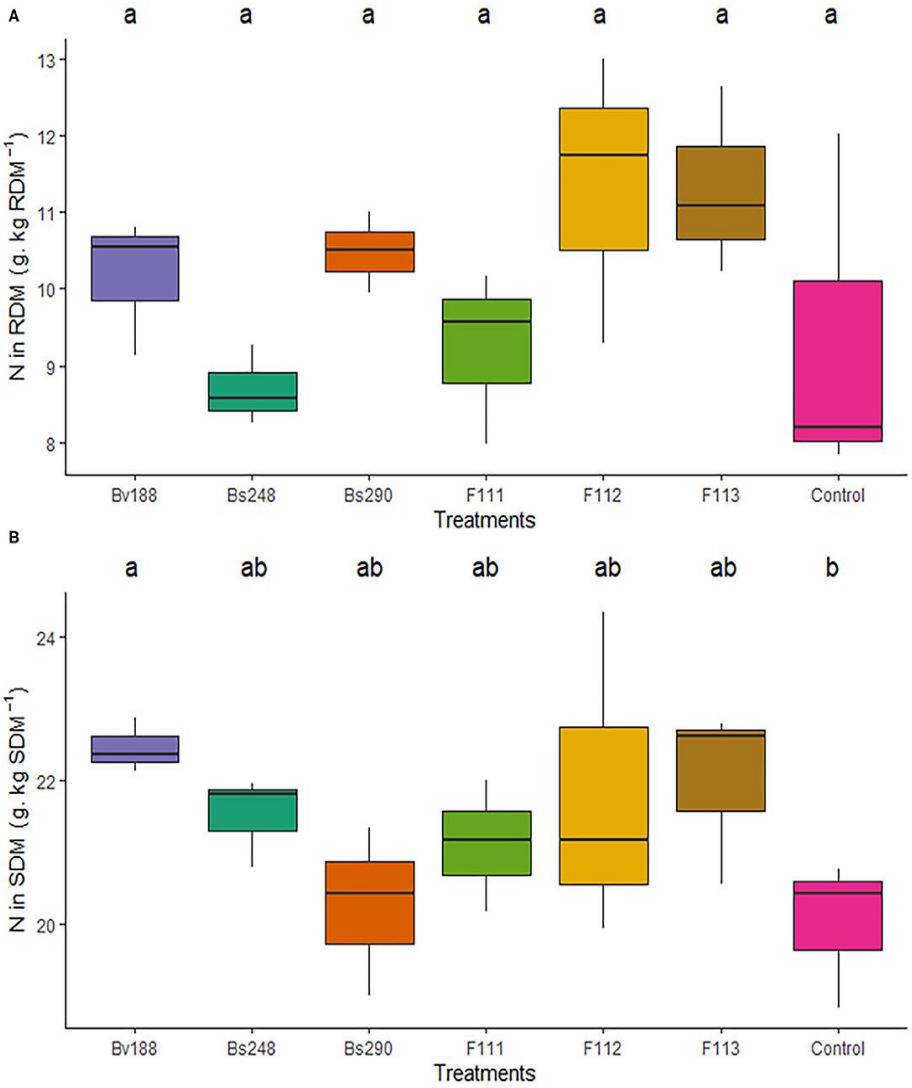

FIGURE 4 | Boxplots (median and quartiles) of the N content in the root **(A)** and shoot **(B)** dry matter of cotton plants inoculated with the treatments. Different letters in the rows indicate significant differences between means (Duncan's, $p < 0.05$). RDM, root dry matter; SDM, shoot dry matter; Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; F111, *Aspergillus brasiliensis*; F112, *Aspergillus sydowii*; F113, *Aspergillus* sp. *versicolor* section; Control, no inoculation.

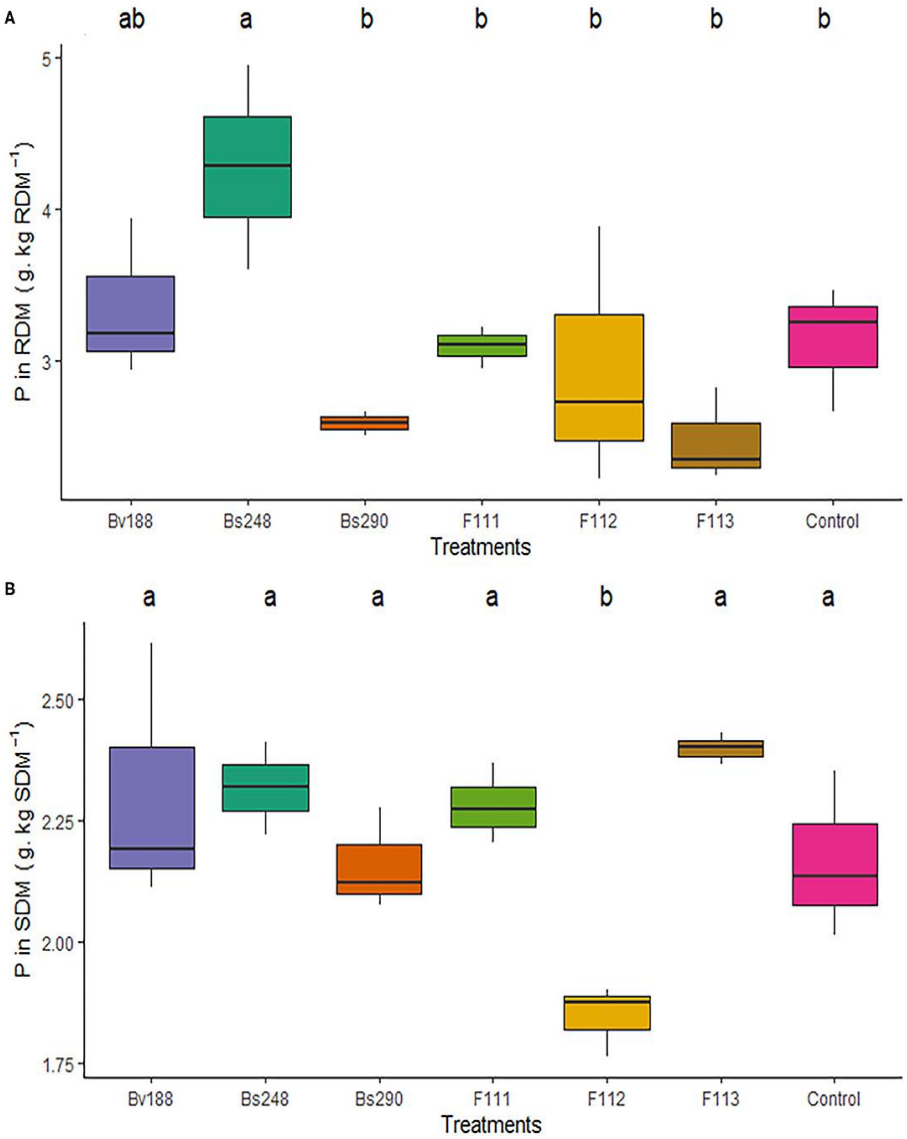

FIGURE 5 | Boxplots (median and quartiles) of P extracted in root **(A)** and shoot **(B)** total dry matter of cotton plants inoculated with the treatments. Different letters in the rows indicate significant differences between means (Duncan's, $p < 0.05$). RDM, root dry matter; SDM, shoot dry matter; Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; F111, *Aspergillus brasiliensis*; F112, *Aspergillus sydowii*; F113, *Aspergillus* sp. *versicolor* section; Control, no inoculation.

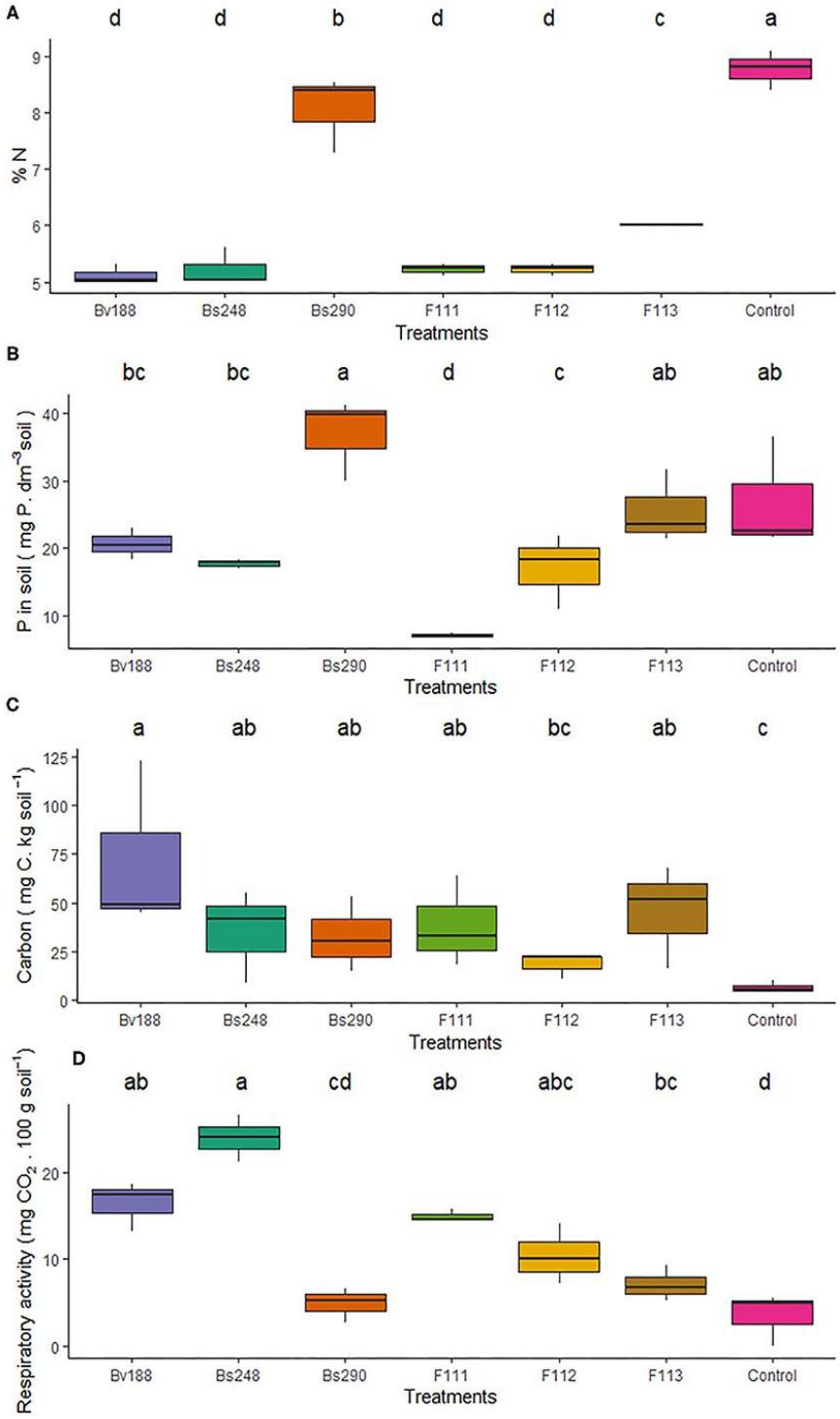

FIGURE 6 | Boxplots (median and quartiles) of the N percentage **(A)**, soluble phosphorus **(B)**, biomass carbon **(C)**, and respiratory activity **(D)** in soil inoculated with the treatments. Different letters in the rows indicate significant differences between means (Duncan's, $p < 0.05$). RDM, root dry matter; SDM, shoot dry matter; TDM, total dry matter; Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; F111, *Aspergillus brasiliensis*; F112, *Aspergillus sydowii*; F113, *Aspergillus* sp. *versicolor* section; Control, no inoculation.

DISCUSSION

The microorganisms used in the present study showed specific abilities to promote growth in cotton plants according to the parameters evaluated. There was no microorganism that improved all the parameters analyzed.

Concerning the shoot and root dry matter, higher values were found for *Aspergillus* sp. versicolor and *B. subtilis* 290, although there was no significant difference between them. When bacteria promote an increase in shoot dry mass, the plant has a great chance to improve its photosynthetic efficiency, producing more metabolites for growth. When root development is increased, plants increase their efficiency in exploring the soil and their capacity to absorb water and nutrients (Hu and Chabbi, 2021). The abilities shown for these two microorganisms are important for plant growth.

Bacillus sp. isolated from the wheat rhizosphere showed the ability to promote plant growth by increasing the shoot and root length, fresh weight, dry weight, production of siderophores, phosphorus solubilization activity, production of IAA, and inhibitory action on the growth of *Fusarium oxysporum* in medicinal plants (Zhao et al., 2015). El-Deeb et al. (2012) isolated *B. subtilis* from roses and evaluated their colonization capacity to increase the content of dry matter, solubilization of phosphorus, production of siderophores and extracellular enzymes, and production of hydrolytic enzymes. *B. subtilis* in the rhizosphere promotes a decrease in soil pH, production of organic acids in the rhizosphere, and anion exchange and chelation, thus demonstrating its potential to improve phosphorus absorption in cotton crops (Ahmad et al., 2021).

In the present study, *B. velezensis* (Bv188) isolated from maize plants promoted an increase in the total and root dry matter, nitrogen absorption in the shoots, and phosphorus solubilization in the roots. Similar results were found by Meng et al. (2016), who demonstrated that inoculation with *B. velezensis* BAC03 promoted greater biomass gain, IAA production, and enzymatic activity in beet, carrot, cucumber, and radish plants.

Bacillus velezensis FZB42, a strain previously classified as *Bacillus amyloliquefaciens* ssp. *plantarum* FZB42, due to the morphology, physiology, chemotaxonomy, and phylogenetic similarity related to them, sharing the same phenotype and genotype (Dunlap et al., 2016). This bacterium isolated from the beet rhizosphere has been investigated as a new species that is widely found in the rhizosphere and has potential for agricultural use due to its inhibitory action on fungi and biocontrol skills (Perron et al., 2020).

The two isolates of the genus *Aspergillus* used in this study were sequenced and identified as *A. brasiliensis* (F111) and *A. sydowii* (F112) by Baron et al. (2018). The results demonstrate that these fungi inoculated in cotton plants favor phosphorus and nitrogen absorption (Figure 6B). Both *A. brasiliensis* and *A. sydowii* showed great biotechnological potential due to the production of enzymes and other substances of interest and can be used to aid in the absorption and solubilization of phosphorus and nitrogen, which are important for plant growth.

Interestingly, *A. brasiliensis* promoted the lowest value of phosphorus in the soil. This result suggests that this fungus has a great ability to mineralize this nutrient, promoting its greater availability to plants. The ability to solubilize phosphorus is important because this nutrient is essential to plant growth, including cotton crops, and easily, this nutrient is absorbed by soil clay, becoming unavailable to plants (Wan et al., 2020). Phosphorus is responsible for the storage and transfer of energy such as glucose, fructose, and ATP (Habib et al., 2015; Mumtaz et al., 2019). Similar results were found by Liang et al. (2020), who demonstrated that phosphorus-solubilizing bacteria also influence the physical, chemical, and biological soil properties by significantly increasing the growth and productivity of tomato plants. Another essential nutrient is nitrogen, which is an important element for life. It is present in the structures of essential biochemicals, such as nucleotides and proteins. Fungi do not have the ability to fix nitrogen from the atmosphere; however, fungi mineralize soil organic matter, making this nutrient available to plants (Baron et al., 2018).

Filamentous fungi belonging to the genera *Aspergillus* and *Trichoderma* are recognized as producers of cellulolytic enzymes used in the bioethanol industry (Zoglowek et al., 2015). Studies have shown that *A. brasiliensis* is grouped in the section of black aspergillus and produces large amounts of citric acid and enzymes,

such as xylanases, thermostable β-xylidasidases, α-glucosidase, and amylases, with enzymes being one of the main factors responsible for the characteristic phosphorus solubilization in soil (Nahas and de Assis, 1992; Schneider et al., 2010; de Oliveira Mendes et al., 2014).

Another important highlight in this work is verified by the biomass (Figure 6C) and microbial respiration (Figure 6D) in the soil. Inoculation positively affected the biological characteristics of the soil, which contributed to an increase in the microbial community (bacterial biomass and respiratory activity).

Biomass carbon is directly proportional to the colonization ability of microorganisms. The microorganisms that stood out in this parameter were *B. velezensis* 188 and *B. subtilis* 248. Another parameter that reinforces this result is the increase in respiratory activity related to soil microbial activity. Ju et al. (2019) demonstrated the beneficial impact of the inoculation of rhizospheric microorganisms on plant growth in addition to the increase in microbial biomass, which positively contributed to the biochemical properties of soils planted with lettuce.

As previously mentioned, no microorganism showed a great ability to increase all parameters. On the other hand, all microorganisms improved one or more parameters compared to the control, which did not receive microbial inoculation.

CONCLUSION

The results suggest that the *Aspergillus* sp. versicolor tends to improve plant growth and development. Nevertheless, *A. brasilense* can improve the phosphorus availability in soil, and *B. subtilis* 248 promoted an increase in phosphorus extracted from the roots and total and phosphorous compounds from the root dry matter and increased the value of soil respiratory activity.

Based on the results, the bacterium *B. subtilis* 248 could be used as an inoculant in cotton crops.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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CHAPTER 3 - Effect of *Aspergillus* and *Bacillus* Concentration on Cotton Growth Promotion[2]

ABSTRACT - There are no studies in literature on the effect of inoculant concentrations on plant growth promotion. Therefore, in the present study, two experiments were carried out, one under pot conditions and the other in the field with cotton crop, in order to verify the effect of *Aspergillus* and *Bacillus* concentrations on the biometric and nutritional parameters of plant and soil, in addition to yield. The pot experiment evaluated the effect of different concentrations, ranging from 1×10^4 to 1×10^{10} colony-forming units per milliliter (CFU mL^{-1}) of microorganisms *Bacillus velezensis* (Bv188), *Bacillus subtilis* (Bs248), *B. subtilis* (Bs290), *Aspergillus brasiliensis* (F111), *Aspergillus sydowii* (F112), and *Aspergillus* sp. *versicolor* section (F113) on parameters plant growth promotion and physicochemical and microbiological of characteristics soil. Results indicated that the different parameters analyzed are influenced by the isolate and microbial concentrations in a different way and allowed the selection of four microorganisms (Bs248, Bv188, F112, and F113) and two concentrations (1×10^4 and 1×10^{10} CFU mL^{-1}), which were evaluated in the field to determine their effect on yield. The results show that, regardless of isolate, inoculant concentrations promoted the same fiber and seed cotton yield. These results suggest that lower inoculant concentrations may be able to increase cotton yield, eliminating the need to use concentrated inoculants with high production cost.

Keywords: rhizobacteria, *Aspergillus sydowii*, *Bacillus* sp., yield, growth promoters, inoculants

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INTRODUCTION

The use of plant-growth promoting microorganisms (PGPMs) has increased in the world as an alternative to the excessive application of mineral fertilizers that can contribute to soil degradation, emission of polluting gases into the atmosphere, and reduction of biodiversity in different ecosystems (Singh et al., 2016).

Inoculants are products that have in their composition live microorganisms capable of promoting plant development with different mechanisms or modes of action, such as production of phytohormones and siderophores, phosphate solubilization, and induction of resistance against abiotic and biotic stresses (Bhattacharyya and Jha, 2012; Malusá and Vassilev, 2014). PGPM application has been carried out in several agricultural cultures, and many studies have been developed to elucidate its mode of action in plants to meet the new requirements of industries in the sector and agricultural producers. The microorganisms most frequently used as inoculants are fungi of the genera *Trichoderma, Purpureocillium, Metarhizium, Beauveria*, and *Aspergillus* (Behie and Bidochka, 2014; Samson et al., 2014; Alori and Babalola, 2018; Baron et al., 2018, 2020; Ahmad et al., 2020), and bacteria of the genera *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, and *Streptomyces* (Kloepper et al., 1989; Okon and Labandera-Gonzalez, 1994; Glick et al., 1999; Tahmatsidou et al., 2006; Marulanda et al., 2009; Pedraza et al., 2010; Diaz et al., 2019).

Under field conditions, PGPMs are applied in the form of formulated products, which contain inerts and additives in addition to the active ingredient, which is the microorganism. The search for new inoculant formulations, which enhance plant development in order to reduce the use of mineral fertilizers, thus contributing to more sustainable agriculture, is increasing (Malusá and Vassilev, 2014; Bizos et al., 2020). These new formulations have included increasing the concentration of microorganisms to be applied in the field. However, despite the advance in the use of inoculants in agriculture, there are few studies that have evaluated the effect of inoculant concentration on plant growth promotion, particularly in cotton. Thus, this theme has become essential to define whether the increase in the concentration of

microorganisms is an important aspect related to product efficiency or whether it is just an aspect of commercial advantage.

In this study, cotton was used because it is a crop that stands out for its high demand for mineral fertilizers and phytosanitary products to ensure good productivity, a situation that causes serious changes in the environment (Michereff and Barros, 2001; Carvalho and Barcellos, 2012).

The aim was to determine the effect of different concentrations of microorganisms *Bacillus velezensis, Bacillus subtilis, Aspergillus brasiliensis, Aspergillus sydowii*, and *Aspergillus sp*. (versicolor section) on the growth of cotton plants under pot conditions in greenhouse and field conditions.

MATERIALS AND METHODS

Study Location

According to the Köppen and Geiger classification, the climate of the region corresponds to a tropical climate with dry season in the winter (Peel et al., 2007). The predominant soil at the site is classified as Red Eutrophic Latosol (Oxisol) with clayey texture (52% clay, 23% silt, and 24% total sand) (EMBRAPA, 2006).

Experiment 1: Determination of the Effect of Inoculation of Microorganisms at Different Concentrations in Greenhouse

Microorganisms and Inoculant Preparation

Microorganisms (bacteria and fungi) used in this study belong to the collection of the Laboratory of Soil Microbiology, UNESP, Campus of Jaboticabal (Table 1) and were selected for presenting growth-promoting characteristics such as phosphorus solubilization, biological nitrogen fixation, and indole acetic acid production (Baron et al., 2018; Diaz et al., 2019; Milani et al., 2019).

Table 1 | Description of microorganisms.

Microorganisms	Code in the collection	GenBank deposit number
Bacillus subtilis	Bs248	MZ133755
B. subtilis	Bs290	MZ133476
Bacillus velezensis	Bv188	MZ133757
Aspergillus brasiliensis	F111	MZ133758
Aspergillus sydowii	F112	MZ133759
Aspergillus sp. (*versicolor* section)	F113	MZ133456
Control	–	–

The microorganisms used in the study were pre-inoculated in Petri dishes containing nutrient agar for bacteria and potato dextrose agar for fungi. Incubation was carried out in BOD oven at 30°C for 24 h for bacteria and at 25°C for 7 days for fungi.

Each bacterial isolate was multiplied in Erlenmeyer flask containing 90 ml of sterile nutrient broth medium inoculated with isolates prepared on Petri dishes. Flasks were incubated at 30°C for 24 h under agitation at 150 rpm. Then, absorbance readings of each isolate were carried out in spectrophotometer at 600 nm to determine the optical density. In addition, 100 µl of each flask with the different isolates was seeded in Petri dishes containing nutrient agar for the determination and adjustment of cell concentrations (Kloepper et al., 1989).

For fungi, conidium suspension was prepared by scraping Petri dishes containing mycelium cultivated on potato dextrose agar for 7–10 days at 25°C. For scraping, 0.1% Tween 80 solution was used. Fungi suspensions obtained were filtered in sterile voile to remove excess mycelium. The determination of the conidium concentration of each fungus was performed by counting in Neubauer chamber. For all microorganisms (bacteria and fungi), concentrations of 1×10^4, 1×10^6, 1×10^8, and 1×10^{10} colony-forming units/ml (CFU mL^{-1}) were standardized for bacteria and conidia ml for fungi.

Seed Inoculation

Cotton seeds were individually inoculated with microorganisms (bacteria or fungi) by immersion for 8 h at 25°C (Jaber and Enkerli, 2016). Immersion was carried out in the dark under agitation at 130 rpm. This procedure was performed for all microorganisms and concentrations. After the immersion period, cotton seeds were sown in pots containing previously sieved soil.

Cotton seedlings were inoculated three times from the beginning to the end of the experiment at 15-day intervals. In each inoculation, 10 ml of suspension containing the respective microorganism at concentrations of 1×10^4, 1×10^6, 1×10^8, and 1×10^{10} CFU mL^{-1} for bacteria and conidia ml for fungi was applied per pot. Inoculations were performed by applying the inoculum at the base and stem of plants using graduated micropipette (Kasvi monocanal premium black k1-1000 PB).

Experimental Design and Experiment Management

The experiment was carried out at the Horticulture Sector of the “Júlio de Mesquita Filho” São Paulo State University (UNESP), Campus of Jaboticabal, São Paulo, Brazil. The experiment was arranged in a randomized block design with 6 × 4 factorial arrangement + 1 additional treatment (control) with five replicates, totaling 125 pots. Microorganism factor sublevels were Bs248, Bs290, Bv188, F111, F112, and F113 (Table 1). Concentration factor sublevels were 1×10^4, 1×10^6, 1×10^8, and 1×10^{10} CFU or conidia ml^{-1}. Pots of 5-L capacity were filled with sieved soil (particles smaller than 1 cm in diameter) and fertilized according to previously performed soil analysis (Table 2) and nutritional recommendations for pot experiments proposed by Malavolta et al. (1997) for cotton crop. Nitrogen (N: 3.33 g urea/pot), phosphorus (P: 5.5 g P_2O_5/pot), potassium (K: 1.66 g KCl/pot), calcium (Ca: 6.25 g super single/pot), magnesium (Mg: 0.5 g MgO/pot), sulfur (S: 3.125 g super single/pot), zinc (Zn: 0.125 g $ZnSO_4$/pot), boron (B: 0.025 g H_3BO_3/pot), molybdenum (Mo: 0.002 g molybdate/pot), copper (Cu: 0.03 g $CuSO_4$/pot), and manganese (Mn: 0.08 g $MnSO_4$/pot) were added. All nutrients were mixed with the sieved soil 1 week before

sowing. The moisture content of pots was kept around 70% of the field capacity with daily irrigations.

Table 2 | Analysis of soil used in greenhouse and field experiments.

pH	OM	P	K	Ca	Mg	H + Al	S.B.	CEC	V
$CaCl_2$	g/dm^3	Mg/dm^3			……$mmol_c/dm^3$……				%
6.9	10	23	0.7	79	13	11	93.4	104.2	90

*OM, organic matter; S.B., Ca + Mg + Na + K; CEC, S.B. + H + Al; V%, (S.B./CEC) * 100.*

Five cotton seeds (*Gossypium hirsutum*–IMA7501 WS) were sown per pot; and 15 days after seedling emergence, thinning was performed, keeping one plant per pot. The experiment was carried out until the flowering of cotton plants, 70 days after emergence.

Evaluated Parameters

Shoot and Root Dry Matter

Plants were collected and separated into shoots and roots, washed in running water, and placed in paper bags for drying in oven with air circulation at 65°C until reaching constant weight. Root and shoot dry matter weight was determined using analytical scale.

Preparation of Soil Samples

Samples were separated into two subsamples of approximately 100 g each. A subsample was sieved and dried at room temperature for chemical analysis, and the other was kept in a refrigerator for microbiological analysis.

Counting Bacteria Present in the Soil

Ten grams of soil was placed in an Erlenmeyer flask containing 95 ml of 0.1% sodium pyrophosphate saline solution. All Erlenmeyer flasks were shaken for 1 h at 130 rpm, and the contents of flasks were used to prepare serial dilutions following methodology proposed by Wollum (1982). Aliquots of 100 µl of obtained dilutions were inoculated into Petri dishes containing nutrient agar medium or potato dextrose agar in triplicate. Plates were kept in BOD oven at 30°C for bacteria and 25°C for fungi. The number of CFU mL^{-1} was verified after 24, 48, and 72 h (Vieira and Nahas, 2000).

Counting of Endophytic Bacteria and Fungi

Plants were separated into leaves and roots and washed with running water. Samples containing 3 g of each vegetative tissue (leaves and roots) were submitted to superficial disinfection to eliminate epiphytic microorganisms. Each tissue (leaf or root) was sequentially immersed in 70% ethanol for 1 min, sodium hypochlorite solution (2.0–2.5% active Cl) for 4 min, and 70% ethanol for 30 s. Subsequently, tissues were washed three times with distilled water. Once washed and disinfected, tissues were macerated with 3 ml of sterile 0.85% saline solution with the aid of a flask and a pestle (de Araújo et al., 2002). The macerated material was used to prepare serial dilutions, and 100 µl of aliquots was seeded in Petri dishes containing tryptone soy agar (TSA) medium for bacterial isolation and potato dextrose agar for fungal isolation. Plates were grown in microbiological greenhouses at constant temperature of 30°C for 24 h for bacterial growth and at 25°C for 7 days in the case of fungal isolation (Caruso et al., 2000). Microorganism counts were performed in separate groups, fungi, and bacteria with their respective controls.

Determination of the Phosphorus Concentration in Plants and Soil

The determination of soluble soil phosphorus was carried out using the method proposed by Watanabe and Olsen (1965). For the determination of phosphorus in plants, phosphorus concentrations in roots and shoots were determined according to

methodology proposed by Haag et al. (1975) and modified by Bezerra Neto and Barreto (2011).

Determination of the Total Nitrogen Concentration in Plants and Soil

The determination of the nitrogen concentration in shoots and roots was performed according to Haag et al. (1975) with sulfuric digestion of plant material to estimate the nitrogen concentration or dose associated with obtaining 90% of dry matter production. For the determination of total nitrogen in soil, the methodology proposed by Bremner and Mulvaney (1983) and modified by Wilke (2005) was used.

Microbial Respiratory Activity

The respiratory activity was determined by the method of quantification of released CO_2 according to Jenkinson and Powlson (1976), using wide-mouth flasks with 100 g of soil (dry or wet). Inside flasks, two beakers (one containing 20 ml of NaOH, and the other 20 ml distilled water) were placed, were then sealed with plastic film, and incubated in the dark for 7 days. Microbial respiration was estimated from the amount of CO_2 released from soil samples in a continuous air flow system free from CO_2 and moisture. After incubation, the remaining NaOH was quantified by titration with HCl.

Microbial Biomass Carbon

Microbial biomass carbon was determined by the irradiation-extraction method (Islam and Weil, 1998; Mendonça and Matos, 2017), using microwave oven. After irradiation, samples were submitted to 0.5 mol/L of potassium sulfate extractor, and microbial biomass carbon was determined by oxidation with 0.066 mol/L of potassium

dichromate followed by titration with 0.033 mol/L of ammonia ferrous sulfate (Brookes et al., 1982).

Statistical Analysis

Prior to analysis of variance, data normality (the Kolmogorov–Smirnov test) and homogeneity of variances (Levene's test) were tested for each parameter evaluated. Data were transformed into $(x + 0.5)^{1/2}$ to comply with assumptions of the analysis of variance. Comparisons of means were performed using Tukey's test ($\alpha \leq 0.05$). Analyses were performed using the R 3.4.1 open software for Windows (R Core Team, 2020).

Experiment 2: Determination of the Effect of Inoculation of Microorganisms on Cotton Plants Under Field Conditions

Cotton Planting

The experiment was carried out at the Teaching, Research and Extension Farm (FEPE) – UNESP, Jaboticabal, São Paulo, during the off season (January–June 2020). The field soil was classified as Red Eutrophic Latosol (Oxisol) with clayey texture. Soil chemical analysis is detailed in Table 2.

Soil fertilization was performed once before sowing using the 8–28–16 of NPK + 0.5% Zn formula, with the amount of nitrogen 80% lower than the requirement to avoid masking the effect produced by microorganisms and their concentrations on cotton yield. Cotton was sown at spacing of 1 m between rows and 8–10 seeds per linear meter. The dimensions of the plot were 5 m in length by 5 m in width with useful area of 15 m^2.

The microorganisms used in the experiment were selected based on results of experiment 1. Microorganisms Bs248, Bv188, F112, and F113 were tested at concentrations of 1×10^4 and 1×10^{10} CFU or conidia ml^{-1}. The multiplication of these microorganisms was performed as previously described in experiment 1. Application

was performed three times, every 15 days, using back sprayer with constant pressure. In this experiment, seeds were not inoculated, and the first application was carried out 7 days after the emergence of cotton seedlings.

Microorganisms were applied at dose of 1 L of suspension per hectare (ha). The amount of water used was 200 L/ha (500 ml per useful area of 15 m^2). The control treatment was sprayed with water only. Cotton was manually harvested 151 days after seedling emergence. Seed cotton was harvested from plants of the useful plot (15 m^2).

Experimental Design and Experiment Management

A randomized block design with 4 × 2 factorial arrangement + 1 additional treatment (control) with four replicates was used. Microorganism factor sublevels were Bs248, Bv188, F112, and F113. Concentration factor sublevels were 1×10^{10} and 1×10^4 CFU mL^{-1}. Crop management was carried out considering commercial management for the region.

Evaluated Parameters

Parameters were evaluated by manual harvesting of plants in useful plots. The weight of seed cotton was measured using analytical scale. After drying in oven with air circulation at 65°C, seeds were manually separated from fibers and weighed on analytical scale. Fiber weight was obtained by the difference between the weight of the cotton harvested and the weight of the seed. Seed weight and fiber weight were estimated in kg/ha.

Data Analysis

Analyses were performed using the R software for Windows (R Core Team, 2020). The normality and homogeneity of variances were assessed using the Shapiro–

Wilk test and Levene's test ($\alpha \leq 0.05$), respectively. Treatments were analyzed using ANOVA, followed by Tukey's test ($\alpha \leq 0.05$) to compare the mean of treatments.

RESULTS AND DISCUSSION

Experiment 1: Determination of the Effect of Inoculation of Microorganisms at Different Concentrations in Greenhouse

The results indicate that there was no interaction between microorganism factor and inoculant concentration for variables shoot, root, and total dry matter in cotton plants. This means that regardless of microorganism, the behavior was the same, given the different inoculant concentrations. Furthermore, there was no effect of the concentration factor on variables shoot, root and total dry matter, nitrogen content in root dry matter, phosphorus in shoot dry matter, and biomass carbon; however, there was a significant effect of the microorganism factor on variables shoot (Figure 1A) and total (Figure 1B) dry matter, highlighting fungi *A. sydowii* and *Aspergillus* sp. *versicolor* section, with values of 30.83 and 33.40 g/plant, respectively, for shoot dry matter, and 47.71 and 51.20 g/plant, respectively, for total dry matter, compared with control treatment, which was 23.40 g/plant for shoot dry matter and 30.04 g/plant for total dry matter.

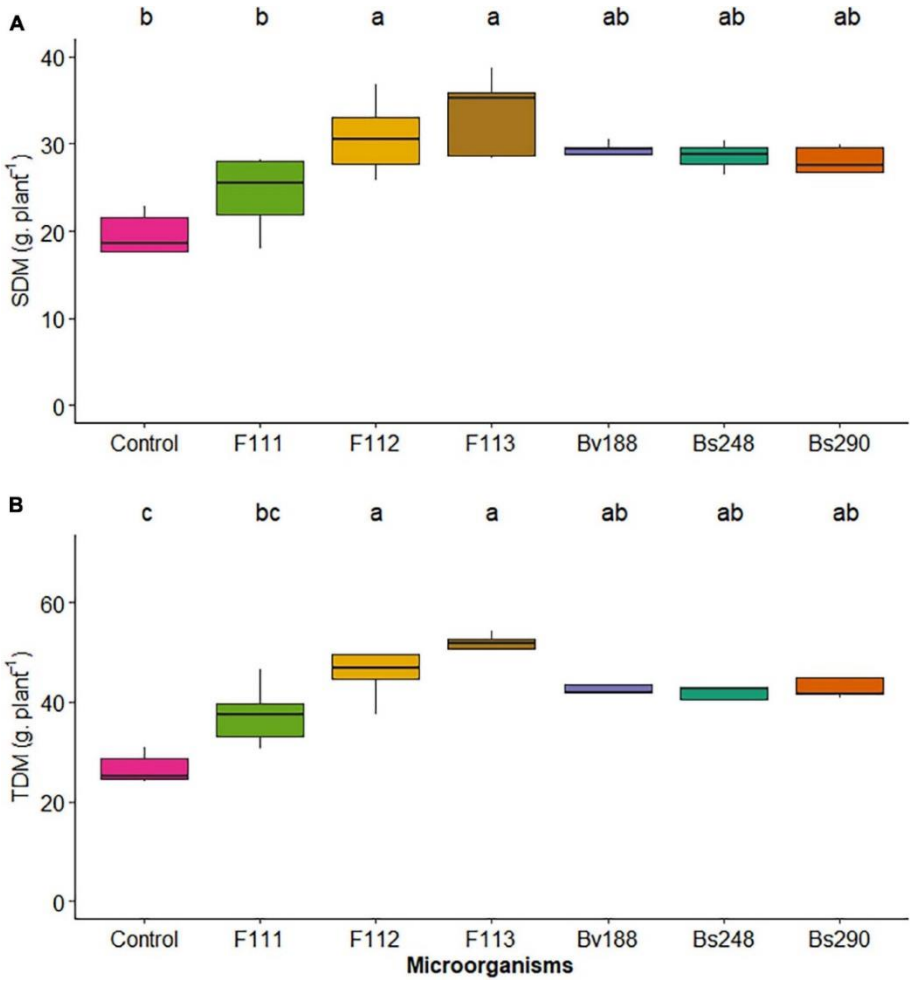

FIGURE 1 | Boxplots (median and quartiles) of SDM **(A)** and TDM **(B)** in cotton inoculated with plant growth-promoting microorganisms. Different lowercase letters in the line indicate statistical difference between means (Tukey, $p < 0.05$). F111, *Aspergillus brasiliensis*; F112, *Aspergillus sydowii*; F113, *Aspergillus* sp.; Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; Ctrl, control; SDM, shoot dry matter; TDM, total dry matter.

Plant–fungus associations are mainly established by two groups of fungi, mycorrhizal and endophytic fungi (Bonfante and Genre, 2010). Endophytic fungi are those capable of living endosymbiotically with plants without causing disease symptoms (Behie and Bidochka, 2014). They can act as plant growth promoters, increase germination rate, improve seedling establishment, and increase plant resistance to biotic and abiotic stresses, producing antimicrobial compounds, phytohormones, and other bioactive compounds. In addition, endophytic fungi are responsible for the acquisition of soil nutrients, including macronutrients such as

phosphorus, nitrogen, potassium, and magnesium, and micronutrients such as zinc, iron, and copper (Behie and Bidochka, 2014; Rai et al., 2014; Khan et al., 2015).

Soil fungi are widely distributed and participate in ecological processes that influence plant growth and soil health. It is considered that the diversity of fungi that inhabit the soil and the rhizosphere can reach more than 200 species in a single soil (Vandenkoornhuyse et al., 2002).

Several *Aspergillus* species are commercially exploited due to their ability to produce and secrete many enzymes and metabolites, such as antibiotics and mycotoxins (Volke-Sepulveda et al., 2016). The ability of fungi of the genus *Aspergillus* to produce secondary metabolites is very important because they play a vital role in survival and adaptation in soil; in addition, they are involved in the degradation of a wide range of natural organic substrates, particularly plant materials (Goldman and Osmani, 2008).

On the other hand, there was interaction between microorganism factor and inoculant concentration with variables nitrogen and phosphorus content in shoot (Figure 2) and root dry matter (Figure 3), soil phosphorus (Figure 4), soil nitrogen percentage (Figure 5), respiratory activity (Figure 6), colony-forming units in leaves (Figures 7, 8), and colony-forming units in roots and soil (Figure 9).

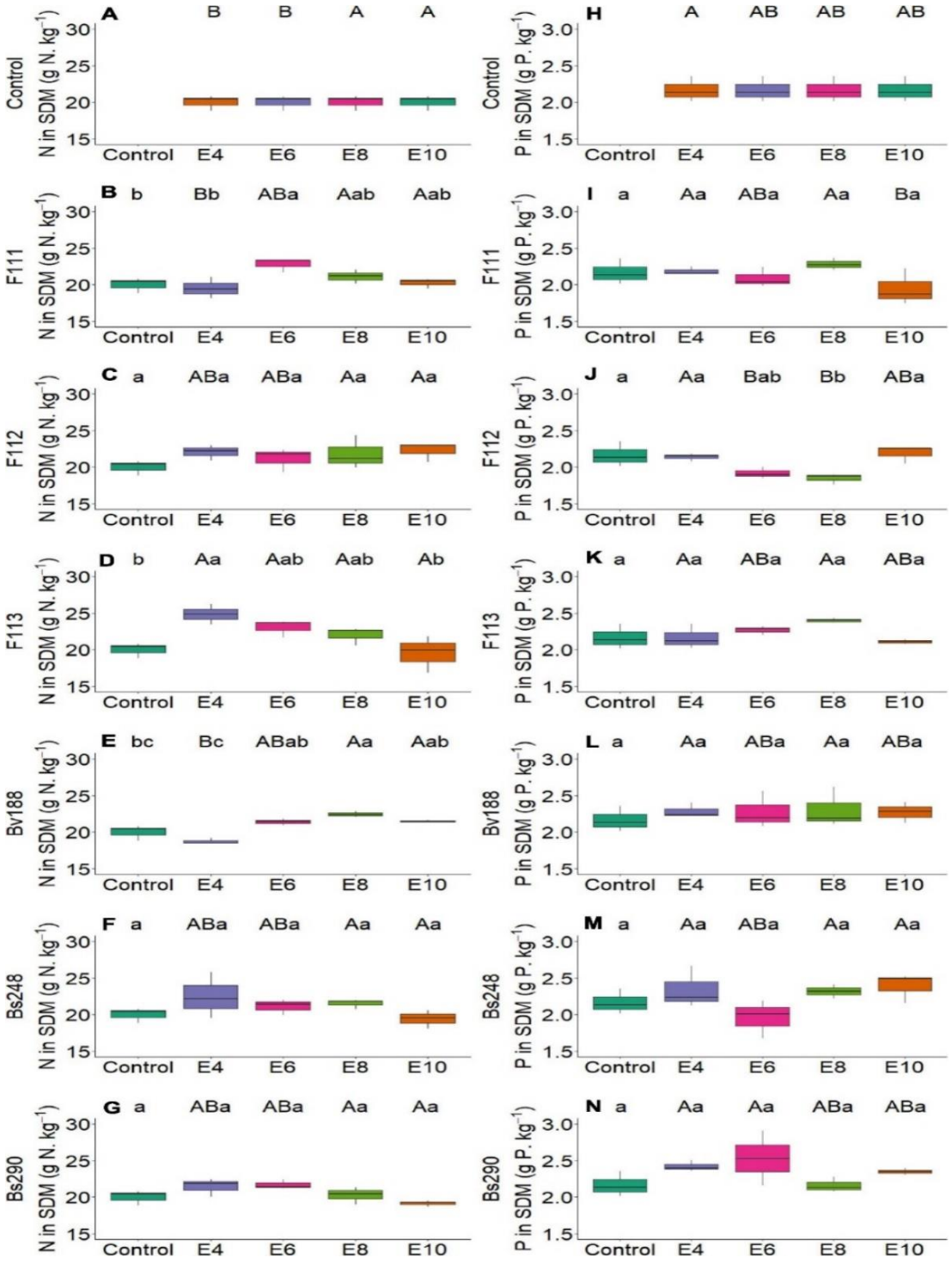

FIGURE 2 | Boxplots (median and quartiles) of nitrogen (A–G) and phosphorus (H–N) content in SDM in cotton inoculated with plant growth-promoting microorganisms. Different lowercase letters in a row and uppercase letters in a column indicate statistical difference between means (Tukey, $p < 0.05$). F111, *Aspergillus brasiliensis*; F112, *Aspergillus sydowii*; F113, *Aspergillus* sp.; Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; E4, 1×10^4; E6, 1×10^6; E8, 1×10^8; E10, 1×10^{10} conidia or CFU mL^{-1}; Ctrl, control; SDM, shoot dry matter.

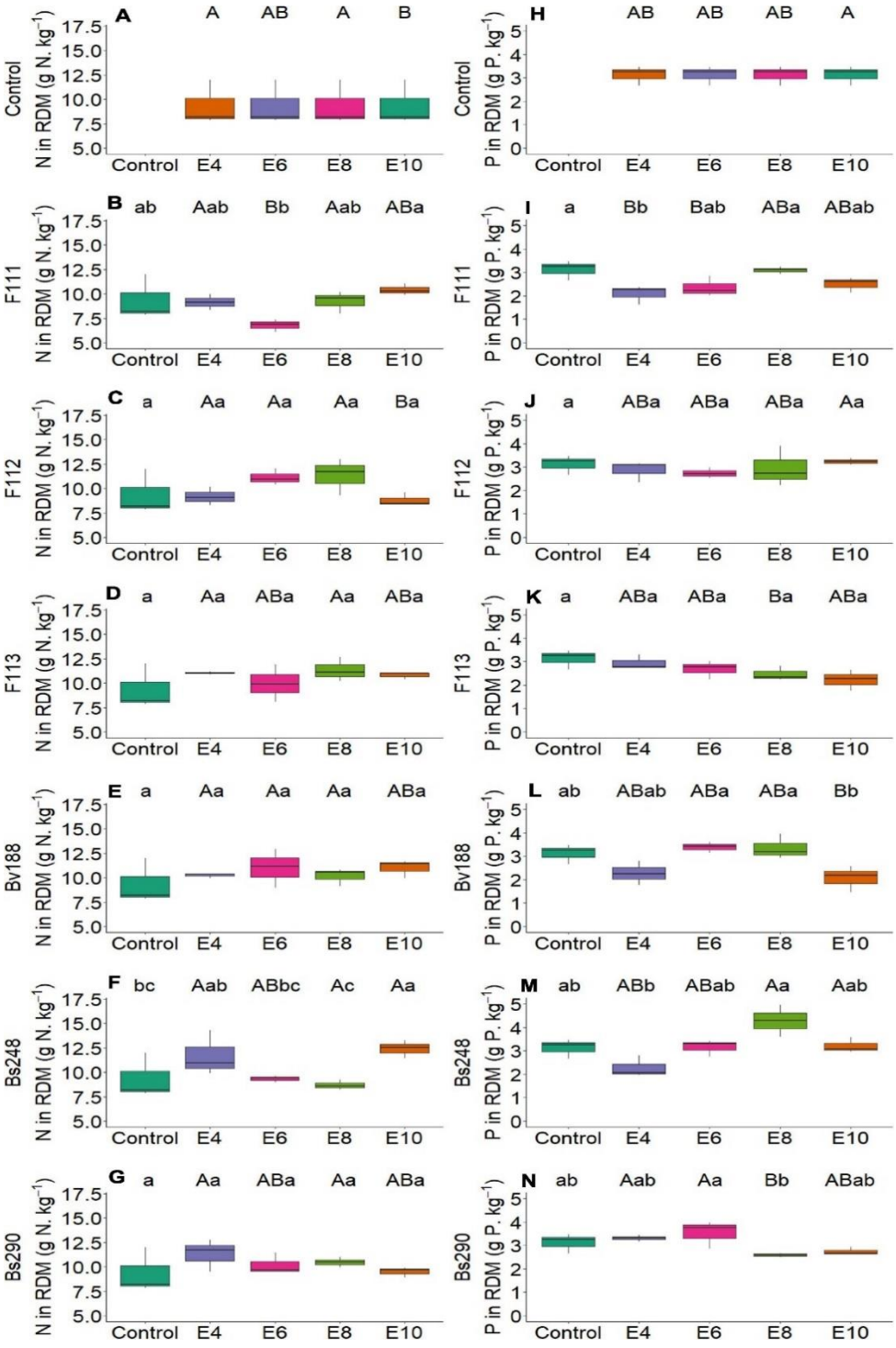

FIGURE 3 | Boxplots (median and quartiles) of nitrogen (A–G) and phosphorus (H–N) content in RDM in cotton inoculated with plant growth-promoting microorganisms. Different lowercase letters in a row and uppercase letters in a column indicate statistical difference between means (Tukey, $p < 0.05$). F111, *Aspergillus brasiliensis*; F112, *Aspergillus sydowii*; F113, *Aspergillus* sp.; Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; E4, 1×10^4; E6, 1×10^6; E8, 1×10^8; E10, 1×10^{10} conidia or CFU mL^{-1}; Ctrl, control; RDM, root dry matter; CFU, colony-forming units.

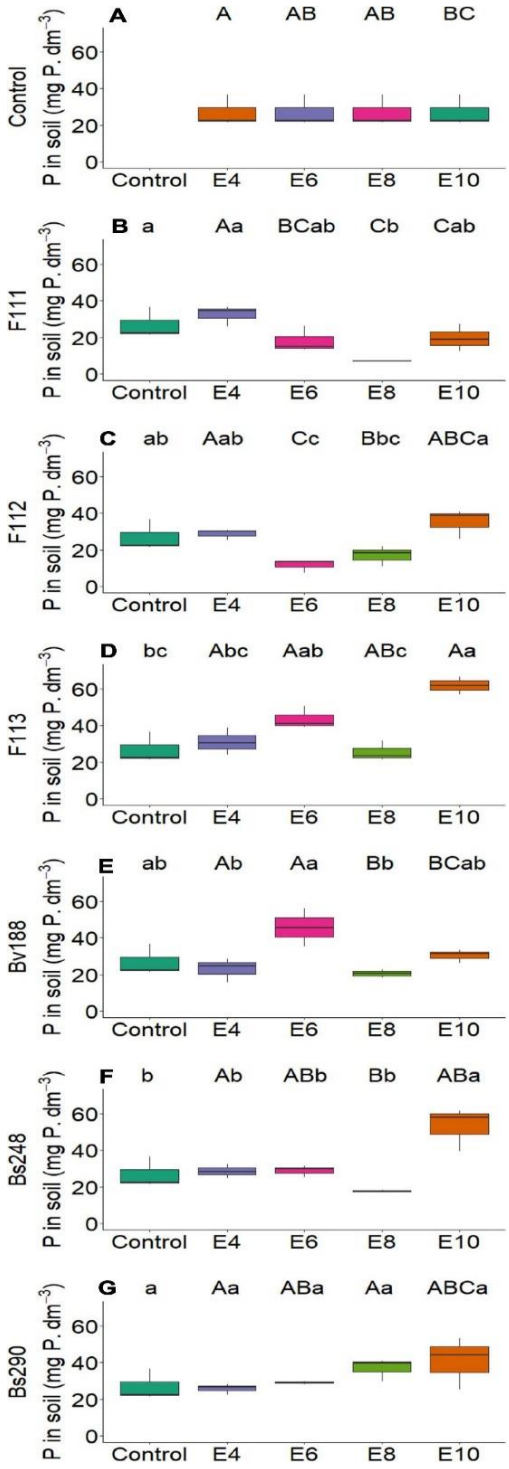

FIGURE 4 | Boxplots (median and quartiles) of phosphorus in soil sown with cotton and inoculated with plant growth-promoting microorganisms: Control (A); F111 (B); F112 (C); F113 (D); Bv188 (E); Bs248 (F); and Bs290 (G). Different lowercase letters in row and uppercase letters in column indicate statistical difference between the means (Tukey, $P < 0.05$). Abbreviations: F111, *Aspergillus brasiliensis*; F112, *A. sydowii*; F113, *Aspergillus* sp.; Bv188, *B. velezensis* strain Bv188; Bs248, *B. subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; E4, 1×10^4; E6, 1×10^6, E8, 1×10^8; E10, 1×10^{10} conidia or CFU/ml; Ctrl, Control; CFU, colony- forming units.

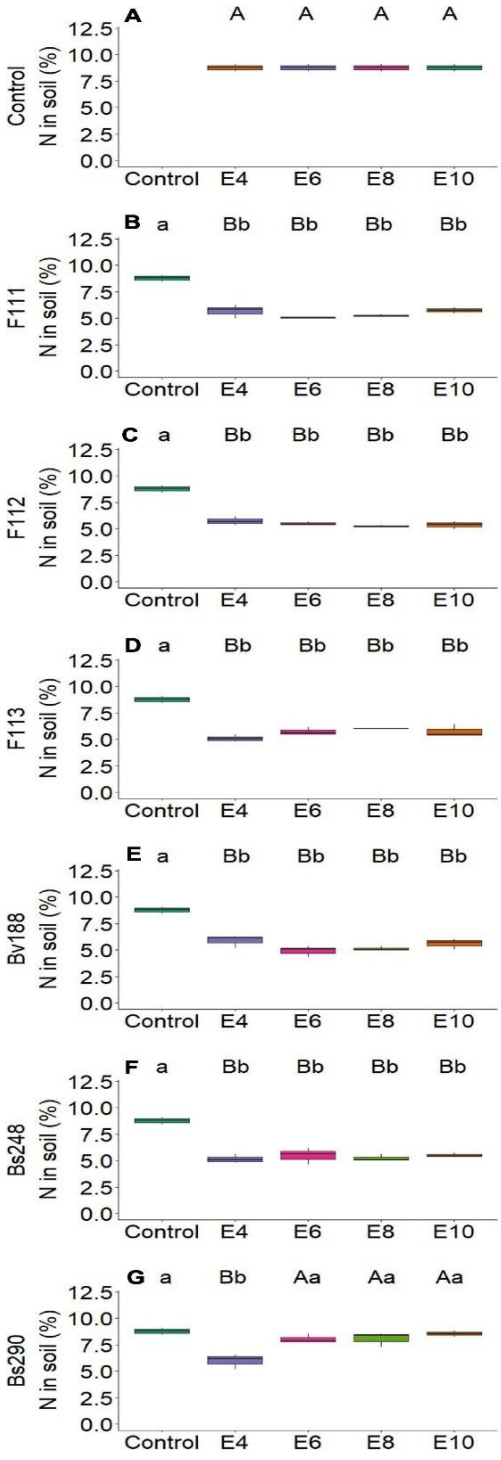

FIGURE 5 | Boxplots (median and quartiles) of percentage of nitrogen in soil sown with cotton and inoculated with plant growth-promoting microorganisms: Control **(A)**; F111 **(B)**; F112 **(C)**; F113 **(D)**; Bv188 **(E)**; Bs248 **(F)**; and Bs290 **(G)**. Different lowercase letters in row and uppercase letters in column indicate statistical difference between means (Tukey, $P < 0.05$). Abbreviations: F111, *Aspergillus brasiliensis*; F112, *A. sydowii*; F113, *Aspergillus* sp.; Bv188, *B. velezensis* strain Bv188; Bs248, *B. subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; E4, 1×10^4; E6, 1×10^6; E8, 1×10^8; E10, 1×10^{10} conidia or CFU/ml; Ctrl, Control; CFU, colony- forming units.

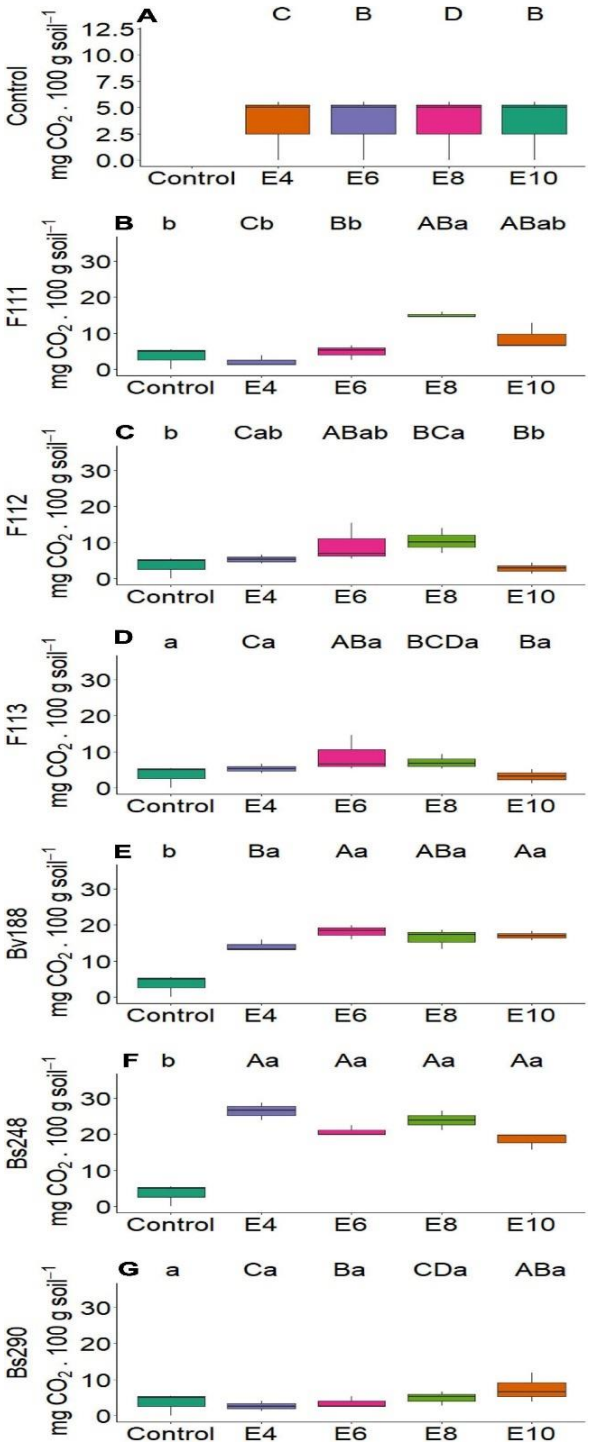

FIGURE 6 | Boxplots (median and quartiles) of respiratory activity in soil sown with cotton and inoculated with plant growth-promoting microorganisms: Control **(A)**; F111 **(B)**; F112 **(C)**; F113 **(D)**; Bv188 **(E)**; Bs248 **(F)**; and Bs290 **(G)**. Different lowercase letters in row and uppercase letters in column indicate statistical difference between means (Tukey, $P < 0.05$). Abbreviations: F111, *Aspergillus brasiliensis*; F112, *A. sydowii*; F113, *Aspergillus* sp.; Bv188, *B. velezensis* strain Bv188; Bs248, *B. subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; E4, 1×10^4; E6, 1×10^6; E8, 1×10^8; E10, 1×10^{10} conidia or CFU/ml; Ctrl, Control; CFU, colony- forming units.

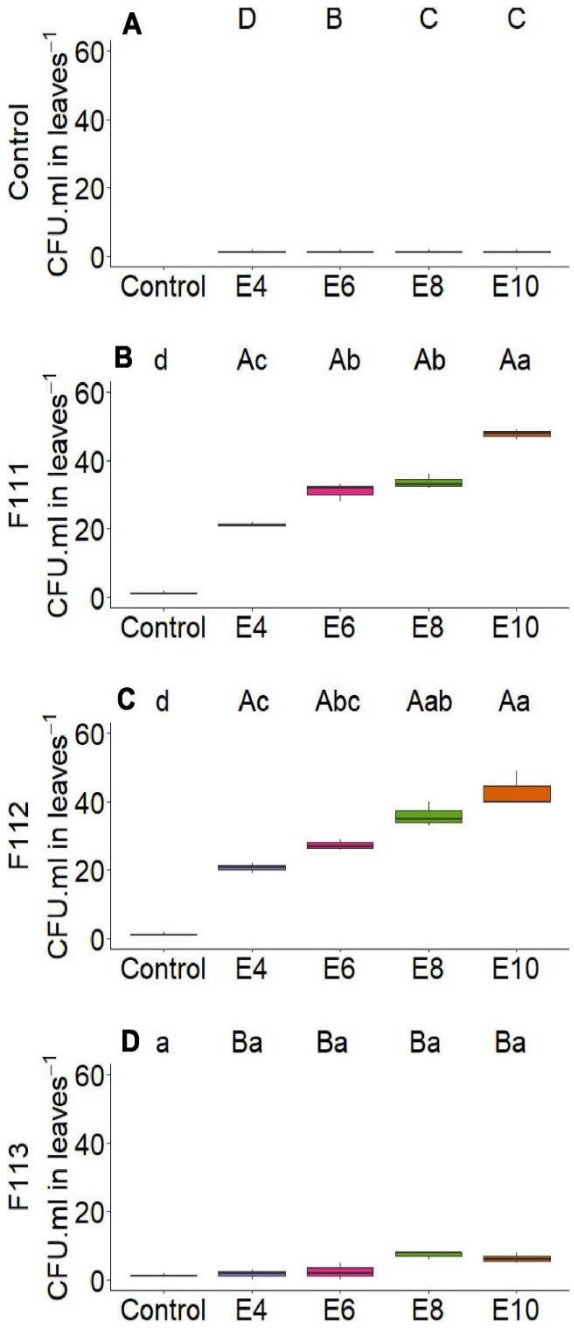

FIGURE 7 | Boxplots (median and quartiles) of CFU in cotton leaves inoculated with **(A)** *Aspergillus brasiliensis* **(B)**, *Aspergillus sydowii* **(C)**, and *Aspergillus* sp. **(D)** in four concentrations. Different lowercase letters in a row and uppercase letters in a column indicate statistical difference between means (Tukey, $p < 0.05$). F111, *Aspergillus brasiliensis*; F112, *A. sydowii*; F113, *Aspergillus* sp.; E4, 1×10^4; E6, 1×10^6; E8, 1×10^8; E10, 1×10^{10} conidia or CFU mL^{-1}; Ctrl, control; and CFU, colony-forming units.

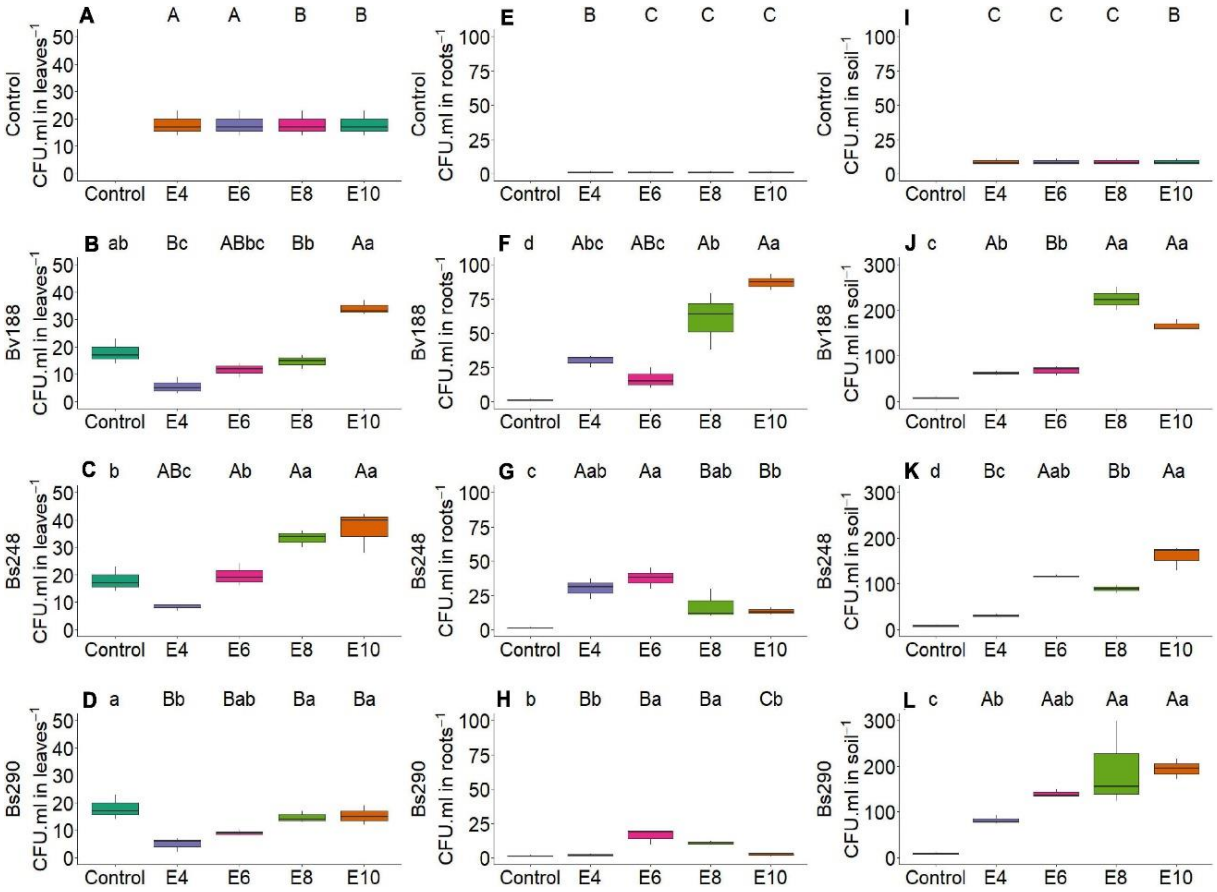

FIGURE 8 | Boxplots (median and quartiles) of CFU in leaves **(A–D)**, root **(E–H)**, and soil **(I–L)** inoculated with plant growth-promoting microorganisms. Different lowercase letters in a row and uppercase letters in the vertical indicate statistical difference between means (Tukey, $p < 0.05$). Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; Bs290, *B. subtilis* strain Bs290; E4, 1×10^4; E6, 1×10^6; E8, 1×10^8; E10, 1×10^{10} conidia or CFU mL^{-1}; Ctrl, control; CFU, colony-forming units.

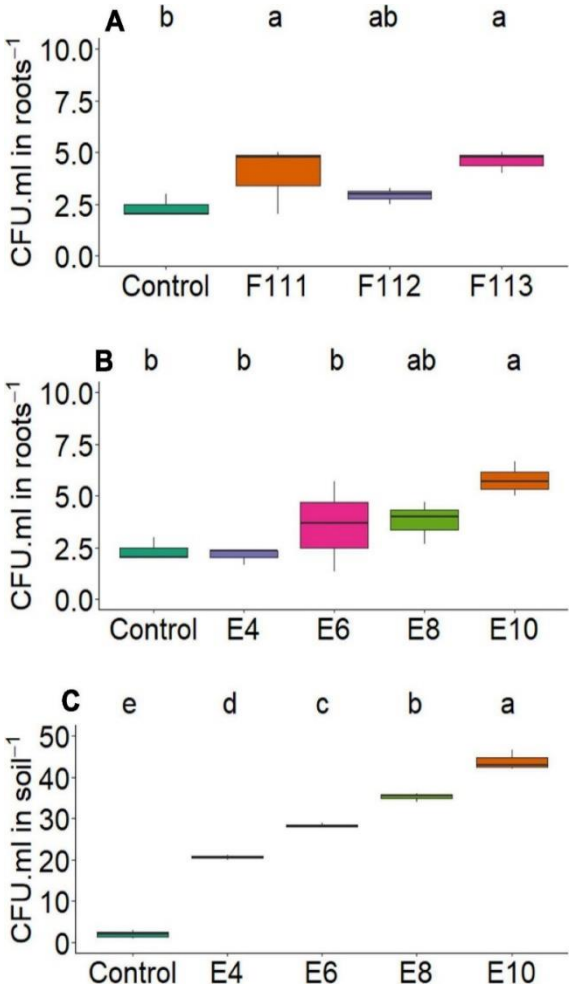

FIGURE 9 | Boxplots (median and quartiles) of CFU in root (A, B) and soil (C) inoculated with *Aspergillus brasiliensis*, *Aspergillus sydowii*, and *Aspergillus* sp. Different lowercase letters in a row indicate statistical difference between means (Tukey, $p < 0.05$). F111, *Aspergillus brasiliensis*; F112, *A. sydowii*; F113, *Aspergillus* sp.; E4, 1×10^4; E6, 1×10^6; E8, 1×10^8; E10, 1×10^{10} conidia or CFU mL^{-1}; Ctrl, control; CFU, colony-forming units.

For fungus *A. brasiliensis*, the unfolding of interactions indicates that inoculation in cotton plants at a concentration of 1×10^6 conidia ml^{-1} favored the increase in shoot nitrogen content (22.75 g N/kg; Figure 2B); root and soil phosphorus contents were lower at concentrations of 1×10^4 and 1×10^8 conidia ml^{-1}, with values of 2.09 g P/kg and 7.10 mg P/dm^3 soil, when compared with controls (3.13 g P/kg and 26.91 mg P/dm^3 soil, respectively) (Figures 3I, 4B). Species of the genus *Aspergillus*, according to Souchie et al. (2006), Pacheco and Damasio (2013), and de Oliveira Mendes et al. (2014), highlight the phosphorus solubilization capacity and its potential for use as solubilizers for different sources of phosphorus in the soil. Schneider et al. (2010)

reported the ability to synthesize organic acids and produce large amounts of citric acid, which is one of the main factors responsible for the solubilization of phosphorus in these fungi. The soil nitrogen percentage was lower than that of control at all inoculant concentrations (Figure 5B). These results suggest that *A. brasiliensis* can serve as hosts for nitrogen-fixing bacteria (endosymbionts) (Paul et al., 2020). These interactions may allow the plant to have absorbed nitrogen fixed and/or contained in the soil. The nitrogen-fixing property is absent in eukaryotes, but they circumvented this deficiency by associating with nitrogen-fixing bacteria (Kneip et al., 2007).

The soil respiratory activity reached the highest value (14.98 mg CO_2/100 g soil) at a concentration of 1×10^8 conidia ml^{-1} compared with control, 3.50 mg CO_2 (Figure 6B); and the number of colony-forming units in leaves was higher for all inoculant concentrations compared with control (Figure 7B). For values of colony-forming units in roots, although presenting no interaction, there was a significant effect of the microorganism factor, where *A. brasiliensis* stood out, with 3.92 CFU mL^{-1} ($p < 0.039$, Figure 9A); in addition, a positive correlation ($p < 0.05$) was observed between inoculant concentration and the number of colony-forming units in roots (Figure 10A). *A. brasiliensis* was isolated from the cotton plant, demonstrating that this fungus was probably able to colonize and enter the plant, showing its effects as an endophytic growth-promoting fungus on cotton. *A. brasiliensis* is described as a fast-growing and sporulating species, with characteristics closely related to *Aspergillus niger* (Varga et al., 2007); and *A. sydowii* is described as one of the fungi most commonly found in the soil (Raper and Fennell, 1965; Klich, 2002) and is used in industry for the production of enzymes such as β-glucosidase, α-galactosidase, cellulase, and xylanase (Tian et al., 2016).

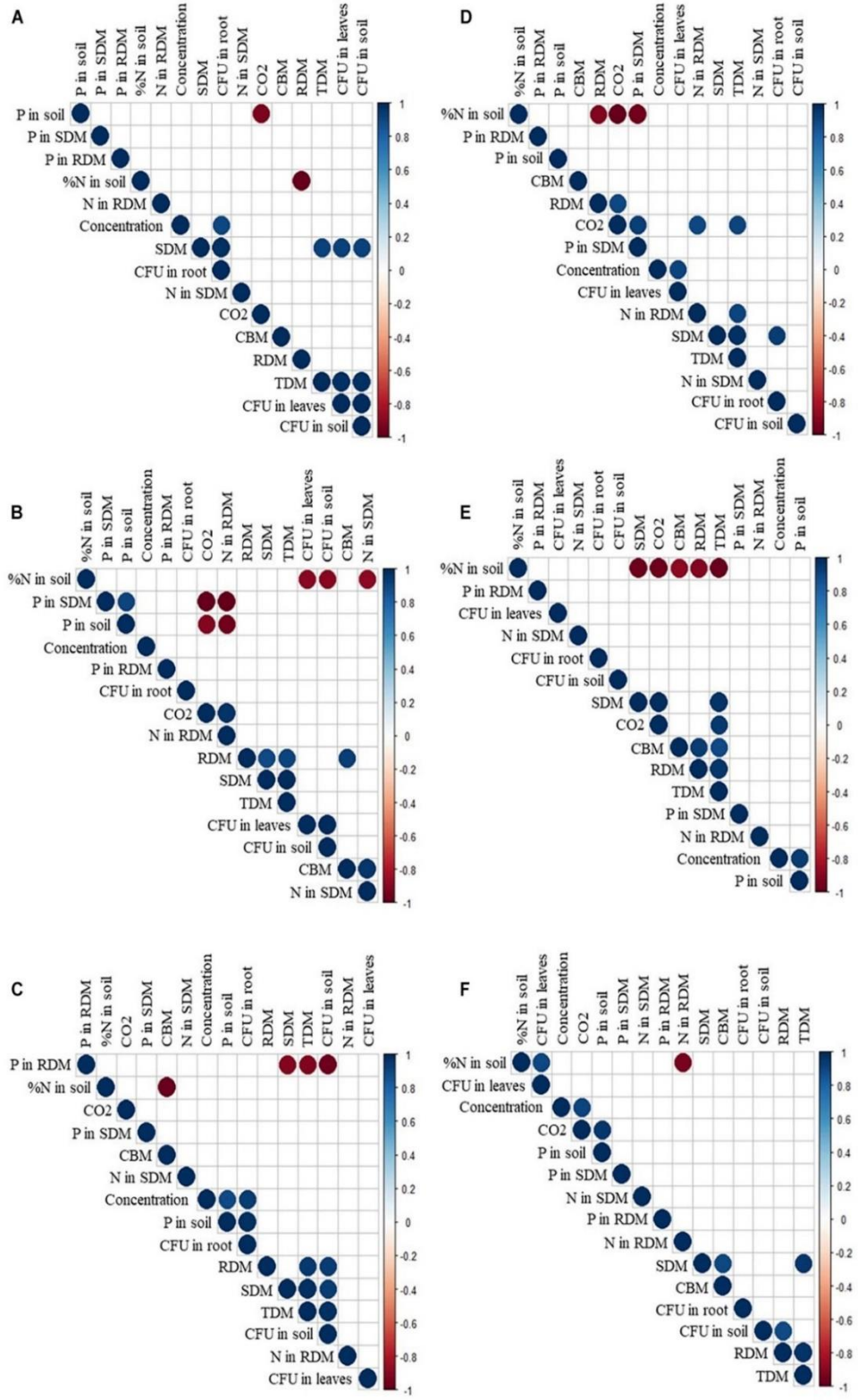

FIGURE 10 | Correlation of growth promotion variables and concentration of *Aspergillus brasiliensis* **(A)**, *Aspergillus sydowii* **(B)**, *Aspergillus* sp. **(C)**, *Bacillus velezensis* **(D)**, and *Bacillus subtilis* strain Bs248 **(E)** and Bs290 **(F)**. P, phosphorus; N, nitrogen; SDM, shoot dry matter; RDM, root dry matter; TDM, total dry matter; CO_2, respiratory activity; CBM, biomass carbon; and CFU, colony-forming units.

For *A. sydowii*, the unfolding of interactions indicates that the shoot phosphorus content presented lower value at a concentration of 1 × 10^8 conidia ml^{-1} (1.85 g P/kg, Figure 2J) when compared with control (2.17 g P/kg); the soil phosphorus content was lower with 11.68 mg P/dm^3 at a concentration of 1 × 10^6 conidia ml^{-1}, and control reached 26.91 mg P/dm^3 (Figure 4C); the nitrogen percentage in soil inoculated with *A. sydowii* at all concentrations was lower than that of control (Figure 5C); the soil respiratory activity was higher (10.43 mg CO_2/100 soil) with inoculation at a concentration of 1 × 10^8 conidia ml^{-1} compared with control, which was 3.5 mg CO_2/100 soil (Figure 6C) and for colony-forming units in leaves, highlighting inoculation of *A. sydowii* at a concentration of 1 × 10^{10} conidia ml^{-1} with 43.00 CFU mL^{-1} compared with control, 1.33 CFU mL^{-1} (Figure 7C).

For *Aspergillus* sp. *versicolor* section, the interaction indicates that the highest nitrogen content in shoot dry matter was obtained at the lowest concentration of 1 × 10^4 conidia ml^{-1} (24.86 g N/kg; Figure 2D), when compared with control, 20.02 g N/kg; there was a positive correlation ($p < 0.05$, Figure 10C) between inoculum concentration and soluble phosphorus in soil, and the largest amount (62.00 mg P/dm^3 soil) was obtained at a concentration of 1 × 10^{10} conidia ml^{-1} (Figure 4D) and control only 26.91 mg P/dm^3 soil; and the soil nitrogen percentage was lower at all concentrations when compared with control (Figure 5D).

For colony-forming units in roots, there was a significant effect ($p < 0.039$, Figure 9A) of the microorganism factor, where *Aspergillus* sp. *versicolor* section stood out from control, with 4.58 CFU mL^{-1}, and a positive correlation ($p < 0.05$) was observed between concentration and the number of colony-forming units in roots (Figure 10C). The greatest amount of CFU mL^{-1} in roots and soil was reached when plants were inoculated at maximum concentration (1 × 10^{10} conidia ml^{-1}), regardless of fungus used (*A. brasiliensis*, *A. sydowii*, and *Aspergillus* sp. *versicolor* section) (Figures 9B,C).

For *A. brasiliensis* and *A. sydowii*, the increase in inoculum concentration had a positive effect on variable colony-forming units in leaves (Figures 7B,C); however, a concentration of 1 × 10^6 conidia ml^{-1} of *A. brasiliensis* proved to be appropriate to obtain higher shoot nitrogen contents (Figure 2B), and a concentration of 1 × 10^8 conidia ml^{-1} of *A. brasiliensis* or *A. sydowii* was suitable for higher respiratory activity values (Figures 6B,C).

The highest inoculant concentrations promoted the highest numbers of CFU mL^{-1} recovered from cotton roots and leaves. Endophytism promotes a more intimate interaction between a microorganism and a host, intensifying the benefits for both (Hardoim et al., 2008; Nadeem et al., 2014; Khan et al., 2015). Interestingly, treatments that presented a greater number of endophytic microorganisms did not necessarily promote greater plant development. Lobo et al. (2019) verified that the treatment that promoted a higher maize yield under field conditions, compared with control, also presented a lower number of recovered CFU mL^{-1}. These results suggest that the growth-promoting effect probably depends more on the abilities of microorganisms and the interaction between microorganism and plant than on higher CFU mL^{-1} values.

According to results of the present study, the hypothesis that the highest *A. brasiliensis* and *A. sydowii* concentrations positively affect microorganism colonization can be confirmed. However, this greater colonization did not reflect in greater plant development. These results also show that *A. brasiliensis* and *A. sydowii* are fungi with endophytic capacity in cotton plants. This characteristic in both fungi is an advantage because the endophytic colonization of plant tissues allows the fungus to establish itself inside the organs for some time without causing apparent damage to the host (Petrini, 1991), in addition to protecting plants against eventual colonization and pathogen infection or pest infestation (Bulgarelli et al., 2013). Studies carried out in China have shown that *A. niger* P85 has the ability to solubilize phosphorus, produce indole acetic acid in maize plants, and increase available phosphorus in the soil (Yin et al., 2015); and in Brazil, similar studies have demonstrated the ability of *A. sydowii* and *A. brasiliensis* as phosphorus solubilizers in maize plants (Baron et al., 2018). *A. brasiliensis* and *A. sydowii* have great potential for use in other agricultural crops of great economic importance.

For *Aspergillus* sp. *versicolor* section, increasing inoculum concentration had a positive effect on soil phosphorus concentration and number of colony-forming units in roots (Figures 10B,C); however, a concentration of 1×10^4 conidia ml^{-1} was suitable for cotton plants to show the highest shoot nitrogen content (Figure 2D).

Aspergillus sp. *versicolor* section are accepted as distinct species based on molecular and phenotypic differences, are isolated from soil, and adapt to form part of the rhizospheric plant community (Zeljko et al., 2012). *Aspergillus* sp. *versicolor* section

are fungi that are part of the microbial community of the rhizosphere of tea plants (Rahi et al., 2009). Similarly, in the present study, *Aspergillus* sp. *versicolor* section showed soil phosphorus solubilization capacity and root colonization. These characteristics are interesting in agriculture because inoculation with higher *Aspergillus* sp. *versicolor* section concentrations could decrease the need for use of mineral fertilizers in the field (Qiao et al., 2019; Caruso et al., 2020) as a consequence of the more efficient use of these fertilizers by plants. Some studies have shown that the association of this fungus with roots promotes abiotic stress tolerance and protection against pathogens (Singh et al., 2012; Begum et al., 2019; Rana et al., 2019).

For *B. velezensis*, the unfolding of interactions indicates that the nitrogen content in shoot dry matter of cotton plants was higher with 22.46 g N/kg at a concentration of 1×10^8 CFU mL^{-1} compared with control, 20.02 g N/kg (Figure 2E); the phosphorus content in the root dry matter and in the soil at all concentrations did not differ from that of control (Figures 3L, 4E); the soil nitrogen percentage was lower at all concentrations compared with that of control (Figure 5E); the respiratory activity was higher at all concentrations when compared with that of control (Figure 6E); the amount of colony-forming units in leaves, roots, and soil was higher at a concentration of 1×10^{10} CFU mL^{-1} (34.00, 93.67, and 163.33 CFU mL^{-1}, respectively; Figures 8B,F,J); in addition, there was a positive correlation between concentration and colony-forming units in leaves ($p < 0.05$, Figure 10D).

For inoculation of *B. subtilis* Bs248, interaction indicates that the concentration of 1×10^{10} CFU mL^{-1} in cotton plants promoted the highest nitrogen content in the root dry matter (12.41 g N/kg) when compared with control (9.35 g N/kg) (Figure 3F); the phosphorus content in the root dry matter was not affected by concentration (Figure 3M); soil phosphorus at a concentration of 1×10^{10} CFU mL^{-1} was approximately double (53.15 mg P/dm^3) that found at concentrations of 1×10^4, 1×10^6, and 1×10^8 CFU mL^{-1} and control (Figure 4F); in addition, there was a positive correlation between variable soil phosphorus and concentration ($p < 0.05$, Figure 10E); soil nitrogen percentage was lower, and the respiratory activity was higher when *B. subtilis* Bs248 was inoculated at any concentration (Figures 5F, 6F). The number of colony-forming units in leaves was higher when inoculum was applied at concentrations of 1×10^8 and 1×10^{10} CFU mL^{-1} (Figure 8C); the number of colony-forming units in roots was greater

when inoculum was applied at a concentration of 1×10^6 CFU mL^{-1} (Figure 8G), and the number of colony-forming units in soil was greater at concentrations of 1×10^6 and 1×10^{10} CFU mL^{-1} (Figure 8K).

For *B. subtilis* Bs290, interaction indicates that the inoculation of cotton plants at a concentration of 1×10^4 CFU mL^{-1} had the lowest nitrogen percentage, 5.97%, when compared with control, which reached 8.77% (Figure 5G), and a smaller amount of colony-forming units in leaves with 5.00 CFU mL^{-1}, when compared with control of 18.00 CFU mL^{-1} (Figure 8D); the number of colony-forming units in roots was higher, with 15.67 and 10.67 CFU mL^{-1}, when the microorganism was inoculated at concentrations of 1×10^6 and 1×10^8 CFU mL^{-1}, respectively (Figure 8H); and the number of colony-forming units in soil was higher, with 192.67 and 194.33 CFU mL^{-1}, when inoculated at concentrations of 1×10^8 and 1×10^{10} CFU mL^{-1}, respectively (Figure 8L). Additionally, a positive correlation was observed between concentration and respiratory activity ($p < 0.05$, Figure 10F).

Most *Bacillus* species are considered plant growth-promoting rhizobacteria and have the ability to colonize roots, improve nutrient availability, reduce abiotic stress, and produce a wide range of biologically active secondary metabolites that can inhibit the growth of pathogens (Ongena and Jacques, 2008; Lugtenberg and Kamilova, 2009; Bhattacharyya and Jha, 2012; Sivasakthi et al., 2014). The increase in inoculum concentration had a positive effect on variable colony-forming units in leaves for *B. velezensis*, soil phosphorus for *B. subtilis* Bs248, and a respiratory activity for *B. subtilis* Bs290.

Bacillus velezensis was previously grouped with *B. subtilis* and *Bacillus amyloliquefaciens*, and in recent years, several isolates of this bacterium have received attention due to their potential in disease control (Fan et al., 2017; Adeniji et al., 2019). Previous studies have determined that *B. velezensis* has the ability to produce indole acetic acid in pepper plants applied at a concentration of 1×10^8 CFU mL^{-1} (Zhang et al., 2019); in addition, it has been shown that metabolites produced have an antagonistic activity against bacterial and fungal pathogens under laboratory and greenhouse conditions in tomato crops (Cao et al., 2018). In the present study, *B. velezensis* showed the ability to colonize cotton leaves as the inoculum concentration increases. These results demonstrate that *B. velezensis* is an endophytic bacterium

with capacity to promote growth through nitrogen content in shoot dry matter; in addition, results of colony-forming units in leaves suggest that *B. velezensis* has potential to inhibit the growth of pathogens in cotton plants.

On the other hand, studies have demonstrated the ability of *B. subtilis* to solubilize phosphate, produce indole acetic acid and siderophores, and increase dry weight in maize and sorghum (Aquino et al., 2019), okra, spinach, and tomato plants, in addition to presenting antagonistic action against *Rhizoctonia solani* (Adesemoye et al., 2009). Regarding colonization, studies carried out with cucumber and tomato plants inoculated with *B. subtilis* at concentrations of 10^5 and 10^6 CFU mL^{-1} of root were enough for the microorganism to be able to colonize and survive in the rhizosphere. Thus, in addition to protecting plants by suppressing *Fusarium oxysporum* from cucumber, *B. subtilis* had an antagonistic effect against *Pseudomonas syringae* after root colonization in tomato plants (Cao et al., 2011; Chen et al., 2013). In the present study, *B. subtilis* strains have shown a correlation between soil phosphorus content and respiratory activity. These results suggest that to improve phosphorus solubilization and respiration in the soil, it is necessary to increase inoculum concentration.

On the other hand, studies have shown that the long-term continuous use of inoculants influences the quantity and quality of microorganisms present in the soil rhizosphere, but this depends on conditions such as organic matter, availability of nutrients (such as phosphorus), and type of soil (Gnankambary et al., 2008; Angelina et al., 2020). Furthermore, it is important to consider that the composition of the soil community is largely influenced by environmental variability and the microbial community present in the soil (Xun et al., 2015).

As one of the most important and essential macronutrients in addition to nitrogen, phosphorus is important for plant development, but it is the nutrient element least mobile in plant and soil. Globally, P is extracted from geological sediments and added to agricultural soils in order to meet critical plant requirements for agronomic productivity. Phosphorus is present in soil in the organic and inorganic forms. The various inorganic forms of the element in the soil are salts with calcium, iron, and aluminum, while the organic forms come from decomposing vegetation and microbial residues. There is great diversity of plant microbiomes (epiphytic, endophytic, and

rhizospheric) and soil microbiomes that have the ability to solubilize insoluble P and make it available for plants. The main solubilization mechanism of inorganic P is by the production of organic acids, which lower soil pH, or by the production of acids and alkaline phosphatases, which cause the mineralization of organic P. P-solubilizing and P-mobilizing microorganisms belong to all three domains: archaea, bacteria, and eukarya. Strains belonging to genera *Arthrobacter*, *Bacillus*, *Burkholderia*, *Natrinema*, *Pseudomonas*, *Rhizobium*, *Serratia*, and *Aspergillus* have been reported as efficient and potential P solubilizers. The use of P solubilizers, alone or in combination with another plant growth-promoting microbe as an ecological microbial consortium, could increase P uptake by plants, increasing their yields for agricultural and environmental sustainability (Kour et al., 2021). However, results have shown that for some treatments, phosphorus concentrations in soil and roots decreased. Factors such as mineral concentration, temperature, and availability of carbon and nitrogen (N) sources can affect the phosphorus solubilization potential of these microorganisms, and these results suggest that there was greater solubilization and absorption of phosphorus from the soil by plants and greater translocation to shoots.

For the field phase, *A. sydowii* was selected for presenting abilities to promote a positive effect on variables shoot and total dry matter, soil respiratory activity, and colony-forming units in leaves and roots; *Aspergillus* sp. *versicolor* section were selected for presenting the ability to promote positive effects on variables shoot and total dry matter, nitrogen content in shoot dry matter, colony-forming units in roots and soil phosphorus; *B. velezensis* (Bv188) was selected for presenting the ability and promoting positive effects on variables nitrogen content in shoot dry matter, respiratory activity, colony-forming units in leaves, roots, and soil; and *B. subtilis* 248 was selected for presenting the ability to promote positive effects on variables root nitrogen content, soil phosphorus, respiratory activity in soil, and colony-forming units in leaves, roots, and soil.

Experiment 2: Determination of the Effect of Inoculation of Microorganisms on Cotton Plants Under Field Conditions

Regarding field yield, there was no interaction of concentration factor and microorganism factor on variables fiber yield (Figures 11A–E) and seed yield, except for *Aspergillus* sp. *versicolor* section (F113), which presented the lowest yield for a concentration of 1×10^{10} CFU mL^{-1} compared with a concentration of 1×10^{4} CFU mL^{-1} (Figure 11H). Fiber yield in cotton plants inoculated with *B. velezensis*, *B. subtilis* 248, *A. sydowii*, and *Aspergillus* sp. *versicolor* section were superior to control, which had 326.94 kg/ha (Figures 11A–F). Inoculation of *A. sydowii* at a concentration of 1×10^{10} conidia ml^{-1} and *Aspergillus* sp. *versicolor* section at a concentration of 1×10^{4} conidia ml^{-1} had the highest seed yield, with 1,131.14 and 1,364.96 kg/ha, respectively (Figures 11G,H). Inoculation with *B. velezensis* at a concentration of 1×10^{4} and 10^{10} CFU mL^{-1} showed no differences when compared with that with control (Figure 11I). Inoculation with *B. subtilis* Bs248 showed no differences between concentrations of 1×10^{4} and 1×10^{10} CFU mL^{-1}, reaching values of 1,118.54 and 1,024.68, respectively (Figure 11J).

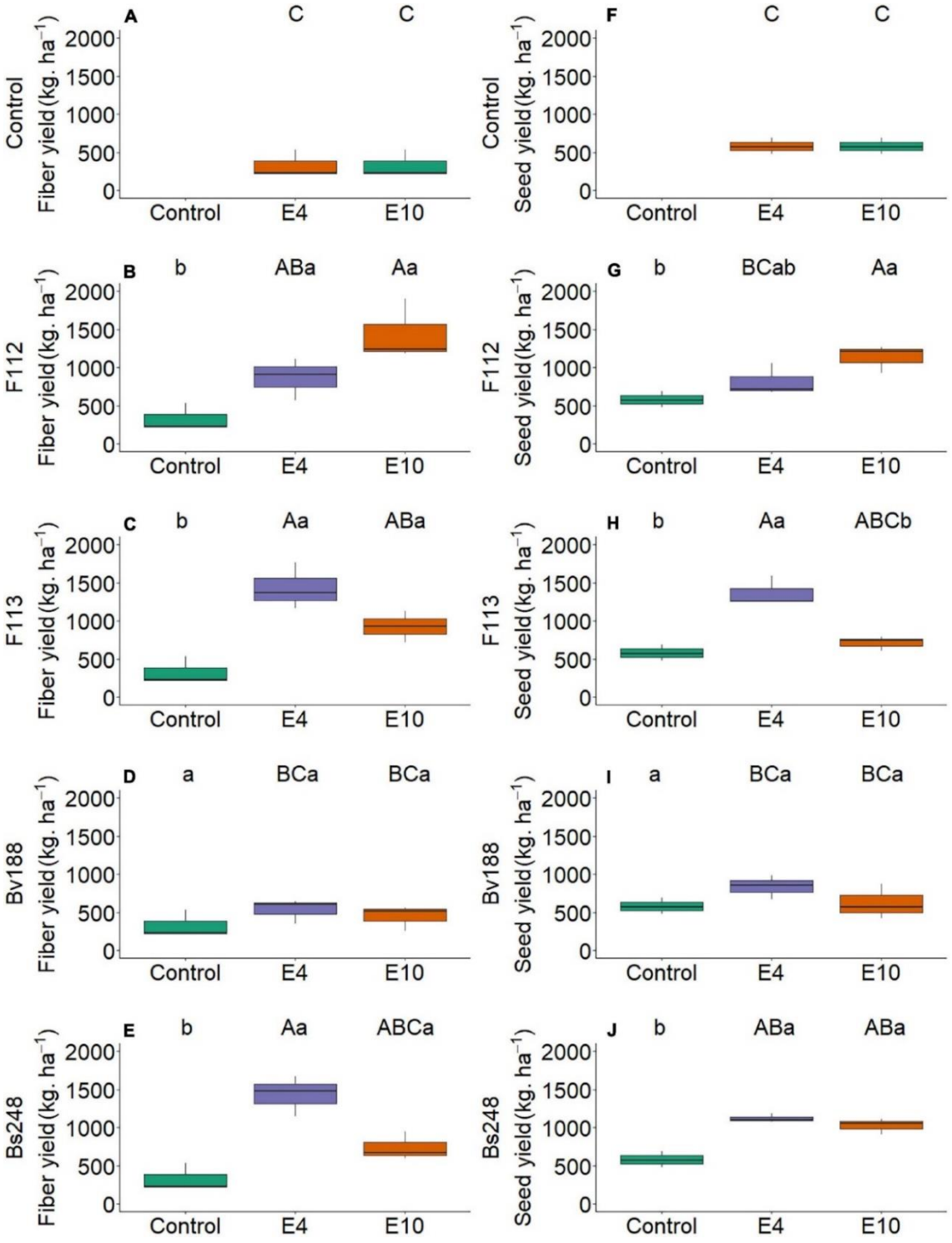

FIGURE 11 | Boxplots (median and quartiles) of fiber **(A–E)** and seed **(F–J)** cotton yield inoculated with plant growth-promoting microorganisms in two concentrations. Different lowercase letters in a row and uppercase letters in a column indicate statistical difference between means (Tukey, $p < 0.05$). F112, *Aspergillus sydowii*; F113, *Aspergillus* sp.; Bv188, *Bacillus velezensis* strain Bv188; Bs248, *Bacillus subtilis* strain Bs248; E4, 1×10^4; E10, 1×10^{10} conidia or CFU mL^{-1}; Ctrl, control; and CFU, colony-forming units.

For *A. sydowii* and *B. subtilis* Bs248, the hypothesis that fiber and seed yield at concentrations of 1×10^4 or 1×10^{10} CFU mL^{-1} are similar is confirmed. Thus, the results of the present study demonstrate that there is no effect of concentration on cotton seed and fiber yield when inoculated with *A. sydowii* and *B. subtilis* Bs248 and that there is no effect of concentration on cotton seed yield when inoculated with *Aspergillus* sp. *Versicolor* section.

Yield studies performed with *A. sydowii* and *Aspergillus* sp. *Versicolor* section in cotton are scarce in scientific literature; for example, studies carried out on chickpea plants have shown the ability of fungi *Aspergillus awamori* and *Penicillium citrinum* inoculated at a concentration of 1×10^6 spores/ml to increase seed weight by approximately twice (Mittal et al., 2008). In addition, *A. niger*, *Aspergillus fumigatus*, and *Penicillium pinophilum* inoculated on wheat and fava beans at a concentration of 2×10^9 spores/ml^{-1} increased yield by 28.9–32.8% and 14.7–29.4%, respectively (Abdul Wahid and Mehana, 2000). Likewise, phosphorus uptake by both cultures increased due to inoculation with tested fungi. Other studies include arbuscular mycorrhizal fungi in maize plants using concentrations of 1×10^3 spores/ml where, in addition to increasing yield by 80%, these fungi are capable of inducing resistance against pathogenic *A. niger* strains (Molo et al., 2019).

For plant-growth promoting bacteria, Tripti et al. (2017) observed increase in the amount of fruits on tomato plants inoculated with *Bacillus* sp. strain A30 and *Burkholderia* sp. strain L2 at a concentration of 10^{10} CFU mL^{-1}. Furthermore, inoculation with *A. brasiliensis* Ab-V5 and *B. subtilis* strain CCTB04 at a concentration of 1×10^8 CFU mL^{-1} positively affected corn yield by 39.5 and 29.1%, respectively (Pereira et al., 2020).

Microorganisms *A. sydowii*, *Aspergillus* sp. *Versicolor* section, and *B. subtilis* Bs248 used at concentrations of 1×10^4 and 1×10^{10} conidia or CFU mL^{-1} in the field phase allow achieving similar results in cotton fiber and seed yield. These results show that lower inoculant concentrations could be used with no damage to plant growth efficiency promoted by the microbial isolate.

CONCLUSION

The parameters that were favored by the highest inoculant concentrations were soil respiratory activity, phosphorus in root dry matter, nitrogen in shoot dry matter, and number of colony-forming units in roots and leaves. Concentrations did not affect nitrogen in root dry matter, phosphorus in shoot dry matter, and microbial biomass carbon. However, other factors such as nitrogen and phosphorus contents in the soil, except for *Aspergillus* sp. *versicolor* section, were negatively affected with the highest inoculant concentrations. Interestingly, inoculant concentrations did not affect cotton fiber or seed yield.

The present study brings results that help in a better understanding of the effect of concentrations of fungi- and bacteria-based inoculants on the biometric parameters of plants, on microbial activities and soil fertility, on the nutritional status of plants, and on cotton crop productivity.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CHAPTER 4 - Final considerations

The present work focused on the concentration of *Bacillus* spp. and *Aspergillus* spp. as biological inoculants in cotton cultivation. In general, the results show that there is no ideal concentration of inoculant based on *Bacillus* spp. and *Aspergillus* spp. for the promotion of cotton plant growth.

Aspergillus brasiliensis and *A. sydowii* inoculated in cotton plants favor nutrient uptake and increase root and total dry mass, showing great potential as growth promoters. However, there was no significant difference for productivity as a function of different inoculant concentrations (*Aspergillus* spp. and *Bacillus* spp.)

In practice, the results suggest that the farmers can use lower concentrations of the evaluated microorganisms to promote cotton growth and increase productivity in the field.

Commercial disputes place inoculant concentration as an essential factor for product quality. The present study comes to demystify this commercial issue.