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CÂMPUS DE ILHA SOLTEIRA**

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**AGRONOMIC BIOFORTIFICATION OF GRAIN CROPS WITH ZINC IN  
ASSOCIATION WITH PLANT GROWTH-PROMOTING BACTERIA**

Ilha Solteira  
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**AGRONOMIC BIOFORTIFICATION OF GRAIN CROPS WITH ZINC IN  
ASSOCIATION WITH PLANT GROWTH-PROMOTING BACTERIA**

Tese apresentada à Faculdade de Engenharia de Ilha Solteira – UNESP como parte dos requisitos para a obtenção do título de doutor em Agronomia. Especialidade: Sistemas de Produção.

**Orientador:** Prof. Dr. Marcelo Carvalho Minhoto Teixeira Filho

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## ***Dedication***

*I dedicate this humble effort to my parents (Noor Jalal and Muntaj Pari), my uncle Ghulam Farooq, my wife and son, my sibling, whose valuable prayers, strong support and unceasing confidence in my abilities helped me a lot in completion of this research.*

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***“In The Name of Allah  
The Most Beneficent,  
The Most Merciful”***

***“Em nome de Alláh, O Mais Beneficente,  
O Mais Misericordioso”***

## ABSTRACT

The intensive cropping system to pursue high grain production and feed the fast-growing global population has disturbed agricultural sustainability and nutritious security due to the imbalance use of chemical fertilizers. There is an urgent need to understand soil micronutrients deficiency and their impact on crop nutrition and productivity. Micronutrient fertilizer management especially zinc (Zn) through the soil and foliar application is crucial agronomic approach for improving agronomic biofortification of staple grains crops. The intervention of plant growth-promoting bacteria (PGPBs) could be one of the sustainable and safe strategies to improve nutrient acquisition and uptake in edible tissues of wheat-maize and common beans to combat Zn malnutrition and hidden hunger in human beings. In this context, this research was developed to understand the interactive effect of inoculation/ co-inoculation with PGPBs and Zn fertilization via soil and foliar to enhance growth performance, yield, and nutrition of wheat-maize and common bean crops for better biofortification of grains under field conditions of tropical savannah. A total of six experiments were performed in a randomized complete block design in Rhodic Haplustox soil under no-tillage system. **Experiment 1** with common bean was conducted in  $7 \times 2$ , co-inoculation with PGPBs (no inoculation, *Rhizobium tropici*, *R. tropici* + *Azospirillum brasilense*, *R. tropici* + *Bacillus subtilis*, *R. tropici* + *Pseudomonas fluorescens*, *R. tropici* + *A. brasilense* + *B. subtilis*, and *R. tropici* + *A. brasilense* + *P. fluorescens*) in association with soil Zn application (without and  $8 \text{ kg ha}^{-1}$ , applied from zinc sulphate) during 2019 and 2020 (May-August). **Experiment 2** with common beans was conducted in  $7 \times 3$  factorial scheme, co-inoculation with PGPBs (no inoculation, *Rhizobium tropici*, *R. tropici* + *Azospirillum brasilense*, *R. tropici* + *Bacillus subtilis*, *R. tropici* + *Pseudomonas fluorescens*, *R. tropici* + *A. brasilense* + *B. subtilis*, and *R. tropici* + *A. brasilense* + *P. fluorescens*) in association foliar nano-Zn doses (0, 1.5 and  $3.0 \text{ kg ha}^{-1}$ , applied from zinc oxide) during 2019 and 2020 (May-August). **Experiment 3** with wheat was conducted in  $4 \times 2$  factorial scheme, inoculations with PGPBs (no inoculation - control, *A. brasilense*, *B. subtilis* and *P. fluorescens*) in association with soil Zn application ( $0$  and  $8 \text{ kg ha}^{-1}$ ) during 2019 and 2020 (May-September). **Experiment 4** with wheat was conducted in  $4 \times 5$  factorial scheme, inoculations with PGPBs (no inoculation - control, *A. brasilense*, *B. subtilis* and *P. fluorescens*) in association with five foliar nano-Zn doses (0, 0.75, 1.5, 3.0 and  $6.0 \text{ kg ha}^{-1}$ ) during 2019 and 2020 (May-September). **Experiment 5** with maize was performed in  $4 \times 2$  factorial scheme, inoculations with PGPBs (no inoculation - control, *A. brasilense*, *B. subtilis* and *P. fluorescens*) in association with soil Zn application ( $0$  and  $8 \text{ kg ha}^{-1}$ ) during 2019–2020 and 2020–2021 (November-March). **Experiment 6** with maize was also performed  $4 \times 2$  factorial scheme, inoculations with PGPBs (no inoculation - control, *A. brasilense*, *B. subtilis* and *P. fluorescens*) in association with two foliar nano-Zn doses ( $0$  and  $3 \text{ kg ha}^{-1}$ ) during 2019–2020 and 2020–2021 (November-March). Co-inoculation with *R. tropici* + *B. subtilis* and *R. tropici* + *P. fluorescens* along with soil and foliar Zn application improve plant growth, grain yield and nitrogen (N), phosphorous (P) and Zn concentrations in shoot and grains of common bean. Zinc use efficiency, applied Zn recovery, and utilization were also increased with co-inoculation of *R. tropici* + *B. subtilis* along with soil Zn application and foliar nano-Zn application at a dose of  $1.5 \text{ kg ha}^{-1}$  whereas agro-physiological efficiency was increased with triple co-inoculation of *R. tropici* + *A. brasilense* + *P. fluorescens*. Inoculation with *B. subtilis* improved maize grain yield (14-17%), while inoculation with *P. fluorescens* improve Zn accumulation in shoot (33-51%) and grains (37-50.7%) of maize under residual Zn and nano-Zn foliar fertilization in wheat-maize succession. In addition, biochemical attributes (chlorophyll a, b and total, carotenoids, total soluble sugar and amino acids) and Zn use efficiency were also increased with inoculation *B. subtilis* and *P. fluorescens* in combination with

soil and nano-Zn foliar application. Inoculation with *B. subtilis* and *P. fluorescens* along with soil Zn and nano-Zn foliar application improved Zn concentration of shoot (18-31%) and grains (16-34%), N concentration of shoot (10-24%) and grains (16-20%), P concentration of shoot (26-32%) and grains (18-26%), and grain yield (4-20.6%) of wheat in tropical savannah. Also, inoculation of *P. fluorescens* with soil and foliar Zn application improved zinc use efficiency and utilization efficiency that increased Zn partitioning to edible grains for higher estimated Zn intake in with daily wheat consumption. Therefore, inoculation of *B. subtilis* and *P. fluorescens* with soil and foliar nano-Zn application is considered one of the most effective, sustainable and environmentally safe strategy for improving biochemical, nutritional and productivity attributes of common beans, maize and wheat cropping system. Hence, it is recommended that seed inoculation with *B. subtilis* and *P. fluorescens* along with Zn fertilization via soil or foliar nano-Zn at a dose 3 kg ha<sup>-1</sup> could be an effective mechanism to improve plant nutrients acquisition and use efficiencies particularly Zn, leading to sustainable production and biofortification of common beans, wheat and maize under tropical savannah regions of Brazil.

**Keywords:** *Phaseolus vulgaris* L.; *Triticum aestivum* L.; *Zea mays* L.; zinc fertilization; zinc uptake; zinc use efficiency; zinc partitioning; productivity; zinc nutrition; inoculation.

# BIOFORTIFICAÇÃO AGRONÔMICA DE CULTURAS GRANÍFERAS COM ZINCO EM ASSOCIAÇÃO COM BACTÉRIAS PROMOTORAS DE CRESCIMENTO DE PLANTAS

## RESUMO

O sistema de cultivo intensivo para buscar alta produção de grãos e alimentar a população global em rápido crescimento tem prejudicado a sustentabilidade agrícola e a segurança alimentar devido ao uso desequilibrado de fertilizantes minerais. Há uma necessidade urgente de entender o baixo teor de micronutrientes no solo e seu impacto na nutrição e produtividade das culturas. O manejo de fertilizantes com micronutrientes, especialmente zinco (Zn) aplicado no solo e via foliar, é uma abordagem agronômica crucial para melhorar a biofortificação agronômica de culturas graníferas. A aplicação de bactérias promotoras de crescimento vegetal (PGPBs) pode ser uma das estratégias sustentáveis e seguras para melhorar a aquisição e absorção de nutrientes em tecidos comestíveis de trigo-milho e feijão comum para combater a desnutrição de Zn e a fome oculta em seres humanos. Nesse contexto, esta pesquisa foi desenvolvida para entender o efeito interativo da inoculação/coinoculação com bactérias promotoras de crescimento de plantas (BPCP) e adubação com Zn via solo e foliar para melhorar o crescimento, produtividade e nutrição das culturas de trigo-milho e feijão, além de averiguar a biofortificação de grãos em condições de campo de Cerrado tropical. Para isso, realizou-se seis experimentos, todos em delineamento de blocos casualizados em Latossolo vermelho de textura argilosa sob sistema plantio direto. O **experimento 1** com feijão comum foi conduzido em esquema fatorial  $7 \times 2$ , sendo co-inoculação com PGPBs (sem inoculação, *Rhizobium tropici*, *R. tropici* + *Azospirillum brasilense*, *R. tropici* + *Bacillus subtilis*, *R. tropici* + *Pseudomonas fluorescens*, *R. tropici* + *A. brasilense* + *B. subtilis* e *R. tropici* + *A. brasilense* + *P. fluorescens*) e aplicação de Zn no solo (sem e  $8 \text{ kg ha}^{-1}$ , como sulfato de zinco) em 2019 e 2020 (maio-agosto). O **experimento 2** com feijão comum foi conduzido em esquema fatorial  $7 \times 3$ , sendo co-inoculação com PGPBs (sem inoculação, *R. tropici*, *R. tropici* + *A. brasilense*, *R. tropici* + *B. subtilis*, *R. tropici* + *Pseudomonas fluorescens*, *R. tropici* + *A. brasilense* + *B. subtilis*, e *R. tropici* + *A. brasilense* + *P. fluorescens*) em associação a doses foliares de nano-Zn (0, 1,5 e  $3,0 \text{ kg ha}^{-1}$  de Zn, como óxido de zinco) em 2019 e 2020 (maio-agosto). O **experimento 3** com trigo foi conduzido em esquema fatorial  $4 \times 2$ , sendo inoculações com PGPBs (sem inoculação - controle, *A. brasilense*, *B. subtilis* e *P. fluorescens*) em associação com aplicação de Zn no solo (0 e  $8 \text{ kg ha}^{-1}$ ) em 2019 e 2020 (maio a setembro). O **experimento 4** foi conduzido com trigo em esquema fatorial  $4 \times 5$ , sendo inoculações com PGPBs (sem inoculação - controle, *A. brasilense*, *B. subtilis* e *P. fluorescens*) em associação com cinco doses foliares de nano-Zn (0, 0,75, 1,5, 3,0 e  $6,0 \text{ kg ha}^{-1}$  de Zn) em 2019 e 2020 (maio-setembro). O **experimento 5** com milho foi realizado em esquema fatorial  $4 \times 2$ , sendo inoculações com PGPBs (sem inoculação - controle, *A. brasilense*, *B. subtilis* e *P. fluorescens*) em associação com aplicação de Zn no solo (0 e  $8 \text{ kg ha}^{-1}$ ) em 2019–2020 e 2020–2021 (novembro-março). Também foi realizado **experimento 6** com milho em esquema fatorial  $4 \times 2$ , sendo inoculações com PGPBs (sem inoculação - controle, *A. brasilense*, *B. subtilis* e *P. fluorescens*) em associação com duas doses foliares de nano-Zn (0 e  $3 \text{ kg ha}^{-1}$  de Zn) em 2019–2020 e 2020–2021 (novembro-março). A coinoculação com *R. tropici* + *B. subtilis* e *R. tropici* + *P. fluorescens* juntamente com a aplicação de Zn no solo e foliar melhoram o crescimento da planta, a produtividade de grãos e as concentrações de nitrogênio (N), fósforo (P) e Zn na parte aérea e nos grãos de feijão comum. As

eficiências de uso do zinco, de recuperação e de utilização do Zn aplicado também foram aumentadas com a coinoculação de *R. tropici* + *B. subtilis* juntamente com a aplicação de Zn no solo e aplicação foliar de nano-Zn na dose de 1,5 kg ha<sup>-1</sup>, enquanto a eficiência agrofisiológica aumentou com a coinoculação tripla de *R. tropici* + *A. brasilense* + *P. fluorescens*. A inoculação com *B. subtilis* aumentou a produtividade de grãos de milho (14-17%), enquanto a inoculação com *P. fluorescens* incrementou o acúmulo de Zn na parte aérea (33-51%) e grãos (37-50,7%) de milho sob Zn residual no solo e nano-Zn adubação foliar na sucessão trigo-milho. Além disso, atributos bioquímicos (clorofila a, b e total, carotenóides, açúcar solúvel total e aminoácidos) e eficiência de uso de Zn também foram aumentados com a inoculação de *B. subtilis* e *P. fluorescens* em combinação com solo e aplicação foliar de nano-Zn. A inoculação com *B. subtilis* e *P. fluorescens* juntamente com a aplicação foliar de nano-Zn e Zn no solo melhorou a concentração de Zn na parte aérea (18-31%) e grãos (16-34%), a concentração de N na parte aérea (10-24%) e grãos (16-20%), concentração de P na parte aérea (26-32%) e grãos (18-26%) e produtividade de grãos (4-20,6%) de trigo em cerrado tropical. Além disso, a inoculação de *P. fluorescens* com Zn aplicado no solo ou aplicação foliar de Zn melhorou a eficiência de uso de zinco e aumentou a partição de Zn em grãos comestíveis para maior ingestão estimada de Zn com consumo diário de trigo. Portanto, a inoculação de *B. subtilis* e *P. fluorescens* com aplicação de Zn no solo ou foliar de nano-Zn são consideradas estratégias mais eficazes, sustentáveis e ambientalmente seguras para melhorar os atributos bioquímicos, nutricionais e de produtividade de feijão, milho e trigo em sistemas agrícolas. Portanto, recomenda-se que a inoculação de sementes com *B. subtilis* e *P. fluorescens* juntamente com a fertilização de Zn via solo ou nano-Zn foliar na dose de 3 kg ha<sup>-1</sup>, por ser um mecanismo eficaz para melhorar a eficiência de aquisição e uso de nutrientes pelas plantas, particularmente Zn, propiciando uma produção mais sustentável e biofortificação de feijão comum, trigo e milho em regiões de cerrado tropical do Brasil.

**Palavras-chave:** *Phaseolus vulgaris* L.; *Triticum aestivum* L.; *Zea mays* L.; fertilização com zinco; absorção de zinco; eficiência de uso do zinco; partição de zinco; produtividade; nutrição de zinco; inoculação.

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## CHAPTER-1

### 1. INTRODUCTION

Agriculture is facing one of the most threatening and challenging of food and nutrition security. In addition, increasing global population and loss of land resources along with climate changes is another challenge to agricultural crop production system that drastically affect growth, yield and nutritional status of crops (PROSEKOV;IVANOVA 2018; RORIZ *et al.*, 2020). Global crop improvement programs have been given satisfactory attention to ensure food security irrespective of nutritional security. Limited research, genetic inheritance and imbalanced fertilization of staple crops (maize, wheat and common bean) has become the primary cause of malnutrition (RORIZ *et al.*, 2020; DEBNATH *et al.*, 2021). Majority of the global population is deficient micronutrients that increase the momentum of sustainable agriculture practices to maintain yield along with nutrition. The persistent micronutrients deficiency is an emerging and challenging health and social concern of the present and prospective population, termed as hidden hunger that has affected more 2 billion or one third individuals worldwide (HARDING *et al.*, 2018; LOWE, 2021). The precedent deficiency of micronutrients especially zinc (Zn) is a silent epidemic, affecting about 50% of cereal cultivable soils and 17% of the global population and considered as 5th health risk factor (KUMSSA *et al.*, 2015; MAXFIELD; CRANE, 2019).

Zinc is widespread micronutrient deficiency due to high pH, oxides, complex insoluble carbonates and bicarbonates as well as weathered soils (LEITE *et al.*, 2019; GALINDO *et al.*, 2021). Majority of the Zn deficient soils either having low available Zn or in the form that is unavailable to plants (SHAIKH; SARAF, 2017) depending on the nature of soil such as calcareous, paddy, sandy and coarse textured soils under elevated level of phosphorous and silicon (CAKMAK, 2008). Zinc fertilization is strongly correlation with cereals growth and production that's why low Zn doses drastically reduce cereal crop production. In addition, conventional cereals like wheat and maize have higher phytate compounds that contribute to high phytate-Zn ratio and reducing Zn concentration in the edible tissues (REHMAN *et al.*, 2017). Zinc is acting as a co-factor and constituent of more than 300 enzymes, thus regulating several cellular, biochemical and physiological processes as well as helping in metabolism and energy transferring activities (HAFEEZ *et al.*, 2013; ULLAH *et al.*, 2020). Hence, Zn fertilization of staples crops like

maize, wheat and common beans promote their growth and productivity, and enriching grains with Zn to favor biofortification to alleviate malnutrition in human population.

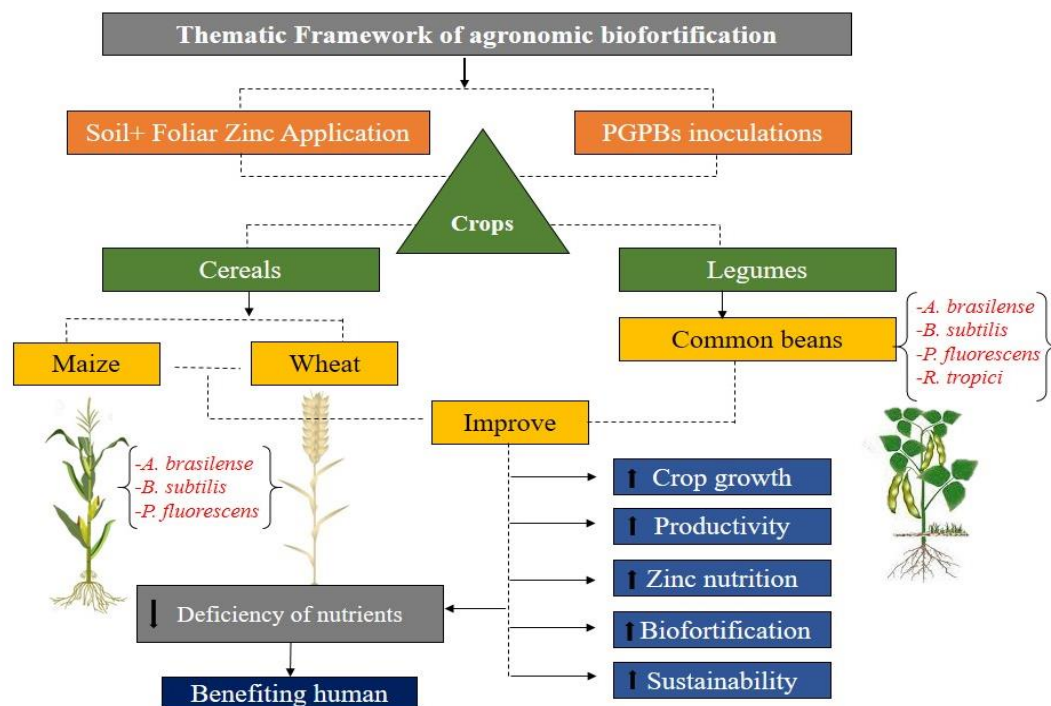
Biofortification is a promising strategy that fulfill food and nutritious security by improving nutrient bioavailability in edible grains. Agronomic biofortification improve mineral nutrients availability in soil, their uptake in above-ground plant parts and mobilization and re-localization into to edible tissues of food crops to overcome malnutrition and proved the most feasible and sustainable strategy (DHALIWAL *et al.*, 2022). It can be brought into practice through mineral fertilization especially Zn via soil, foliar or seeds priming to increase concentration of targeted nutrient in the grains of chosen crop (ULLAH *et al.*, 2020). Agronomic biofortification is up-scaling nutrient bioavailability by lowering dietary risk of Zn in marginal and weathered soils. Malnutrition of global population has reduced in the last decades due to awareness of appropriate fertilizer application and several research initiatives, especially on agronomic biofortification of Zn in staple crops (RAHMAN *et al.*, 2020). Zinc biofortified crops have affirmative inherent abilities to compete with abiotic stresses, enhancing productivity and social-economic returns of crops under different environmental constraints (RAHMAN *et al.*, 2020; ULLAH *et al.*, 2020). Satisfying the requirements of the highly increasing population under limited agricultural land resources and fertilizers loading into the soils can be achieved under sustainable agriculture (FAO, 2022). Sustainable agriculture ensures global food and nutritional security and increasing economic benefits of communities by protecting and promoting ecosystem and reducing environmental risks (CAMPANHOLA; PANDEY, 2018). Therefore, it is unprecedented to adopt sustainable agriculture practices to deal with challenge of food and nutritional security under low environmental risks.

Biofortification depends on the bioavailability of nutrients in the soils, plant species and role of rhizosphere microbial population in nutrient dynamics (VAN DER STRAETEN *et al.*, 2020). Plant growth-promoting bacteria (PGPBs) multiply in rhizosphere of plants to promote soil fertility, crop productivity, nutrients bioavailability and food security, thus contributing to sustainable agriculture (SINGH *et al.*, 2022). These PGPBs are contributing to several ecological processes including decomposition of organic matter, homeostasis, nutrient cycling to support crop growth and health (JALAL *et al.*, 2022). Diverse genera of PGPBs including species of *Rhizobium*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Gluconacetobacter*, *Bacillus*, *Burkholderia*, *Klebsiella*, *Enterobacter* and many others are being reported to promote plant growth and nutrients

bioavailability (RANA *et al.*, 2020; NITU *et al.*, 2020; ULLAH *et al.*, 2020). These PGPBs promote growth, yield and nutrients acquisition through nitrogen fixation, nutrient solubilization, enzyme activation and phyto-hormones production (direct mechanisms) as well as siderophores production and inhibition of phyto-pathogens (indirect mechanisms) (KUMAR *et al.*, 2019). In addition, PGPBs are significantly acting on the roots elongation and root physiology that could increase absorption of available Zn for the better plant growth and yield (SINGH *et al.*, 2017).

Currently, cereals (maize and wheat) and legume (common beans) are selected for increasing of targeted nutrient (Zn), which are staple food crop of the most regions of the world and helpful in elimination of Zn malnutrition. Inoculation with PGPBs under adequate fertilizers management is a promising sustainable alternative that can facilitate nutritional status and productivity of different crops. However, there is a research gap to study the integrated effect of inoculations with PGPBs (*Azospirillum brasilense*, *Bacillus subtilis* and *Pseudomonas fluorescens*) and soil and foliar Zn in maize and wheat cropping system under tropical savannah. Also, there is lacking of literature on the interaction of co-inoculation (*Rhizobium tropici* with *Azospirillum brasilense*, *Bacillus subtilis* and *Pseudomonas fluorescens*) with soil and foliar Zn fertilization in common beans under tropical savannah. Based on the mechanisms of actions of PGPBs in plants, it is expected that there is synergistic interaction between PGPBs and Zn fertilization either in soil or foliar application, which improve nutrition and productivity of cereals and legume in this study, presented in the thematic framework (Figure 1).

**Figure 1.1.** Thematic framework of treatments application and their benefits to respective crops in current study.



**Source:** Author

In this scenario, new approach of these PGPBs inoculation/co-inoculation with soil and foliar is needed to be performed to alleviate Zn malnutrition and increase productivity and Zn daily intake with common beans, wheat and maize grains. In other words, this study tried to increase common bean, wheat and maize yields with the integrated use of PGPBs inoculation and Zn doses to stimulate nutrient availability for obtaining desired nutrient-enriched grains of these crops. Therefore, the hypothesis of this study was that there may be a synergetic association of different PGPBs with soil and foliar Zn application on Zn availability on plant and soil, yield, Zn use efficiency and intake of fortified common bean, wheat and maize in the tropical savannah of Brazil. In this context, the objectives of the study were to evaluate the effect of seed inoculation and co-inoculation with PGPBs in combination soil and foliar Zn application on common bean, wheat and maize growth and yield. Additionally, the effect of inoculation and co-inoculations and Zn fertilization on Zn accumulation in plant shoot and grains, Zn intake, and Zn use efficiencies for sustainable biofortification in the tropical savannah of Brazil.

## 1.2 Outline and thesis organization

The structure of this thesis consists of eight chapters.

- **Chapter 1 – Introduction:** introduced background information, problem of the research and importance and research gap as well as objectives of the study.
- **Chapter 2 – Review of literature:** comprehensively discussed the concept and proposed methodology of the study. Also, shed light on the interaction of the applied treatments and their implementation, also their contribution to sustainable agriculture and benefits to human.
- **Chapter 3 - Common bean yield and zinc use efficiency in association with diazotrophic bacteria co-inoculations** (Published: Agronomy- doi.10.3390/agronomy11050959).
- **Chapter 4 - Agronomic biofortification and productivity of common bean with nano-zinc and plant growth-promoting bacteria.**
- **Chapter 5 - Agronomic biofortification and productivity of wheat with soil zinc and diazotrophic bacteria under tropical savannah** (Published: Crop and Pasture Science, doi.10.1071/CP21457)
- **Chapter 6 - Integrated use of plant growth-promoting bacteria and nano-zinc foliar spray is a sustainable approach for wheat biofortification, yield and zinc use efficiency.**
- **Chapter 7 - Diazotrophic bacteria is an alternative strategy for increasing grain biofortification, yield and zinc use efficiency of maize** (Published: Plants- doi.10.3390/plants11091125)
- **Chapter 8 - Nano-zinc and plant growth-promoting bacteria improve biochemical and metabolic attributes of maize in tropical Cerrado** (Published: Frontiers in Plant Science- doi. 10.3389/fpls.2022.1046642).

## CHAPTER-2

### 2. REVIEW OF LITERATURE

#### 2.1 Plant Mineral nutrition: unlocking plant-soil interaction

Plant mineral nutrition refers to the application of mineral nutrients to soil, translocation and absorption through roots system, and mobilization and utilization in the shoots of plants (FAGERIA *et al.*, 2010). The nutrients that are essential for plant growth and development are divided into two main categories; macronutrients and micronutrients (KATHPALIA; BHATLA, 2018). Plants acquire most of the nutrients through root from soil rhizosphere to maintain functions like uptake, sequestration and metabolism for intercellular interference. Nutrients are one of the challenging environmental factors that confer productivity of almost all-natural ecosystems, lead to global nutrients limitation (LAMBERS; OLIVEIRA, 2019). Nutrients mobilization and re-localization from soils to plants is another major concern of the plant growth and development, which can be controlled through internal signals (DOTANIYA; MEENA, 2015). Plants adapt several strategies to improve nutrients bioavailability in edible tissues, which can be determine through nutrients in the soil roots under nutrients deficiency and high availability, and amount of nutrient required by the plant. These strategies are involved root-shoot signaling that allow the plants to use available nutrients to fulfill physiological needs of the above ground plant attributes (OLDROYD; LEYSER, 2020).

The suboptimal amount of nutrients that fail to fulfill its functional roles lead to a state of deficiency, which is typically used to characterize nutritional status and fertilization optimization (BANG *et al.*, 2021). Plants experience several deficiencies during their life-cycle, which may range from mild to severe and chronic, leading to poor yield and quality of food and feed. Besides this, plants interactions with a wide range of abiotic and biotic growth factors can also cause nutrient-based disorders. Nutrient availability is a crucial environmental factor next to water that effectively support plants to withstand and survive during climate change and cope with environmental stress, varies with plant species (LAMBERS; OLIVEIRA, 2019; TERRER *et al.*, 2019). Plant nutrition preserves soil fertility in order to deliver nutrients at right time and quantity for adequate plant growth (WITHERS *et al.*, 2018). Soil conditions are the major challenging factors that affecting nutrients delivery and plant growth. Roots modification and

rhizosphere conditions are playing a crucial role in the adaptation of plants to adverse soil conditions (VIVES-PERIS *et al.*, 2020).

Plant-soil interaction is recognized as an important environmental factor, where plant must compete with environmental changes that may be stressful or unsuitable for plant growth like fluctuation in the availability of macro- and micronutrients (BOUAIN *et al.*, 2019). Plant growth and development are determined by nutrients availability and their dynamics of uptake, transportation, assimilation and biological interactions (KUMAR *et al.*, 2021). Plant roots are the unique organs which are responsible for nutrients transportation from soil to plant via xylem as a principal pathway. Roots are improving soil organic matter, retaining soil carbon and forming stable soil aggregates that contribute to healthy plant growth and development under harsh environmental conditions (HAVLIN, 2020). Generally, roots contribute around 10-20% of the total plant dry matter, determined in term of root length, density and mass. In addition, nutrients acquisition and efficiency are affecting by several factors related to soil such as water content, chemistry, composition, pH and soil compaction that can make harder for the plants to uptake nutrients (FAGERIA; MOREIRA, 2011).

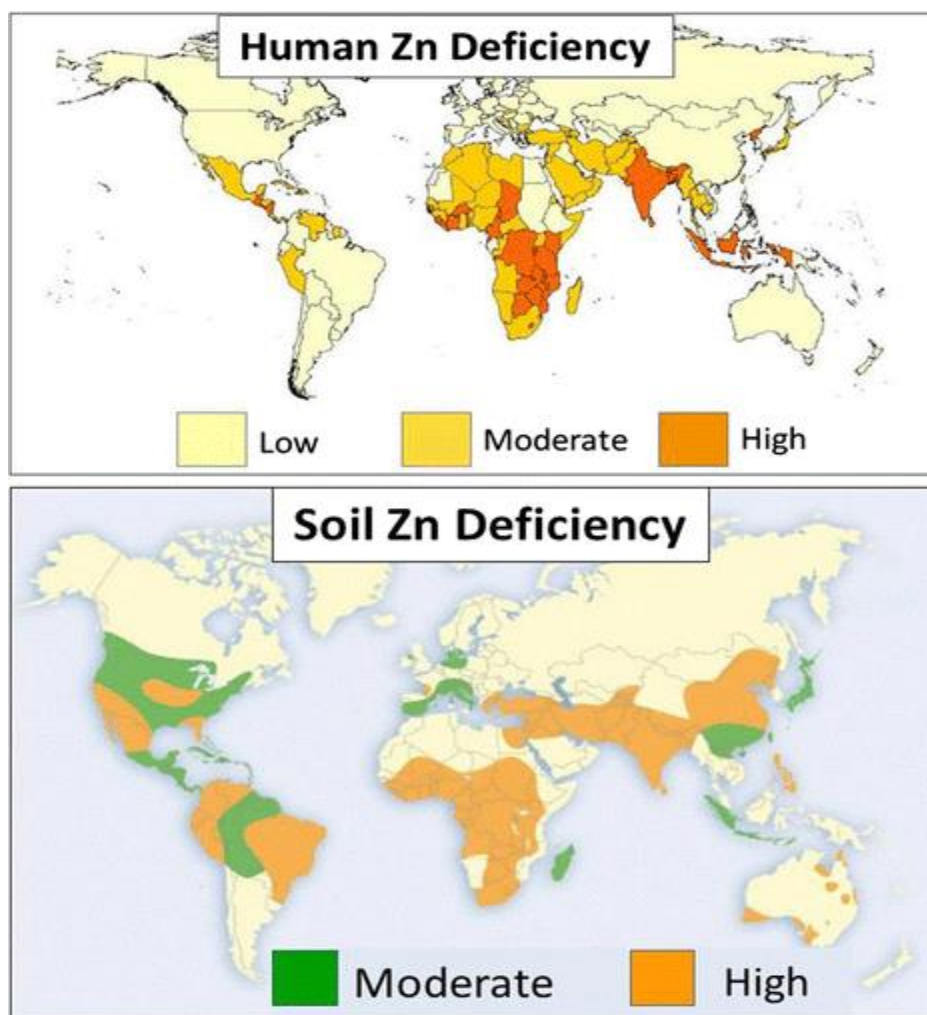
Plant-soil interactions can control around 50% of the total ecosystem CO<sub>2</sub> as result of complex association among root, litter and soil microbial community, that lead to optimization of carbon and nutrients in ecosystem (WANG *et al.*, 2016; TIAN *et al.*, 2019). Plant roots release a diverse range of chemicals that stimulate microbial growth, enzymatic activities and soil organic matter decomposition (GUYONNET *et al.*, 2017). Under these processes, plant species develop several strategies to use soil resources but there are still some abiotic factors that affect plant-soil interactions. In addition, variations in nutrient and water resources can provide an insight understanding of plant competition or plant-soil interaction and a necessary step in the selection of plant species and sustainable development of soil resources (WARING *et al.*, 2015; WU *et al.*, 2020). Mineral nutrient supplementation depending on nutrient, resource, concentration, and environmental conditions can influence plants root system. Mineral nutrition is a cost-effective strategy that sustain crop productivity under reduced environmental risk, and high nutrients availability, contributing to positive plant-soil interaction (RAHMAN *et al.*, 2018). Thus, ensuring adequate nutrients delivery at right time can confer the most challenging issues to crop productivity under climate change (SCHJOERRING *et al.*, 2019). For example, addition of multicomponent

fertilizers (containing Zn, iron, manganese, and copper) can enhance quality and yield of cereal grain crops, when applied in proper fertilization methods and time (KLIKOCA; MARKS, 2018).

## 2.2 Zinc and its dynamics in different cropping system

The widespread zinc (Zn) deficiency is due to low Zn distribution in cultivatable soils. The low Zn solubility in soil solutions lead to the global Zn deficiency in crop plants, which causes stunted growth and malformed leaves, leading to lower productivity and malnutrition of grains and fruits (SUDHA; STALIN, 2017). Global Zn deficiency index indicated that Brazil is still under low prevalence of Zn deficiency in humans and high in soils (Figure 2.1).

**Figure 2.1.** Global distribution of human Zn deficiency and soil Zn deficiency



**Source:** Modified from Wessells and Brown (2012) and Alloway (2008) respectively.

Basically, Zn is an immobile nutrient in the soil solution and its mobility is further reduced by high soil pH, calcium content, intensive agriculture, excessive use of fertilizers, and adsorption to soil colloids (TAHIR *et al.*, 2018). Zinc deficiency is widespread in the surface layer of the soils, which restrict cereal production in tropical agriculture system. The higher phosphorous demand in the tropical agriculture is another major constrain that can availability and functions of several macronutrients, stimulating reactive oxygen species and affecting crop growth and yield (KHATUN *et al.*, 2018).

Zinc is an essential and important nutrient for the normal growth and reproduction of crop plants and required in small amount for proper functions of physiological, enzymatic and metabolic processes of plants (SUGANYA; SARAVANAN, 2016). Zinc is a structural component and regulatory cofactor of several enzymes and proteins of different biochemical pathways such as carbohydrates synthesis, photosynthesis, protein metabolism, pollen formation, cell membranes, and resistance to infection by certain pathogens (DRISSI *et al.*, 2017; SUGANYA *et al.*, 2020). Zinc influences root development, architecture and biomass, and transported through symplast pathways (GUPTA *et al.*, 2016). Zinc transportation from soil to plant is a complex factory of different mechanisms with secretion of phyto-siderophores. Zinc transportation is facilitated via xylem to phloem and then remobilizing from aged to young leaves, where Zn is loaded into plasmodesmata and stored into endosperm cavity (AIQING *et al.*, 2021). Alterations in Zn concentration activate different genes to prevent excessive or poor absorption and uptake in plant tissues through transcriptional factors, enzymes, channels, and transporters (SINCLAIR; KRÄMER, 2012). Several low molecular weights chelates and proteins are involved in Zn homeostasis in cytosol and storage in intracellular compartments. Zinc homeostasis is a complex mechanism in subcellular compartments such vacuole, cell wall, vesicles, where Zn storage in vacuole offers tolerance to Zn toxicity. Although, Zn remobilization from the subcellular compartments is crucial during deficiency/ senescence or abscission (SINCLAIR; KRÄMER, 2012; GUPTA *et al.*, 2016).

### **2.2.1 Maize-wheat cropping systems**

Maize (*Zea mays* L.) was cultivated for the first time in Mexico's Tehuacan Valley and is a main crop of United State (FAO, 2018). Brazil is the third largest producer with total harvested area (19.5 million hectares) and production (105 metric million tons), especially Mato Grosso do

Sul roughly produce one third of the total production of Brazil. This country has three maize crop seasons, second is the main maize cultivation season in relation to the first and third one with increment of 16.5% in production due to favorable environmental conditions (USDA, 2021; CONAB, 2021). Maize has economic and social importance, as food for human and feed for animals and also used in the production of ethanol in Brazil (EMBRAPA, 2018).

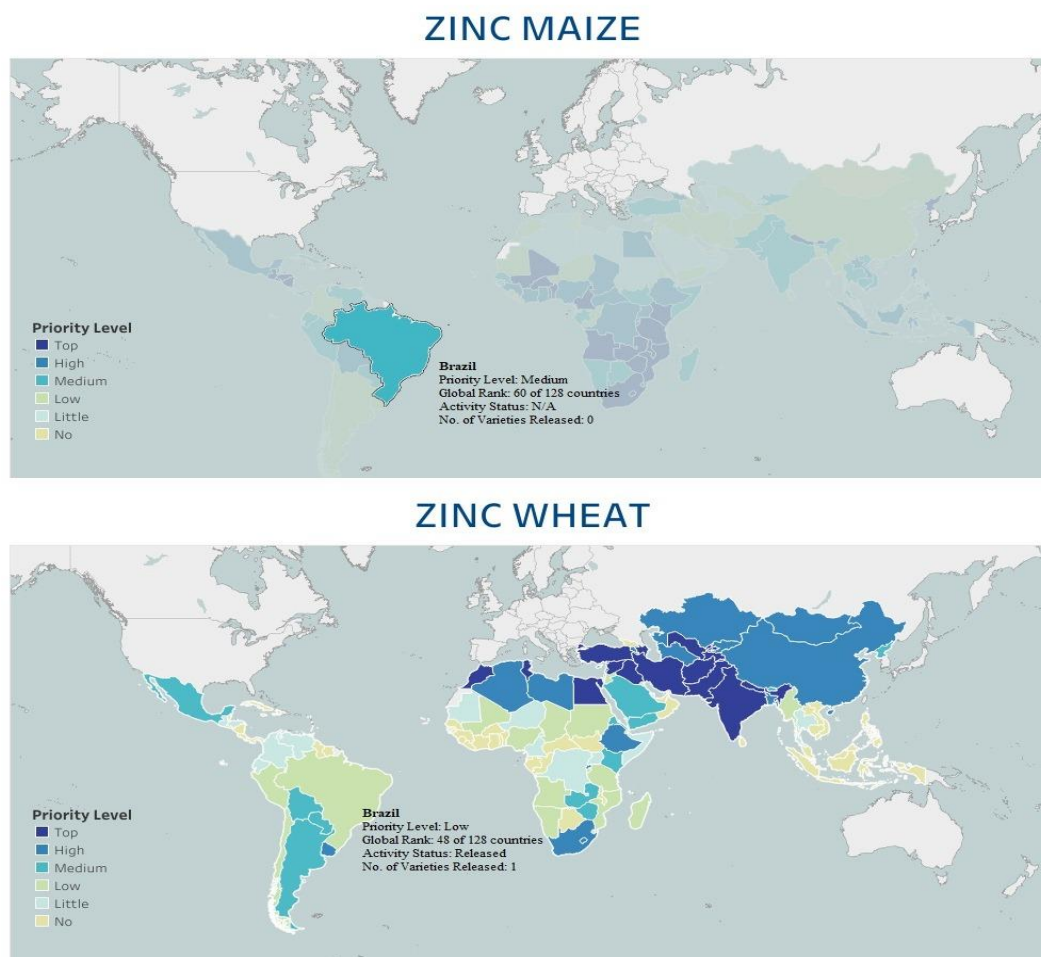
In addition, Wheat (*Triticum aestivum* L.) is widely famous cereal crop and also a major source of staple food for most of regions of the world. Wheat is not the first growing crop of Brazil. However, it is grown on 2.34 million hectares with the production of 7.7 million tons in 2021-22 cropping season with increase of 21% from the production of 2020-21 season, which is still not enough for the country consumption (CONAB, 2022). Wheat is easily digested source of carbohydrates, minerals, vitamins, lipids and protein for human and animals (BRAUN *et al.* 2010).

Zinc fertilizer improve Zn delivery for cereals by enhancing Zn bioavailability and accumulation that lead to higher crop productivity (MANZEKE *et al.*, 2017; CAKMAK; KUTMAN, 2017; JALAL *et al.*, 2020). Cereal plants develop different mechanisms to acquire Zn for their optimal growth, which varies for different species depending on nutrient availability, accumulation and translocation (AKHTAR *et al.*, 2019). Adequate Zn dose-response management is considered as a crucial agronomic strategy under high fertilizer costs and low crop economics (CAKMAK; KUTMAN, 2017). The establishment of better Zn management can improve productivity and agronomic use efficiency of Zn in maize-wheat cropping system (GALINDO *et al.*, 2021). Zinc fertilization is one of the viable options to fulfill crop demands as well as improve Zn concentration in the stem and edible tissues under Zn deficient soils (LIU *et al.*, 2017).

Zinc deficiency is hitting almost all countries of the planet, especially developing countries (FAO, 2019). Particularly, around half (50%) of the global cereal cultivated soils are being reported with Zn deficiency (ALLOWAY, 2010). The widespread Zn deficiency in sequential cereal cropping system is due to high pH, oxides, complex insoluble carbonates and bicarbonates as well as weathered soils (LEITE *et al.*, 2019; GALINDO *et al.*, 2021). There is strong and positive correlation between Zn fertilizers and cereals growth and production, low Zn doses drastically reduce cereal crop production. In addition, conventional cereals like wheat and maize produce higher phytate compounds that can increase phytate-Zn ratio, reducing Zn concentration in the edible tissues (REHMAN *et al.*, 2017). Zinc deficiency initially appear in the young leave due to its immobile nature, which gradually affect different bio-physiological aspects of crop

plants. Wheat and maize are being considered the most susceptible crops to Zn scarcity due to their inherently low Zn concentration, decreasing grain quality (ZOU *et al.*, 2012). Prevalence of maize-wheat Zn deficiency distribution priority is presented in Figure 2.2.

**Figure 2.2.** Prevalence of maize-wheat zinc deficiency priority, status in the world and zinc efficient released cultivars in Brazil



**Source:** Harvest plus ([https://bpi.harvestplus.org/bpi\\_cropmaps.html?id=c8](https://bpi.harvestplus.org/bpi_cropmaps.html?id=c8)); International Maize and Wheat Improvement Center (CIMMYT) and International Institute of Tropical Agriculture (IITA)).

Zinc is an effective nutrient to enhance germination, cell membrane stability, stomatal, photosynthesis and respiration regulation, also interacting with stimulating antioxidant enzymes and proteins synthesis, that lead to better plant health and productivity. However, its scarcity reduce chlorophyll content and protein synthesis (UMAIR HASSAN *et al.*, 2020). Both soil and

foliar Zn fertilization improve maize-wheat nutritional status by enhancing Zn grain concentrations. Although, foliar Zn application enhanced grain Zn recovery and grain Zn concentration in both maize and wheat cropping seasons and consider as one of the economical methods of Zn application (WANG *et al.*, 2012).

Ma *et al.* (2017) performed multiple experiments with soil and foliar Zn fertilization under different environmental conditions to determine wheat physiological indices. It was reported that both soil and foliar Zn fertilizations had positive impacts on leaf antioxidant level, photosynthetic pigments, Zn concentration and grain yield under optimal and least water conditions in wheat crop. Some other studies also indicated that soil, foliar and soil + foliar improved grain Zn concentration and its bioavailability in wheat under the Zn deficit soils, leading to higher grain yield and human Zn nutrition irrespective of the agro-climatic and agronomic inputs (ZOU *et al.*, 2012; ZHAO *et al.*, 2014).

Wheat grain filling is the driving stage for increasing Zn mobilization from root to sink with the involvement of nitrogenous compounds. Nitrogen nutrition helps Zn translocation and mobilization in wheat root-shoot via xylem and phloem through leaves to grains. Zinc applied with NPK highlighted that foliar Zn stability and its impact on wheat nutritional quality. Zinc application alone or in combination with NPK increased grain yield and nutritional quality of wheat that ultimately improve human diet (WANG *et al.*, 2021).

Integrated application of Zn with NPK increased grain yield of maize 130% as compared to single NPK fertilization (MANZEKE *et al.*, 2014). In addition, Amanullah *et al.* (2014) evaluated the effect of P and Zn at booting and silking stages of maize. Foliar application of P and Zn at the rate of 3 and 0.3% at booting stage of maize enhanced morphological and yield indices of maize under irrigated conditions. Zinc applied at dose of 1 and 2% to seed and leaf maize hybrids were noted with greater morphological and yielding characters and grain Zn accumulation (MOHSIN *et al.*, 2014). The co-application of potassium and Zn are interacting with soil and plant mechanisms, increasing maize productivity and physiological traits such as cob length, 1000-grain weight, dry weight, relative water content, photosynthesis and transpiration rate, and stomatal conductance (RAZA *et al.*, 2021). Foliar Zn application from chelated and non-chelated sources alleviate drastic effects of drought stress by interacting with nitrogen and copper concentration in maize shoot, increasing productivity and stabilizing resource utilization (WEISANY *et al.*, 2021).

Further research is required to study physiological and molecular insights of Zn interaction with other macronutrients in cereal cropping system.

### **2.2.2 Common bean cropping system**

Common bean (*Phaseolus vulgaris* L.) originated in central Mexico and now grown globally especially in Central and South America (DESIDERIO *et al.*, 2013). Common beans have imperative traditional, historical and nutritional profile (proteins, amino acid, minerals as well as antioxidants and polyphenols) which fulfill the nutritional diet of humans in many regions of the world as staple food (SPARVOLI *et al.*, 2015; RIVERA *et al.*, 2018). The annual beans production is about 26.5 million tons around the world (FAOSTAT, 2018). However, Brazil produced around 3.15 million tons of dry beans in 2018-19 cropping season, cultivated in three distinctive seasons 2018-19 (CONAB, 2019). Legumes could improve soil fertility through biological nitrogen fixation and enhancing microbial activity, thus decreasing dependency on chemical fertilizers and promoting sustainable environment and production (SORATTO *et al.*, 2013).

Common bean is an important grain legume, normally grown for dry seeds that fulfill nutritional demands of several regions of the world as a staple food (RIVERA *et al.*, 2018; PHILIPO *et al.*, 2020b). Common bean seeds contain relatively high Zn concentration and consider as an important source of Zn nutrition among other staple foods (GŁOWACKA *et al.*, 2015). Common bean is widely produced and consumed crop in Brazil but most of the consumed varieties had low Zn concentration, that may be either due to low soil Zn concentration or Zn deficient genotypes (SAHA *et al.*, 2017; CAMBRAIA *et al.*, 2019). Zinc fertilization in common beans is considered more effective to alleviate Zn deficiency as compared to other grains crops, since, common bean is not undergoing milling process and consumed as a whole grain (PHILIPO *et al.*, 2021). Common bean have 2–3 times more Zn than maize and wheat with an average range of 18–77 mg kg<sup>-1</sup> (MURUBE *et al.*, 2021).

This legume plant is being considered sensitive to Zn deficiency, where Zn uptake is correlated with Zn concentration in old and young plant portions. Common bean cultivation on Zn insufficient soils was noted with low dry matter production of young plant portions than old ones (BEYGI *et al.*, 2012). Zinc fertilization is playing dual functions, improving grain Zn concentration and reducing phytic acid concentration (anti-nutritional factor) therefore, increasing Zn bioavailability human diet (HOPPLER *et al.*, 2014). Zinc soil fertilization from zinc sulphate

has been reported with 100% increase in common bean Zn concentration while foliar Zn application from the same source increased grain Zn concentration by 14.7% in common beans in Brazil (CAMBRAIA *et al.*, 2019). Zinc application from chelated and non-chelated sources increased vegetative growth, yield and qualitative characterization of common bean (MAHDIEH *et al.*, 2018). Zinc efficient common bean cultivars adapt several physiological mechanisms to cope with Zn deficiency as well as increasing Zn efficiency under Zn deficient soils. Zinc is responsible for the mitigation of reactive oxygen species by reducing lipid peroxidation and antioxidant metabolism in common bean plants (RAM *et al.*, 2016). Zinc soil and foliar fertilization at different physiological stages of common beans is considered as one the best Zn management strategy to improve several metabolisms' characterizations, thus leading to high productivity and biofortification (KACHINSKI *et al.*, 2022).

### **2.3 Zinc management approaches**

Different strategies are being adapted to manage Zn fertilization, such as right source, time, rate, place and method of application. The most common Zn fertilization strategies are; 1) soil-Zn application, 2) foliar-Zn, and 3) seed priming, which are viable alternatives that allow plants to survive by preventing growth and yield under Zn insufficient soils (MAQBOOL; BESHIR, 2019).

#### **2.3.1 Zinc form and functions in soil**

Zinc is the 23<sup>rd</sup> most abundant element in the earth crust in the form of Polly-metallic mines (KNIJNENBURG *et al.*, 2019). Zinc in soil can be found in a wide variety and forms including water soluble, organically adsorbed Zn, exchangeable Zn, chelated and soil solutions Zn. Soil parent material is one of the important factors for the bioavailability of Zn in soil. Volcanic rocks contain ~70–130 mg kg<sup>-1</sup> of soil Zn concentration while carbonated rocks and sandstone contain ~20 and ~16 mg kg<sup>-1</sup> soil Zn concentrations respectively (ALLOWAY, 2013). Soil Zn is available to plants in the form of Zn<sup>2+</sup>, ZnOH<sup>+</sup>, and Zn complexes with soluble organic materials that can be affected by high carbonates, bicarbonates, pH, phosphorous content, and imbalanced macronutrients fertilizers (ALLOWAY, 2009; LEITE *et al.*, 2019). Zinc availability to the plants and leaching vary with soil clay and organic matter content. Zinc is not uniformly adsorbed to the clay particles, where high Fe and Al oxides, organic matter contents contribute to the reduction of Zn availability (MAŁECKI *et al.*, 2015). Soil pH is anti-proportional to the availability of Zn in

soil solution for plants uptake. High soil pH renders Zn desorption clay and organic matter particles, thus decreasing  $Zn^{2+}$  phyto-availability (SALINITRO *et al.*, 2020).

### **2.3.2 Zinc fertilization and sources**

Fertilizer management with Zn containing fertilizers is one the best option to deal with Zn deficiency in soil and plants (LIU *et al.*, 2020). Zinc fertilizers are applied as broadcast and sprayed on soil, banded application, foliar sprays, seed priming or dipping roots of transplanted seedlings (HAFEEZ *et al.*, 2013). There are different types of Zn fertilizers, each one is used on the basis of its effectiveness for particular crops. There is a wide range of Zn fertilizers including Zn sulphate ( $ZnSO_4$ ), Zn oxide ( $ZnO$ ), and Zn chloride ( $ZnCl_2$ ), Zn-coated urea/ superphosphate and Zn oxy-sulphate. Among these, Zn sulphate and Zn oxides are mostly usually used Zn fertilizers around the world. Use of Zn oxide is recently increased due to its potential advantages to agriculture and specially crop production system (RAI *et al.*, 2018; DIMKPA *et al.*, 2020). Zinc fertilization has high response in the soil with low Zn concentration, grain Zn concentration is higher in the soils with low Zn content as compared to high Zn content soils.

### **2.3.3 Zinc application methods and timings**

Zinc can be extracted from the soil in soil solution of different compounds. Zinc fertilizer needed when plants can't uptake soil supplied nutrients due to leaching and adsorption on soil particles, leading to the inhibition of plant development. Soil Zn application is one of the mostly used mechanism for increasing grain Zn concentration and crop productivity (YASEEN; HUSSAIN, 2020). Soil applied Zn go through different transformation mechanisms depending on soils, crops, and environmental conditions. Agricultural soils reserve numerous Zn fractions that can uniformly contribute to the uptake of available Zn level to plants (LIU *et al.*, 2018). The fractions of Zn in soils depend on leaching from soil, extractability, plants uptake, adsorption, and association with other minerals. Zinc fractions concentrations in alkaline soils were increased under wheat-maize and wheat-rice cropping systems (LIU *et al.*, 2020; LAKSHMI *et al.*, 2021), that can allow the crops to uptake residual Zn fractions for higher grains Zn concentration and yield. An understanding on the effectiveness of Zn application to soils, plants and environment as well as its distribution and mobility in soil solution is very crucial (LIU *et al.*, 2020). Soil Zn application is less effective due to low mobility, absorption in soil (ALLOWAY, 2008), high

phosphorous fertilization in tropical agricultural soils, carbonates and oxides complexions, which deteriorate Zn nutrition and productivity (PENN; CAMBERATO, 2019; ALWAHIBI *et al.*, 2020).

Nutrient foliar application deals as a novel solution to address current challenges of agricultural production system. Foliar Zn application has got more attention in recent decades due to its benefits of quick absorption by plants, cost-effective, and low influence on soil health deal with malnutrition (AZIZ *et al.*, 2019). Foliar Zn can apply at vegetative stages of crop plants which is absorbed by leaves and quickly used for plant metabolic processes (FAGERIA *et al.*, 2009). Foliar Zn absorption rate can be different for different sources of Zn application to leaf surface, enter into leaves through stomata and transported via apoplastic and symplastic pathways to different plant parts. Zinc is absorbed by epidermal cells and transported via vascular bundle/phloem to grain tissues (HONG *et al.*, 2021). Foliar Zn oxide is applied in small amount that can improve tolerance against oxidative stress by alleviating and defending structure of plant cell membrane (BURMAN *et al.*, 2013).

In addition, priming of targeted crop seeds in Zn solution is used for uniform Zn application that is important for better crop establishment under adverse soil and environmental conditions (RAI-KALAL; JAJOO, 2021). It is a sustainable technique that rapidly increase seed establishment and quality attributes that subsequently enhance plant growth. Several studies have been reported that seed priming increase seed germination and emergence, and growth by effecting water use efficiency, nutrient uptake and providing resistance to biotic and abiotic stresses (DUTTA, 2018; SALAM *et al.*, 2022).

Dipping of roots of transplanted crops in Zn solution/ suspension has driven impact on the root system, which has simulative effect on growth, photosynthetic efficiency and antioxidant system of plants (FAIZAN *et al.*, 2018).

#### **2.3.4 Timing of zinc application**

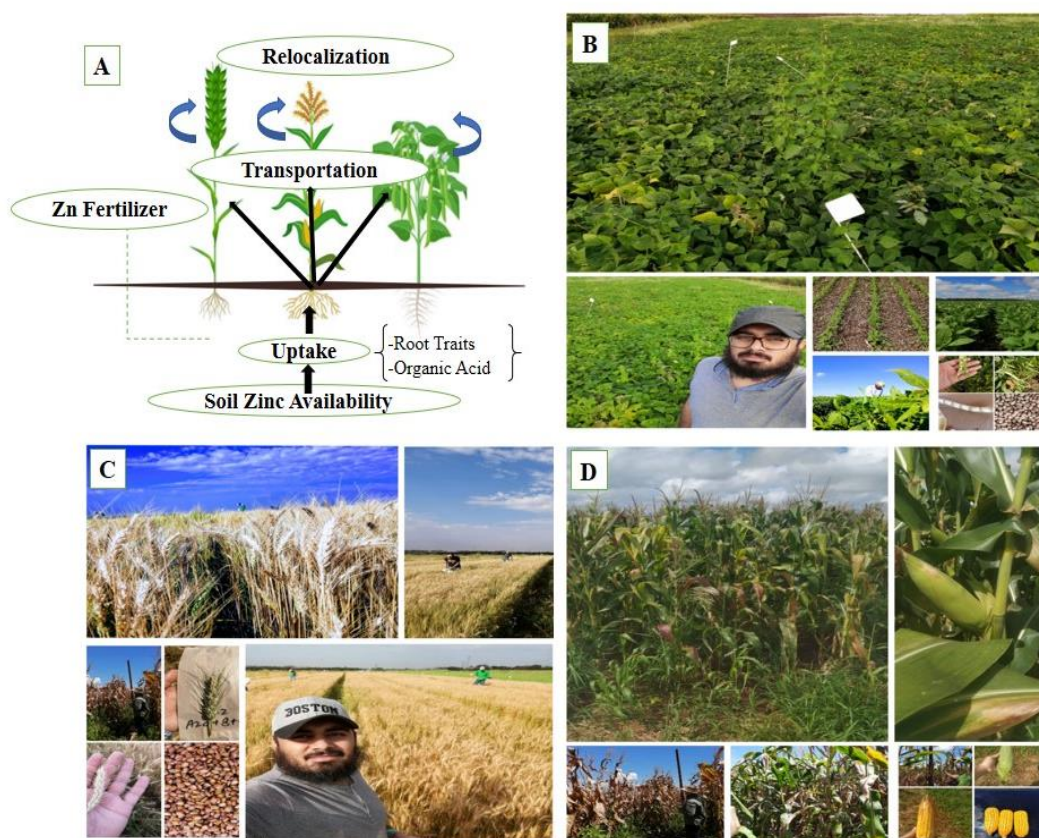
The effectiveness of Zn fertilization is better defined by the time of application. Zinc fertilizer can be applied at planting on to soil and foliar spray during vegetative, flowering and grain-filling stages. Although, stage of foliar Zn fertilization and number of applications are important for Zn loading into the grains (CAKMAK, 2008; JALAL *et al.*, 2020). Soil Zn fertilization followed by foliar spray at grain filling stage improved Zn loading into grain tissues and grain yield as compared by single Zn fertilization (CHATTHA *et al.*, 2017). Dual foliar Zn

fertilizations at flowering and grain filling stages is more efficient to improve rice grain Zn concentration and Zn use efficiency under Zn deficient soils (MABESA *et al.*, 2013). Zinc broadcasting + foliar spray (tasseling and flowering stage) increased grain and shoot yield of maize. Foliar or mutual soil + foliar Zn fertilization are useful alternative strategies increase Zn uptake in plants and reducing Zn malnutrition. Foliar Zn application or urea-coated-Zn is also considered one of the best strategies as compared to soil Zn application (SHIVAY; PRASAD, 2014). The combined soil and foliar Zn fertilization at dough stage of wheat can increase yield by 10-13% and can provide 1.5 times more bioavailable Zn to the grains under sustainability guidelines (CHAKRABORTY *et al.*, 2022).

#### **2.4 Agronomic approaches to manage Zn in crop production system**

Understanding of the effect of fertilizers and fertilization management can change macro- and micronutrients concentration in soil and plants and enhance plant performance. Modern agricultural system uses a wide range of fertilizers to increase yield and micronutrient contents especially, Zn and iron (KLIKOCKA; MARKS, 2018). Appropriate agronomic management practice (fertilization, crop rotation, crop residues, and selection of Zn efficient varieties) can enable the plants to survive under Zn deficient soils and avoid reduction in crop yield (MOUSAVI *et al.*, 2013). However, unawareness and least accessibility to Zn fertilizers reduced small and poor farmer's interest. Therefore, friendlier and less expensive approaches needed to adopt to efficiently increase grain yield of crops. For this purpose, combining Zn fertilization with agronomic management practices can have synergistic effect on grain Zn concentration, yield under reduced labor cost. A little amount of Zn fertilizers is required under proper agronomic practices for the proper physiological processes and crop production under Zn deficient soils. Soil Zn fertilization helps in Zn transportation from roots to above-ground plant and re-localization to grains (Figure 2.3A), that improve plant growth, yield and biofortification of dietary crops like common bean, wheat, and maize under tropical savannah (Figure 2.3B, 3C, 3D).

**Figure 2.3.** The possible mechanism of Zn availability, transportation and uptake from soil to grains (A) in common bean (B), winter wheat (C), and maize (D).



**Source:** Original photographs of each planted crop in tropical savannah conditions, Mato Grosso do Sul, Brazil, captured by Arshad Jalal.

### 2.4.1 Tillage management

Tillage management one of the most critical factors of crop production system. Tillage tends to alter soil physical characterizations and also influence nutrients conservation and release. Zero or no-tillage is known for the reduction of degradation of soil physical to sustain soil fertility and productivity when compared with frequent mechanized tillage system. No-tillage soils had high water retention and low temperature and porosity as compared to conventional tillage system (AGBEDE, 2010; ZHANG *et al.*, 2018). Adaptation of no-tillage in long-term crop production system reduce soil pH and increasing root biomass and microbial activities that are important for nutrients and water absorption. However, no-tillage under crop residues can improve soil quality

by decreasing physical breakdown of soil aggregates, resisting soils against precipitation and increasing nutrient cycling and soil organic carbon, as well as reducing soil erosion (BUSARI *et al.*, 2015; ZHANG *et al.*, 2018).

Zinc availability, transportation and uptake by plants from the upper soil profile can be influenced by tillage practices. The greater root system and decomposition of residues in the upper soil profile can increase distribution and availability of residual Zn to the successive crops. Zinc fertilization under no-tillage can improve soil health and productivity of cereal crops and also retain an effective residual Zn content for the successive crops (NADEEM *et al.*, 2020). Soil Zn application at a dose of 6 kg ha<sup>-1</sup> under direct planted system can be effective for weed management and increasing grain and biomass yield of rice (KUMAR *et al.*, 2022). Zinc fertilization to common bean in no-tillage plantation increased nutrients exportation especially Zn nutrition in tropical savannah of Brazil (KACHINSKI *et al.*, 2020). No-till system sustain more Zn content in the upper rhizosphere as compared to conventional tillage system due to crop residues decomposition that could increase water retention, regulate temperature and soil pH, thus improving Zn phyto-availability (DASAPPAGOL *et al.*, 2017; NARESH *et al.*, 2017).

### **2.4.2 Irrigation**

Irrigation and fertilizer application to crops are strongly correlated, as water supply dissolve applied fertilizers to increase their availability to the crops for adequate growth and development. Irrigation water plays a critical role in the availability, transportation and absorption of nutrient applied to crop plant, which is impaired under insufficient irrigation water supply (ANDERSON *et al.*, 2012). Zinc starvation can occur due to several climatic factors, such as imbalance distribution and amount of precipitation and temperature are among them. Zinc deficiency and irrigation are considered the most limiting agronomic factors that affect plant growth and yield of rice (ALI *et al.*, 2016). Thus, combination of proper irrigation and nutrient management can be viable alternative to crop production system of tropical and sub-tropical regions (CAKMAK *et al.*, 2008).

Water irrigation is required almost at all critical growth stages of crop for better performance and greater productivity. Irrigation at branching + pod development and at branching + pre-anthesis + pod development stages in combination with fertilization of zinc sulphate increased chlorophyll content, yield and grain Zn uptake of chickpea (SAREN *et al.*, 2018). Higher

Zn fertilizers doses under heavy irrigation increase grain P/Zn and Fe/Zn ratios, Ca/Mg ratio of grain and shoot, and grain and shoot yield of crop (JAKHAR; YADAV, 2016). Also, Peykarestan *et al.* (2018) conducted an experiment with Zn fertilizer and irrigation methods in maize hybrid cultivation and reported that Zn fertilization from zinc sulphate with alternating furrows irrigation reduced water consumption by the crop and increase grain Zn concentration. In addition, irrigation with low water supply helps the farmers in proper crop cultivation as well as increasing water and nutrient use efficiency (AHMADI *et al.*, 2013).

### **2.4.3 Crop rotation**

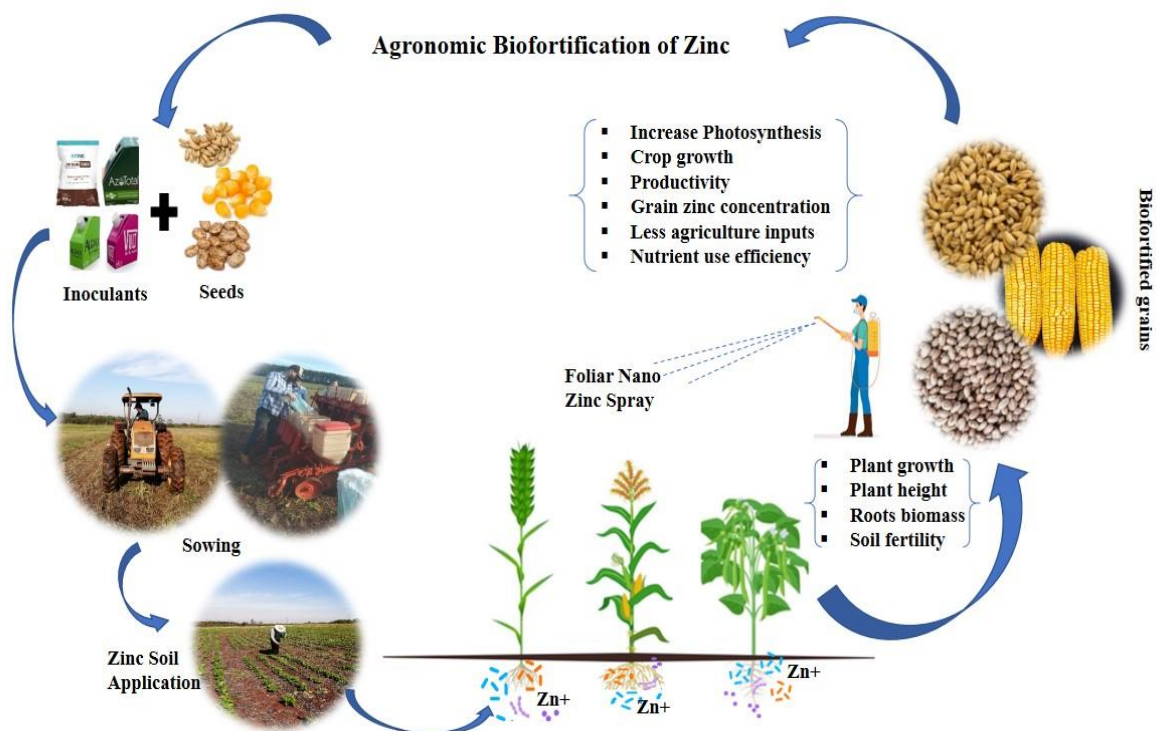
Crop rotation is a key strategy to improve performance, yield and quality of successive crop. Cereal-cereal and cereal-legumes are the most common crop rotation system, where individual crop specie can utilize different or same resources in more effective manner to increase yield per unit area (ULLAH *et al.*, 2020). Nutrient management is very important for higher productivity, long-term sustainability and environmental safety. Soil-plant nutrient cycling and nutrient management in tropical regions are influenced by soil fertility, fertilizers application, quantity and quality of crop residues and crop productivity (CHAUHAN *et al.*, 2012; HAZRA *et al.*, 2014). Crop rotation with legumes in cereal-cereal rotation can efficiently upscale soil quality, improve nutrients available and residual status especially nitrogen, phosphorous, zinc and iron (XUE *et al.*, 2016; ULLAH *et al.*, 2020). Crop rotation is an integral strategy of sustainable agriculture and regulate nitrogen status in plant tissue for higher productivity and nutrient re-localization and transportation. Higher nitrogen concentration is linked to phytosiderophores production and Zn uptake and passage by roots for higher deposition in grain tissues (CAMAK *et al.*, 2010c; ACIKSOZ *et al.*, 2011).

### **2.5 Biofortification**

Malnutrition or micronutrients deficiency is the most threatening issue of the world, occur due to inadequate/ insufficient intake of nutritious food, known as “Hidden Hunger” (NISSAR *et al.*, 2019), affecting half of the global population. Biofortification is considered one of the sustainable and cost-effective strategy for the production of nutrient-enriched crops that sustain in human bodies for long-term and eliminate malnutrition. Biofortification is considered a key technique to generate and release micronutrients and vitamins enriched high quality food to deal

with nutritious security. It's a technique to make nutrient-rich cereal crops by improving their biological functions to supply quality and nutritional food to general public (YOUNAS *et al.*, 2022). Biofortification is a long run approach with a one-time investment, precise and cost effective for correcting nutrients deficiency in staple crops. Biofortification is considered an ideal intervention that benefit the poor and rural community with high nutrient dense edible grains through agronomic and genetic approaches for sustainable and durable profits (OBAID *et al.*, 2022). Agronomic biofortification is mediated with the use of mineral fertilizers, applied via soil and foliar or with inoculation of beneficial microbes to improve Zn availability for plant uptake to increase crop productivity and nutritional status of plant with targeted nutrients for better human nutrition (Figure 2.4).

**Figure 2.4.** Schematic presentation of agronomic biofortification and its outcomes in the current field experiments of wheat, maize and common beans.



Sources: Author.

### **2.5.1 Agronomic Biofortification**

Agronomic biofortification is a successful, straightforward, quick and least labor-intensive technique to provide general public with nutrient-rich food to overcome micronutrients and vitamins deficiencies (PRASAD; SHIVAY, 2020). Agronomic biofortification is increasing nutrients accumulation in edible plant tissue through fertilizers or triggering factors. The lack of micronutrients in human can cause several health problems. Iron and Zn deficiency is one the global challenging problems that directly impacts more than two billion people around the world especially developing countries (JALAL *et al.*, 2020). Agronomic biofortification deal with malnutrition through fertilization via soil, foliar, priming and seed coating, as well as microbial inoculation, which enables the plants to uptake available micronutrients directly and increasing growth and productivity. Zinc is a common micronutrient deficiency, affecting one third of global population, which cause several health problems such poor growth and immunity system, affecting sexual organs, increasing infection susceptibility, damaging DNA and nervous system therefore, agronomic biofortification of Zn in crops can deal as multi-purpose approach to several human diseases (CHOI *et al.*, 2018; PRASAD, 2020).

### **2.5.2 Mineral biofortification**

Mineral fertilizers are composed of essential minerals that can improve soil characterizations and micronutrients concentration in plant for quality production. The availability of minerals nutrients in the soil can be improve with application of highly soluble and mobile mineral fertilizers. Mineral biofortification provide a variety of fortified foods including cereals, legumes and vegetables, commonly ingested foods (JALAL *et al.*, 2022; PRASAD; SHIVAY, 2020). Micronutrient supplementation to staple crops is adopted as a promising strategy to improve micronutrients content in edible organs of plants all over the world, especially in developing countries of Asia and Africa (KIRAN *et al.*, 2022). Cereals crops including rice, wheat and corn are the most consumed grains in the world and being adopted an excellent tool of agronomic biofortification to counter malnutrition (CAKMAK *et al.*, 2020; SIGNORELL *et al.*, 2019).

Zinc deficiency impairs growth and development, biochemical and metabolic activities of plants, which cause nutritious and food security (ULLAH *et al.*, 2020). Zinc deficiency in plants

normally appears after 2-3 weeks in young and meristems tissues under low-Zn growth conditions. Soil Zn deficient conditions cause pollen sterility and severely affect shoot-root growth that led to low grain yield (CAKMAK *et al.*, 2020; ULLAH *et al.*, 2020). Zinc fertilization via soil and leaf are key techniques to alleviate Zn deficiency in plants. Zinc fertilization via soil and foliar improves performance, grain Zn concentration and yield of crop plants (GONÇALVES *et al.*, 2019; KANDALI *et al.*, 2021). Soil Zn fertilization allows the plants to uptake, translocate and redistribute Zn in plant stem and grains tissues (BHARTI *et al.*, 2013). Zinc is the most applied from mineral fertilizers such as zinc sulfate, zinc oxide, and chelates that significantly enhance plant growth, yield and Zn nutrition (LIU *et al.*, 2020; PHILIPO *et al.*, 2021). In addition, high Zn content in straw may favor subsequent crops with its residual effect and organic matter decomposition (DAI *et al.*, 2020). Foliar Zn fertilization counters Zn scarcity in standing crops, Zn spray at tasseling and milking growth stages improve grain development and grain Zn concentration (JALAL *et al.*, 2022).

Zinc biofortification of cereals reported some excellent results by improving plants vigor and nutritional status under limited water and nutrients availability, especially Zn in soil (CANDAN *et al.*, 2018). Biofortification of legumes is an excellent and interesting strategy to improve human nutrition especially in African countries, where legumes are consumed as staple food (DHALIWAL *et al.*, 2022). Maize Zn biofortification is used as a dual-purpose crop, as Zn enriched grains and stem could directly improve human body Zn bioavailability while feeding this Zn enriched maize to animals can indirectly improve Zn bioavailability in human through meat consumption (GARCÍA-LARA; SERNA-SILDIVAR, 2019; KUMAR; RAM, 2021). In addition, wheat is one of the staple food of the most of the global population, which can better addressed malnutrition by Zn biofortification with aim to provide fortified food (YASSEN; HUSSAIN, 2021).

### **2.5.3 Microbes-mediated biofortification**

Microbial application to enhance Zn availability in soil and plant is comparatively less explored. Biofortification through microbes is an environmentally safe and cost-effective strategy for increasing nutrients bioavailability in dietary food crops by reducing phytic acids in grains (SINGH *et al.*, 2020). Microbes adopt several mechanisms including acidification, oxidation and reduction, solubilization, chelation as well as modify root architecture and physiology to facilitate

nutrients availability and biofortification. Soil microbial activities are responsible for increasing Zn nutritional status by solubilization and re-localization into plants, thus linking microbes with higher grain Zn biofortification of cereal crops (MUMTAZ *et al.*, 2017; ADELE *et al.*, 2021; GALINDO *et al.*, 2021). Use of microbes is a possible alternate strategy to convert soil fixed Zn into available Zn crop plants to eliminate Zn malnutrition. Several plant growth-promoting bacteria genera of *Azotobacter*, *Azospirillum*, *Bacillus*, *Gluconacetobacter*, *Acinetobacter* and *Pseudomonas* and plant growth-promoting fungi groups of *Arbuscular mycorrhizae*, *Trichoderma* and *Piriformospora indica* have been reported to increase micronutrient solubilization in root rhizosphere of plants (MUMTAZ *et al.*, 2017; DINESH *et al.*, 2018; COCCINA *et al.*, 2019; ADELE *et al.*, 2021). Since, the focus of the study is role of plant growth-promoting bacteria (PGPBs) on Zn biofortification in wheat, maize and common beans.

The presence of PGPBs in the soil-root ecosystem can reduce toxic effect of Zn by increasing bioaccumulation in plant tissues through roots, thus improving plant health (ADELE *et al.*, 2018). These interaction of PGPBs in soil-root ecosystem adopt several mechanisms such as phytohormones synthesis, secretion of Zn chelated compounds, prevention of excessive ethylene secretion, nitrogen fixation, and biogeochemical cycle to promote nutrient use efficiency through solubilization, bioavailability, remobilization and translocation of Zn (GONTIA-MISHRA *et al.* 2017; ZEB *et al.* 2018; KAUR *et al.*, 2020). Plant growth-promoting bacteria produce phytohormones like IAA and cytokinin, which influence root biomass and architecture to promote nutrient use efficiency (KAUR *et al.*, 2020). These PGPBs stimulate multiple direct and indirect mechanisms in soil rhizosphere to improve plant health, nutrients cycling and homeostasis. These direct mechanisms include biological nitrogen fixation, nutrients solubilization and enzymes synthesis whereas the indirect mechanism include resistance to pathogens infestation by producing siderophores and antibiotics (LAMBRESE *et al.*, 2018; YAHAGHI *et al.*, 2019). Thus, using all the mechanisms made these PGPBs suitable alternative tool for enhancing micronutrient nutrient acquisition from soil rhizosphere and crop biofortification of targeted nutrient.

## **2.6 Zins interaction with root mechanisms of plants growth-promoting bacteria**

Rhizosphere is an active and confined surrounding of soil-root interaction that is characterized by microbial diversity and root exudates to facilitate different metabolic processes of plants and microbes (HELLIWELL *et al.*, 2017). The microbial population of rhizosphere

benefit the plants through a series of mechanisms including nitrogen fixation, nutrients mobilization, altering and producing phytohormones, increasing stress tolerance, bioremediation, and also acts as a bio-control agent (SINGH *et al.*, 2022). Rhizosphere is colonized by plants growth-promoting bacteria (PGPBs) where they share metabolites and extracellular matrix to establish biofilm on root surface and increase diversity of native microbiota for improving plant health (SUN *et al.*, 2021). These rhizospheres microbial population are used to recover soil damage through a direct association with root exudates and plant root system, contributing to the alleviation of negative impacts of nutrient-deficient soils on plant growth (YANG *et al.*, 2009; HACQUARD *et al.*, 2015).

Zinc, due to poor native availability and root depletion zone is not mobile in the soil and plants mostly uptake it by diffusion (HAVLIN *et al.*, 2005). Thus, Zn proximity to the roots can either be achieved by exogenous Zn fertilization or by increasing root growth and surface area. Inoculation of PGPBs (*Arthrobacter* sp. and *Bacillus* sp.) increase thickness of root cortex, volume and diameter of vascular bundles and peri-cycle that improve Zn uptake and biofortification of wheat grains (SINGH *et al.*, 2018a). Inoculation with bacterial strains of *Pseudomonas* and *Enterobacter* sp. were observed in the wheat rhizosphere with the tendency of Zn mobilization and producing exo-polysaccharides, siderophores, 1-aminocyclopropane-1-carboxylic acid, antimycotic activities and phosphorus solubilization, thus improving biofortification of wheat grains (ULLAH; YUSUF, 2019).

### **2.6.1 Zinc and inoculation with PGPBs**

Plant growth-promoting bacteria can be used as a prospective alternative to replace less-effective synthetic fertilizers for eliminating Zn malnutrition in a sustainable and environmentally safe manner. Plant growth-promoting bacterial strains like *Bacillus* sp. and *Pseudomonas* sp., generally known as Zn solubilizers, which can solubilize unavailable Zn by the production of chelators, secretion of organic acids, amino acids, vitamins and phytohormones and oxidation-reduction systems and proton extrusion (MUMTAZ *et al.*, 2017; BASHIR *et al.*, 2021; KARNWAL, 2021). The production of organic acid by microbial strains is one of the main mechanisms of Zn solubilization. Among these organic acids, production of 2-ketogluconic and gluconic acid by PGPBs is responsible for Zn solubilization (COSTEROUSSE *et al.*, 2017; YASMIN *et al.*, 2021; REHMAN *et al.*, 2022). Several genera of PGPBs including *Pseudomonas*,

*Rhizobium*, and *Azospirillum* (NAZ *et al.*, 2016; SINGH *et al.*, 2022), *Bacillus* sp. (MUMTAZ *et al.*, 2017), *Burkholderia cenocepacia* (Pawar *et al.*, 2015), *Arthrobacter* sp. (SINGH *et al.*, 2017) are being recognized and characterized for modulating growth, yield and zinc biofortification of cereal and legume crops.

Zinc solubilizing bacteria improved growth and quality of wheat through siderophore and exopolysaccharide production, Zn solubilization and transportation from soil to above-ground plant. Among these bacteria genera, inoculation with *Rhizobium* sp. increased shoot length, root-shoot biomass while inoculation with *Pseudomonas* sp. promote root biomass and grain Zn bioavailability in wheat crop (KAMRAN *et al.*, 2017). Inoculation of Zn deficient and efficient wheat cultivars with *Bacillus subtilis* and *Arthrobacter* sp. were observed with significant influence on root morphology that led to higher Zn uptake in grains and different Zn use efficiencies (SINGH *et al.*, 2017). Inoculation of wheat seeds with *Pseudomonas protegens* increased plant growth-promoting mechanisms such as synthesis of siderophore, indole acetic acid, exopolysaccharide, ACC deaminase, and ammonia that all contribute to amelioration of Zn in soil, plant and grains to ensure sustainable production (SINGH *et al.*, 2022). Inoculation with *Pseudomonas* sp. and Zn fertilization reduced phytic acid concentration and improving Zn concentration in embryo, aleurone and endosperm, better contributing to agronomic biofortification (REHMAN *et al.*, 2017). Seeds inoculation of wheat with *Bacillus subtilis* under Vertisol and Alfisol type of soils improved rhizosphere microbiota, phosphorous delivery to plants and Zn bioavailability in wheat grains, promoting biofortification (MORENO-LORA *et al.*, 2019). Inoculation with *Azospirillum brasilense* in combination with Zn fertilizer has reported for enhancing grain yield and Zn use efficiency of wheat-maize crops under tropical conditions (GALINDO *et al.*, 2021).

### **2.6.2 Zinc and co-inoculation with PGPBs**

The global inoculants market has been looking for new strains with new formulations and validation of application methods. In last decade, the idea of combined application of different species of microorganisms contributed to different plant processes and known as mixed inoculation or co-inoculation. Currently, a variety of co-inoculants consisting of symbiotic rhizobia together with PGPBs are present in market for many crops (SANTOS *et al.*, 2019). The inoculants of co-inoculation have synergetic interaction with one another that could effectively

support plant growth, yield and nutrient use efficiency by increasing root morphological traits and phytohormones production (RIBEIRO *et al.*, 2022). Co-inoculation with *Azotobacter* sp. + *Azospirillum* sp. and *Bacillus* sp. + *Pseudomonas* sp. favored the growth, the increase in the number of branches, the productivity of seeds, leaves and essential oil in the two cuts of the basil plants, contributing to the reduction in the use of mineral fertilizers (TAHAMI *et al.*, 2017). Co-inoculation of *Andrographis paniculata* with *Azotobacter chroococcum*, *Bacillus megaterium*, *Pseudomonas monteilii* and *Glomus intraradices* improved soil chemical properties, phosphatase, plant growth, yield and grain quality (KHAN *et al.*, 2015).

The co-formulation or consortia inoculation of beneficial microorganisms has proved the most practical and greener technique that alleviate effects of climate changes and indiscriminate use of fertilizers in agricultural soils (BACKER *et al.*, 2018). Co-inoculation with strains of *Azospirillum* sp. and *Bacillus* sp. are being observed for synergistic impact on wheat growth promotion and reduced disease index (BAGHERI *et al.*, 2019). Co-inoculation of several PGPBs improved root activities by stimulating different physiological mechanisms including root hair formation and elongation, and meristems cell multiplication that led to better utilization of soil and increasing tolerance to biotic and abiotic stresses (BAGHERI *et al.*, 2019; MORETTI *et al.*, 2020).

Co-inoculation is an effective strategy dealing with multifaceted plant growth mechanisms in cost-effective, environmentally friendly and sustainable manner. Several combinations are so far reported with enhancing growth-promoting and quality traits of different crops. Co-inoculation of *Bacillus megaterium* with *Rhizobium* improved root-shoot biomass of common bean plants that lead to the alleviation of biotic and abiotic stresses, when compared with single inoculation (KORIR *et al.*, 2017). Co-inoculation of different *Bacillus* sp. strains enhanced Zn uptake in wheat-soybean cropping system that led to higher growth, yield and improved biofortification of wheat and soybean (RAMESH *et al.*, 2014). Co-inoculation of sorghum seeds with *Bacillus mojavensis* and *Bacillus cereus* increased root-shoot biomass,

Co-inoculation of *Rhizobium* sp. strains with *Pseudomonas* sp. strains increased nodulation, chlorophyll content, and nutrients uptake, which contribute to higher growth, and quality grains production of different legume crops (STAJKOVIC *et al.*, 2011; MISHRA *et al.*, 2012). Co- inoculation with *Rhizobium tropici* and *Azospirillum brasilense* along with nitrogen doses stimulate root nodulation that contribute to higher growth and productivity of common bean under topical conditions (STEINER *et al.*, 2019). In another detailed study under field conditions,

JALAL *et al.* (2021) reported that co-inoculation of *Rhizobium tropici* with *Azospirillum brasilense*, *Bacillus subtilis* and *Pseudomonas fluorescens* and soil Zn fertilization are being observed the most effective combinations for improving growth, yield and nutrient use efficiency that elevated Zn uptake in shoot and grains, thus lead to biofortification and higher estimated Zn intake to benefit human, especially Brazilian population.

## 2.7 Plant growth-promoting bacteria and sustainable agriculture

The devastating climate changes and increasing human population is a challenging threat to food security, which increase the demand of sustainable agriculture. In addition, United Nation also developed sustainable goals to attain food and nutritional security under sustainable agricultural practices and eliminate hunger and malnutrition (UNITED NATION, 2015). Induction of microbes alone or in combination with synthetic fertilizers is considered a cost-effective alternative that can sustainably enhance soil quality, productivity and regulating natural vegetation (PRATHAP *et al.*, 2022). This can only be achieved by developing interaction of plant growth-promoting bacteria (PGPBs) and plant rhizosphere that assist nutrients acquisition and cycling to improve plant health and prevent biotic and abiotic stresses (PATHANIA *et al.*, 2020). Multiple genera of PGPBs such as *Azotobacter*, *Azospirillum*, *Acinetobacter*, *Agrobacterium*, *Arthrobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Serratia*, *Streptomyces*, *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Frankia*, and *Thiobacillus* are contributing to sustainable agriculture production through stimulation of different enzymatic, phyto-hormonal and nutrients solubilization as well as inhibiting phyto-pathogen infestation (WANI; GOPALAKRISHNAN, 2019).

The success of PGPBs on growth promotion under different environmental conditions depend on the progressive interaction of plants and microbes. There exists a complex plant–microbe interactions below-ground regions like microbial biodiversity, growth-promoting attributes, mechanisms of action, interaction with already existing population, all these are key function to understand for appropriate plant growth and health while maintaining sustainable agriculture system. There exists a great variation in plant response to PGPBs under controlled and field conditions. Therefore, the consortium of multi-strains inoculation in the field condition can be more effective to compete with indigenous diversity and population to support partner microbes and promote plant health (AHMAD *et al.*, 2008). The combination of these PGPBs develop a synergetic interaction within rhizosphere to increase nutrients acquisition, stimulate physio-

biochemical activities of the host plants. The consortia or inoculation of *Azospirillum*, *Azotobacter*, *Pseudomonas*, and *Bacillus* sp. inoculants promote plant growth, root-shoot biomass, biochemical and physiological aspects, enzymatic activities, which are effectively alleviating stressful conditions and achieve food security and maintain sustainable agriculture practices (VISHWAKARMA *et al.*, 2020).

In recent decades, several strains of PGPBs are being introduced against the stressed environments to enhance fertilizers use efficiency and promote growth and yield of different crops around the world. Inoculation of crops with PGPBs is the most promising technology and ready to replace synthetic fertilizers and pesticides through symbiotic association, improved nutrition and resistance against biotic and abiotic stresses (AHIRWAR *et al.*, 2020). In addition, several Zn solubilizing bacteria are being reported a sustainable alternative by producing Zn, phosphorous and potassium solubilization, nitrogen fixation, and production of phytohormones (kinetin, indole-3-acetic acid, and gibberellic acid), as well as synthesis of siderophores, hydrogen cyanide, and ammonia. All these factors together discourage synthetic fertilizers application by promoting Zn solubilization, growth, productivity and soil fertility status (KUMAR *et al.*, 2019). Thus, inoculation with PGPBs could be one of the best transmission tools to optimize harnesses of climate changes to better understand the establishment of sustainable agriculture.

## CHAPTER-3

### 3. COMMON BEAN YIELD AND ZINC USE EFFICIENCY IN ASSOCIATION WITH DIAZOTROPHIC BACTERIA CO-INOCULATIONS

#### 3.1 Introduction

Common bean (*Phaseolus vulgaris* L.) originated in central Mexico and is now grown globally, especially in Central and South America (LONG *et al.*, 2022). Beans have imperative traditional, historical, and nutritional profiles (proteins, amino acid, and minerals, as well as antioxidants and polyphenols) that fulfill the nutritional needs of human diet in many regions of the world as a staple food source (HAYAT *et al.*, 2014; RIVERA *et al.*, 2018). The global annual average bean production is about 26.5 million tons (FAO, 2018). The mean production of beans in Brazil has increased by 3.15 million tons in the preceding 20 years, being adjusted to Brazil demand (CONAB, 2018). Legumes improve soil fertility by fixing nitrogen (N), enhancing microbial activity, and decreasing dependency on mineral fertilizers, which helps to promote a sustainable environment and production (SORATTO *et al.*, 2013).

Zinc (Zn) is one of the most important micronutrients for all living organisms (ESTRADA-DOMÍNGUEZ *et al.*, 2020), including plants, humans, and micro-flora (COSTA *et al.*, 2019) and is required throughout their life cycles in small quantities to orchestrate a complete array of physiological functions (YASEEN *et al.*, 2021). Zinc deficiency has frequently been reported in tropical crops (MELO *et al.*, 2018), concurrently being declared as “hunger of the day”, with several health issues (BHATT *et al.*, 2020), especially in developing countries (PONIEDZIAŁEK *et al.*, 2020). Zinc deficiency affects almost 17.3% of the global population and 30% of South Asian countries (MAXFIELD; CRANE 2019), and therefore affects 2 billion people within the global population, and it is ranked as the fifth highest health risk factor in developing countries (WHO, 2007; BOLLINEDI *et al.*, 2020). The inadequate dietary status of Zn is anti-proportional to human health and leads to several diseases (GUPTA *et al.*, 2020), including immune deficiency syndrome, pneumonia, memory disorder, cancer, respiratory and cardiovascular disorder, and diarrhea in humans (GAMMOH; RINK, 2017; Uwitonze *et al.*, 2020). In the current scenario, it is interesting that Zn deficiency may be one of the predisposing factors for the infection and progression of COVID-19 (SKALNY, 2020).

Zinc deficiency in crop plants is an alarming concern and is the most effective micronutrient limiter in legume yields. Zinc plays a critical role in several plant physiological processes, including protein synthesis (KRYVORUCHKO, 2017), energy production, maintenance of membrane integrity, and cell growth and multiplication (DOOLETTE *et al.*, 2019). Zinc also has a role in different photosynthetic and enzymatic activities such as peptidases, dehydrogenases, phosphohydrolases, and pollen fertility (REHMAN *et al.*, 2019; ULLAH *et al.*, 2020). It is therefore important to combat Zn deficiency in soil, plants, and most importantly, in human beings (FAROOQ *et al.*, 2018; MONTANHA 2020). Soil Zn application is the most practiced strategy in field crops to improve yield and grain Zn concentration (HAIDER *et al.*, 2018). The deficiency of Zn cannot be treated solely with Zn application for better crop establishment, productivity, and Zn use efficiency, especially in tropical regions, due to complexation with carbonates and oxides (ZHANG *et al.*, 2017). Therefore, new alternatives and sustainable strategies need to be adapted for better nutrient replenishment and productivity with higher Zn use efficiency (ZnUE).

Nutrients enriched plant rhizosphere environments significantly stimulate several ecological processes such as decomposition of organic matter, homeostasis, and nutrient cycling to reduce crop dependency on synthetic fertilizers to support sustainable and stable ecosystems (HAKIM *et al.*, 2021). Plant growth-promoting bacteria (PGPBs) or diazotrophic bacteria adapt several direct and indirect mechanisms to improve plant growth (KHOSHRU *et al.*, 2020). They improve nutrient availability by playing a role in biological nitrogen fixation (BNF), nutrient solubilization, and enzymes synthesis through direct mechanisms (KHOSHRU *et al.*, 2020; SEDLAKOVA-KADUKOVA *et al.*, 2019) while inhibiting pathogen infestation by producing siderophores and antibiotics (YAHAGHI *et al.*, 2019; LAMBRESE *et al.*, 2018). Zinc solubilizing bacteria could be applied in one or more of the above-mentioned mechanisms to increase Zn solubility through production of organic and inorganic acids and several chelators (KHOSHRU *et al.*, 2020). A diverse range of bacteria including species of *Rhizobium*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Acinetobacter*, and many others may solubilize or tolerate Zn and plant growth promoters (ULLAH *et al.*, 2020; MUMTAZ *et al.*, 2017; KUSHWAHA *et al.*, 2021).

The effects Zn biofortification of the interaction of diazotrophic bacteria in co-inoculation with soil applied Zn is lacking in the literature. There is a research gap on the association of

diazotrophic bacterial co-inoculation and Zn fertilization on Zn nutrition, ZnUE, and yields of common bean. It is necessary to determine such strategies to improve the Zn nutritional quality of common bean to increase the dietary intake of the population. Therefore, the hypothesis of this study was that there may be a synergetic association of different diazotrophic bacteria with soil Zn application on Zn availability on plant and soil, ZnUE, yield, and intake of fortified common bean in the tropical savannah of Brazil. The objectives of the study were to evaluate the effect of soil Zn application in combination with seed inoculation of different diazotrophic bacteria on common bean growth and yield. Additionally, the effect of co-inoculations and Zn fertilization on Zn accumulation in plant shoot and grains, Zn intake, and Zn use efficiencies for sustainable biofortification in the tropical savannah of Brazil.

## **3.2 Materials and Methods**

### **3.2.1 Experimental area and location**

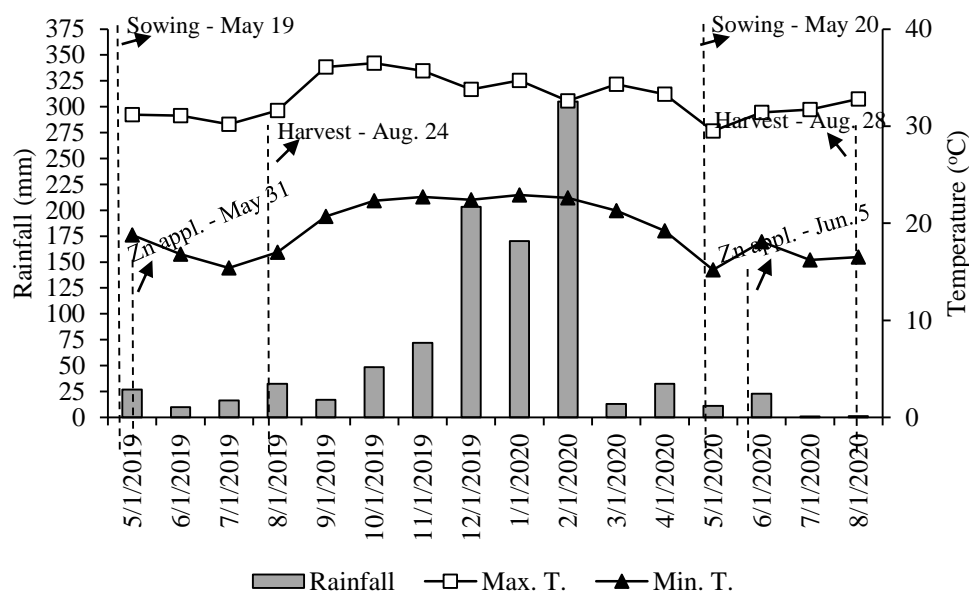
A field experiment on common bean was performed during two consecutive cropping years (2019 and 2020) at the research farm of the School of Engineering (UNESP) at the geographical coordinates 20°22' S, 51°22' W, and 335 m altitude (Figure 3.1), located in Mato Grosso do Sul, Brazil. The soil is classified as Rhodic Haplustox with clay texture (USDA, 2019). The experimental site has been used for the cultivation of an annual crop (cereals and legumes) for more than 28 years, the last 12 being under no tillage. The climate of the experimental area is classified as Aw according to Köppen classification, and the data of both cropping seasons are summarized in Figure 3.2.

**Figure 3.1** Location of the experimental area at the Research and Extension Farm, UNESP—Ilha Solteira Campus, at Selvíria—Mato Grosso do Sul state, Brazil (20°22' S, 51°22' W, altitude of 335 m) in 2019 and 2020 crop seasons.



**Source:** The map was created by using geographic information system (QGIS) software and the Google Earth program. The QGIS Development Team (2021). Open-Source Geospatial Foundation project. <http://qgis.osgeo.org>. Accessed on: 9<sup>th</sup> March, 2021. Projection System WGS 84/UTM 200DC [EPSG: 4326]. This image was taken from the Google Earth program, Google Company (2021). Map data: Google, Maxar Technologies.

**Figure 3.2.** Rainfall maximum, average, and minimum temperatures and air relative humidity were acquired from the weather station of the Education and Research Farm of the Faculty of Engineering—UNESP during the common bean cultivation period from May to August, 2019 and May to August, 2020.



### 3.2.2 Soil analysis

A composite soil sample was collected from the experimental site in the soil layer of 0–0.20 m depth for the pre-experimental soil physio-chemical analysis, following the standard procedures of Raij *et al.* (2001). Table 1 (Appendix A) indicates the physio-chemical attributes of the study area, which is clayey and slightly acidic with a minimal Zn concentration.

### 3.2.3 Experimental design and treatments

The experimental design for the common bean crop was randomized, with complete blocks having a  $7 \times 2$  factorial scheme with four replications. The experimental factors were comprised of bacterial seeds inoculations (1—No inoculation, 2—*Rhizobium tropici*, 3—*R. tropici* + *Azospirillum brasilense*, 4—*R. tropici* + *Bacillus subtilis*, 5—*R. tropici* + *Pseudomonas fluorescens*, 6—*R. tropici* + *A. brasilense* + *B. subtilis*, and 7—*R. tropici* + *A. brasilense* + *P. fluorescens*), without and with soil applied Zn (0 and 8 kg Zn ha<sup>-1</sup>).

The inoculation of common beans with *R. tropici* was carried out using a commercial peat inoculant, strain SEMIA 4080, with  $2 \times 10^9$  colony forming units (CFU)  $\text{g}^{-1}$  at a dose of 200 g for every 100 kg of seeds. To facilitate inoculant adhesion with the seeds, a 10% sugar solution was used to mix the seeds properly and homogeneously. The product is commercially registered with the Ministry of Agriculture and Livestock, Brazil. The inoculation of bacterium *A. brasilense* strains Ab-V5 and Ab-V6 (strains CNPSo 2083 = Ab-V5 and CNPSo 2084 = Ab-V6 with a guarantee of  $2 \times 10^8$  CFU  $\text{mL}^{-1}$ ) was performed at a dose of 300 mL of inoculant (liquid) per hectare of sown seeds. The inoculation of *B. subtilis* (strain CCTB04 with a guarantee of  $1 \times 10^8$  CFU  $\text{mL}^{-1}$ ) and *P. fluorescens* (strain CCTB03 with a guarantee of  $2 \times 10^8$  CFU  $\text{mL}^{-1}$ ) were applied at a dose of 150 mL  $\text{ha}^{-1}$ , according to the recommendation of the inoculant providing company @Total Biotechnology, Curitiba, Brazil. Inoculation was performed an hour before plantation of the crop.

Zinc was applied from a source of zinc sulfate (21% Zn and 11% of S) to the soil surface by side dressing cover. Zinc doses (0 and 8 kg  $\text{ha}^{-1}$ ) were manually applied with an even distribution and without incorporation on the soil surface. The Zn dose applied to the soil is based on (FAGERIA *et al.*, 2002; FAGERIA *et al.*, 2004), who recommended 5 to 30 kg  $\text{ha}^{-1}$  to the soil. The amount of Zn per treatment per plot was applied at the V1/V2 stage (1/2 trifoliolate leaves completely unfolded) in both 2019 and 2020 cropping seasons. The experimental area was irrigated with a central pivot irrigation system (14 mm) to evenly incorporate the Zn fertilizer into the soil.

### 3.2.4 Plant materials

The sowing bed was sprayed with systemic (selective) herbicide (2, 4-D—670 g  $\text{ha}^{-1}$  of the active ingredient (AI)) and a broad-spectrum herbicide (glyphosate, 1800 g  $\text{ha}^{-1}$  of AI) 15 days before the experiment implementation for controlling already emerged narrow and broad-leaved weeds. The common bean cultivar (IPR—Campos Gerais) with potential quality and production was sown in May, 2019, and repeated in May, 2020, on no-tillage beds using the drill sowing method. The seeds of beans were treated with piraclostrobin (2.5%  $\text{m v}^{-1}$ ), thiophanate methyl (22.5%  $\text{m v}^{-1}$ ), and fipronil (71.3%  $\text{m v}^{-1}$ ), based on the recommended dose of the cultivar. The plots were composed of six lines with a plot size of  $2.7 \times 4.5$  m, totalizing 12.15  $\text{m}^2$ . The recommended dose of NPK was applied at the time of sowing, with 20 kg N  $\text{ha}^{-1}$  from urea source,

80 kg P<sub>2</sub>O<sub>5</sub> ha<sup>-1</sup> from triple superphosphate source, and 40 kg K<sub>2</sub>O ha<sup>-1</sup> as potassium chloride. The recommended dose of 90 kg N ha<sup>-1</sup> was applied from ammonium sulphate fertilizer after 30 days of emergence. The experimental area was classified with inadequate B content as per the interpretation of Campinas Agronomic Institute (IAC) (RAIJ *et al.*, 2001). To cope with B deficiency, total experimental area was treated with foliar spray of 1 kg ha<sup>-1</sup> of B from the source of boric acid (18% of B) at flower stage through a tractor sprayer machine. Irrigation by sprinkling was done with a central-pivot irrigation system, according to the crop need.

### **3.2.5 Evaluations and analysis**

#### **3.2.5.1 Zinc soil and plant nutritional analysis**

The Zn soil analysis was performed three days after harvest in both the 2019 and 2020 study years. Five samples were collected with an auger (0.0–0.20 m) from each treatment of each replication and mixed together to obtain a uniform sample of each treatment. The homogeneous sample was collected in separate and already labeled bags. The collected samples were air dried, sieved with a sieving net of 2 mm, and stored at room temperature until the Zn analysis. The Zn analyses were performed as described by Raij *et al.* (2001).

The plant material (leaf, straw, and grain) was collected in proper labeled paper bags and dried in an air-tight oven at 60 ± 5 °C for 72 h until it attained a uniform humidity. The material was then ground in a stainless-steel Wiley knife mill by passing it through a 10-mesh sieve and was then placed in labeled plastic containers. Each sample was weighed (0.25 g), digested with nitroperchloric digestion (HNO<sub>3</sub>:HClO<sub>4</sub> solution), and quantified by atomic absorption spectrophotometry. The analysis was developed following the methodology of Malavolta *et al.* (1997).

#### **3.2.5.2 Shoot dry matter and yield**

Plant height at maturity was determined with a ruler from the ground to the upper apex. Shoot dry matter was determined after harvest of four useful central lines. Common bean were harvested and packed in jute bags and then dried in the shade for approximately 1 week. Each plot sample was threshed with an electric thresher to attain the weight of the processed grains for calculating yield ha<sup>-1</sup> (productivity at 13% moisture content). After drying, the beans were ground in a Wiley mill for analysis of nutrients.

### 3.2.5.3 Zinc plant accumulation

The Zn accumulation in shoot and grains ( $\text{g ha}^{-1}$ ) was calculated via the following formula:

$$\text{ZnSA} = \frac{\text{Zinc concentration in shoot} \times \text{dry matter}}{1000} \quad 1)$$

$$\text{ZnGA} = \frac{\text{Zinc concentration in grains} \times \text{grain yield}}{1000} \quad 2)$$

Where; ZnSA = Shoot Zn accumulation and ZnGA = Grain Zn accumulation.

### 3.2.5.4 Zinc partitioning and intake

Zinc partitioning index (ZPI) toward grains and intake were calculated following standard methodology of (LESSA *et al.*, 2019; FERREIRA, 2011):

$$\text{ZPI} = \frac{\text{Zinc concentration in grains}}{\text{Zinc concentration in shoot}} \times 100 \quad 3)$$

$$\text{Zn intake} = [\text{Zn}] \times \text{C} \quad 4)$$

where Zn intake ( $\text{g person}^{-1} \text{ day}^{-1}$ ) is the daily Zn intake of an estimated person<sup>-1</sup>, [Zn] ( $\text{g kg}^{-1}$ ) is the Zn concentration in biofortified grains from the current study results, and C ( $\text{kg person}^{-1} \text{ day}^{-1}$ ) is the mean consumption of common bean grains per person in Brazil.

### 3.2.5.5 Zinc use efficiency

The following Zn use efficiencies were calculated following measurements based on the standards of (FAGERIA *et al.*, 2011) via the formula:

$$\text{ZnUE} = \frac{\text{Grain yield ZnF} - \text{Grain yield ZnW}}{\text{Applied Zn dose}} \quad 5)$$

$$APE = \frac{\text{Grain yield ZnF} - \text{Grain yield ZnW}}{\text{ZnA in grain and shoot ZnF} - \text{ZnA in grain and shoot ZnW}} \quad 6)$$

$$RAZn (\%) = \frac{\text{ZnA in grain and shoot ZnF} - \text{ZnA in grain and shoot ZnW}}{\text{Applied Zn dose}} \quad 7)$$

$$UE = PE \times RAZn \quad 8)$$

Where ZnUE = Zinc use efficiency, APE = Agro-physiological efficiency, RAZn = Recovery of applied Zn, UE = Utilization efficiency, PE = physiological efficiency, ZnF = Zn fertilized treatments, ZnW = without Zn fertilized treatments, and ZnA = Zn accumulated.

### 3.2.6 Statistical analysis

All data were initially tested for normality using Shapiro and Wilk tests, which showed that the data were normally distributed ( $W \geq 0.90$ ). Levene's homoscedasticity tests ( $p \leq 0.05$ ) were performed to assess the equality of variances. Afterwards, data were subjected to an analysis of variance (F test). The Zn soil application and diazotrophic bacterial inoculations and their interactions were considered fixed effects in the model. When a main effect or interaction was observed to be significant by the F test ( $p \leq 0.05$ ), the Tukey test ( $p \leq 0.05$ ) was used for comparison of the means of Zn soil application, whereas the Scott Knott test ( $p \leq 0.05$ ) was used for comparison of diazotrophic bacterial inoculations using the ExpDes package in R software (R DEVELOPMENT CORE TEAM, 2015). The graphics are made in sigma-plot 12.5.

Pearson correlation analysis ( $p \leq 0.05$ ) was performed using R software (R Development Core Team). To create a heatmap, the corrplot package was used, using the "cor" and "cor.mtest" functions to calculate the coefficients and  $p$ -value matrices. Asterisks were added to the heatmap cells to identify significant correlations.

## 3.3 Results

### 3.3.1 Zinc nutrition in soil, plants and grains

The plant and grain Zn concentration of common bean were improved with the application of Zn to the soil in top dressing and co-inoculations of different diazotrophic bacteria (Table 3.1).

**Table 3.1.** Zinc concentration in soil, leaf, shoot, and grain of common bean under the influence of diazotrophic bacteria and soil applied zinc (Zn) doses.

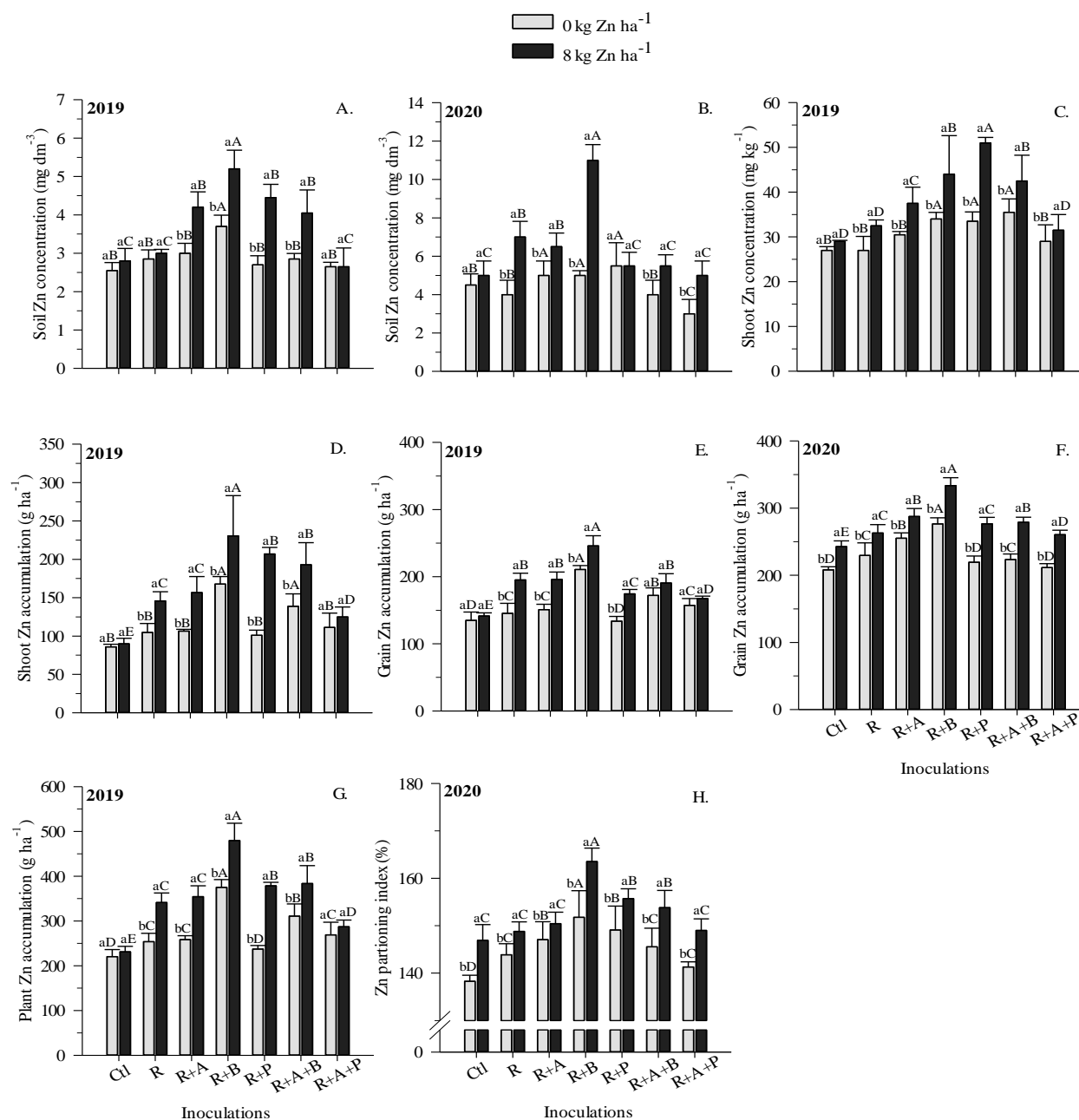
Treatments	Zn-soil		Zn-leaf		Zn-shoot		Zn-grains	
	concentration		concentration		concentration		concentration	
	mg dm <sup>-3</sup>				mg kg <sup>-1</sup>			
	2019	2020	2019	2020	2019	2020	2019	2020
<b>Zn application (kg ha<sup>-1</sup>)</b>								
0	2.8 b	4.6 b	35.9 b	42.7 b	30.7 b	41.1 b	47.4 b	54.0 b
8	3.7 a	6.5 a	38.2 a	46.4 a	38.6 a	46.6 a	48.6 a	56.5 a
<b>Inoculations (I)</b>								
Without	2.7 c	4.8 c	33.3 b	40.6 b	27.8 c	41.2 d	43.4 e	52.5 d
<i>R. tropici</i>	2.7 c	5.6 b	36.7 b	42.6 b	29.8 c	43.4 c	47.0 c	54.5 c
<i>R. tropici</i> + <i>A. brasilense</i>	3.5 b	5.7 b	40.4 a	56.7 a	33.7 b	45.5 b	49.3 b	57.0 b
<i>R. tropici</i> + <i>B. subtilis</i>	4.6 a	7.8 a	42.8 a	45.5 b	39.6 a	48.5 a	54.5 a	60.7 a
<i>R. tropici</i> + <i>P. fluorescens</i>	3.5 b	5.5 b	36.6 b	43.5 b	42.0 a	43.7 c	48.8 b	53.7 d
<i>R. tropici</i> + <i>A. brasilense</i> + <i>B. subtilis</i>	3.5 b	4.8 c	35.2 c	43.3 b	39.8 a	42.8 c	47.1 c	55.2 c
<i>R. tropici</i> + <i>A. brasilense</i> + <i>P. fluorescens</i>	2.7 c	4.2 c	34.5 b	39.8 b	30.0 c	41.6 d	45.8 d	53.1 d
<b>F-values</b>								
Zn	86.2 **	112 **	6.7 *	9.1 *	85.4 **	239 **	5.3 *	67.3 **
I	25.3 **	23 **	9.2 **	12.4 **	25.9 **	28.4 **	24.1 **	48.1 **
Zn x I	7.8 **	20 **	0.9 ns	0.4 ns	5.8 *	1.2 ns	1.8 ns	1.6 ns
CV (%)	10.7	12.3	8.6	10.2	9.2	3.0	4.35	2.09

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ ); \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; ns—non-significant, by F-test.

The soil Zn application and diazotrophic bacterial co-inoculation and their interactions significantly influenced soil Zn concentration after crop harvest for the study years of 2019 and 2020 (Table 3.1). The concentration of Zn in the soil after crop harvest was elevated with soil Zn application in combination with diazotrophic bacterial inoculations. Soil Zn application in side dressing had increased soil Zn concentration by 31.1 and 42.2% when compared with no Zn application in 2019 and 2020, respectively. Inoculation of seeds with *R. tropici* + *B. subtilis* prominently increased soil Zn concentration by 67 and 62% in both years, respectively, in

comparison to non-inoculated treatments. The interaction of soil applied Zn and bacterial inoculation were also significant (Figure 3.3A, B) in both 2019 and 2020. In addition, co-inoculation of *R. tropici* + *B. subtilis* in 2019 resulted in higher soil Zn concentration, irrespective of Zn fertilization, whereas in 2020, higher soil Zn concentration was observed for *R. tropici* + *P. fluorescens* in the treatments without Zn application (Figure 3.3A,B).

**Figure 3.3.** Influence of soil Zn and co-inoculations of different diazotrophic bacteria on soil Zn concentration, plant and grain Zn concentration and accumulation, and Zn partitioning index in common bean. (A and B) Soil Zn concentration ( $\text{mg dm}^{-3}$ ) in 2019 and 2020, respectively; (C) Shoot Zn concentration ( $\text{mg}$ ) in 2019; (D) Shoot Zn accumulation ( $\text{g kg}^{-1}$ ) in 2019; (E,F) Grain Zn accumulation ( $\text{g kg}^{-1}$ ) in 2019 and 2020, respectively; (G) Plant Zn (straw + grain) accumulation in 2019; and (H) Zn partitioning index (%) in 2020.



**Source:** Author. Ctl (No inoculation); R (*R. tropici*); R + A (*R. tropici* + *A. brasilense*); R + B (*R.*

*tropici* + *B. subtilis*); R + P (*R. tropici* + *P. fluorescens*); R + A + B (*R. tropici* + *A. brasilense* + *B. subtilis*); and R + A + P (*R. tropici* + *A. brasilense* + *P. fluorescens*). The uppercase letters are used for inoculation interactions within each level of soil Zn application, whereas lowercase letters are used for the unfolding of Zn levels within each inoculation treatment. The identical alphabetic letters do not differ from each other, as analyzed by Tukey (Zn application;  $p \leq 0.05$ ) and Scott–Knott (inoculations;  $p \leq 0.05$ ) tests for 2019 and 2020, respectively. Error bars indicate the standard error of the mean ( $n = 4$  replications). Selvíria, 2020.

The analysis indicated that Zn leaf concentrations were different in both of the study years. The leaf Zn concentration of common bean was significantly influenced by soil Zn application and diazotrophic bacteria co-inoculations, whereas their interactions were insignificant (Table 3.1). The leaf Zn concentration was higher by 8.6% with soil Zn application in 2020 and 6.2% in 2019, in comparison to plots without Zn being applied. The effect of diazotrophic bacteria co-inoculations was also significant and indicated that Zn leaf concentration was increased by 29 and 40%, respectively, with co-inoculation of *R. tropici* + *B. subtilis* in both studied years. The leaf Zn concentration was 38% higher in 2020 than in 2019 for the treatments of *R. tropici* + *B. subtilis*. In both years, the interaction of soil Zn application and bacterial co-inoculations was not significant for leaf Zn concentration.

Shoot Zn concentration was improved with soil applied Zn and co-inoculation of diazotrophic bacteria in both of the 2019 and 2020 cropping seasons (Table 3.1). Application of Zn in side dressing improved shoot Zn concentration by 26 and 13.3% in 2019 and 2020, respectively, when compared to the treatments without Zn application. The co-inoculation of *R. tropici* + *P. fluorescens* improved Zn shoot concentration by 50.6% in 2019, which is statistically similar to values obtained with co-inoculations of *R. tropici* + *A. brasilense* + *B. subtilis* and *R. tropici* + *B. subtilis*. The Zn shoot concentration in 2020 was improved by 17.6% with co-inoculation of *R. tropici* + *B. subtilis*. The interaction of soil applied Zn and bacterial co-inoculations were significant for Zn shoot concentration in 2019 (Figure 3.3C), whereas the interactions of 2020 were not significant (Table 3.1). However, triple co-inoculation of *R. tropici* + *A. brasilense* + *B. subtilis* was also observed to have higher shoot Zn concentration in the treatments without soil Zn application (Figure 3.3C).

Grain Zn concentration of common bean was significantly influenced by soil Zn fertilization and co-inoculations of different diazotrophic bacteria in both 2019 and 2020 (Table

3.1). The application of Zn in side dressing was noted to have higher grain Zn concentration (increased in by 2.7 and 4.7%, respectively) as compared to untreated plots. The co-inoculation of *R. tropici* + *B. subtilis* was observed to have higher Zn grain concentrations (25.7 and 15.7% for 2019 and 2020, respectively) in comparison to the control treatments. The interactions for grain Zn concentrations in both years were not significant (Table 3.1).

### **3.3.2 Zinc accumulation in plant and grains, and partitioning index (ZPI)**

Zinc accumulation in shoot, plant, and grains and the Zn partitioning index of common beans had a positive relation with soil Zn application and co-inoculation of different diazotrophic bacteria in both cropping seasons (Table 3.2).

The treatment with soil Zn application in side dressing significantly improved shoot Zn accumulation in 2019 and 2020 by 43.1 and 17.4%, respectively, as compared to plots without Zn being applied. The co-inoculation of *R. tropici* + *B. subtilis* increased shoot Zn accumulation by 127% in 2019, whereas in 2020, shoot Zn accumulation was improved by 18% with co-inoculation of *R. tropici* + *A. brasilense*, which is statistically similar to co-inoculation of *R. tropici* + *B. subtilis*. The interaction of Zn and the co-inoculation of bacteria in 2019 was significant (Figure 3.3D), although it was not significant in 2020 (Table 3.2). The co-inoculation of *R. tropici* + *B. subtilis* also lead to greater shoot Zn accumulation in the treatments without soil Zn application (Figure 3.3D).

**Table 3.2.** Zinc accumulation in shoot, grain, and plant (straw + grain), and Zn partitioning index of common bean, as influenced by diazotrophic bacteria and soil Zn application.

Treatments	Zn-shoot		Zn grain		Zn-plant		Zn partitioning	
	accumulation		accumulation		accumulation		index	
	----- g ha <sup>-1</sup> -----							
	2019	2020	2019	2020	2019	2020	2019	2020
<b>Zn application (kg ha<sup>-1</sup>)</b>								
0	116 b	161 b	159 b	233 b	276 b	394 b	122 b	145 b
8	166 a	189 a	187 a	277 a	340 a	466 a	133 a	152 a
<b>Inoculations (I)</b>								
Without	89 d	162 c	140 e	223 e	244 f	386 d	126 b	142 e
<i>R. tropici</i>	124 c	176 b	173 b	250 c	295 d	426 c	126 b	146 d
<i>R. tropici</i> + <i>A. brasilense</i>	133 c	191 a	170 b	271 b	300 d	461 b	123 b	149 c
<i>R. tropici</i> + <i>B. subtilis</i>	202 a	188 a	228 a	307 a	386 a	496 a	137 a	158 a
<i>R. tropici</i> + <i>P. fluorescens</i>	153 b	174 b	155 d	246 c	318 c	420 c	125 b	152 b
<i>R. tropici</i> + <i>A. brasilense</i> + <i>B. subtilis</i>	170 b	173 b	180 b	252 c	338 b	425 c	129 b	149 c
<i>R. tropici</i> + <i>A. brasilense</i> + <i>P. fluorescens</i>	119 c	163 c	162 c	234 d	277 e	397 d	129 b	144 d
<b>F-values</b>								
Zn	122 **	196 **	97.1 **	341**	208 **	507 **	54.3 **	138 **
I	38 **	17 **	55.6 **	75.9 **	60.5 **	79**	5.0 *	39 **
Zn x I	7.3 **	1.5 ns	6.5 *	3.7 *	13.6 **	1.56 ns	1.42 ns	2.7 *
CV (%)	12.01	4.24	6.16	3.50	5.35	2.78	4.38	1.57

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ ); \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; and ns—non-significant, by F-test.

Grain Zn accumulation was improved in 2019 and 2020 by 17.6 and 18.9%, respectively, with soil Zn application compared to without soil Zn application. The co-inoculation of *R. tropici* + *B. subtilis* increased grain Zn accumulation by 62.8% in 2019 and 37.7% in 2020, respectively, compared to un-inoculated treatments. The interactions of soil applied Zn and co-inoculation of bacteria were also significant for both the years (Figure 3.3E, F). The co-inoculation of *R. tropici* + *B. subtilis* with and without soil Zn application was noted for higher grain Zn accumulation,

whereas co-inoculation of *R. tropici* + *P. fluorescens* in the absence of Zn application resulted in lower grain Zn concentrations in both 2019 and 2020 (Figure 3.3E,F).

Plant (straw + grain) Zn accumulation was influenced by soil Zn application and co-inoculation of different diazotrophic bacteria in both of the 2019 and 2020 study years (Table 3.2). Higher plant Zn accumulations (23.2 and 18.3%) was noted in the plots treated with 8 kg Zn ha<sup>-1</sup> as compared to the treatments without Zn application in 2019 and 2020, respectively. The co-inoculation of *R. tropici* + *B. subtilis* also increased plant Zn accumulation in 2019 and 2020 (58.2 and 28.5%, respectively) in comparison to there being no inoculation treatments. The interactions of soil Zn application and bacterial co-inoculation were found significant for plant Zn accumulation in 2019 (Figure 3.3G), whereas it was not significant in 2020 (Table 3.2).

The Zinc partitioning index (ZPI) of the grain was significantly increased with soil Zn application and co-inoculation by different diazotrophic bacteria in 2019 and 2020 (Table 3.2). The interaction of soil Zn application and co-inoculation of bacteria was not significant in 2019, although, in 2020, it was significant for the ZPI (Figure 3.3H). The side dressing of Zn at a rate of 8 kg Zn ha<sup>-1</sup> significantly increased ZPI by 9 and 5% in 2019 and 2020, respectively, compared to there being no Zn applied treatments (Table 3.2). The co-inoculation of *R. tropici* + *B. subtilis* predominantly boosted ZPI by 8.7 and 17.5% in 2019 and 2020, respectively, when compared to non-inoculated plots. In addition, co-inoculation of *R. tropici* + *B. subtilis* was also observed to have higher ZPI, even in the absence of Zn application, whereas the lower ZPI was observed in the control treatments (Figure 3.3H).

### 3.3.3 Nitrogen and phosphorous concentration in shoot and grains

Nitrogen and phosphorous concentration in shoot and grains of common beans were positively influenced by soil Zn application and co-inoculations (Table 3.3). Shoot N concentration in 2019 crop season was not significantly affected by soil Zn application. In addition, co-inoculation with *R. tropici* + *P. fluorescens* increased shoot N concentration by 28.6 and 34% in 2019 and 2020 wheat cropping seasons.

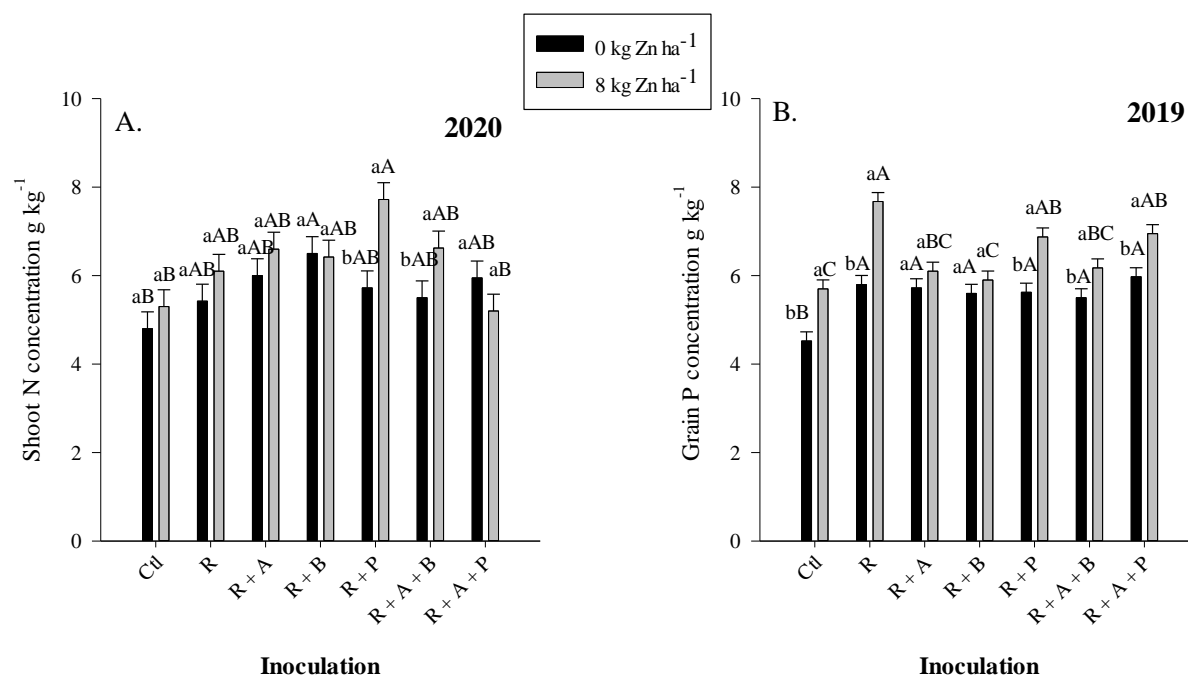
**Table 3.3.** Nitrogen and phosphorous concentration in shoot and grains of common bean, as influenced by diazotrophic bacteria and soil Zn application. Selvíria—MS, Brazil, 2019 and 2020.

Treatments	N-shoot concentration		P-shoot concentration		N- grain concentration		P-grain concentration	
	-----g kg <sup>-1</sup> -----							
	2019	2020	2019	2020	2019	2020	2019	2020
<b>Zinc application (kg ha<sup>-1</sup>)</b>								
0	7.4	5.7 b	0.61 b	1.1 b	28.6 b	29.4 b	5.5 b	4.5 b
8	7.9	6.3 a	0.83 a	1.4 a	30.4 a	31.3 a	6.5 a	5.0 a
<b>Inoculations (I)</b>								
Without (Control)	7.0 ab	5.0 b	0.52 b	1.0 b	26.9 b	27.1 b	5.1 d	4.3 c
<i>R. tropici</i>	7.4 ab	5.7 ab	0.72 ab	1.2 b	30.1 a	31.7 a	6.7 a	4.7 abc
<i>R. tropici</i> + <i>A. brasilense</i>	7.5 ab	6.3 a	0.64 ab	1.2 b	30.7 a	29.9 ab	5.9 bc	4.8 abc
<i>R. tropici</i> + <i>B. subtilis</i>	6.3 b	6.5 a	0.85 a	1.2 b	29.9 ab	31.1 a	5.7 c	4.6 ab
<i>R. tropici</i> + <i>P. fluorescens</i>	9.0 a	6.7 a	0.79 a	1.6 a	29.8 ab	30.7 a	6.2 abc	4.7 abc
<i>R. tropici</i> + <i>A. brasilense</i> + <i>B. subtilis</i>	7.9 ab	6.1 ab	0.77 a	1.3 ab	28.7 ab	30.6 a	5.8 bc	5.0 ab
<i>R. tropici</i> + <i>A. brasilense</i> + <i>P. fluorescens</i>	8.3 ab	5.5 ab	0.74 ab	1.4 ab	30.0 ab	31.2 a	6.5 ab	5.1 a
<b>F- values</b>								
Zn	1.4 ns	8.8**	29.5**	12.1**	10.5**	14.8**	74.8**	31.9**
I	2.6*	4.5**	3.8**	5.0**	3.1*	5.7**	13.5**	5.1**
Zn x I	1.7 ns	2.6*	0.6 ns	1.5 ns	0.8 ns	0.9 ns	3.6**	1.0 ns
<b>CV (%)</b>	20.2	12.7	21.5	18.5	6.9	5.9	6.8	6.5

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ ); \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; and ns—non-significant, by F-test.

The interactive effect of soil Zn application  $\times$  co-inoculation for shoot N concentration was significant in 2020 crop season (Figure 3.4). Co-inoculation with *R. tropici* + *P. fluorescens* in the presence of soil Zn increased shoot N concentration, which was statistically similar with co-inoculation of *R. tropici* + *B. subtilis* in the absence of Zn application. The lowest shoot N concentration was noted in the treatments without inoculation and co-inoculation with *R. tropici* + *A. brasilense* + *P. fluorescens* under no soil Zn application (Figure 3.4).

**Figure 3.4.** Influence of soil Zn and co-inoculations of different diazotrophic bacteria on shoot N concentration in 2020 (A) and grain P concentration in 2019 (B).



**Source:** Auhtor. Ctl (No inoculation); R (*R. tropici*); R + A (*R. tropici* + *A. brasilense*); R + B (*R. tropici* + *B. subtilis*); R + P (*R. tropici* + *P. fluorescens*); R + A + B (*R. tropici* + *A. brasilense* + *B. subtilis*); and R + A + P (*R. tropici* + *A. brasilense* + *P. fluorescens*). The uppercase letters are used for inoculation interactions within each level of soil Zn application, whereas lowercase letters are used for the unfolding of Zn levels within each inoculation treatment. The identical alphabetic letters do not differ from each other, as analyzed by Tukey (Zn application;  $p \leq 0.05$ ) and Scott–Knott (inoculations;  $p \leq 0.05$ ) tests for 2019 and 2020, respectively. Error bars indicate the standard error of the mean (n = 4 replications). Selvíria, 2020.

The interactions of soil Zn application  $\times$  co-inoculation were not significant for shoot P and grain N concentrations in both crop seasons (Table 3.3). Soil Zn application increased shoot P concentration by 36 and 27.3% in 2019 and 2020 respectively as compared to control. Co-inoculation with *R. tropici* + *B. subtilis* and *R. tropici* + *P. fluorescens* increased shoot P concentration of common bean by 63.5 and 60% respectively in 2019 and 2020 crop seasons. In addition, grain N concentration was increased by 6.3 and 6.5% with soil Zn application in first and second crop season. Co-inoculation with *R. tropici* + *A. brasilense* and *R. tropici* increased grain N concentration by 14.1 and 16.9% respectively as compared to without inoculations (Table 3.3).

Grain P concentration of common bean was increased with soil Zn application and co-inoculation with diazotrophic bacteria in both crop seasons (Table 3.3) while the interaction was only significant in 2019 crop season (Figure 3.4B). Inoculation with *R. tropici* under soil Zn application was observed with higher grain P concentration, which statistically at per with co-inoculation of *R. tropici* + *A. brasilense* + *P. fluorescens* and *R. tropici* + *P. fluorescens*. The treatments with co-inoculations in combination with soil Zn application were observed with higher grain P concentration. The lowest grain Concentration was noted in the treatments without inoculations (Figure 3.4B).

### 3.3.4 Plant height, dry matter, grain yield and zn intake

Plant height of common bean was one of the determining attributes that was significantly influenced by soil Zn application and co-inoculations of different bacteria in 2019, whereas in 2020, plant height was not significantly influenced (Table 3.4). The plots treated in 2019 and 2020 with Zn fertilizer were seen to have taller plants (7.36 and 5.43%, respectively) compared to those not fertilized with Zn. In 2019, the co-inoculation with *R. tropici* + *B. subtilis* led to taller plants (13%), which was significantly similar to co-inoculation with *R. tropici* + *A. brasilense* and *R. tropici* + *A. brasilense* + *B. subtilis* (12.6%). The plant height in 2020 was statistically not different; however, taller plants were observed in the control and the triple co-inoculation of *R. tropici* + *A. brasilense* + *P. fluorescens*. The interaction for plant height in 2019 was significant (Figure 3.5A), and that of 2020 was insignificant (Table 3.4). In addition, treatments in the absence of Zn fertilizer were observed, with taller plants under co-inoculation of *R. tropici* + *A. brasilense* and shorter plants with co-inoculation of *R. tropici* + *P. fluorescens* (Figure 3.5A).

**Table 3.4.** Plant height, shoot dry matter, grain yield, and Zn intake in common bean under the influence of diazotrophic bacteria and soil applied zinc doses. Brazil, 2019 and 2020.

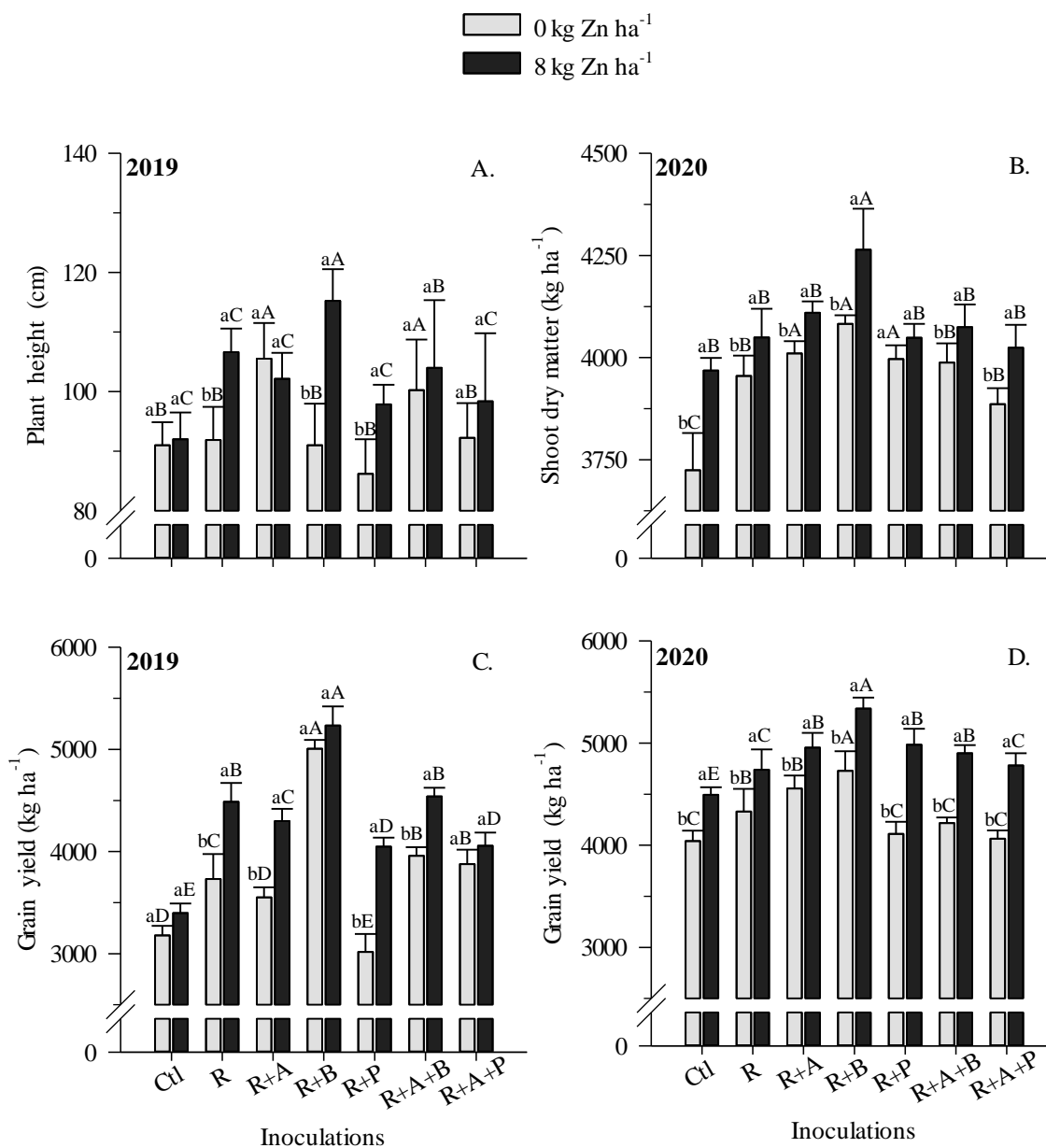
Treatments	Plant height		Shoot dry matter		Grain yield		Zn intake	
	cm		kg ha <sup>-1</sup>		g Person <sup>-1</sup> Day <sup>-1</sup>			
	2019	2020	2019	2020	2019	2020	2019	2020
Soil Zn application (kg ha <sup>-1</sup> )								
0	95 b	92 a	3934 b	3930 b	3758 b	4303 b	6.73 b	7.68 b
8	102 a	97 a	4040 a	4075 a	4269 a	4888 a	6.92 a	8.04 a
Inoculations (I)								
Without (Control)	92.1 b	99.8 a	3843 d	3818 d	3268 e	4252 e	6.16 e	7.46 d
<i>R. tropici</i>	98.6 b	94.1 a	3952 c	3989 c	4121 b	4590 c	6.68 c	7.75 c
<i>R. tropici</i> + <i>A. brasilense</i>	103.7 a	95.4 a	4060 b	4063 b	3899 c	4748 b	7.01 b	8.11 b
<i>R. tropici</i> + <i>B. subtilis</i>	104.1 a	93.0 a	4207 a	4164 a	5069 a	5052 a	7.75 a	8.64 a
<i>R. tropici</i> + <i>P. fluorescens</i>	92.2 b	90.1 a	3978 c	4018 b	3542 d	4564 c	6.95 b	7.64 d
<i>R. tropici</i> + <i>A. brasilense</i> + <i>B. subtilis</i>	103.7 a	94.1 a	3962 c	4012 b	4234 b	4558 c	6.70 c	7.85 c
<i>R. tropici</i> + <i>A. brasilense</i> + <i>P. fluorescens</i>	96.0 b	98.1 a	3905 d	3955 c	3962 c	4405 d	6.52 d	7.55 d
<b>F-values</b>								
Zn	24.1 **	3.4 ns	23.6 **	74.9 **	161 **	306 **	5.3 *	67.2 **
I	7.2 **	0.85 ns	16.4 **	22.7 **	115 **	33.1 **	22.1 *	48.2 **
Zn x I	4.8 *	0.84 ns	0.13 ns	2.68 *	10.2 **	5.1 **	1.8 ns	1.7 ns
CV (%)	5.71	10.44	2.06	1.56	3.76	2.72	4.35	2.09

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ );

\* and \*\*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; and ns—non-significant, by F-test.

Soil Zn application at sowing and co-inoculation of different diazotrophic bacteria significantly influenced the shoot dry matter of common bean in both of the 2019 and 2020 study years (Table 3.4). Shoot dry matter was increased by 2.7 and 3.6% in the presence of Zn fertilization in 2019 and 2020, respectively, compared to the treatments without Zn application. The co-inoculation of *R. tropici* + *B. subtilis* increased shoot dry matter by 9.5 and 9.1% in 2019 and 2020, respectively, compared to the control. The interaction for shoot dry matter in the first season was not significant (Table 3.4), whereas, in the second crop season, interaction was significant (Figure 3.5B).

**Figure 3.5.** The influence of soil Zn and co-inoculations on common bean growth attributes and grain yield: (A) Plant height (cm) in 2019; (B) Shoot dry matter ( $\text{kg ha}^{-1}$ ) in 2020; (C, D) Grain yield ( $\text{kg ha}^{-1}$ ) in 2019 and 2020, respectively.



**Source:** Author. Ctl (No inoculation); R (*R. tropici*); R + A (*R. tropici* + *A. brasilense*); R + B (*R. tropici* + *B. subtilis*); R + P (*R. tropici* + *P. fluorescens*); R + A + B (*R. tropici* + *A. brasilense* + *B. subtilis*); and R + A + P (*R. tropici* + *A. brasilense* + *P. fluorescens*). The uppercase letters are used for inoculation interactions within each level of soil Zn application, whereas lowercase letters are used for the unfolding of Zn levels within each inoculation treatment. The identical alphabetic

letters do not differ from each other, as analyzed by Tukey (Zn application;  $p \leq 0.05$ ) and Scott–Knott (inoculations;  $p \leq 0.05$ ) tests for 2019 and 2020, respectively. Error bars indicate the standard error of the mean ( $n = 4$  replications). Selvíria, 2019 and 2020.

Grain yield of common bean was significantly influenced by soil Zn application in combination with different diazotrophic bacteria in 2019 and 2020 (Table 3.4). The interactions of Zn and co-inoculations of bacteria were significant for both years (Figure 3.5C, D). Grain yield of common bean was increased by 13.6 and 13.5% in 2019 and 2020, respectively, under the treatments with soil applied Zn when compared to the treatments without Zn application. The co-inoculation of *R. tropici* + *B. subtilis* increased the grain yield of common bean by 54.3 and 18.9% in 2019 and 2020, respectively, compared to the control plots. In addition, co-inoculation of *R. tropici* + *B. subtilis* was observed for higher grain yield, irrespective of Zn application in both study years (Figure 3.5C, D). However, lower grain yield was observed with co-inoculation of *R. tropici* + *P. fluorescens* in 2019 (Figure 3.5C) and without inoculation in 2020 (Figure 3.5D).

The estimation of daily Zn intake in Brazil was increased with soil Zn application and co-inoculations of diazotrophic bacteria in 2019 and 2020 (Table 3.4). The daily consumption of beans in Brazil ( $\sim 142.2$  g person<sup>-1</sup> day<sup>-1</sup>) and the results of present grain Zn concentration and soil Zn application, along with different diazotrophic co-inoculations, are being calculated to attain Zn intake (g person<sup>-1</sup> day<sup>-1</sup>). The treatments with soil applied Zn were observed to have higher estimated Zn intake in 2019 and 2020 (2.83 and 4.69%, respectively). The co-inoculation of *R. tropici* + *B. subtilis* increased estimated Zn intake by 25.8% in 2019 and 15.8% in 2020, compared to the control. The interaction of soil Zn application and diazotrophic bacteria was not significant for Zn intake in both of the study years.

### 3.3.5 Zinc Efficiencies

Zinc use efficiency (ZnUE) was increased in the treatments of different diazotrophic bacteria in combination with soil Zn application (Table 3.5). The effect of bacterial co-inoculation was significant for ZnUE in both years. The co-inoculation of *R. tropici* + *B. subtilis* improved ZnUE in 2019 and 2020 (941.6 and 172%, respectively), compared to control treatments.

**Table 3.5.** Zinc efficiencies of common beans under the influence of diazotrophic bacteria and soil applied zinc doses. Selvíria—MS, Brazil, 2019 and 2020.

Treatments	ZnUE		APE		UE		AZnR	
	----- kg kg <sup>-1</sup> -----							
	2019	2020	2019	2020	2019	2020	2019	2020
<b>Inoculations (I)</b>								
Without (Control)	24 d	64 d	6.95 a	5.15 c	41 d	133 c	3 e	12 d
<i>R. tropici</i>	168 b	104 c	9.88 a	6.36 b	209 b	183 b	17 c	16 c
<i>R. tropici</i> + <i>A. brasilense</i>	140 b	123 b	7.68 a	5.90 b	228 b	208 b	18 c	21 b
<i>R. tropici</i> + <i>B. subtilis</i>	250 a	174 a	7.17 a	6.56 b	447 a	273 a	35 a	26 a
<i>R. tropici</i> + <i>P. fluorescens</i>	106 c	132 b	4.96 a	7.66 a	212 b	204 b	21 b	17 c
<i>R. tropici</i> + <i>A. brasilense</i> + <i>B. subtilis</i>	167 b	121 b	7.68 a	7.02 a	238 b	198 b	22 b	17 c
<i>R. tropici</i> + <i>A. brasilense</i> + <i>P. fluorescens</i>	106 c	104 c	10.20 a	7.48 a	148 c	175 b	10 d	14 d
<b>F-values</b>								
I	51 *	16 *	2.96	9.10 *	44.2 *	13.4 *	62.2 *	32.4 *
CV (%)	14.2	13.9	26.7	8.9	16.8	11.8	13.6	9.2

ZnUE = Zinc use efficiency, APE = Agro-physiological efficiency, UE = Utilization efficiency, and AZnR = Applied zinc recovery. Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ ); \* —significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; and ns—nonsignificant, by F-test.

The co-inoculations of different bacteria along with Zn application had not significantly affected agro-physiological efficiency (APE) in 2019, whereas the effect was significant in 2020 (Table 3.5). The highest APE was observed with the triple co-inoculation of *R. tropici* + *A. brasilense* + *P. fluorescens*, which was 46.7% higher than the control, whereas the lowest APE was observed with *R. tropici* + *P. fluorescens*, which was 0.71% less than the control in 2019. The APE was improved by 48.7% with triple co-inoculation of *R. tropici* + *A. brasilense* + *P. fluorescens*, which is statistically similar to co-inoculation of *R. tropici* + *P. fluorescens* (45.2%) and *R. tropici* + *A. brasilense* + *B. Subtilis* (36.3%) in 2020, compared to the control plots.

The utilization efficiency (UE) was significantly improved with co-inoculations of different diazotrophic bacteria along with soil Zn application in 2019 and 2020 (Table 3.5). The co-inoculation of *A. brasilense* + *B. subtilis* prominently increased utilization efficiency in 2019 and 2020 by 990.2 and 105.2%, respectively, compared to the control.

The co-inoculations of different diazotrophic bacteria along with soil applied Zn improved applied Zn recovery (AZnR) in 2019 and 2020 (Table 3.5). The co-inoculation of *R. tropici* + *A. brasilense* in 2019 and 2020 significantly improved AZnR by 167 and 117%, respectively, along with soil application of Zn, compared to the control.

### **3.3.6 Pearson's linear correlation among zn content in tissues and soil, Zn efficiencies and common bean grain yield**

Pearson's linear correlation was positive between Zn content in soil; Zn concentration in leaf tissue, shoot and grain; Zn accumulated in shoot, grain, and aerial part with shoot biomass; and grain yield. Similarly, Pearson's correlation was positive between Zn use efficiency, agro-physiological efficiency, applied Zn recovery, and utilization efficiency with shoot biomass and common bean grain yield (Appendix B; Figure 1).

## **3.4 Discussion**

Zinc soil application is one the most adaptable strategies to boost Zn assimilation in plant tissues and grains (Haider *et al.*, 2018) in Zn deficient soil ranging from 0.0 to 0.3 mg dm<sup>3</sup> (VAN RAIJ *et al.*, 1997). In addition, Zn agronomic application improves different physiological functions and results in better growth, Zn use efficiency, and high productivity (KRYVORUCHKO *et al.*, 2017; REHMAN *et al.*, 2019; HAIDER *et al.*, 2018). The positive Pearson's correlation between Zn uptake (Zn concentration in soil and tissues and Zn accumulated in aerial part) and Zn efficiencies with common bean shoot dry matter and grain yield (Appendix B; Figure 1) support current hypothesis.

The inoculation of diazotrophic bacteria can improve Zn solubilization and availability to plants through the production of several phytohormones and enzymes and the biological fixation of nitrogen (ULLAH *et al.*, 2020; KHOSHRU *et al.*, 2020). These bacteria facilitate Zn assimilation and accumulation through carboxylation and solubilization, unlike sole Zn applications that generate Zn toxicity (REHMAN *et al.*, 2018). Zinc is available in different ways over time, which needs further and extensive study to understand the co-inoculation and Zn dose effect, which will lead to better nutrition, plant physiology, and yield. The soil Zn application with diazotrophic bacteria sustainably increased plant adaptation in tropical regions, leading to the better Zn nutrition, accumulation, and yield of common bean.

This field study indicated that soil Zn application and co-inoculations of diazotrophic bacteria improved Zn soil concentration after common bean harvest (Figure 3.3A, B) in 2019 and 2020. This improvement might be due to certain metabolic mechanisms that bacteria could adopt to dissolve unavailable soil Zn through synthesis of reactive products from non-reactive materials (HUSSAIN *et al.*, 2020). The increase in soil Zn concentration may be due to the positive relationships of different bacteria, including *Bacillus* and *Pseudomonas* sp., which potentially improve soil Zn concentration through carboxylation and different organic acids production (MUMTAZ *et al.*, 2017; IMRAN *et al.*, 2014).

The concentration of Zn in shoots and grains were improved with soil applied Zn and co-inoculation of different bacteria (Figure 3.3C; Table 3.1). The reason for this might be the structural role of bacteria in different metabolic, enzymatic, and biochemical processes (ULLAH *et al.*, 2019), which should proactively increase Zn and other nutrients availability to plants (FAGERIA *et al.*, 2011). Plants-microbe interaction in root rhizosphere increased Zn solubilization and availability in the plant tissues of legume crops (SAINI *et al.*, 2021). Previous studies have shown that the sequencing of bacterial inoculants (especially *Bacillus* sp.) had the potential to better solubilize Zn insoluble complexes, including phosphate, oxides, and carbonates, and make them available for plant uptake and therefore improve the growth attributes of leguminous crops (KUSHWAHA *et al.*, 2021; PAWAR *et al.*, 2015). Several previous studies have indicated that different strains of *Bacillus*, *Pseudomonas*, and *Rhizobium* sp. improved Zn solubilization and availability in different legumes and cereal crops (SARAVANAN *et al.*, 2007; YADAV *et al.*, 2002). The co-inoculation of these microorganisms with synthetic Zn fertilizers increased nutrient concentration and uptake, leading to healthy and quality grains with rich nutrient accumulation in bean cultivars (KHANDE *et al.*, 2017). The indigenous Zn solubilizing bacteria are more effective, with potentially better improvements in different phytohormones than in exogenous ones, and are able to sustainably sustain plant and grain concentrations with higher growth, yield, and soil fertility status (SINDHU *et al.*, 2019). In this way, our study also showed that shoot (Figure 3.3C) and grain (Table 3.1) Zn concentrations were improved with co-inoculation of *R. tropici* + *B. subtilis* along with 8 kg ha<sup>-1</sup> of soil applied Zn. The activities and redistribution of different *Bacillus* strains in rhizosphere Zn pools potentially increased plant Zn availability, growth, and assimilation to the grains of legumes and cereals, allowing them to overcome malnutrition (KUMAR *et al.*, 2019).

The accumulation of Zn in the shoots, grains, and plants of common bean (Figure 3.3D, G) were improved with soil applied Zn and co-inoculation of *R. tropici* + *B. subtilis* bacteria. Both the accumulation and concentration of Zn in plant tissues are directly related to the population, availability, and composition of soil micro-biota in the root rhizosphere (RAMESH *et al.*, 2014), which, therefore, with some specific strains, increased Zn availability and uptake (KUMAR *et al.*, 2019). The crops on Zn concentrated soils may uptake a lower concentration of Zn due to its oxides and carbonates (RANA *et al.*, 2012), which should be potentially increased with root-rhizosphere Zn solubilizing microbes. There have been several previous studies with different crops where *Bacillus* strains, alone or in combination with organic and inorganic fertilizers, increased Zn and macronutrients availability and uptake (SINGH *et al.*, 2005; SHARMA *et al.*, 2012), which can potentially combat Zn scarcity and enhanced the productivity and Zn accumulation in plants (GOTETI *et al.*, 2013).

The present study also showed that soil Zn application, along with co-inoculation of *R. tropici* and *B. subtilis*, increased Zn partitioning to common bean grain in two consecutive cropping seasons (Table 3.2; Figure 3.3H). Different strains of *Rhizobium* sp. are one of the PGPBs that potentially convert unavailable mineral nutrients into an available form for plant uptake (SINGH *et al.*, 2017). A previous study, with a range of bacterial isolates, indicated that *Bacillus* and *Pseudomonas* are the strains with the most potential to solubilize Zn soon after their inoculation (GONTIA-MISHRA *et al.*, 2017). Different genera of *Bacillus* are widely known for Zn solubilization and mobilization from soil to plant and, subsequently to grains (MUMTAZ *et al.*, 2017). Therefore, our study also confirmed that Zn partitioning to the grains was improved with co-inoculation of *R. tropici* and *B. subtilis*. A study described that the Zn partitioning index was regulated up and down with inoculation of *Bacillus* in legume and cereal crops due to the up and down regulation of Zn transporter genes and, therefore, *Bacillus* is one of the most promising candidate bacteria for the bioavailability and bio-fortification in several crop (GOTETI *et al.*, 2013; KHANGHAHI *et al.*, 2018).

The plant height, dry matter, and grain yield (Figure 3.5A, D) of common bean were also increased with soil Zn application and co-inoculation of diazotrophic bacteria. Zinc is one of the most imperative nutrients of its kind that affects crop growth and development (SUNITHAKUMARI *et al.*, 2016), is known for having indispensable functions in cell division, and contains ribosomal stabilization carbohydrate and several other growth-promoting enzymes.

The application of Zn favored plant growth by favoring cell division in a better way, Zn being an imperative component of several plant biochemical processes (ULLAH *et al.*, 2020). Zinc has a proactive role in the maintenance of cell integrity, elongation, and multiplication (DOOLETTE *et al.*, 2018), along with enzyme activities (REHMAN *et al.*, 2019) that benefit plant physiology and biomass production with high yielding potential (ULLAH *et al.*, 2020; JALAL *et al.*, 2020). The present study supports these previous studies, demonstrating that, in common bean crops, the application of Zn resulted in taller plants with higher dry matter production and yield.

The inoculation of diazotrophic bacteria, especially Zn solubilizing bacteria, play an outstanding role in maintaining a sustainable and eco-friendly environment (Hakim *et al.*, 2021). This micro-biota biodiversity adopts several mechanisms to not only regulate the environmental cause but also improve plant growth, physiology, and yield, with a better accumulation of nutrients in grains for human benefits (HUSSAIN *et al.*, 2020; GONTIA-MISHRA *et al.*, 2017; JALAL *et al.*, 2020). The same strategy has been followed in our study, with soil Zn application and *R. tropici* and *B. subtilis*, for the increment of shoot dry matter, grain yield (Figure 3.5B, D) of common bean, and estimated Zn intake. It has been reported that *Bacillus* strains potentially decrease phytic-P concentration in legume grains, which improves Zn bioavailability in seeds for assimilation and consumption by humans, with a high feed efficiency (BOLETA *et al.*, 2020). It should also be considered that Zn rates tested for Zn concentration and intake in the present study are not of concern in term of toxicity for plants, which are recognized as being safe for common bean biofortification. This is also be due to the bio-activation of several zinc-soil-microbes-plant mechanisms to solubilize unavailable soil Zn and improve plant Zn uptake and biofortification (SARAVANAN *et al.*, 2007; KUMAR *et al.*, 2019; RAMESH *et al.*, 2020). Therefore, our results also highlight that soil Zn application has synergistically improved the nutritional status and yield of common bean when co-inoculated with *R. tropici* and *B. subtilis* (Figure 3.5C, D).

The Zn efficiencies were improved with soil Zn application and bacterial co-inoculation (Table 3.5). Zinc efficiency is determined in term of Zn availability in the grains of low Zn available soils (FAGERIA; STONE, 2004; FAGERIA, 2011). Studies determining the impact of applying Zn from 0 to 10 kg ha<sup>-1</sup> have demonstrated the potentially improved nutritional quality of bean grains, without toxicity symptoms, in tropical soil (VAID *et al.*, 2014). The sole application of Zn may lead to a negative relation with Zn use efficiencies (CAMBRAIA *et al.*, 2019); however, inoculant strains of *Bacillus* sp. and *Pseudomonas* sp. are considered to have high dissolution

properties regarding Zn sulphide, oxides, and carbonates (SARAVANAN *et al.*, 2007). These rhizospheres micro-biota adopt several mechanisms to promote the plant root system, which therefore increase nutrient uptake and utilization. Hence, our results have exhibited that *R. tropici* and *B. subtilis* improved ZnUE, AZnR, and Zn utilization (Table 3.5). The better Zn use efficiencies with Zn application might be due to better crop establishment and grain yield (Table 4), which was further increased with Zn assimilation to grains under co-inoculation of these diazotrophic bacteria (Figure 3.3H). Our study also indicated that agro-physiological were improved with triple co-inoculation (*R. tropici* + *A. brasilense* + *P. fluorescens*) along with soil Zn application (Table 3.5). This positive improvement in Zn efficiencies with Zn application may be in response to the low available concentration of Zn in the tropical savannah (FAGERIA *et al.*, 2011). Therefore, inoculation of Zn solubilizing bacteria increased Zn partitioning and accumulation irrespective of Zn use efficiency and proved a sustainable and integrated strategy for grain biofortification and productivity of the common bean, the most important leguminous crop, which could improve human nutrition in a sustainable way.

### 3.5 Conclusions

Soil zinc application is one feasible strategy to enrich and fortify the grains of crops. The application of diazotrophic bacteria, including Zn solubilizing bacteria, is an interesting alternative sustainable strategy to encourage Zn use efficiencies in an eco-friendly way. Our results indicated that soil Zn application with different diazotrophic bacteria improved Zn concentrations in the soil, as well as in the plants and grains of common bean. It was also concluded that soil Zn application in combination with co-inoculation of *R. tropici* and *A. brasilense* to common bean sustainably improved Zn-leaf. The co-inoculation of *R. tropici* and *B. subtilis* in combination with Zn better improved Zn concentration and accumulation in shoots and grains, with a promising effect on grain yield and the estimated Zn intake of the common bean.

The Zn use efficiencies were prominently improved with co-inoculation, irrespective of Zn application. The Zn use efficiency, applied Zn recovery, and Zn utilization efficiency were higher with co-inoculation of *R. tropici* and *B. subtilis* in comparison to all other inoculations. Therefore, co-inoculation of *R. tropici* and *B. subtilis* could be the most effective method, in association with soil Zn application, for improvement in the acquisition of plant nutrients and their use efficiencies, especially Zn, for the better biofortification of common bean grains in tropical regions. Further

studies aimed at improving Zn utilization and recovery in combination with diazotrophic bacteria and their impact on legumes biofortification, sequencing, and physiological processes should be performed under different environmental and edaphic conditions to better understand Zn solubilizing bacteria under field conditions.

## CHAPTER-4

### 4. AGRONOMIC BIOFORTIFICATION AND PRODUCTIVITY OF COMMON BEAN WITH NANO-ZINC AND PLANT GROWTH-PROMOTING BACTERIA

#### 4.1 Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important legume crops and being a part of daily diet for human consumption around the world. Brazil is one of the largest global producers of common bean, ranked third in terms of grain production (3.2 million metric tons) and second in terms of cultivated area (2.9 million hectares) during three major sowing seasons (CONAB, 2021). It is one of the most consumed grain foods in Brazilian nutrition for being a source of important protein, vitamins, minerals and amino acids (REZENDE *et al.*, 2017). Agricultural production systems and practices are responsible to ensure food and nutrition security to increasing population without environmental risks, which link soil, plants and animals to human health for maintaining sustainable agriculture (YAN *et al.*, 2022). Climatic extremes and vulnerabilities are disturbing sustainable agriculture production, availability, utilization, and stability of food in both developed and developing countries, leading to global hunger (FAO, 2018). In addition, crop cultivation is accompanied by intensive use of synthetic fertilizers to meet food demand of increasing global population which contributes to the deficiency of most common nutrients including iron (Fe), zinc (Zn), selenium (Se) and iodine (I) in human blood plasma (BOUIS, 2018).

Zinc (Zn) malnutrition is a global dietary concern and most vulnerable to agriculture soil, crop production and nutritional quality of staple field crops. Zinc is one of the most persistent deficiencies in tropical regions that lead to serious health issues especially in low-income countries (SILVA *et al.*, 2021). Plant-based Zn deficiency could impair a series of human metabolic reactions and affect more than 2 billion people, being ranked as the second most common deficiency and health concern (HAIDER *et al.*, 2021; SILVA *et al.*, 2021). The inadequate Zn supply has affected around 17% of the global population and ranked as 5<sup>th</sup> health risk factor with a prevalence of deficiency in least developed or developing countries (MOREIRA *et al.*, 2018; WHO, 2021). To feed the rising population of the world with both sufficient and nutritious food is itself one of the sustainable development goals of the United Nations (<https://sdgs.un.org/goals/goal3>). Thus, it is considered that agronomic biofortification is one of the best and prompt alternatives to enrich grain

crops with targeted deficient nutrient in the soil and fulfill nutritional requirements (MENGIST *et al.*, 2021; JALAL *et al.*, 2022).

Biofortification with nano-fertilizer could enhance nitrogen metabolism, productivity and nutrition into the edible tissues of targeted plant crops to address global malnutrition (YUSEFITANHA *et al.*, 2020). Biofortification could better be define by the time of foliar spray, choice and growth stage of crop as well as source, formulation and particle size of applied fertilizers (FERNÁNDEZ; BROWN 2013; AFSHRAF *et al.*, 2020). Foliar spray of nano Zn fertilizer has gained more attention and interest in agriculture sector for increasing crop nutrition and productivity in ecofriendly manner (MANIVANNAN *et al.*, 2021; JALAL *et al.*, 2022). Nano zinc oxide (ZnO) is widely use Zn fertilizer that could quickly absorb via stomata and cuticles, distributed and translocated to chloroplast thus contributing to plant nutrition and production (SU *et al.*, 2019; WEISANY *et al.*, 2021). The efficacy of nano ZnO could be recognized by their physical characteristics, composition and size of particle that potentially increase nutrient use efficiency and reduce dependency on synthetic fertilizers, promoting Zn concentration in stem and edible tissues, and other growth characteristics of different crops (MUNIR *et al.*, 2018; WANG *et al.*, 2018). However, excessive use of synthetically produced nanoparticles may confer plant growth and productivity depending on surface and size of particulate (SUBBAIAH *et al.*, 2016). Therefore, sustainable alternative techniques need to be adapted to improve production and nutritional status of crop plants, and minimize devastating environmental impacts of current agriculture techniques (DROBEK *et al.*, 2019).

Plant growth-promoting bacteria (PGPBs) application is one of the sustainable and greener approach that are enhancing food quantity without compromising quality and productivity (CHOUHAN *et al.*, 2021). These PGPBs can improve nutrient use efficiency and tolerance against biotic and abiotic stresses by contributing to root architecture, improving soil fertility, and enhancing solubilization of macro- and micronutrient that all together can lead to higher plant productivity (ROUPHAEL; COLLA 2020). Several genera of PGPBs including *Bacillus* sp., *Pseudomonas*, *Enterobacter* sp., *Rhizobium* sp., *Acinetobacter* and *Klebsiella* sp. are being reported and studied for improving nutrient use efficiency and productivity, reducing use of synthetic fertilizers in different crops (ULLAH *et al.*, 2020; JALAL *et al.*, 2021; ROUSTRAY *et al.*, 2021; ROSA *et al.*, 2022). Plant growth-promoting bacteria support plant health and performance by production and regulation of phyto-hormones (VACHERON *et al.*, 2013;

YAHAGHI *et al.*, 2019), solubilization of nutrients (PATEL; ARCHANA 2017; HAKIM *et al.*, 2021), biosynthesis of siderophores and antibiotic (GOSWAMI *et al.*, 2016; LAMBRESE *et al.*, 2018) to resist biotic and abiotic stresses. The interaction of PGPBs with soil and plant tissues enhance fitness of the plant to drastic environmental conditions by regulating plants metabolic and physiological traits (RAVANBAKHSH *et al.*, 2019).

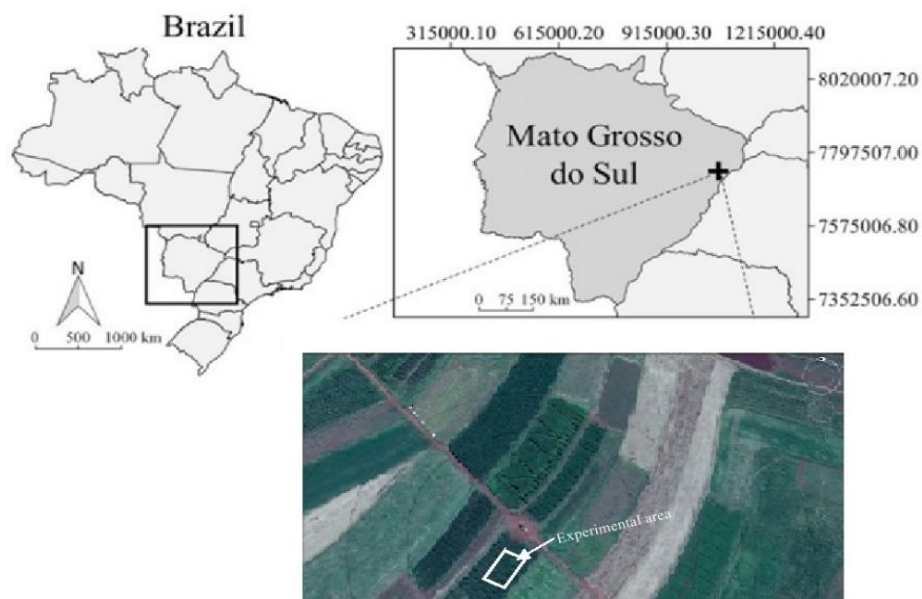
Sustainable nutrient management in field crops is challenging issue in tropical savannah. Despite this, Brazil is being spotted in the list of Zn deficient countries (<http://www.harvestplus.org>). Several strategies have been adapted to increase nutrient use efficiency and productivity however, there still exist research gap on the use of nano Zn foliar spray in association with PGPBs for improving nutrition, productivity and biofortification of common bean. In this context, the hypothesis of the current study was that PGPBs and nano Zn foliar may improve nutrition, productivity, Zn use efficiencies and biofortification of common bean edible tissues. Hence, the study aimed to determine the best performing PGPBs inoculation with nano Zn foliar application on growth, yield, concentration of Zn in shoot and grains, Zn use efficiencies and estimated Zn intake in common beans under tropical savannah of Brazil.

## **4.2 Materials and Methods**

### **4.2.1 Description of experimental site**

Field experiment with a test crop common bean were conducted during May-August of 2019 and 2020 respectively, at the Research and Extension Farm of School of Engineering, São Paulo State University (UNESP) in Selvíria, state of Mato Grosso do Sul, Brazil. The geographical coordinates of the site are 20°22' S latitude, 51°22' W longitude, and an altitude of 335 m (Figure 4.1). The experimental soil is classified as Rhodic Haplustox (SOIL SURVEY STAFF, 2014) and Red Dystrophic according Brazilian Soil Classification System (SANTOS *et al.*, 2018), being cultivated with cereal-legume cropping system for last 30 years, and no-tillage for last 13 years.

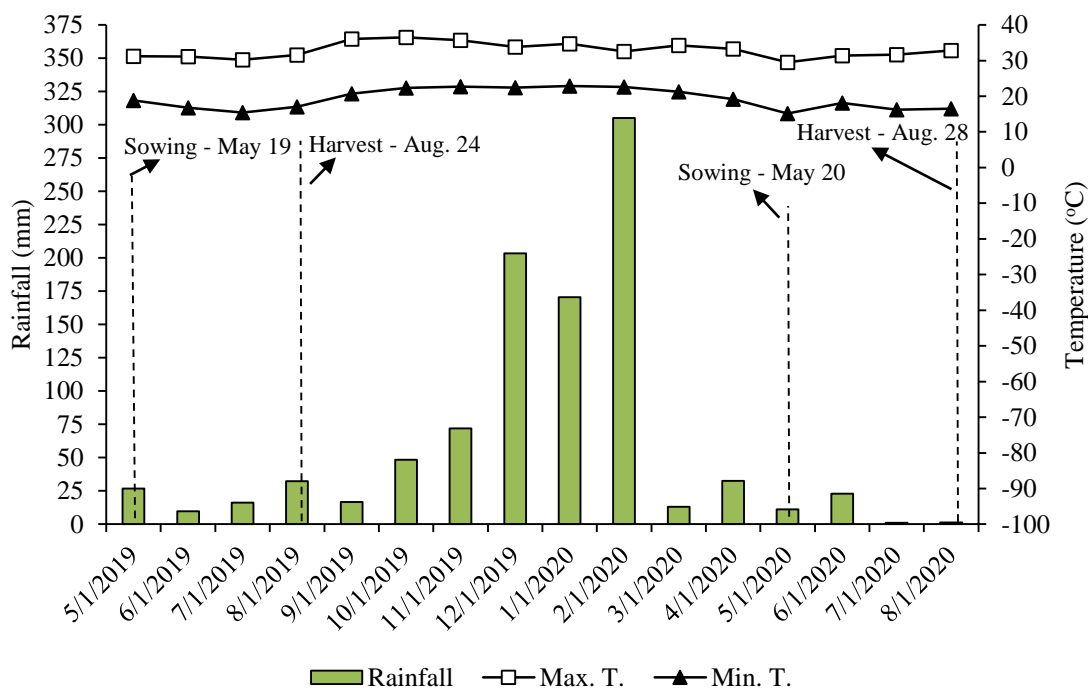
**Figure 4.1.** Geographical location of field trail at Research and Extension Farm, UNESP—Ilha Solteira at Selvíria, Mato Grosso do Sul, Brazil ( $20^{\circ}22' S$ ,  $51^{\circ}22' W$ , altitude of 335 m) during 2019 and 2020 cropping seasons.



**Source:** Author. The map was composed using geographic information system (QGIS) and Google Earth program (The QGIS Development Team 2021) and Open-Source Geospatial Foundation project. <http://qgis.osgeo.org>. Accessed on: 12<sup>th</sup> September, 2022. Projection System WGS 84/UTM 200DC [EPSG: 4326]. This image was taken from Google Earth program, Google Company (2021). Map data: Google, Maxar Technologies.

The climate of the region is characterized as Aw- Köppen with rainy summer, dry winter and humid tropical with relative humidity 70-80% (ALVARES *et al.*, 2013). The climatic data during current experiment in both cropping seasons is summarized in Figure 4.2.

**Figure 4.2.** Rainfall, and maximum and minimum temperatures during common bean cultivation, acquired from the weather station of Research and Extension Farm of UNESP from May to August 2019 and 2020.



#### 4.2.2 Soil Analysis

Twenty random soil samples were collected with the help of cup-auger from a soil layer of 0.00–0.20 m before initiation of common bean experiments during both cropping seasons. The samples were mixed to make a composite sample and determined for chemical characterizations (RAIJ *et al.*, 2001). The physio-chemical characterizations of soil are summarized in Table 2 (Appendix A).

#### 4.2.3 Experimental Design and Treatments

The experiments were designed in a randomized complete block design in a 7 x 3 factorial scheme and four replications. The treatments were consisted of seven kind of seeds inoculations with PGPBs (1- No inoculation, 2- *Rhizobium tropici*, 3- *R. tropici* + *Azospirillum brasilense*, 4- *R. tropici* + *Bacillus subtilis*, 5- *R. tropici* + *Pseudomonas fluorescens*, 6- *R. tropici* + *A. brasilense* + *B. subtilis* and 7- *R. tropici* + *A. brasilense* + *P. fluorescens*), and three foliar nano-zinc oxide spray (0.0, 1.5 and 3 kg ha<sup>-1</sup>), applied 50% at R5 (flowering) and R8 (grain filling)

stage of common bean according to International Center for Tropical Agriculture-CIAT (FERNÁNDEZ *et al.*, 1986).

Seeds of common bean were manually inoculated by mixing each inoculant of PGPBs and seeds in a plastic bag an hour before to sowing. The seeds were inoculated with *R. tropici*, using commercial peat inoculant, strain SEMIA 4080 with  $2 \times 10^9$  colony forming units (CFU)  $\text{g}^{-1}$  at a dose of 200 g per 100 kg seeds. A 10% sugar solution was used to facilitate inoculant adhesion with seeds. *R. tropici* is a commercially registered inoculant with Ministry of Agriculture and Livestock, Brazil for common bean cultivation. *A. brasilense* strains Ab-V5 (CNPSO 2083) and Ab-V6 (CNPSO 2084) seed inoculation was carried out at a dose of 200 mL liquid inoculant per 24 kg seeds with guarantee of  $2 \times 10^8$  CFU  $\text{mL}^{-1}$ . Inoculations with *B. subtilis* strain (CCTB04), guarantee of  $1 \times 10^8$  CFU  $\text{mL}^{-1}$  and *P. fluorescens* strain (CCTB03), guarantee of  $2 \times 10^8$  CFU  $\text{mL}^{-1}$  at dose of 150 mL  $\text{ha}^{-1}$  per 24 kg seeds were manually performed by following the procedures of inoculants providing company (Total Biotechnology<sup>®</sup>), Curitiba, Paraná State, Brazil. It has been reported that *A. brasilense* strains Ab-V5 and Ab-V6 are carrying *fix* and *nif* genes to enhance nutrients availability and recycling, also has a role in biological nitrogen fixation, auxin production and induce plant tolerance against biotic and abiotic stresses (FUKAMI *et al.*, 2017; FUKAMI *et al.*, 2018b; GALINDO *et al.*, 2021). *B. subtilis* is first gram positive bacterium carrying non-ribosomal peptide synthetases and a beta-glucanase as phyto-pathogens resistant as well as *zntR* as Zn transporter that assist plant growth-promotion while resisting phyto-pathogens and heavy metals absorption (CHAOPRASID *et al.*, 2015; REKHA *et al.*, 2017; MUÑOZ-MORENO *et al.*, 2018). *P. fluorescens* is considered the most efficient bio-control agent with the synthesis of antibiotics, volatile organic compounds to resist soil pathogens, produce gluconic acid, and solubilize nutrients and fixing N (DAVID *et al.*, 2018; JING *et al.*, 2020).

Nano Zn foliar spray was carried out during morning from a liquid Zn source (Nano R1 zinco) of ALLPLANT<sup>™</sup> fertilizers industry, São Paulo-Brazil, being registered with Ministry of Agriculture, Brazil. This nano Zn is characterized as fluid suspension with 50% p/p Zn, 1000 g  $\text{L}^{-1}$  solubility and 2.0 density and successfully studies for improving plant growth and productivity (NAKAO *et al.*, 2018; JALAL *et al.*, 2022c). All the doses of foliar nano Zn were applied in two split applications (50% at R5 and 50% at R8 stage) of common bean according to International Center for Tropical Agriculture-CIAT (FERNÁNDEZ *et al.*, 1986). The application was performed through manual sprayer pump with water capacity of 6.0 L (300 L  $\text{ha}^{-1}$  of volume

spray). The field was thoroughly visited soon after foliar nano Zn spray and no leaf damage was observed.

#### 4.2.4 Field Management

The field site was sprayed with herbicides 2,4-D + glyphosate (670 + 1800 g ha<sup>-1</sup> of a.i.) for controlling pre-experiment emerged weeds. Common bean cultivar (IPR – Campos Gerais belonging to commercial Carioca group), erect and indeterminate type-II with an average life cycle of 88 days (IAPAR, 2019). Field experiment with common bean were planted in the first half of May, 2019 and 2020 under a no-tillage system. Each plot was consisted of 5 m long 6 lines with plot size of 2.7 m x 5 m, totalizing 13.5 m<sup>2</sup>. The rows were spaced by 0.45 m and regulated 12 seeds m<sup>-1</sup>. The treatments in both experiments were performed at the same phenological stages.

All the treatments were applied with a basal dose of NPK according to initial soil analysis (Table 1). A total amount of nitrogen (N: 20 kg ha<sup>-1</sup>) from a source of urea, phosphorus (P<sub>2</sub>O<sub>5</sub>: 80 kg ha<sup>-1</sup>) from triple superphosphate and potassium (K<sub>2</sub>O: 40 kg ha<sup>-1</sup>) from potassium chloride were applied at the time of plantation. Despite this, a recommended dose of 90 kg ha<sup>-1</sup> of N was applied from ammonium sulphate in covering after 5 weeks of plantation. The region of the experiment was interpreted as boron deficient (RAIJ *et al.*, 2001). Therefore, the entire field was sprayed with 1 kg boron ha<sup>-1</sup> from the source of boric acid (18% of B) through a tractor sprayer at flowering stage of common bean. The crop was irrigated with a central-pivot sprinkler irrigation system (14 mm water volume on a shift of 72 h) and all cultural practices were performed, when necessary. The common bean were manually harvested on 24<sup>th</sup> and 28<sup>th</sup> August, 2019 and 2020 respectively.

#### 4.2.5 Assessments and Evaluations

Plant height was determined at physiological maturity by measuring plant length from ground surface to upper apex of plants. Plants from four central rows were harvested, labeled, dried in shade and weighed with analytical balance for shoot dry matter. Each sample was mechanically threshed and transferred into kg ha<sup>-1</sup> at 13% humidity to quantify grain yield of common bean.

Zinc concentrations in shoot and grains were determined with nitro-perchloric digestion and quantified by atomic absorption spectrophotometry, following the procedure of Malavolta *et al.*, (1997). In addition, uptake of Zn in shoot and grains were estimated from the ratio of Zn

concentration in shoot and grains, and shoot dry matter and grain yield respectively. Zinc partitioning index (ZPI) was derived from grains and shoot Zn concentration (Rengel; GRAHAM 1996) while estimated Zn intake with common bean grains in Brazil was calculated from the biofortified common bean grains of the present study (DE LIMA LESSA *et al.*, 2019).

$$\text{ZPI} = \frac{\text{Grain Zn concentration} - \text{Shoot Zn concentration}}{\text{Shoot Zn concentration}} \times 100 \quad \text{Equation 1}$$

$$\text{Zinc intake} = [\text{Zn}] \times C \quad \text{Equation 2}$$

where: Zn intake ( $\text{g person}^{-1} \text{ day}^{-1}$ ) is daily Zn intake estimation  $\text{person}^{-1}$ , [Zn] ( $\text{g kg}^{-1}$ ) is Zn concentration in biofortified grains in the present study and C ( $\text{kg person}^{-1} \text{ day}^{-1}$ ) is the mean consumption of common bean grains per person in Brazil, which is  $\sim 142.2 \text{ g person}^{-1} \text{ day}^{-1}$  (IBGE, 2019).

Zinc use efficiency (ZnUE), agro-physiological efficiency (APE), applied Zn recovery (AZnR) and utilization efficiency were derived in the fractions acquired from Fageria *et al.*, (2011) via the following equations;

$$\text{ZnU} = \frac{\text{GYF} - \text{GYC}}{\text{Applied Zn dose}} \quad \text{Equation 3}$$

$$\text{APE} = \frac{\text{GYF} - \text{GYC}}{\text{Grain} + \text{Shoot ZnUF} - \text{Grain} + \text{Shoot ZnUC}} \quad \text{Equation 5}$$

$$\text{RAZn} (\%) = \frac{\text{Grain} + \text{Shoot ZnUF} - \text{Grain} + \text{Shoot ZnUC}}{\text{Applied Zn dose}} \quad \text{Equation 5}$$

$$\text{UE} = \text{PE} \times \text{RAZn} \quad \text{Equation 6}$$

Where GYF = grain yield with nano-Zn foliar spray, GYC = grain yield in without nano Zn foliar, ZnF = with nano Zn foliar spray, ZnC = without nano Zn foliar spray, ZnUF = grain + shoot Zn uptake in nano-Zn sprayed treatments, ZnUC = grain + shoot Zn uptake in without nano Zn foliar treatments and PE = physiological efficiency.

#### 4.2.6 Statistical analysis

The entire data were tested for normality with Shapiro–Wilk test and Levene's homoscedasticity test ( $p \leq 0.05$ ) which showed that data were to be normally distributed ( $W \geq 0.90$ ). Then, data were subjected to analysis of variance (F test). Foliar nano-Zn doses, PGPBs inoculations and their interactions were considered fixed and replication was random effect in the model. When a main effect or interaction was observed significant by F test ( $p \leq 0.05$ ), then Tukey

test ( $p \leq 0.05$ ) was used for means comparison of nano-Zn spray and Scott Knott test ( $p \leq 0.05$ ) for PGPBs inoculations using ExpDes package of R software (R CORE TEAM, 2015).

The Pearson correlation analysis ( $p \leq 0.05$ ) was calculated and heatmap was created using corrplot package of "cor" and "cor.mtest" functions to calculate coefficients and evaluate relationship among growth, yield, nutritional, biochemical and metabolic attributes of maize using R software (R CORE TEAM 2015).

## **4.3 Results**

### **4.3.1 Growth and grain yield of common bean**

Plant height of common bean was positively influenced by nano Zn foliar spray and co-inoculation with plant growth-promoting bacteria (PGPBs) in 2019 while their effect in 2020 was not significant (Table 4.1). Nano Zn foliar application at a dose of  $1.5 \text{ kg ha}^{-1}$  increased plant height by 6.4% as compared to control in first cropping season. In addition, co-inoculation with *R. tropici* + *B. subtilis* was observed with increasing plant height by 9.7% as compared to without inoculation treatments in 2019 crop season. The interaction of nano Zn foliar spray and inoculations with PGPBs were not significant in both cropping seasons (Table 4.1).

**Table 4.1.** Plant height, shoot dry matter and grain yield of common bean as a function of plant growth-promoting bacteria and nano Zn foliar doses in 2019 and 2020 cropping seasons.

Treatments	Plant height		Dry matter		Grain yield	
	----- cm -----		----- kg ha <sup>-1</sup> -----			
	2019	2020	2019	2020	2019	2020
<b>Inoculations (I)</b>						
Without (Control)	93 d	93.3	3861 e	3908 d	3521	4076
<i>R. tropici</i>	95 d	91.8	3982 d	3982 d	3761	4199
<i>R. tropici</i> + <i>A. brasilense</i>	98 c	92.6	4081 c	4070 c	3902	4347
<i>R. tropici</i> + <i>B. subtilis</i>	102 a	97.1	4318 a	4265 a	4196	4602
<i>R. tropici</i> + <i>P. fluorescens</i>	100 b	93.4	4157 b	4120 b	3999	4452
<i>R. tropici</i> + <i>A. brasilense</i> + <i>B. Subtilis</i>	98 c	91.0	4036 c	4052 c	3860	4340
<i>R. tropici</i> + <i>A. brasilense</i> + <i>P. fluorescens</i>	96 c	91.3	3948 d	4018 c	3708	4298
<b>Nano Zn foliar spray (kg ha<sup>-1</sup>)</b>						
0.0	94 c	90.6	4007 b	3961 b	3530	4153
1.5	100 a	93.8	4119 a	3146 a	4097	4533
3	98 b	94.3	4041 b	4071 a	3921	4305
<b>F- values</b>						
I	13 **	1.3 ns	67 **	9.9 **	38 **	17 **
Nano Zn	31 **	2.8 ns	23 **	15.9 **	161 **	50 **
I × nano Zn	0.24 ns	1.0 ns	1.4 ns	0.15 ns	3.2 **	2.5 *
<b>CV (%)</b>	3	7	2	3.1	3	3.3

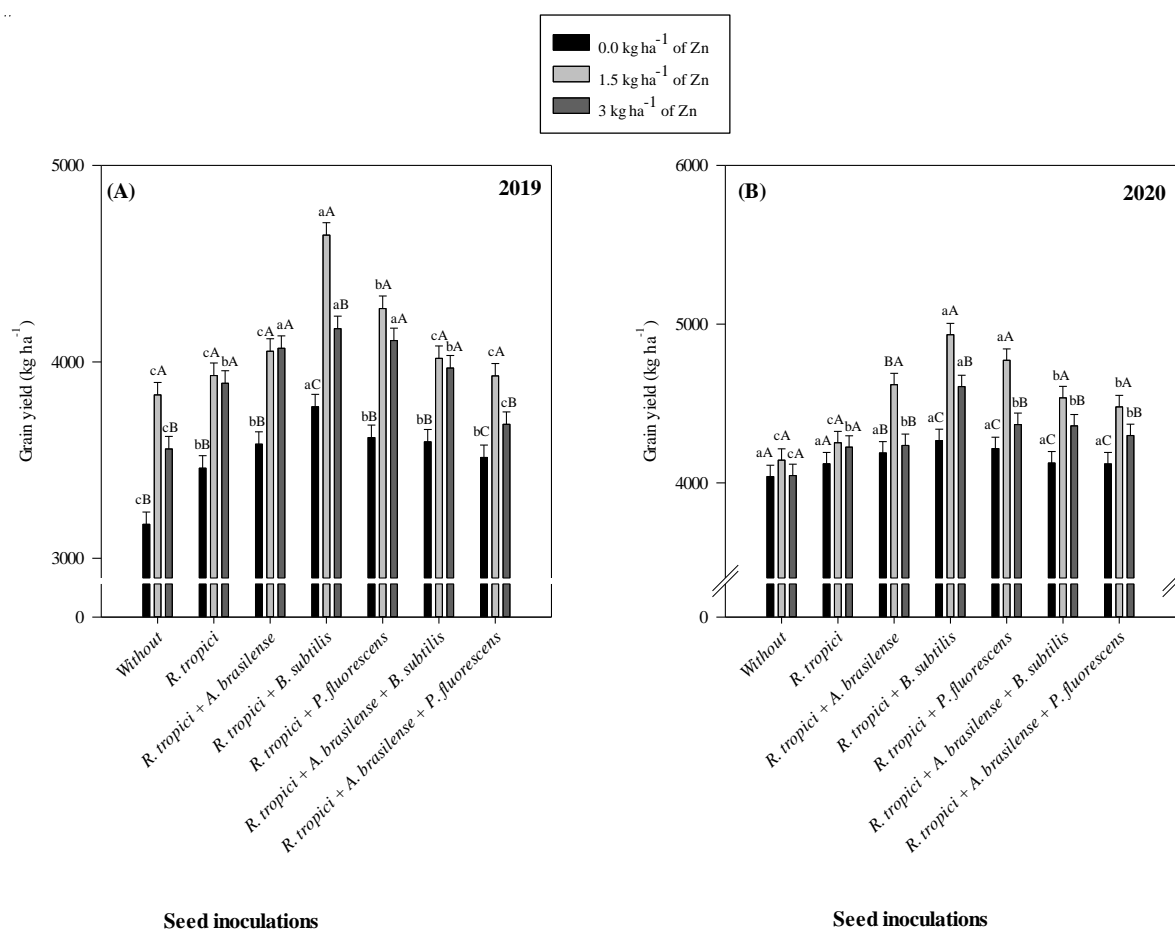
Means in the column followed by similar letters are statistically not different by Tukey test ( $p \leq 0.05$ ) for foliar nano Zn dose and Scott Knott test for PGPBs ( $p \leq 0.05$ ). \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$  respectively while ns—non-significant by F-test.

Shoot dry matter of common bean was significantly influenced by nano Zn foliar spray and inoculation with PGPBs whereas their interaction was not significant in both crop seasons (Table 4.1). Shoot dry matter was increased by 2.8% with application of 1.5 kg ha<sup>-1</sup> of foliar nano Zn in 2019 while an increase of 2.7% was observed with 3 kg ha<sup>-1</sup> of foliar nano Zn application, which was statistically at par with 1.5 kg ha<sup>-1</sup> of foliar nano Zn in 2020 as compared without nano Zn foliar application treatments. In addition, co-inoculation with *R. tropici* + *B. subtilis* increased shoot dry matter of common bean by 11.8 and 9.2% in first and second crop seasons respectively in comparison of without inoculation treatments (Table 4.1).

The single effect of nano Zn foliar doses and inoculations with PGPBs and their interaction were significant for grain yield of common bean in both 2019 and 2020 cropping seasons (Table 4.1). The treatments with co-inoculation of *R. tropici* + *B. subtilis* at a dose of 1.5 kg ha<sup>-1</sup> of nano Zn foliar application were observed with higher grain yield as compared to without and other

inoculations treatments in 2019 and 2020 common bean cropping seasons (Figure 4.3A,B). The treatments with nano Zn foliar application and inoculations with PGPBs were observed with greater grain yield as compared to without inoculation and Zn application. The treatments with 1.5 kg ha<sup>-1</sup> of nano Zn foliar application was observed with greater grain yield under all inoculations treatments as compared to other Zn doses (Figure 4.3A,B). The treatments without inoculations of PGPBs and nano Zn foliar application was observed with the lowest grain yield in 2019 and 2020 in relation to other treatments (Figure 4.3A-B).

**Figure 4.3.** Grain yield of common bean in 2019 (A) and 2020 (B) cropping seasons as a function of foliar nano Zn application and co-inoculations of plant growth-promoting bacteria.



**Source:** Author. The upper case letters are used for the interactions of PGPBs inoculations within each dose of foliar nano Zn application, whereas lower case letters are used for the interaction of foliar nano Zn doses within each inoculation treatment. The identical alphabetic letters are statistically similar with each other as analyzed by Tukey test for foliar Zn doses ( $p \leq 0.05$ ) and

Scott-Knott test for PGPBs inoculations ( $p \leq 0.05$ ) in 2019 and 2020 respectively. Error bars indicate standard error of the means ( $n = 4$  replications).

#### 4.3.2 Shoot and grain Zn nutrition, and Zn daily intake

Shoot Zn concentration of common bean was increased with nano Zn foliar doses and co-inoculations with PGPBs while the interactions were not significant in both 2019 and 2020 (Table 4.2). Nano foliar Zn application at the dose of  $1.5 \text{ kg ha}^{-1}$  increased shoot Zn concentration of common bean by 31.3 and 14.4% in 2019 and 2020 cropping season in relation to the treatments without nano Zn foliar application. Shoot Zn concentration was increased by 38.2 and 23.6% with co-inoculation of *R. tropici* + *B. subtilis* in 2019 and 2020 common bean crop seasons respectively as compared to without inoculation.

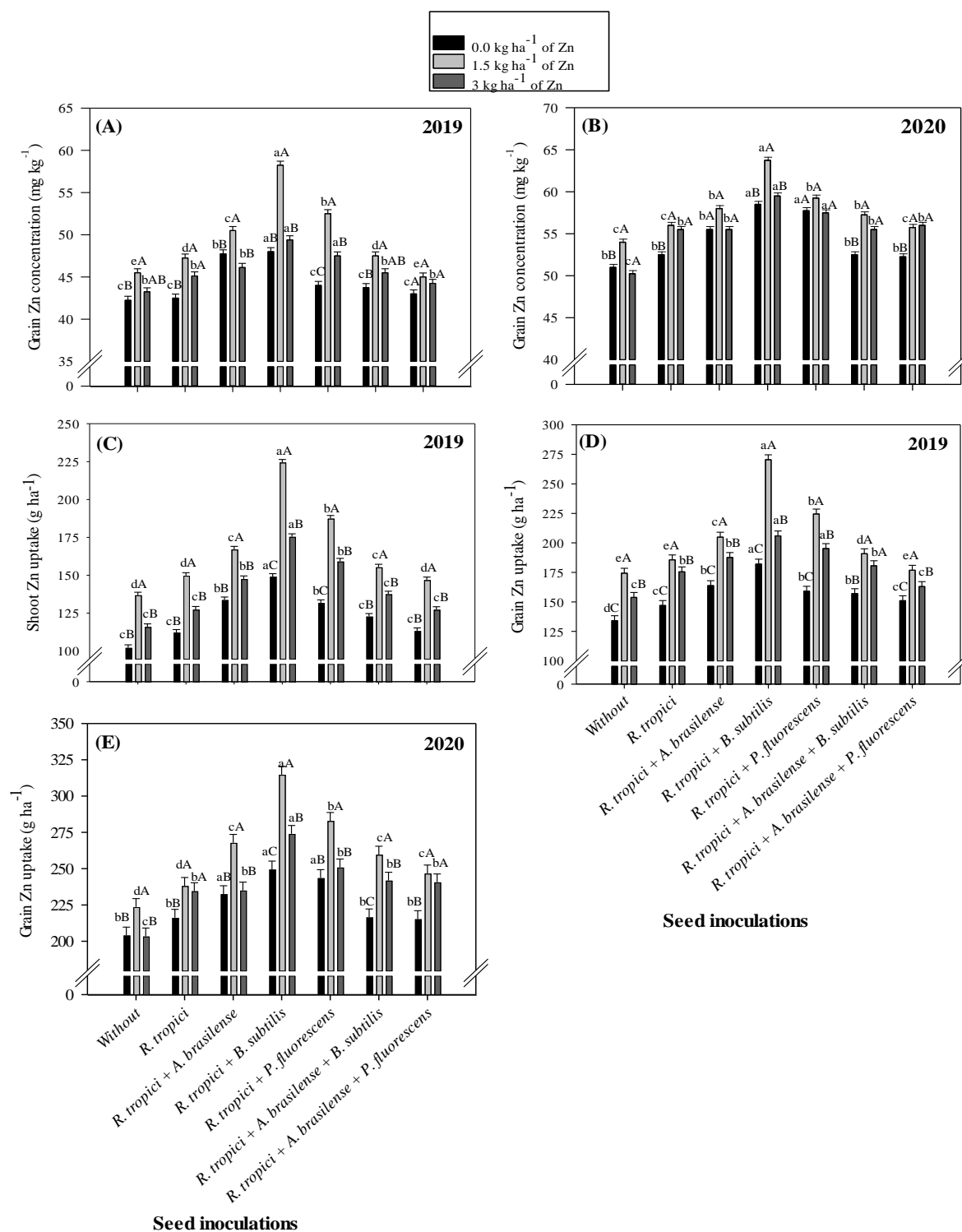
**Table 4.2.** Zinc concentration and accumulation in shoot and grains tissues of common beans as a function of plant growth-promoting bacteria and nano Zn foliar doses in 2019 and 2020 cropping seasons.

Treatments	Shoot Zn concentration		Grain Zn concentration		Shoot Zn uptake	
	----- mg kg <sup>-1</sup> ----- g ha <sup>-1</sup> -----					
	2019	2020	2019	2020	2019	2020
<b>Inoculations (I)</b>						
Without (Control)	30.6 d	39.9 d	43.6	51.7	118	156 d
<i>R. tropici</i>	32.5 c	42.0 c	44.9	54.6	130	167 c
<i>R. tropici</i> + <i>A. brasilense</i>	36.6 b	44.7 b	47.6	56.3	149	182 b
<i>R. tropici</i> + <i>B. subtilis</i>	42.3 a	49.3 a	51.9	60.6	183	210 a
<i>R. tropici</i> + <i>P. fluorescens</i>	38.3 b	45.8 b	48.0	58.2	159	189 b
<i>R. tropici</i> + <i>A. brasilense</i> + <i>B. subtilis</i>	34.3 c	42.1 c	45.6	55.1	139	171 c
<i>R. tropici</i> + <i>A. brasilense</i> + <i>P. fluorescens</i>	32.7 c	40.8 d	44.1	54.6	129	164 c
<b>Nano Zn foliar spray (kg ha<sup>-1</sup>)</b>						
0.0	30.7 c	41.0 c	44.2	54.3	123	163 c
1.5	40.3 a	46.9 a	49.5	57.7	167	195 a
3	34.9 b	42.6 b	45.8	55.7	141	174 b
<b>F- values</b>						
I	31 *	31 *	43.5 **	44 **	57 **	39 **
Nano Zn	105 *	62 *	88.9 **	38 **	129 **	71 **
I × nano Zn	1.6 ns	0.74 ns	4.9 **	2.3 *	3 *	0.9 ns
<b>CV (%)</b>	7	4.7	3.3	2.6	7	6

Means in the column followed by similar letters are statistically not different by Tukey test ( $p \leq 0.05$ ) for foliar nano Zn dose and Scott Knott test for PGPBs ( $p \leq 0.05$ ). \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$  respectively while <sup>ns</sup>—non-significant by F-test.

The interaction of nano foliar Zn doses and inoculation with PGPBs was significant for grain Zn concentration in both 2019 and 2020 common bean crop seasons (Table 4.2). The treatments applied with  $1.5 \text{ kg ha}^{-1}$  foliar nano Zn in combination with co-inoculation of *R. tropici* + *B. subtilis* were observed with higher grain Zn concentration in 2019 and 2020 cropping seasons as compared with all other treatments (Figure 4.4A,B). The treatments with  $1.5 \text{ kg ha}^{-1}$  foliar nano Zn was observed with higher grain concentration regardless of the inoculations. The treatments with no foliar nano Zn application and without inoculation was observed with lowest grain Zn concentration in 2019 while  $3 \text{ kg ha}^{-1}$  of foliar nano Zn and without inoculation treatments were noted with lowest grain Zn concentration in 2020 as compared to other treatments (Figure 4.4A,B).

**Figure 4.4.** Grain zinc (Zn) concentration in 2019 (A) and 2020 (B), shoot Zn uptake in 2019 (C), grain Zn uptake in 2019 (D) and 2020 (E) common bean cropping seasons as a function of foliar nano Zn application and co-inoculations of plant growth-promoting bacteria.



**Source:** Author. The upper case letters are used for the interactions of PGPBs inoculations within each dose of foliar nano Zn application, whereas lower case letters are used for the interaction of foliar nano Zn doses within each inoculation treatment. The identical alphabetic letters are statistically similar with each other as analyzed by Tukey test for foliar Zn doses ( $p \leq 0.05$ ) and Scott-Knott test for PGPBs inoculations ( $p \leq 0.05$ ) in 2019 and 2020 respectively. Error bars indicate standard error of the means ( $n = 4$  replications).

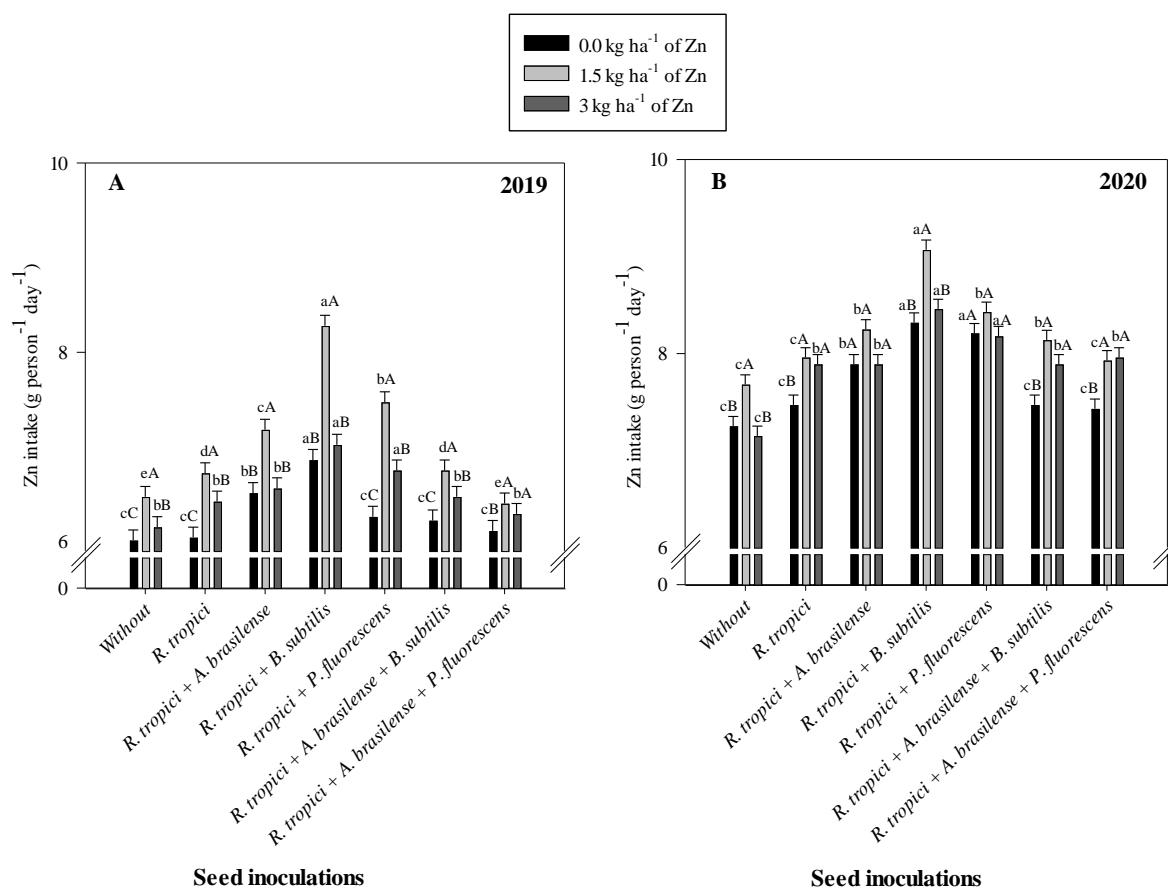
Nano Zn foliar doses and inoculations with PGPBs had positively improved shoot Zn uptake in 2019 and 2020 cropping seasons. The interaction of nano Zn foliar doses and inoculations with PGPBs for shoot Zn uptake was significant in first season while non-significant in the second common crop season (Table 4.2). Shoot Zn uptake was improved by 35.8 and 19.6% with foliar nano Zn at a dose of  $1.5 \text{ kg ha}^{-1}$  in first and second cropping seasons respectively in relation to without nano Zn fertilization. The treatments with co-inoculation of *R. tropici* + *B. subtilis* improved shoot Zn uptake by 55 and 34.6% in 2019 and 2020 respectively as compared to without inoculation treatments. In addition, the interactive effect of  $1.5 \text{ kg ha}^{-1}$  nano Zn foliar application and co-inoculation of *R. tropici* + *B. subtilis* was observed with higher shoot Zn uptake in first common bean growing season as compared with other treatments (Figure 4.4C). In general, the treatments with foliar nano Zn application and inoculations of PGPBs were observed with higher shoot Zn uptake as compared to control. The treatments without foliar application of nano Zn and without inoculation were observed with low shoot Zn uptake as compared to other treatments (Figure 4.4C).

The single and interaction effects of nano Zn foliar doses and inoculations with PGPBs were positive for grain Zn uptake in both studied years (Appendix A; Table 3). Grains Zn uptake was improved with foliar nano Zn at a dose of  $1.5 \text{ kg ha}^{-1}$  in association with co-inoculation of *R. tropici* + *B. subtilis* in both common bean cropping seasons (Figure 4.4D,E). Interesting, the treatments with foliar nano Zn application and inoculations with PGPBs were observed with higher grain Zn uptake as compared to control. In addition, treatments without foliar nano Zn application and without inoculation were observed with low grain Zn uptake in 2019 (Figure 4.4D), while foliar nano Zn at a dose of  $3 \text{ kg ha}^{-1}$  and without inoculation was noted with low Zn uptake in common bean grains in 2020 as compared with other treatments (Figure 4.4E). In general, the treatments without inoculations of PGPBs were observed with low grain Zn uptake regardless of the foliar nano Zn applications in both cropping seasons (Figure 4.4D,E).

Foliar nano Zn doses and inoculation with PGPBs had positively increased partitioning of Zn to common bean grains while their interactions were not significant in 2019 and 2020 cropping seasons (Appendix A; Table 3). Foliar nano Zn application at a dose of 1.5 kg ha<sup>-1</sup> increased Zn partitioning index by 7 and 11.3% in 2019 and 2020 respectively as compared to control. The treatments with co-inoculation of *R. tropici* + *B. subtilis* were observed with 13.9 and 11.4% higher Zn partitioning to common bean grains in 2019 and 2020 respectively, as compared to without inoculations treatments.

The interactions and single effect of foliar nano Zn doses and inoculation with PGPBs were significant for estimated daily Zn intake in Brazil with consumption of common bean grains in 2019 and 2020 (Appendix A; Table 3). The treatments with foliar nano Zn application at a dose of 1.5 kg ha<sup>-1</sup> in association with co-inoculation of *R. tropici* + *B. subtilis* were observed with greater daily Zn intake in first and second cropping seasons respectively in relation to control (Figure 4.5A,B). The treatments with foliar nano Zn application and inoculation with PGPBs were observed with greater daily Zn intake as compared to control treatments. The treatments in the absence of foliar nano Zn application were noted with low daily Zn intake regardless of the inoculation in both studied years. However, least Zn intake with daily consumption of common bean grains was observed without foliar nano Zn fertilization and inoculation in 2019 cropping season (Figure 4.5A). In addition, foliar nano Zn at a dose of 3 kg ha<sup>-1</sup> and without inoculation was observed with lowest daily Zn intake in 2020 cropping season, which was statistically similar with co-application of 0.0 kg ha<sup>-1</sup> foliar nano Zn and without inoculation, inoculation with *R. tropici*, co-inoculation with *R. tropici* + *A. brasilense* + *B. Subtilis* and *R. tropici* + *A. brasilense* + *P. fluorescens* (Figure 4.5B).

**Figure 4.5.** Daily zinc (Zn) intake with common bean grains in 2019 (A) and 2020 (B) cropping seasons as a function of foliar nano Zn application and co-inoculations of plant growth-promoting bacteria.



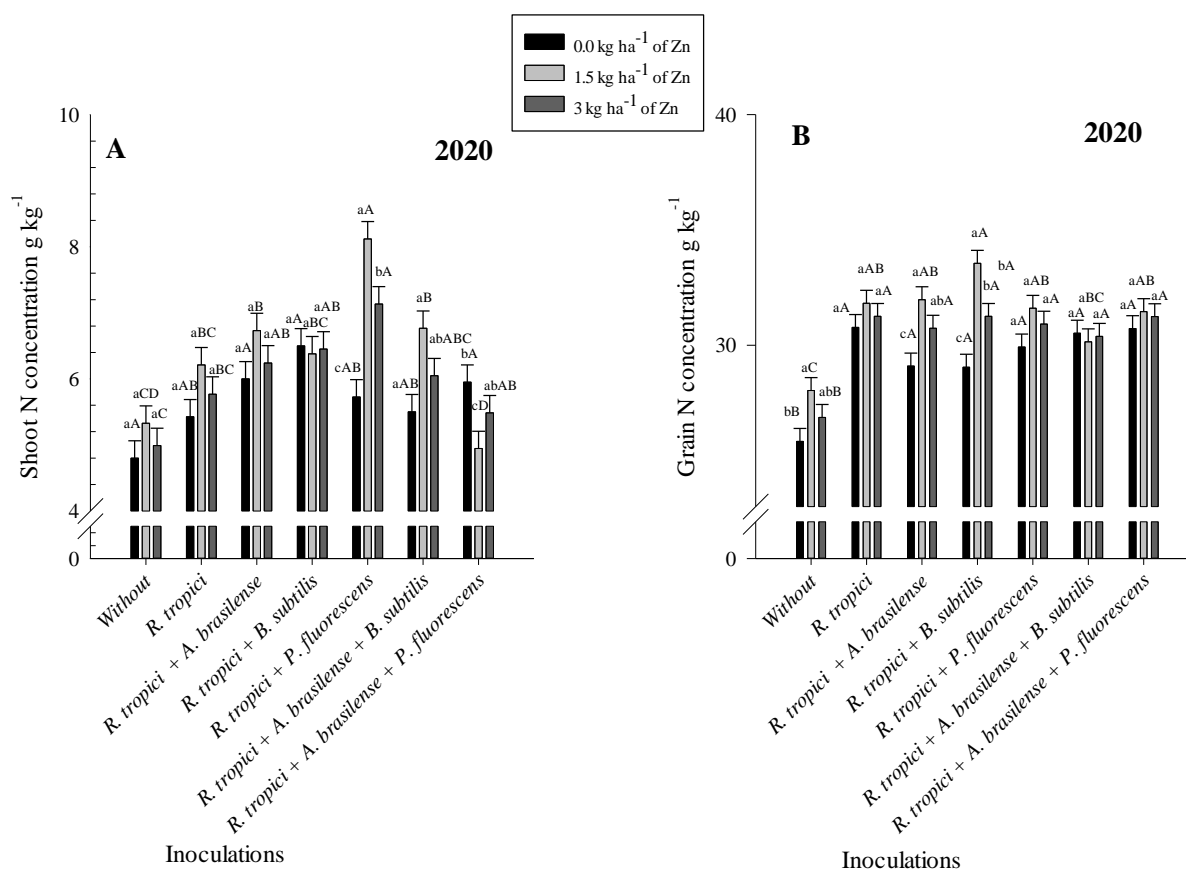
**Source:** Author. The upper case letters are used for the interactions of PGPBs inoculations within each dose of foliar nano Zn application, whereas lower case letters are used for the interaction of foliar nano Zn doses within each inoculation treatment. The identical alphabetic letters are statistically similar with each other as analyzed by Tukey test for foliar Zn doses ( $p \leq 0.05$ ) and Scott-Knott test for PGPBs inoculations ( $p \leq 0.05$ ) in 2019 and 2020 respectively. Error bars indicate standard error of the means ( $n = 4$  replications).

### 4.3.3 Nitrogen and phosphorous shoot and grain concentration

Nitrogen and phosphorous shoot and grain concentrations were increased with co-inoculations and nano-Zn foliar Zn application (Table 4.3). Shoot N concentration was increased by 41.3% with co-inoculation of *R. tropici* + *P. fluorescens*, which was statistically similar with

co-inoculation of *tropici* + *B. subtilis* in 2019 common bean cropping season. In addition, nano-Zn foliar at a dose of 1.5 kg ha<sup>-1</sup> increased shoot N concentration by 2.7% as compared to control. The interaction of nano-Zn foliar doses and co-inoculations was significant in 2020 crop season (Figure 4.6A). Co-inoculation with *R. tropici* + *P. fluorescens* along with nano-Zn foliar fertilization was observed the best dose as compared to the rest of applied treatments. The lowest shoot N concentration was noted in the treatments without foliar nano-Zn application and inoculations (Figure 4.6A).

**Figure 4.6.** Shoot and grain N concentration (A) and (B) respectively in 2020 cropping seasons as a function of foliar nano Zn application and co-inoculations of plant growth-promoting bacteria.



**Source:** Author. The upper case letters are used for the interactions of PGPBs inoculations within each dose of foliar nano Zn application, whereas lower case letters are used for the interaction of foliar nano Zn doses within each inoculation treatment. The identical alphabetic letters are statistically similar with each other as analyzed by Tukey test for foliar Zn doses ( $p \leq 0.05$ ) and

Scott-Knott test for PGPBs inoculations ( $p \leq 0.05$ ). Error bars indicate standard error of the means ( $n = 4$  replications).

Interaction was not significant. Shoot P concentration was increased by 60.4 and 60.3% with co-inoculation of *R. tropici* + *P. fluorescens* in 2019 and 2020 common bean cropping season as compared to without inoculation treatments (Table 4.3). Foliar nano-Zn foliar fertilization at a dose of 1.5 kg ha<sup>-1</sup> increased shoot P concentration by 20 and 20.5% as compared to control in 2019 and 2020 crop seasons.

**Table 4.3.** Zinc concentration and accumulation in shoot and grains tissues of common beans as a function of plant growth-promoting bacteria and nano Zn foliar doses in 2019 and 2020 cropping seasons.

Treatments	Shoot N concentration		Shoot P concentration		Grain N concentration		Grain P concentration	
	----- g kg <sup>-1</sup> -----							
	2019	2020	2019	2020	2019	2020	2019	2020
<b>Inoculations (I)</b>								
Without (Control)	6.3 ab	5.0	0.48 c	1.01 c	26.2 c	26.9 b	4.8 d	4.2 c
<i>R. tropici</i>	7.4 abc	5.8	0.67 ab	1.26 b	30.2 ab	31.2 a	6.2 a	4.6 b
<i>R. tropici</i> + <i>A. brasilense</i>	7.4 abc	6.3	0.56 ab	1.21 bc	30.6 a	30.6 a	5.8 bc	4.8 ab
<i>R. tropici</i> + <i>B. subtilis</i>	5.9 c	6.4	0.79 a	1.29 b	29.5 ab	31.3 a	5.6 c	4.6 b
<i>R. tropici</i> + <i>P. fluorescens</i>	8.9 a	6.9	0.77 a	1.62 a	29.3 ab	30.8 a	5.8 abc	4.7 b
<i>R. tropici</i> + <i>A. brasilense</i> + <i>B. subtilis</i>	7.7 ab	6.1	0.68 ab	1.30 b	28.0 ab	30.3 a	5.6 b	5.0 a
<i>R. tropici</i> + <i>A. brasilense</i> + <i>P. fluorescens</i>	8.8 a	5.4	0.66 ab	1.38 b	29.2 ab	31.1 a	6.2 ab	5.1 a
<b>Nano-Zn foliar spray (kg ha<sup>-1</sup>)</b>								
0	7.4	5.7	0.60 b	1.17 b	28.6	29.4 c	5.5 c	4.5 c
1.5	7.6	6.3	0.72 a	1.41 a	29.6	31.2 a	6.0 a	4.9 a
3	7.5	6.0	0.66 ab	1.30 a	29.0	30.3 b	5.7 b	4.7 b
<b>F- values</b>								
I	8.7**	18.4**	6.9**	12.9**	7.0**	23.1**	27.3**	22.8**
Nano Zn	0.2 ns	10.4**	4.5*	13.7**	1.5 ns	18.3**	15.2*	32.0**
I × nano Zn	0.3 ns	4.1**	0.1 ns	1.5 ns	0.12 ns	2.0*	0.74 ns	1.4 ns
<b>CV (%)</b>	17.3	8.7	21	13.5	6.6	3.7	5.5	4.1

Means in the column followed by similar letters are statistically not different by Tukey test ( $p \leq 0.05$ ) for foliar nano Zn dose and Scott Knott test for PGPBs ( $p \leq 0.05$ ). \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$  respectively while <sup>ns</sup>—non-significant by F-test.

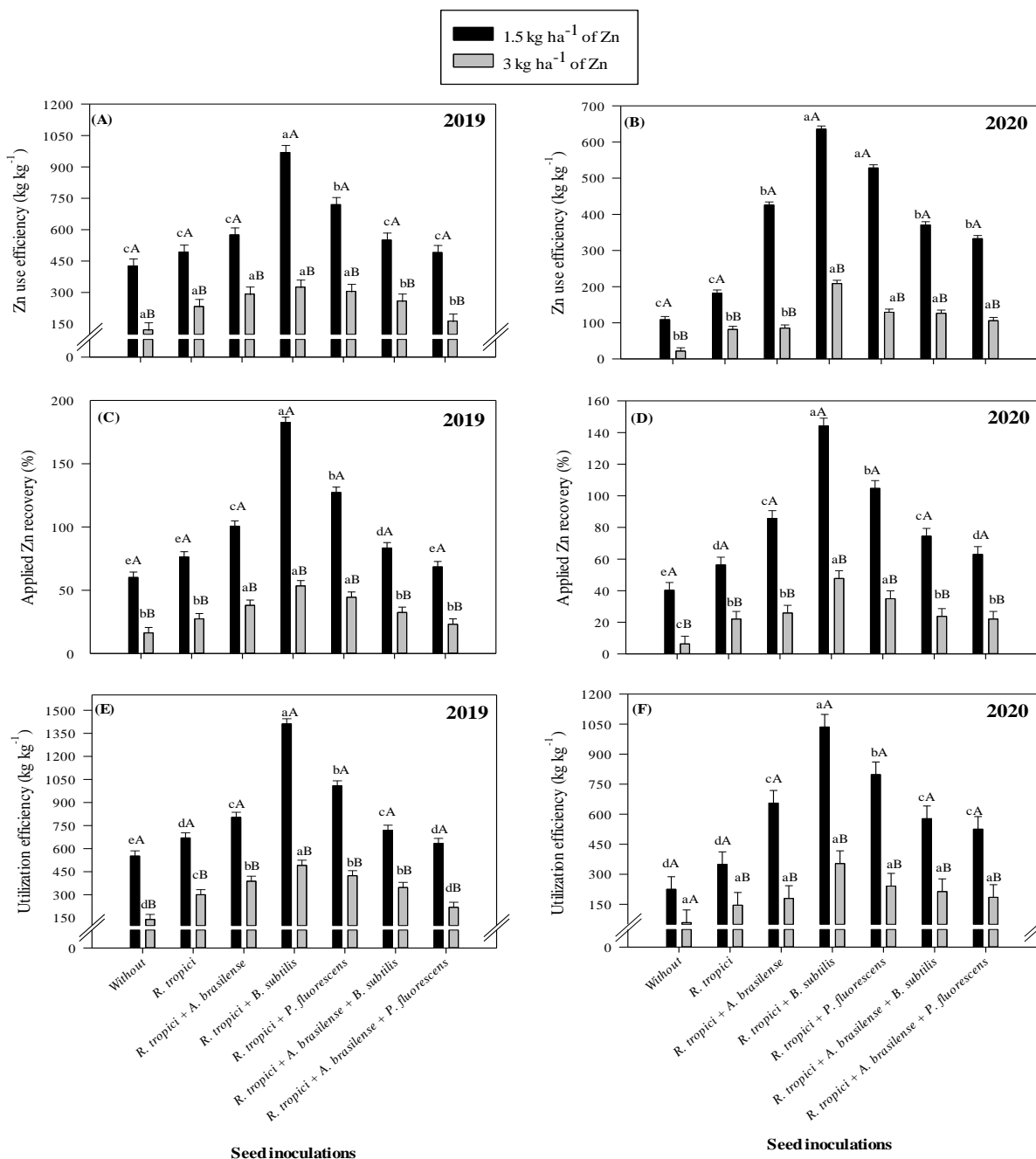
Co-inoculation with *R. tropici* + *A. brasilense* increased grain N concentration of common bean by 15.2% in 2019 as compared to without inoculation treatments (Table 4.3). In addition, interaction of nano-Zn foliar doses and co-inoculation with PGPBs was significant (Figure 4.6B). Co-inoculation with *tropici* + *B. subtilis* along with nano-Zn foliar fertilization at a dose of 1.5 kg ha<sup>-1</sup> increased grain N concentration as compared to without inoculation in 2020 crop season (Figure 4.6B). Overall co-inoculation increased grain N concentration as compared to without inoculation regardless of foliar Zn fertilization. The lowest grain N concentration was noted in control treatments.

Grain P concentration was increased with co-inoculation of *R. tropici* and *R. tropici* + *A. brasilense* + *P. fluorescens* in 2019 and 2020 crop seasons respectively, which were statistically similar with *R. tropici* + *A. brasilense* + *B. subtilis* (Table 4.3). In both cropping years, nano-Zn foliar fertilization at a dose of 1.5 kg ha<sup>-1</sup> increased grain N concentration as compared to other applied foliar nano-Zn doses.

#### 4.3.4 Zinc use efficiencies

Foliar nano Zn and inoculation with PGPBs, and their interactions were significant for Zn use efficiency in both 2019 and 2020 cropping seasons (Appendix A; Table 4). The co-application of foliar nano Zn at a dose of 1.5 kg ha<sup>-1</sup> with *R. tropici* + *B. subtilis* increased Zn use efficiency as compared with 3 kg ha<sup>-1</sup> and other inoculations treatments in both cropping seasons of common bean (Figure 4.7A,B). Interestingly, the treatments with 1.5 kg ha<sup>-1</sup> foliar nano Zn application was observed with higher Zn use efficiency regardless of the inoculations in relation to 3 kg ha<sup>-1</sup> foliar nano Zn in both cropping seasons (Figure 4.7A,B). The lowest Zn use efficiency in both common bean cropping seasons was observed at 3 kg ha<sup>-1</sup> foliar nano Zn application under no-inoculation treatments (Figure 4.7A,B).

**Figure 4.7.** Zinc (Zn) use efficiency (A,B), applied Zn recovery (C,D), and utilization efficiency (E,F) in 2019 and 2020 common bean cropping seasons respectively, as a function of foliar nano Zn application and co-inoculations of plant growth-promoting bacteria.



**Source:** Author. The upper case letters are used for the interactions of PGPBs inoculations within each dose of foliar nano Zn application, whereas lower case letters are used for the interaction of

foliar nano Zn doses within each inoculation treatment. The identical alphabetic letters are statistically similar with each other as analyzed by Tukey test for foliar Zn doses ( $p \leq 0.05$ ) and Scott-Knott test for PGPBs inoculations ( $p \leq 0.05$ ) in 2019 and 2020 respectively. Error bars indicate standard error of the means ( $n = 4$  replications).

Agro-physiological efficiency (APE) of common bean was positively influenced by foliar nano Zn doses and inoculation with PGPBs only in 2019 while the treatments effect and interaction were not significant in 2020 cropping season (Appendix A; Table 4). The treatments with foliar nano Zn at a dose of  $3 \text{ kg ha}^{-1}$  increased APE by 19% in 2019 cropping season. The treatments with inoculation of *R. tropici* were observed with higher APE, which was statistically at par with triple inoculation of *R. tropici* + *A. brasilense* + *B. subtilis* and *R. tropici* + *A. brasilense* + *P. fluorescens*, co-inoculation of *R. tropici* + *A. brasilense*, and without inoculation treatments (Appendix A; Table 4).

The interactions and treatment effect of foliar nano Zn and inoculation with PGPBs were significant for applied Zn recovery (AZnR) in 2019 and 2020 (Appendix A; Table 4). The treatments with foliar nano Zn at a dose of  $1.5 \text{ kg ha}^{-1}$  along with co-inoculation of *R. tropici* + *B. subtilis* in both cropping seasons as compared to other treatments (Figure 4.7C,D). Interestingly, AZnR was increased with  $1.5 \text{ kg ha}^{-1}$  foliar nano Zn application under inoculation, co-inoculation and without inoculation treatments as compared with  $3 \text{ kg ha}^{-1}$  foliar nano Zn application. The lowest AZnR in the first cropping season was observed without inoculation and foliar nano Zn application at a dose of  $3 \text{ kg ha}^{-1}$  as compared to other treatments (Figure 4.7C,D).

Foliar nano Zn doses, inoculation with PGPBs and their interaction had positively improved Zn utilization efficiency (UE) of common bean in both studied years (Appendix A; Table 4). The highest Zn UE was observed with foliar nano Zn at a dose of  $1.5 \text{ kg ha}^{-1}$  in combination with co-inoculation of *R. tropici* + *B. subtilis* in both 2019 and 2020 cropping seasons as compared to other treatments (Figure 4.7E,F). In general, the treatments with  $1.5 \text{ kg ha}^{-1}$  foliar nano Zn application performed better within all inoculation, co-inoculation and without inoculation treatments as compared with  $3 \text{ kg ha}^{-1}$  foliar nano Zn application. The lowest Zn UE was noted with  $3 \text{ kg ha}^{-1}$  foliar nano Zn application under without inoculation treatments as compared to other treatments (Figure 4.7E,F).

#### 4.3.5 Pearson's correlation

There were positive and significant correlations between zinc use efficiency and plant height, shoot dry matter, shoot-grain Zn concentration and accumulation, Zn partitioning index, Zn intake in Brazil under common beans cultivation in 2019 regardless the treatments applied (Appendix B: Figure 2A). A positive correlation was observed between Zn partitioning index and shoot and grain Zn accumulation, applied Zn recovery, shoot dry matter, and grain yield of common beans in 2020 cropping season (Appendix B: Figure 2B).

#### 4.4 Discussion

Plants growth and productivity are affected by a variety of environmental factors, thus several sustainable strategies have been adapted to maintain quantity and quality of crop plants under harsh tropical conditions (GALINDO *et al.*, 2021). In this context, nano Zn fertilizers have attracted the attention by providing nutrients to plants in more technical manner to minimize leaching and adsorption as well as improving fertilizer efficiency and grain tissue assimilation (SALAMA *et al.*, 2019; JALAL *et al.*, 2022). Plant growth-promoting bacteria (PGPBs) are being used as sustainable and eco-friendly approach to Zn availability to plants through several direct and indirect mechanisms (PATEL AND ARCHANA 2017; LAMBRESE *et al.*, 2018; YAHAGHI *et al.*, 2019), leading to better grains biofortification (JALAL *et al.*, 2022). Thus, the positive interaction between foliar nano Zn and inoculation/co-inoculation with PGPBs (Appendix B: Figure 2) endorsed the hypothesis of the present study.

The present results indicated that foliar nano Zn and co-inoculation with *R. tropici* + *B. subtilis* were observed with taller plants and greater shoot dry matter (Table 4.1), and grain yield of common bean (Figure 4.3). The increase might be driven by the direct mechanisms of PGPBs that could enhance nutrients availability by either fixation, solubilization or alteration of hormonal activities and Zn involvement in maintaining cell division, elongation and photosynthesis and regulatory co-factor in protein synthesis (ROSSI *et al.*, 2019; SWARNALAKSHMI *et al.*, 2020). Previous studies have reported that inoculation of PGPBs either alone or with Zn could alter different enzymatic activities of soil and plant to enhance plant growth, yield and biofortification of edible tissues (TANVEER *et al.*, 2022; JALAL *et al.*, 2021). Plant growth-promoting bacteria could increase vegetative growth of crop plants that contribute to higher productivity at later reproductive stage (SOUSA *et al.*, 2021). In addition, Zn has critical role in regulation of cell

multiplication and elongation as well as several biochemical function of plant that could ultimately increase shoot dry matter and productivity of different crops (DOOLETTE *et al.*, 2018; Jalal *et al.*, 2020). The co-application of Zn and PGPBs could increase pod formation through pollen development that could contribute to better growth and yield of legumes (ULLAH *et al.*, 2019c, 2020).

Zinc deficiency is an alarming issue in agricultural soils that caused stagnation in agronomic biofortification and productivity (JALAL *et al.*, 2020). However, Zn foliar spray could be an efficient alternative strategy to cope Zn edaphic deficiency by ameliorating its bioavailability in edible tissue, leading to biofortification (MISHRA, 2022). In this context, the present study indicated that treatments with foliar Zn and inoculation of PGPB were observed with higher Zn, N and P concentration and uptake in shoot and grains of common bean (Table 4.2, 4.3). Foliar nano Zn and co-inoculation with *R. tropici* + *B. subtilis* were noted with higher shoot and grain Zn concentration (Table 4.2, Figure 4.4A, B) and also shoot and grain Zn uptake (Figure 4.4C, D, E). This might be due to the positive interaction of applied inoculant with native microbial community in roots rhizosphere that could articulate root architecture and stimulating nutrients availability for plant uptake (SINGH; PRASANNA, 2020). In addition, PGPB have the ability to produce organic acids, chelating agents and siderophores that could not only promote plant growth and productivity but also increasing Zn assimilation to the edible tissue to sustain biofortification (UPADHAYAY *et al.*, 2022; VERMA *et al.*, 2022). The co-application of PGPB with foliar/soil Zn could reduce phytic acid concentration in the edible tissue, thus contributing to higher Zn concentration in embryo, aleuronic, endosperm and whole grains of cereal (REHMAN *et al.*, 2018).

Zinc partitioning index (Appendix A; Table 3) and daily Zn estimated intake in common bean grains were improved with foliar nano Zn fertilization along with co-inoculation of PGPBs (Figure 4.5A, B). It might be due to the positive interaction of plants and microbes that could biosynthesis several compounds including phenolic acid, siderophores, organic acids and phytohormones to contribute different biochemical and metabolic functions of plants (REHMAN *et al.*, 2018; KHOSHRU *et al.*, 2020a). In addition, foliar Zn has also been reported a rapid strategy that could improve translocation and redistribution of applied Zn into newly generated grains to deal with human malnutrition (DOOLETTE *et al.*, 2020; FIRDOUS *et al.*, 2020). It has also reported in previous studied that Zn and PGPB contributed to the biosynthesis of nodules and leg-hemoglobin, where root nodulation could improve transportation of Zn in sucrose from leaves to

nodules and leg-hemoglobin could optimize plant growth (KRYVORUCHKO, 2017; ULLAH *et al.*, 2020).

The combined application of Zn with PGPBs has the ability to increase Zn efficiency by dissolving carbonates and oxides of Zn as compared to individual Zn fertilization (SARAVANAN *et al.*, 2007). Zinc efficiencies are being defined by the availability of Zn in edible tissues in Zn deficient soils (FAGERIA *et al.*, 2011), where foliar Zn fertilization is considered a better option for biofortification and higher yield (FAGERIA *et al.*, 2012). Therefore, the present results indicated that combined application of foliar nano Zn with co-inoculations of PGPBs improved Zn use efficiency (Figure 6A, B), agro-physiological efficiency (Appendix A; Table 3), applied Zn recovery (Figure 6C, D), and utilization efficiency (Figure 6E, F) in tropical savannah of Brazil. The possible reason of higher Zn efficiencies might be due to higher Zn concentration and uptake in shoot and grains (Table 4.2, Appendix A; Table 3) and greater biomass and grain yield (Table 4.1). Plant growth-promoting bacteria adapt several mechanism including nutrients solubilization, nitrogen fixation, organic acids production and enzymatic activities that could possibly increase nutrient absorption and re-localization to stem and grains tissues (ABADI *et al.*, 2021; HAKIM *et al.*, 2021). It has also previously reported that Zn in combination with diazotrophic bacteria could improve bioavailability and translocation of Zn to shoot and grains, leading to higher Zn use efficiencies (ULLAH *et al.*, 2020; JALAL *et al.*, 2021). Hence, current results are forefront step to understand influence of foliar nano Zn fertilization and inoculation with PGPBs on growth, yield and biofortification of common bean, emphasizing on the integrated use of foliar Zn and PGPBs to ameliorate Zn concentration, yield and Zn use efficiencies in tropical savannah.

#### **4.5 Conclusions**

Foliar nano zinc (Zn) fertilization is an efficient and rapid strategy of nutrients delivery that enhance plant performance and nutrient use efficiencies in a sustainable manner. The multifaceted functions of plant growth-promoting bacteria (PGPBs) to increase productivity and nutrition of crop plants while maintaining sustainability of agriculture is not deniable. Hence, it has derived from the current results that foliar nano Zn application along with co-inoculation of PGPBs improved plant growth, yield and, shoot and grain nutrition of common bean. Zinc partitioning index and estimated daily Zn intake as well as Zn efficiencies including use efficiency, agro-physiological efficiency, applied Zn recovery and utilization efficiency were also increased

with foliar nano Zn and co-inoculation of PGPBs in common bean cultivation. It has concluded that foliar nano Zn application at a dose of  $1.5 \text{ ha}^{-1}$  along with co-inoculation of *R. tropici* + *B. subtilis* was observed the most effective for improving performance and yield, Zn nutrition and its efficiencies, leading to biofortification of common bean grains in tropical savannah. In addition, nitrogen and phosphorous shoot and grain concentrations were increased with co-inoculation of *R. tropici* + *P. fluorescens* and *R. tropici* + *B. subtilis* under nano-Zn foliar fertilization. Therefore, it is recommended that applying nano Zn via foliar along with co-inoculation of PGPBs could improve productivity and nutrition of common bean in more sustainable manner. The present study showed integrated use of synthetic fertilizers with PGPBs however, we believe that it's not remain an ambitious to highlight that PGPBs/biostimulants have the potential to replace synthetic fertilizers in near future. The molecular and physiological behavior of these PGPBs would attract more attention of agriculturists, which will bring the technology out of developing stage and can highly improve sustainability and agriculture under harsh environmental conditions.

## CHAPTER-5

### 5. AGRONOMIC BIOFORTIFICATION OF WHEAT WITH ZINC IN ASSOCIATION WITH DIAZOTROPHIC BACTERIA UNDER TROPICAL SAVANNAH

#### 5.1 Introduction

Zinc (Zn) is a micronutrient for all living organisms including plants, humans and microflora (ESTRADA-DOMINGUEZ *et al.*, 2020; YASEEN; HUSSAIN, 2020) which is required to better tackle their metabolic and physiological activities (SHAIKH; SARAF, 2017). Zinc is one of the persistent deficiencies in tropical crops (MELO *et al.*, 2018) and suffering almost 17.3% (2 billion) of the world population (MAXFIELD; CRANE 2019; BOLLINEDI *et al.*, 2020). It has been ranked 5<sup>th</sup> health affecting factor and concurrently declaring as “hidden hunger” with several health issues (WHO, 2007; PONIEDZIAŁEK *et al.*, 2020), especially in developing countries (BHATT *et al.*, 2020). The inadequate dietary status of Zn is anti-proportional to human health and leads to several diseases (GUPTA *et al.*, 2020) including immune deficiency syndrome, pneumonia, memory disorder, cancer, respiratory, and cardiovascular disorder and diarrhea etc. in human (GAMMOH; RINK 2017; UWITONZE *et al.*, 2020). In current scenario, Zn deficiency is interestingly one of the predisposing factors that could increase COVID-19 infection and progression (SKALNY *et al.*, 2020; WESSELS *et al.*, 2020).

The deficiency of Zn in the crop plants is one of the alarming concerns of today and the most effective micronutrient encountering cereals productivity (WANG *et al.*, 2012). Zinc plays critical role in several plant physiological processes, protein synthesis (KRYVORUCHKO, 2017), energy production and maintenance of membrane integrity (Drissi *et al.*, 2017). It can also enhance photosynthesis and enzymes activities such as peptidases, dehydrogenases and phosphohydrolases, and pollen fertility (REHMAN *et al.*, 2019; LIU *et al.*, 2020), and cell growth and multiplication (DAVARPANA *et al.*, 2016; DOOLETTE *et al.*, 2018). Thus, it is required to adopt inexpensive and easy agronomic mechanisms (soil, foliar, seed priming, and synthetic solution) to deal with Zn deficiency in soil, plant and human beings (FAROOQ *et al.*, 2018; ULLAH *et al.*, 2019c). The most commonly adapted mechanism for higher Zn concentration and yield in field crops is soil Zn application which could improve effectiveness of nutrition and dietary intake for targeted population (HAIDER *et al.*, 2018; AHSIN *et al.*, 2020). However, sole Zn

application may not be enough to combat Zn deficiency and also increase crop establishment, productivity, Zn grain concentration and Zn use efficiencies in especial, tropical regions due to complexation with carbonates and oxides (ZHANG *et al.*, 2017; JALAL *et al.*, 2021). Hereby, environmentally friendly and sustainable strategies such as diazotrophic bacteria inoculation should a better mechanism to tackle biofortification of cereals with better nutrients replenishment, productivity and Zn use efficiencies.

Nutrients enriched plant rhizosphere environment significantly stimulate several ecological processes like, decomposition of organic matter, homeostasis, nutrient cycling to reduced crop dependency on synthetic fertilizers to support sustainable and stable ecosystem (LALITHA, 2017; HAKIM *et al.*, 2021; JALAL *et al.*, 2020). Plant growth-promoting bacteria (PGPBs) or diazotrophic bacteria are adapting several direct and indirect mechanism to improve plant growth (Khoshru *et al.*, 2019; JALAL *et al.*, 2021). They improve nutrients availability by playing role in biological nitrogen fixation, nutrients solubilization and enzymes synthesis through direct mechanisms (KHOSHURU *et al.*, 2019; SEDLAKOVA-KADUKOVA *et al.*, 2019), whereas inhibiting pathogens infestation by producing siderophores and antibiotics (LAMBRESE *et al.* 2018). Zinc solubilizing bacteria could be apply in one or another above mention mechanisms to increase Zn solubility through production of organic and inorganic acids and several chelators (Idayu *et al.*, 2017; Khoshru *et al.*, 2020). A diverse range of bacteria including species of *Rhizobium*, *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Acinetobacter* and many others may solubilize or tolerate Zn and plant growth promoters (MUMTAZ *et al.*, 2017; ULLAH *et al.*, 2020; JALAL *et al.*, 2021; KUSHWAHA *et al.*, 2021).

Wheat is being cultivated as a staple crop worldwide with higher nutritional quality for human beings (LIU *et al.*, 2021) as compared to other staple crops (CAKMAK, 2008). The food security, increasing production demands and weathered soils in tropical Savannah are the most challenging issues that lead to vulnerable agriculture inputs, low organic matter, malnutrition (specifically micronutrients as like Zn) (GOMES *et al.*, 2019). In addition, the higher required phosphorous constraint Zn nutrition in cereal crops in tropical agriculture systems through formation Al and Fe-oxides (PENN; CAMBERATO, 2019; AMANULLAH *et al.*, 2020). Thereby to better under the accumulation and distribution of Zn, biofortifying staple crops (especially wheat) with micronutrients (especially Zn) has been grabbed the attention of the world to combat with “hidden hunger” in a sustainable way.

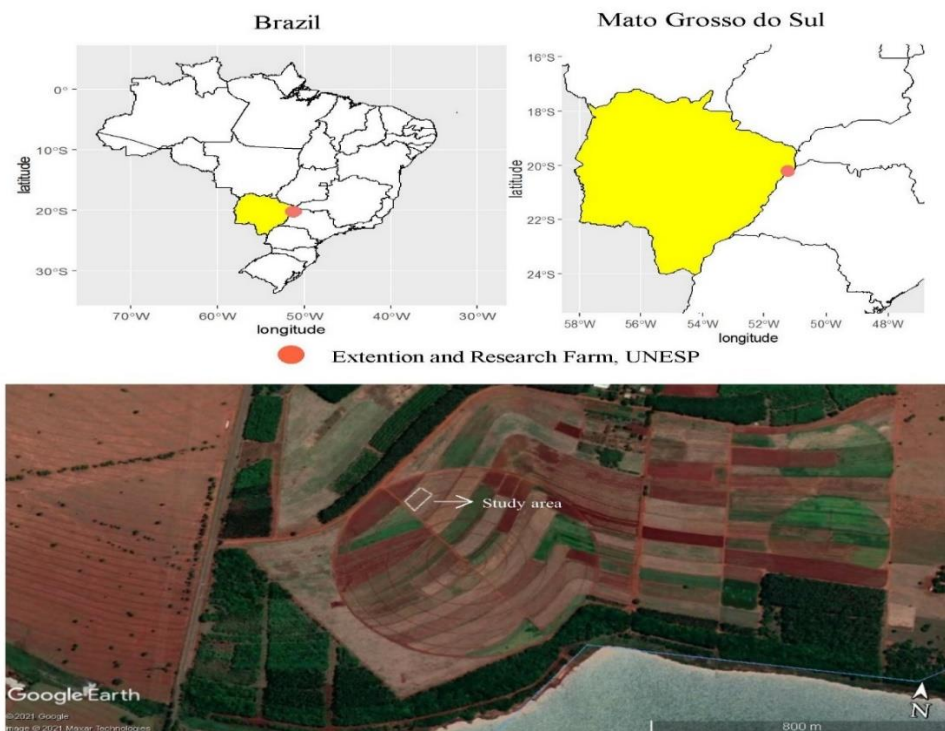
The literature is lacking with the effect of Zn biofortification under the interaction of diazotrophic bacteria and soil applied Zn in tropical Savannah. There is a research gap on the association of diazotrophic bacterial inoculation and Zn fertilization on Zn nutrition, Zn use efficiency (ZnUE) and yield of wheat crop. Therefore, we hypothesized that inoculations of different diazotrophic bacteria might have synergetic relation with soil Zn application on plant and grain concentrations, growth, yield, ZnUE and daily intake of biofortified wheat grains in tropical Savannah of Brazil. Therefore, the objectives of the current were to evaluate the better performing inoculant of diazotrophic bacteria in presence and absence of soil Zn application on wheat growth, leaf and grains Zn concentration and accumulation, yield and Zn use efficiencies in two consecutive growing seasons.

## **5.2 Materials and Methods**

### **5.2.1 Experimental Area and Location**

The field experiment on wheat crop were carried out for two consecutive cropping seasons (2019 - 2020) at the research farm of School of Engineering (UNESP) under the geographical coordinates (20° 22' S, 51° 22' W, and 335 m altitude: Figure 5.1), at Selvíria in Mato Grosso do Sul (MS), Brazil.

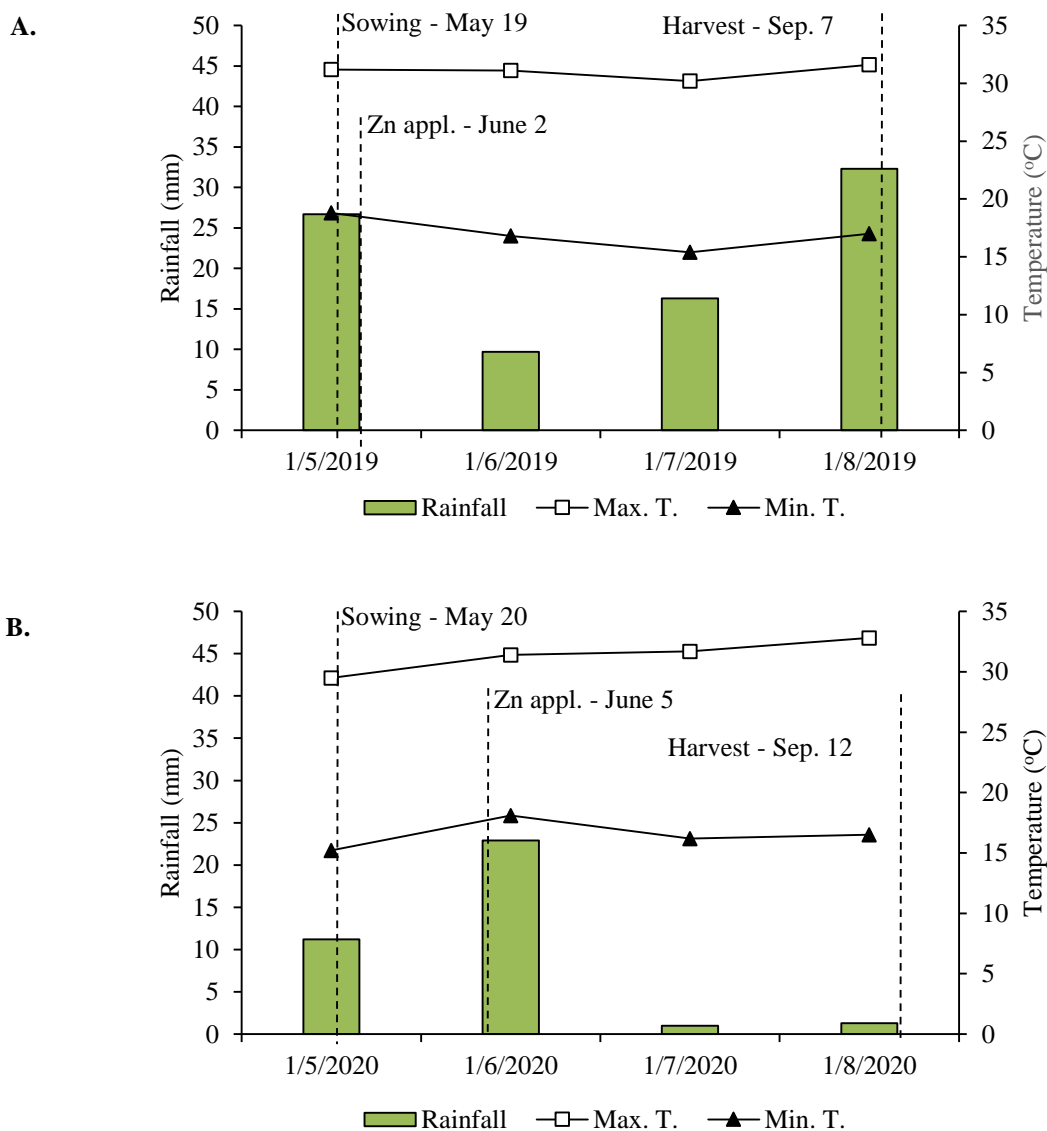
**Figure 5.1.** Location of the experimental area at Selvíria - Mato Grosso do Sul state, Brazil (20°22'S, 51°22'W, altitude of 335 m) in 2019 and 2020 crop seasons.



**Source:** Author. The map was created using `pacot`, `geobr` and `ggplot` with R software (R software (R DEVELOPMENT CORE TEAM, 2015). Projection System WGS 84/UTM 200DC [EPSG: 4326]. This image was taken from Google Earth program, Google Company (2021). Map data: Google, Maxar Technologies.

The soil was classified as Rhodic Haplustox with clay texture (SOIL SURVEY STAFF, 2010). The experimental site used for the cultivation of annual crop (cereals and legumes) for more than 28 years, the last 13 being under no tillage. The climate of experimental area is classified as Aw according to Köppen classification (ALVARES *et al.*, 2013) and data of both cropping seasons are summarized in Figure 5.2.

**Figure 5.2.** Rainfall maximum, average and minimum temperatures and air relative humidity are acquired from the weather station of Research Farm of Faculty of Engineering- UNESP during wheat cultivation period from May to September, 2019 (A) and May to September, 2020 (B).



### 5.2.2 Soil Analysis

A composite soil sample from 0- 0.20 m depth was collected for physical and chemical properties of the soil before the implantation of the experiment, following standard procedures of

van Raij *et al.* (2001). The physical and chemical properties are indicated in Table 5.1 with clayey texture and slightly acidic having minimal Zn concentration.

**Table 5.1.** Analyzed results of soil sample (0 – 0.20 m) from experimental site before experiment implementation.

pH (CaCl <sub>2</sub> )	P (resin)	S-SO <sub>4</sub> <sup>2-</sup>	K <sup>+</sup>	Ca <sup>2+</sup>	Mg <sup>2+</sup>	Al <sup>3+</sup>	H+Al	
---	--- mg dm <sup>-3</sup> ---	----- mmol <sub>c</sub> dm <sup>3</sup> -----						
5.2	37	5.0	2.3	27	17	0.01	35	
CEC*	BS*	OM*	B (Hot water)	Zn (DTPA)*	Fe (DTPA)	Mn (DTPA)	Cu (DTPA)	
mmol <sub>c</sub> dm <sup>-3</sup>	---%---	g kg <sup>-1</sup>	-----mg dm <sup>-3</sup> -----					
71.5	52	19	0.15	0.7	29	41	4.0	

\* CEC: Cation exchange capacity at pH 7.0; BS: Bases saturation; OM: Organic matter; DTPA: Diethylenetriaminepentaacetic acid

### 5.2.3 Experimental design and treatments

The experimental design for the wheat crop was randomized complete block having 4 x 2 factorial scheme with four replications. The experimental factors were comprised of bacterial seeds inoculations (1- No inoculation - Control, 2- *Azospirillum brasilense*, 3- *Bacillus subtilis*, 4- *Pseudomonas fluorescens*), and without and with soil applied Zn (0 and 8 kg Zn ha<sup>-1</sup>).

The inoculation of bacterium *A. brasilense* strains Ab-V5 and Ab-V6 (strains CNPSo 2083 =Ab-V5 and CNPSo 2084 =Ab-V6 with guarantee of 2x10<sup>8</sup> CFU mL<sup>-1</sup>) was performed at the dose of 300 mL ha<sup>-1</sup> of inoculant (liquid) of sown seeds. The inoculation of *B. subtilis* (strain CCTB04 with guarantee of 1x10<sup>8</sup> CFU ml<sup>-1</sup>) and *P. fluorescens* (strain CCTB03 with guarantee of 2x10<sup>8</sup> CFU mL<sup>-1</sup>) were applied at the dose of 150 mL ha<sup>-1</sup> of inoculant (liquid) according to the recommendation of inoculants providing company (Total Biotechnology®), Curitiba, Brazil. The inoculations were performed an hour before plantation of the crop. These inoculations were oriented and repeated in both cropping seasons of 2019 and 2020.

Zinc was applied from the source of zinc sulfate (21% Zn and 11% of S) to the soil surface by side dressing cover. Zinc application (0 and 8 kg ha<sup>-1</sup>) was manually performed with even distribution and irrigated with central pivot irrigation system (14 mm) to incorporate Zn fertilizer into the soil. The amount of Zn as per treatment per plot was applied in V1/V2 stage (1/2 leaves completely unfolded), 14 days after sowing (DAS) in 2019 and 16 DAS in 2020 cropping seasons.

The Zn dose applied to the soil is based on Fageria *et al.*, (2002) who recommended 5 to 30 kg/ha of soil Zn fertilization.

#### 5.2.4 Plant materials

The experimental site was thoroughly sprayed with herbicides (glyphosate, 1800 g ha<sup>-1</sup>; carfentrazone, 40 g ha<sup>-1</sup> and cletodim, 240 g ha<sup>-1</sup> of a.i.) for controlling all narrow and broad leaves weeds 15 days before experiment implementation. The seeds were chemically treated with Standak Top® [co-formulation of fungicides {carbendazim + thiram (45 g + 105 g of active ingredient (a.i.))} and insecticides {imidacloprid + thiodicarb (45 g + 135 g of a.i.)}] per 100 kg seed before inoculation and plantation. This chemical seed treatment is a common agriculture practice in Brazil before growing cereal crops. The seeds after chemical treatment were let to dry and then inoculated manually an hour before planting in each respective season.

The wheat cultivar (TBIO SOSSEGO) with potential quality and production was sown on 11<sup>th</sup> May, 2019 and 3<sup>rd</sup> May, 2020 respectively on a no-till with drill sowing method. A basal dose of 270 kg ha<sup>-1</sup> of 08-28-16 (N-P<sub>2</sub>O<sub>5</sub>-K<sub>2</sub>O) was performed at sowing according with soil analysis results with the recommendation of Cantarella *et al.* (1997). Each plot was comprised of 13 rows, 5 m long and 0.17 m apart, totalizing 12.15 m<sup>2</sup> with a useful area of 9.35 m<sup>2</sup>. The recommended dose of 120 N kg/ha was applied from ammonium sulphate fertilizer after 30 days of emergence. The experimental area was applied with Boron of 1 kg ha<sup>-1</sup> as boric acid (18% of B) through tractor sprayer machine on the basis of soil analysis (Table 5.1) with the interpretation of Campinas Agronomic Institute (IAC) (RAIJ *et al.*, 2001). The crop was irrigated with sprinkler central-pivot irrigation system (14 mm in average) according to crop need. The crop was manually harvested on 7<sup>th</sup> September, 2019 (with 111 days cycle) and 12<sup>th</sup> September, 2020 (with 114 days cycle).

#### 5.2.5 Evaluations and Analysis

The plant material (leaf, shoot and grain) was collected in proper labeled paper bags and was dried in an air-tight oven at 60 ± 5 °C for 72 h until attained a uniform humidity. The material was then ground in a stainless-steel Willey knife mill by passing through a 10-mesh sieve and placed in labeled plastic. Each sample was weighed (0.25 g), digested with nitroperchloric digestion (HNO<sub>3</sub>:HClO<sub>4</sub> solution) and quantified by atomic absorption spectrophotometry. The analysis was developed by following methodology of Malavolta *et al.* (1997).

Plant height at maturity was determined with ruler meter from the ground to the upper apex. Shoot dry matter was determined after harvest of useful four central lines. The wheat was harvested and packed in jute bags and then dried in the shade for approximately 1 week. Each plot sample was threshed with electric thresher to attain weight of processed grains for calculating yield into  $\text{kg ha}^{-1}$  (productivity at 13% moisture content). After drying, the grains were ground in a Willey mill (Marconi, Brazil) for analysis of nutrients.

The Zn accumulation in shoot and grains ( $\text{g ha}^{-1}$ ) were calculated in equations 1 and 2:

$$\text{ZnSA} = \frac{\text{Zinc concentration in shoot} \times \text{dry matter}}{1000} \quad (1)$$

$$\text{ZnGA} = \frac{\text{Zinc concentration in grains} \times \text{grain yield}}{1000} \quad (2)$$

Where: ZnSA = Shoot Zn accumulation and ZnGA = Grain Zn accumulation.

Zinc partitioning index (ZPI, %) toward grains and Zn intake in the world and Brazil were calculated following standard methodology of Rengel and Graham (1996) and Lessa *et al.* (2019) respectively.

$$\text{ZPI} = \frac{\text{Zinc concentration in grains}}{\text{Zinc concentration in shoot}} \times 100 \quad (3)$$

$$\text{Zn intake} = [\text{Zn grain}] \times C \quad (4)$$

Where: Zn intake ( $\text{g/person/day}$ ) is daily Zn consumption estimation per person, [Zn grain] ( $\text{g kg}^{-1}$ ) is Zn concentration in biofortified grains from the current study results and C ( $\text{g/person/day}$ ) is the mean consumption of wheat grains per person in world and Brazil (USDA, 2020).

The following Zn use efficiencies were calculated following the procedures based on standards formula of Fageria (2009).

$$\text{ZnUE} = \frac{\text{Grain yield ZnF} - \text{Grain yield ZnW}}{\text{Applied Zn dose}} \quad (5)$$

$$\text{APE} = \frac{\text{Grain yield ZnF} - \text{Grain yield ZnW}}{\text{ZnA in grain and shoot ZnF} - \text{ZnA in grain and shoot ZnW}} \quad (6)$$

$$\text{RAZn} (\%) = \frac{\text{ZnA in grain and shoot ZnF} - \text{ZnA in grain and shoot ZnW}}{\text{Applied Zn dose}} \quad (7)$$

$$UE = PE \times RAZn \quad (8)$$

Where: ZnUE = Zinc use efficiency; APE = Agro-physiological efficiency; RAZn = Recovery of applied Zn; UE = Utilization efficiency; PE = physiological efficiency; ZnF = Zn fertilized treatments and ZnW = without Zn fertilized treatments; ZnA = Zn accumulated.

### 5.2.6 Statistical analysis

All data were initially tested for normality using Shapiro and Wilk test which showed normal data distribution ( $W \geq 0.90$ ). The data were submitted to analysis of variance (F test). The Zn soil application, diazotrophic bacterial inoculations and their interactions were considered fixed effects in the model. When a main effect or interaction was observed significant by F test ( $p \leq 0.05$ ) and Tukey test ( $p \leq 0.05$ ) was used for comparison of means of soil Zn application. The Tukey test ( $p \leq 0.05$ ) was used for comparison of diazotrophic bacterial inoculations using the ExpDes package in R software (R DEVELOPMENT CORE TEAM, 2015).

The Pearson correlation analysis ( $p \leq 0.05$ ) was performed using R software (R Development Core Team). To create a heatmap, the corrplot package was used, using the "cor" and "cor.mtest" functions to calculate the coefficients and  $p$ -value matrices. Asterisks were added to the heatmap cells to the identification of significant correlations.

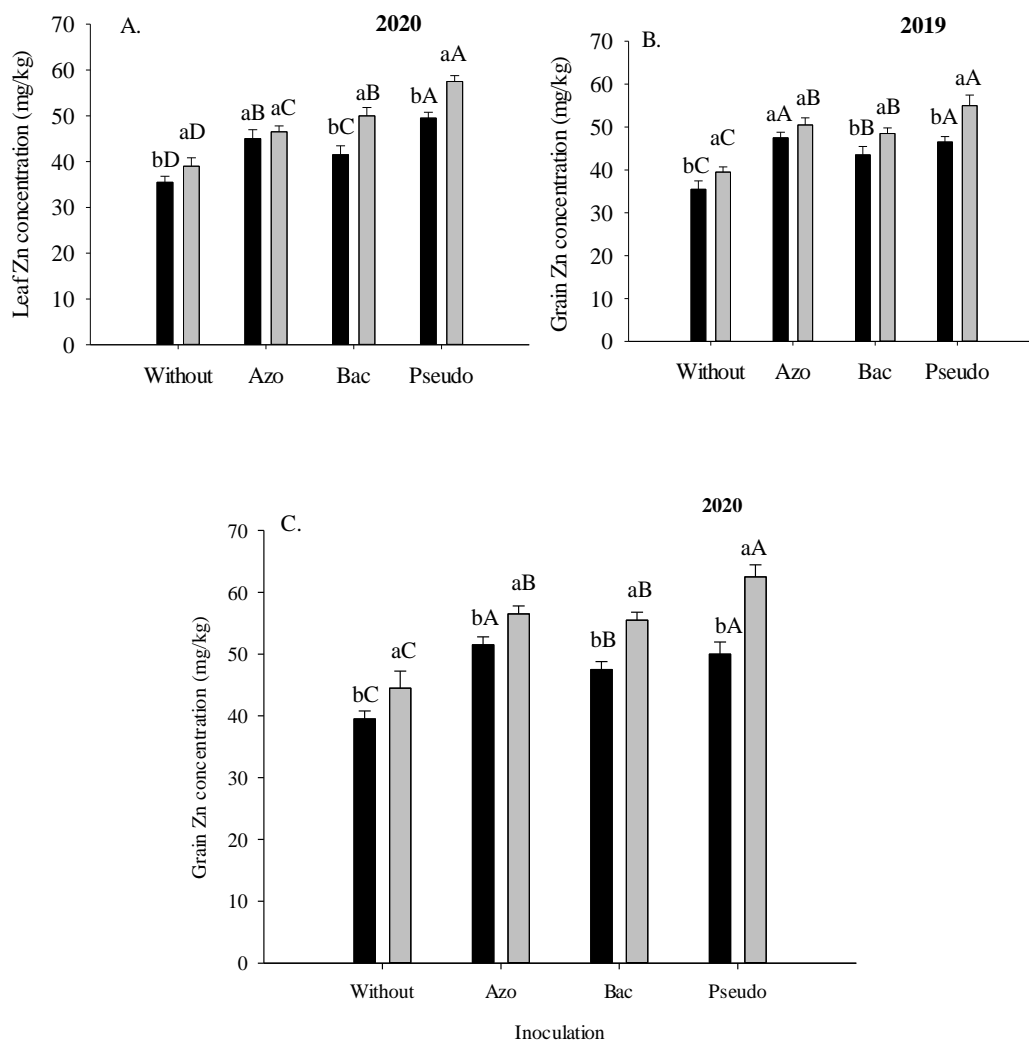
## 5.3 Results

### 5.3.1 Zinc concentration and accumulation in shoot and grains of wheat

The seed inoculation of bacteria with soil zinc (Zn) application was observed with positive influence on Zn concentration and accumulation in wheat plant (leaf diagnose and shoot) and grains (Appendix A; Table 5, Figure 5.3). The inoculation of diazotrophic bacteria along with soil Zn application increased leaf, shoot and grain Zn concentrations in wheat for both cropping seasons (Appendix A; Table 5, Figure 5.3). Leaf Zn concentration was increased by 15 and 11.6% in 2019 and 2020 as compared to without soil Zn application (Appendix A; Table 5, Figure 5.3 A). The inoculation of *P. fluorescens* along with soil Zn application increased leaf Zn concentration by 38.8 and 45.9% in 2019 and 2020 respectively when compared with non-inoculated treatments - control. The interaction of inoculation and soil Zn application also significantly influenced leaf Zn concentration in 2020 (Figure 5.3A) whilst found non-significant

in 2019 (Appendix A; Table 5). In addition, the inoculation of *A. brasilense* was observed with higher leaf Zn concentration under without soil applied Zn treatments (Figure 5.3A).

**Figure 5.3.** The influence of soil Zn and inoculations of diazotrophic bacteria on leaf and grain tissues Zn concentration in wheat. (A) Leaf Zn concentration (mg) in 2020 and (B, C) Grain Zn concentration (mg) in 2019 and 2020 respectively.



**Source:** Author. Without (No inoculation); Azo (*A. brasilense*); Bac (*B. subtilis*); Pseudo (*P. fluorescens*). The uppercase letters are used for inoculation interactions within each level of soil Zn application, whereas lowercase letters are used for the interaction of Zn levels within each inoculation treatment. The identical alphabetic letters do not differ from each other, as analyzed by Tukey (Zn application;  $p \leq 0.05$ ) and Scott–Knott (inoculations;  $p \leq 0.05$ ) tests for wheat

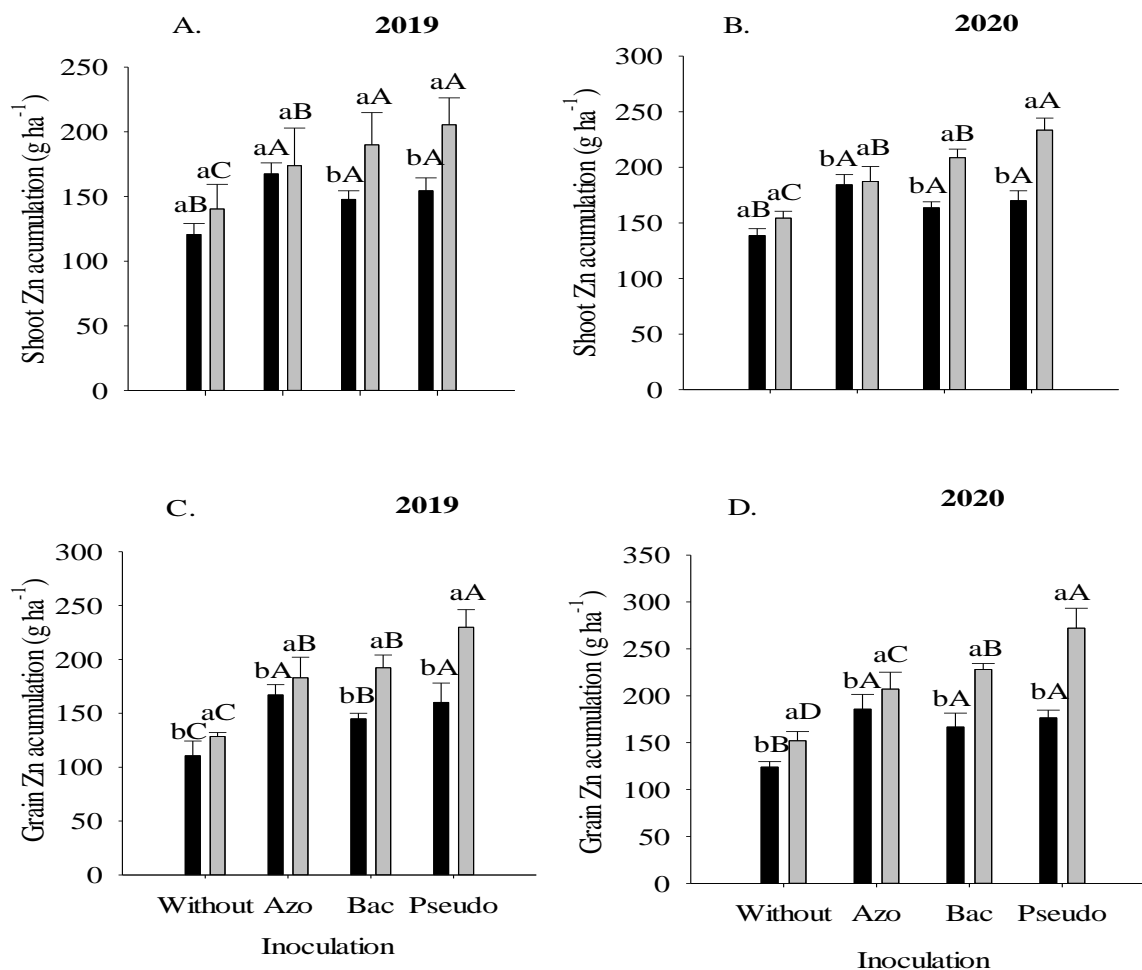
cropping year 2019 and 2020 respectively. Error bars indicate the standard error of the mean ( $n = 4$  replications). Selvíria, 2020.

Shoot Zn concentration of wheat was positively increased by soil applied Zn and diazotrophic bacterial inoculation (Appendix A; Table 5). The soil Zn application elevated shoot Zn concentration by 16.6 and 15.6% in 2019 and 2020 respectively. The inoculation of *P. fluorescens* together with soil Zn application increased shoot Zn concentration by 25% in 2019 which was statistically similar to the results obtained with inoculation of *A. brasilense* and *B. subtilis* as compared to control. The shoot Zn concentration in 2020 was increased by 31% when inoculated with *P. fluorescens* in comparison of non-inoculated treatments. The interactions for both cropping seasons were not significant (Appendix A; Table 5).

Grain Zn concentration was increased with inoculation of *P. fluorescens* and soil Zn application in both of the study years (Appendix A; Table 5). The interaction for grain concentrations in both cropping seasons were also significant (Figure 5.3B, C). The soil Zn fertilization elevated grain Zn concentration by 11 and 17% when compared to control in 2019 and 2020 respectively. The inoculation of *P. fluorescens* along with soil Zn application increased grain Zn concentration by 34% in 2019 (Figure 5.3B) and 33.3% in 2020 (Figure 5.3C) as compare to non-inoculated treatments. The inoculation of *A. brasilense* and *P. fluorescens* in the absence of Zn application was observed with higher grain Zn concentrations in both of the study years (Figure 5.3B, C).

Accumulation of Zn in shoot and grains were positively improved with soil applied Zn diazotrophic bacteria inoculations and their interactions in both cropping seasons (Appendix A; Table 5, Figure 5.4A, B, C and D). The shoot and grain Zn accumulations were improved by 19.6 and 25.3% in 2019, 21 and 32% in 2020 respectively under soil application of Zn (Appendix A; Table 5). In addition, inoculation of *P. fluorescens* was observed with greater shoot Zn accumulation (37.4%) which was statistically similar with inoculation of *A. brasilense* and *B. subtilis* in 2019, when compared with control (Figure 5.4A). Although, inoculation of *P. fluorescens* in 2020 was found with greater shoot Zn accumulation (44%) in comparison with control (Figure 5.4B). The shoot Zn accumulation in absence of soil applied Zn was greater with inoculation of *A. brasilense* in both of the cropping seasons (Figure 5.4A, B).

**Figure 5.4.** Influence of soil Zn and inoculations of diazotrophic bacteria on shoot and grain tissues Zn accumulation in wheat. (A, B) Shoot Zn accumulation ( $\text{g kg}^{-1}$ ) in 2019 and 2020 respectively; (C, D) Grain Zn accumulation ( $\text{g kg}^{-1}$ ) in 2019 and 2020, respectively.



**Source:** Author. Without (No inoculation); Azo (*A. brasilense*); Bac (*B. subtilis*); Pseudo (*P. fluorescens*). The uppercase letters are used for inoculation interactions within each level of soil Zn application, whereas lowercase letters are used for the interaction of Zn levels within each inoculation treatment. The identical alphabetic letters do not differ from each other, as analyzed by Tukey (Zn application;  $p \leq 0.05$ ) and Scott–Knott (inoculations;  $p \leq 0.05$ ) tests for wheat cropping year 2019 and 2020 respectively. Error bars indicate the standard error of the mean (n = 4 replications). Selvíria, 2020.

The inoculation of *P. fluorescens* in the presence of applied Zn significantly improved grain Zn accumulation (64 and 62.3%) in 2019 and 2020 (Figure 5.4C, D). In addition, under the absence

of Zn application, grain Zn accumulation was improved with inoculation of *A. brasilense* which was statistically similar to *B. subtilis* in 2019 (Figure 5.4C), whereas greater grain Zn accumulation was noted with *B. subtilis* which was similar to *A. brasilense* (Figure 5.4D).

### 5.3.2 Nitrogen (N) and phosphorous (P) concentration in shoot and grains

Shoot N and P concentration of wheat was positively influenced by soil Zn application and inoculation with diazotrophic bacteria (Table 5.2). Soil Zn application at the dose of 8 kg ha<sup>-1</sup> increased shoot N concentration by 10.3% only in 2020 wheat cropping season. While, inoculation with *P. fluorescens* increased shoot N concentration by 45.6 and 40.8% in 2019 and 2020 wheat cropping seasons respectively as compared to without inoculation treatments.

**Table 5.2** Nitrogen and phosphorous concentration in shoot and grains of wheat under the influence of diazotrophic bacteria with and without soil zinc (Zn) application.

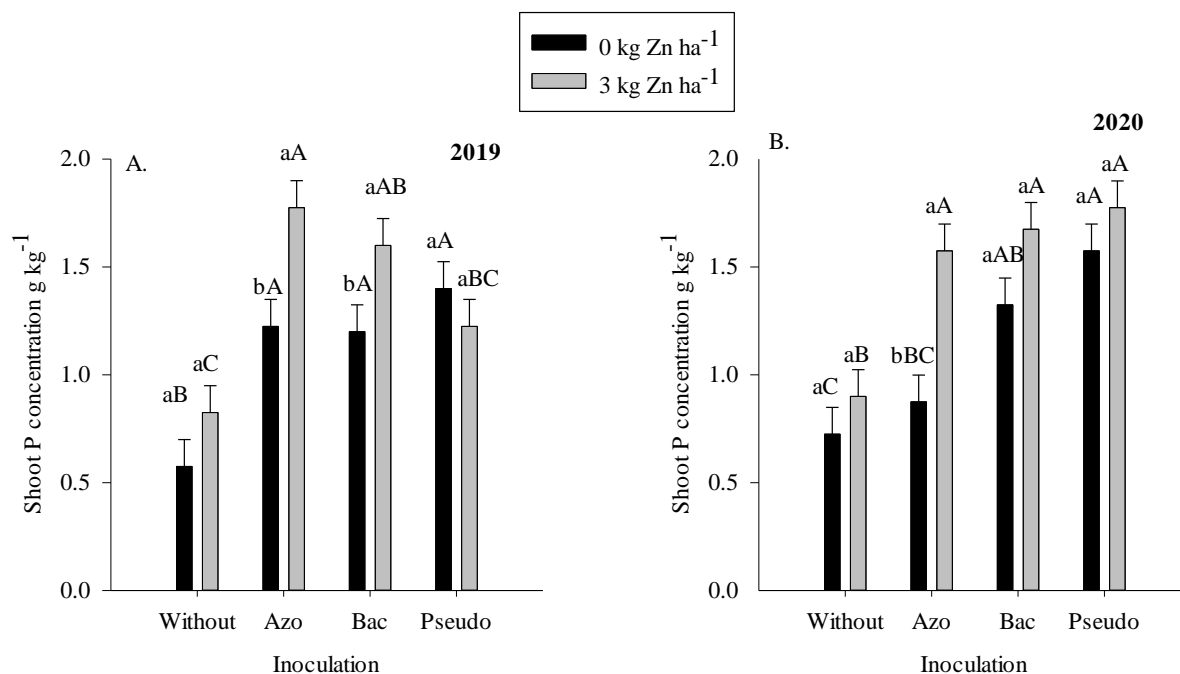
Treatments	Shoot N concentration		Shoot P concentration		Grain N concentration		Grain P concentration	
	----- g kg <sup>-1</sup> -----							
	2019	2020	2019	2020	2019	2020	2019	2020
<b>Zn application (kg ha<sup>-1</sup>)</b>								
0	5.7	5.8 b	1.1 b	1.1 b	23.5 b	24.5 b	3.8 b	2.6 b
8	6.0	6.4 a	1.4 a	1.5 a	24.9 a	28.3 a	4.5 a	4.7 a
<b>Inoculations (I)</b>								
Without	4.6 b	4.9 b	0.7 b	0.8 b	21.6 b	24.2 b	3.4 b	3.0 c
<i>A. brasilense</i>	5.5 b	6.0 a	1.5 a	1.3 a	24.6 a	27.0 a	4.3 a	3.6 bc
<i>B. subtilis</i>	6.6 a	6.5 a	1.4 a	1.5 a	26.0 a	28.1 a	4.6 a	4.3 a
<i>P. fluorescens</i>	6.7 a	6.9 a	1.3 a	1.6 a	24.6 a	26.2 ab	4.2 a	3.7 ab
<b>F- values</b>								
Zn	1.3 ns	4.4*	8.4**	16.4**	4.4*	41.6**	15.4**	185**
I	14.6**	10.7**	16.5**	15.3**	7.7**	8.1**	10.5**	11**
Zn x I	0.17 ns	0.40 ns	3.1*	4.9*	1.4 ns	1.8 ns	0.98 ns	1.6 ns
<b>CV (%)</b>	12.2	12.5	20.4	19	7.6	6.2	10.3	11.6

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ ); \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; ns—non-significant, by F-test.

Interaction of soil Zn application and inoculations was significant for shoot P concentration in both 2019 and 2020 wheat cropping seasons (Table 5.2). Inoculation with *A. brasilense* and *B. subtilis* under soil Zn application increased shoot P concentration in both cropping seasons (Figure 5.5). This was statistically similar with *P. fluorescens* inoculation treatments under the absence of

soil applied Zn application in 2019 and in the presence of soil Zn application in 2020 cropping season of wheat. All the treatments with inoculations performed better as compared to without inoculation regardless of the soil Zn application in both crop seasons (Figure 5.5).

**Figure 5.5.** Influence of soil Zn and inoculations of diazotrophic bacteria on shoot P concentration of wheat. (A, B) Shoot P concentration ( $\text{g kg}^{-1}$ ) in 2019 and 2020 respectively. Without (No inoculation); Azo (*A. brasilense*); Bac (*B. subtilis*); Pseudo (*P. fluorescens*).



**Source:** Author. The uppercase letters are used for inoculation interactions within each level of soil Zn application, whereas lowercase letters are used for the interaction of Zn levels within each inoculation treatment. The identical alphabetic letters do not differ from each other, as analyzed by Tukey (Zn application;  $p \leq 0.05$ ) and Scott–Knott (inoculations;  $p \leq 0.05$ ) tests for wheat cropping year 2019 and 2020 respectively. Error bars indicate the standard error of the mean ( $n = 4$  replications). Selvíria, 2020.

The interactions of soil Zn application and inoculation for grain N and P concentrations were not significant (Table 5.2). Soil Zn application increased grain N concentration by 5.9 and 15.5% while grain P concentration by 18.4 and 80.7% in 2019 and 2020 wheat cropping seasons respectively, when compared to control. In addition, inoculation with *B. subtilis* increased grain N

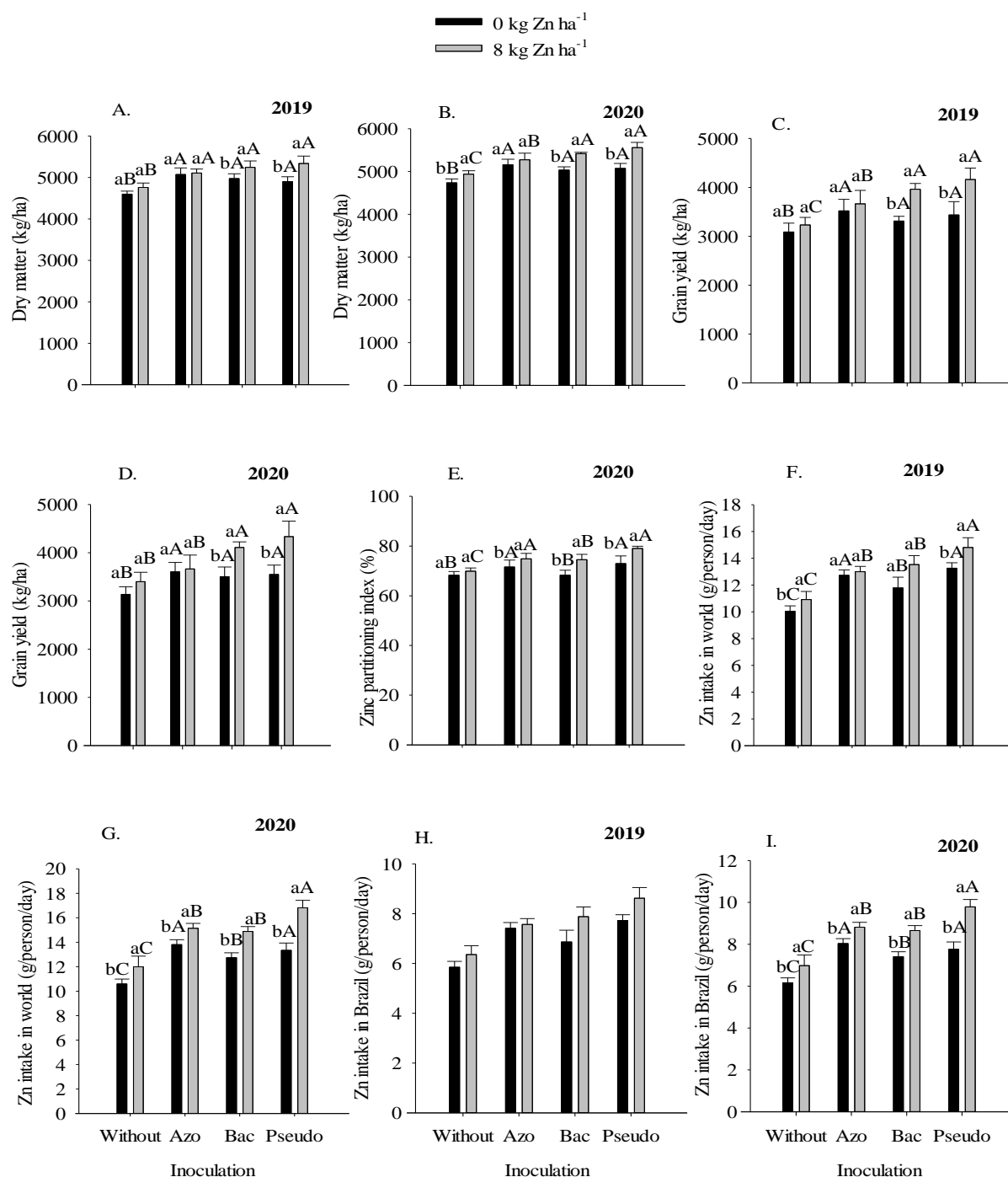
concentration by 16.1 and 20.3% and grain P concentration by 35.3 and 43.3% in first and second crop season (Table 5.2).

### 5.3.3 Wheat plant height, dry matter and yield

Plant height, dry matter and grain yield were greater with soil applied Zn in both cropping seasons. Plant height was significantly increased with Zn application and diazotrophic bacteria inoculations whereas their interaction were not significant (Appendix A; Table 6). The application of Zn produced lengthy plants (taller by 5.5 and 5.3%) in 2019 and 2020 respectively when compared with control. The inoculation of *P. fluorescens* produced taller plants (11.3 and 13.7%) in both of the study years which were statistically at par with inoculation of *B. subtilis* in both 2019 and 2020.

Dry matter of wheat was significantly greater by 4.6 and 6% with soil applied Zn in 2019 and 2020 respectively (Appendix A; Table 6). The treatments when applied soil Zn and inoculated with *P. fluorescens* produced greater dry matter (an increase of 9.4 and 9.9%) as compared to control. However, these results were statistically similar to the values obtained with *B. subtilis* and *A. brasilense* in 2019 and 2020 respectively (Appendix A; Table 6; Figure 5.6A, B). Also, the treatments with inoculation of *A. brasilense* in the absence of Zn application produced greater dry matter in both cropping seasons (Figure 5.6A, B).

**Figure 5.6.** The influence of soil Zn and inoculations of diazotrophic bacteria on plant height, dry matter, grain yield, zinc partitioning index, estimated zinc intake in the world and Brazil with wheat. (A, B) Dry matter ( $\text{kg ha}^{-1}$ ) in 2019 and 2020; (C, D) Grain yield ( $\text{kg ha}^{-1}$ ) in 2019 and 2020; (E) Zn partitioning index (%) in 2020; (F, G) Zn intake in wheat consumption in the world ( $\text{g person}^{-1} \text{ day}^{-1}$ ) in 2019 and 2020; (H, I) Zn intake in wheat consumption in Brazil ( $\text{g person}^{-1} \text{ day}^{-1}$ ).



**Source:** Author. Without (No inoculation); Azo (*A. brasilense*); Bac (*B. subtilis*); Pseudo (*P. fluorescens*). The uppercase letters are used for inoculation interactions within each level of soil Zn application, whereas lowercase letters are used for the interaction of Zn levels within each inoculation treatment. The identical alphabetic letters do not differ from each other, as analyzed by Tukey (Zn application;  $p \leq 0.05$ ) and Scott–Knott (inoculations;  $p \leq 0.05$ ) tests for wheat cropping year 2019 and 2020 respectively. Error bars indicate the standard error of the mean ( $n = 4$  replications).

Grain yield is the most authentic attributes of a wheat crop which was significantly influenced by Zn fertilization, diazotrophic bacteria inoculation and their interactions (Appendix A; Table 6; Figure 5.6C, D). Greater yield was increased by 12.5 and 12.4% with soil Zn application in both cropping seasons respectively (Appendix A; Table 6). The inoculation of *P. fluorescens* provided greater grain yield (increased by 20.3%) in comparison of control which was statistically similar with yield of *B. subtilis* and *A. brasilense* applied treatments in 2019 (Figure 5.6C). Also, grain yield in 2020 follow the same trend with an increase of 20.6% as compared to control. However, it was statistically not different from the inoculated treatments with *B. subtilis* (Figure 5.6D). The treatments without Zn application performed better under the inoculation of *A. brasilense* (Figure 5.6C, D).

### 5.3.4 Zinc partitioning index and intake in wheat

Zinc partitioning and intake are related to higher grain Zn concentration and accumulation. The application of soil applied Zn increased Zn partitioning (5.8 and 7%) to the grains (Appendix A; Table 6) as a result of greater Zn concentration in grains (Figure 5.3B, C). The inoculation of *P. fluorescens* was recognized with higher Zn partitioning (12 and 10%) under Zn applied treatments in first and second cropping seasons respectively (Appendix A; Table 6, Figure 5.6E). However, the results from *P. fluorescens* in the study year 2019 were statistically not different from the results of *A. brasilense* and *B. subtilis* (Appendix A; Table 6). In addition, the treatments without soil applied Zn when inoculated with *A. brasilense* increased Zn partitioning to the wheat grains (Figure 5.6E).

The estimated daily Zn intake in the world and Brazil were significantly increased with soil Zn application, diazotrophic bacteria inoculations and their respective interactions (Appendix A; Table 6; Figure 5.6F, G, H, I). The treatments applied with Zn were observed with greater

estimated Zn intake values in the world (10.1 and 16.6%) and Brazil (10.1 and 17.8%) in 2019 and 2020 respectively (Appendix A; Table 6), regardless the inoculation. The estimated Zn intake tended to greater in the world and in Brazil with inoculation of *P. fluorescens* (Appendix A; Table 6; Figure 5.6F, G, H, I). The estimated Zn intake in worldwide wheat consumption was 34.3 and 33.6% greater (Figure 5.6F, G) while in Brazil Zn intake was 34.4 and 33.3% greater with inoculation of *P. fluorescens* as compared to without inoculated treatments (Figure 5.6H, I). In the absence of Zn application, estimation of Zn intake in the world and Brazil were greater with inoculation of *A. brasilense* in all wheat harvests (Figure 5.6F, G, H, I).

### 5.3.5 Zinc efficiencies in wheat

Zinc use efficiency (ZnUE), utilization efficiency (UE), and applied zinc recovery (AZnR) were enhanced with diazotrophic bacteria inoculation under soil applied Zn (Table 5.3). The agro-physiological efficiency (APE) responded differently to different inoculation and soil Zn application in 2019 and 2020 (Table 5.3). The inoculation of *P. fluorescens* when applied with soil Zn increased ZnUE by 644 and 283% which were not statistically different from the results obtained with *B. subtilis* (511 and 215%) in 2019 and 2020 as compared to control (Table 5.3).

**Table 5.3.** Zinc efficiencies of wheat under the influence of diazotrophic bacteria and soil applied zinc doses.

Treatments	ZnUE		APE		UE		AZnR	
	----- kg kg <sup>-1</sup> -----		----- % -----					
	2019	2020	2019	2020	2019	2020	2019	2020
Diazotrophic bacterial inoculations (I)								
Without (Control)	18 c	41 c	3.4 a	6.4 a	23 b	14 c	5 c	6 d
<i>A. brasilense</i>	72 b	74 b	4.7 a	4.1 b	66 a	56 b	16 b	18 c
<i>B. subtilis</i>	110 a	129 a	5.8 a	6.1 a	83 a	74 a	19 b	21 b
<i>P. fluorescens</i>	134 a	157 a	5.3 a	4.9 b	95 a	91 a	26 a	32 a
F-values								
I	15 *	29 **	1.2 ns	4.1 *	15 *	24 *	23 *	142**
CV (%)	31	19	41	20	24	23	22	9

ZnUE = Zinc use efficiency, APE = Agro-physiological efficiency, UE = Utilization efficiency, and AZnR = Applied zinc recovery.

Means in the column followed by different letters are significantly different ( $p \leq 0.05$ );

\*\* and \*Significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively and ns- non significant by F-test respectively.

Agro-physiological efficiency in 2019 was positively improved with diazotrophic bacteria inoculation. The greater agro-physiological efficiency was greater with inoculation of *B. subtilis* which was not statistically different from the inoculants and control (without inoculation) in 2019 (Table 5.3). In 2020, the agro-physiological was contradictory greater in control plots than all applied inoculants.

The inoculation of *P. fluorescens* in the presence of soil Zn application has increased utilization efficiency of Zn by 313 and 550% as compared to control in 2019 and 2020 respectively (Table 5.3). However, utilization efficiency in both of the cropping seasons were statistically similar to *B. subtilis* inoculation. In addition, applied Zn recovery was statistically greater with inoculation as compared to without inoculation treatments (Table 5.3). The inoculation of *P. fluorescens* under soil Zn application performed statistically better than control and increased applied Zn recovery by 420 and 500% in 2019 and 2020 (Table 5.3).

### 5.3.6 Pearson's Linear Correlation for the analyzed evaluations of wheat

Pearson's linear correlations were positive and significant for most of the evaluated attributes in 2019 and 2020 (Appendix B; Figure 5). The coefficient correlation between agro-physiological efficiency and grain Zn accumulation, plant height, dry matter and grain yield was significant (Appendix B; Figure 5A). There was a positive but not significant correlations between agro-physiological efficiency and leaf Zn concentration, grain Zn concentration, shoot Zn concentration, shoot Zn accumulation, Zn partitioning index and Zn intake (Appendix B; Figure 5A). Similarly, a positive correlation was observed between agro-physiological efficiency and all evaluated attributes are significant (Appendix B; Figure 5B).

## 5.4 Discussion

Zinc (Zn) has a prominent role in several biological processes and essential for growth and production of plants and animals (REHMAN *et al.*, 2018a). In addition, Zn is acting as a constituent element in starch synthesis, pollen fertility, cell elongation and multiplication (DAVARPANA *et al.*, 2016; ULLAH *et al.*, 2020) that could better increase grains filling and grains Zn concentration (JALAL *et al.*, 2020). The inoculation of diazotrophic bacteria can improve Zn solubilization and availability to the plants through production of several phyto-

hormones and enzymes, carboxylation, and biological fixation of nitrogen (MITTER *et al.*, 2013; REHMAN *et al.*, 2019b). The positive correlation among Zn concentration and accumulation in plant and grain tissues, and Zn use efficiencies with dry matter and yield of wheat crop endorsed our hypothesis (Appendix B; Figure 5). Thus, soil Zn application together with diazotrophic bacteria has sustainably improved plant adaptation in tropical region with better Zn nutrition and accumulation, grain yield and estimated intake of wheat crop. Although, Zn availability is claimed in different ways over time and therefore, more extensive research with inoculation and co-inoculation of microbes and Zn dose effect is required to better understand plant nutrition, physiology and yield.

This field study indicated Zn concentration and accumulation in plant and grains tissues were increased with soil applied Zn and inoculation of different bacteria (Appendix A; Table 5 and Figure 5.3). Plants-microbes interaction adapt several mechanisms to enhance biochemical and metabolic functions of plants by producing phenolic acid, siderophores, organic acid in root exudates, auxin and gibberellic acid (REHMAN *et al.*, 2018; PANKIEVICZ *et al.*, 2019; KHOSHRU *et al.*, 2020), which should proactively increase Zn and other nutrients availability to the plants (FAGERIA *et al.*, 2011). The inoculation of diazotrophic bacteria with soil Zn application enhanced nutrients concentration, uptake and leading to healthy, quality and nutrients enriched grains (JALAL *et al.*, 2020; JALAL *et al.*, 2021). Our results are remarkable step to understand the role bacterial inoculation in combination with soil Zn application on growth, yield and biofortification of wheat and emphasized on the integration of Zn application with inoculation to elevate Zn concentration, yield and Zn use efficiencies.

The accumulation and concentration of Zn in plant tissues are directly related to population, availability and composition of soil microbiota in root rhizosphere (DOTANIYA; MEENA, 2015) which can dissolve Zn oxides and carbonates and therefore, increase Zn availability and uptake (AKETI *et al.*, 2014). The current results indicated that Zn concentration in leaf, shoot and grains tissues (Appendix A; Table 5; Figure 5.3A, B, C) and accumulation in shoot and grains tissues (Appendix A; Table 5; Figure 5.4A, B, C, D) of wheat were potentially improved with inoculation *P. fluorescens* and soil Zn application in both study years. The concentration and uptake of Zn in plant tissues are directly associated with consortia, disposal, mobility and configuration of soil micro-biota in root rhizosphere (DOTANIYA; MEENA, 2015) and therefore, some specific strain increase Zn availability and uptake (AKETI *et al.*, 2014;

KUMAR *et al.*, 2019). Several previous studies indicated that different strains of *Bacillus*, *Pseudomonas*, *Rhizobium* and *Azospirillum* sp. improved Zn solubilization and availability in different cereal crops (ABAID-ULLAH *et al.*, 2015; KHANDE *et al.*, 2017; GALINDO *et al.*, 2021).

The application of Zn along with bacterial had evident influence on plant growth, dry matter and yield of wheat in tropical savannah (Appendix A; Table 6). Shoot and grain N and P concentration were also increased with inoculation and soil Zn application (Table 5.2; Figure 5.5). The inoculation of diazotrophic bacteria like *A. brasilense*, *B. subtilis* and *P. fluorescens* can improve nutrient uptake and plant growth through the production of several phytohormones and enzymes and biological fixation of nitrogen (PEREIRA *et al.*, 2020; ROSA *et al.*, 2020). The inoculation *P. fluorescens* under soil application better performed instead of Zn deficient soils, produced taller plants, greater dry matter (Figure 5.6A, B) and grain yield (Figure 5.6C, D) of wheat. It has reported elsewhere that inoculation with *Pseudomonas* sp. increased architecture, branching and proliferation of roots which could increase Zn uptake and fitness of plants in harsh environment (ABADI *et al.*, 2021). In addition, Zn being an important component and catalyst of several enzymes, cell division and elongation (DAVARPANA *et al.*, 2016) maintaining plants biochemical activities (ULLAH *et al.*, 2019) that lead to better crop growth and physiology (JALAL *et al.*, 2020a), fortified grains and increased yield (JALAL *et al.*, 2020b).

The inoculation of Zn solubilizing bacteria are playing an excellent role in maintaining sustainable and eco-friendly environment (KHOSHRU *et al.*, 2020). These bacteria can increase root dry matter of cereal crops where soil Zn is dissolved and absorbed by plants and act as gateway for better nutrition of grains and productivity (BOLETA *et al.*, 2020; GALINDO *et al.*, 2021). In this content, our results also described that application of Zn with *P. fluorescens* has potentially increased Zn partitioning and dietary intake of wheat grains (Appendix A; Table 6; Figure 5.6E, F, G, H, I). It had also reported that Zn solubilizing bacteria like *Pseudomonas* and *Bacillus* sp. strains activate different soil-plant Zn mechanisms to convert insoluble soil into plant available to improve accumulation, grain biofortification and greater yield (HAFEEZ *et al.*, 2013; RAMESH *et al.*, 2014; SINGH *et al.*, 2017a; SINGH *et al.*, 2017b; JALAL *et al.*, 2021). Zinc solubilizing bacteria had potentially increased Zn bioavailability, assimilation in plant and grains tissues and consumption by human beings through reduction in phytic-P concentration (RAMESH *et al.*, 2011; MUMTAZ *et al.*, 2017).

It has been reported that Zn efficiencies are expressed in term of grain Zn concentration in lower soil Zn content where sole Zn intended to be anti-proportional to Zn use efficiency (SHIVAY *et al.*, 2008). While, in another study application of Zn in combination with inoculation of *Pseudomonas* sp. and *Bacillus* sp. has potentially dissolve Zn sulphide, oxides and carbonates (SARAVANAN *et al.*, 2004) to increase Zn use efficiency in better way than sole application. In this context, our study indicated that Zn use efficiency (ZnUE), applied Zn recovery (AZnR) and utilization efficiency (UE) were increased with inoculation of *P. fluorescens* along soil Zn application whereas Agro-physiological efficiency (APE) was not statistically different (Table 5.3). The greater Zn efficiencies might be the results of better Zn accumulation, growth and yield (Appendix A; Table 5, 6). Some other previous studies described that Zn solubilizing bacteria like *Pseudomonas* and *Bacillus* are one the promising inoculants to increase Zn bioavailability and translocation to grains (SINGH *et al.*, 2017a; REHMAN *et al.*, 2018; ULLAH *et al.*, 2020). In our previous studies, it was also reported that Zn application with co-inoculation of different diazotrophic bacteria has improved Zn efficiencies in common bean, wheat and maize (JALAL *et al.*, 2021; GALINDO *et al.*, 2021). Hence, inoculation of these diazotrophic bacteria enhanced Zn partitioning and accumulation regardless of Zn efficiencies and has proven an integrated and sustainable strategy for grain biofortification and productivity of wheat crop.

## 5.5 Conclusions

Soil Zn application is an inexpensive and easy way of agronomic biofortification and greater grains productivity however, sole Zn application is not enough to meet the goal. Therefore, diazotrophic bacterial inoculation is a sustainable, implementable and alternative mechanism that further increase Zn partitioning to the grains and Zn use efficiency. It was verified from our results that diazotrophic bacteria inoculation when performed with soil Zn application increased plant and grain Zn concentrations, growth and yield of wheat. Seed inoculation with *P. fluorescens* under soil applied Zn increased leaf, shoot and grain Zn concentrations and accumulations while plant height, dry matter and grain yield of wheat were increased with *P. fluorescens* and *B. subtilis*. Although, inoculation with *A. brasilense* performed better in the absence of Zn.

Nitrogen and phosphorous shoot and grain concentrations were increased with inoculation of *B. subtilis* under soil Zn application. Zinc partitioning index and estimated daily consumption were also increased with inoculation of *P. fluorescens* and soil Zn application. The Zn use

efficiencies were interestingly greater with inoculation of *P. fluorescens* and *B. subtilis* under the treatments of soil applied Zn. In this context, inoculation of *P. fluorescens* under soil Zn application could be a better and efficient alternative mechanism to improve plant nutrients acquisition and use efficiencies particularly Zn, for sustainable production and biofortification of wheat grains in tropical savannah. Prospective research aiming to improve Zn use efficiency and recovery with inoculation and co-inoculation of diazotrophic bacteria and their influence cereal biofortification, physiological and molecular aspects is required to carry out in different edaphic conditions to better understand Zn solubilizing bacteria under field conditions.

## CHAPTER 6

### 6. INTEGRATED USE OF PLANT GROWTH-PROMOTING BACTERIA AND NANO-ZINC FOLIAR SPRAY IS A SUSTAINABLE APPROACH FOR WHEAT BIOFORTIFICATION, YIELD AND ZINC USE EFFICIENCY

#### 6.1 Introduction

Zinc (Zn) malnutrition and deficiency is a persistent and challenging health and social concern around the world for affecting almost 17.5% of population (MAXFIELD; CRANE 2019; BOLLINEDI *et al.*, 2020). It is ranked as 11<sup>th</sup> global health risk factor and 5<sup>th</sup> in developing countries, declared “hidden hunger” (BHATT *et al.*, 2020; PONIEDZIAŁEK *et al.*, 2020). Zinc deficiency is strongly correlated with cereals growth and production as well as cereal like wheat has higher phytate compounds that contribute to high phytate-Zn ratio and reduced Zn concentration in edible tissues (REHMAN *et al.*, 2017). Wheat consuming countries are the most prevalent ones to Zn malnutrition due to high phytate-Zn content ration in the grains, which is further increased by cultivating this crop under Zn deficient soils (CHATTHA *et al.*, 2017). Zinc in plants is acting as a co-factor and constituent of more than 300 enzymes and regulating several cellular, biochemical and physiological processes as well as helping in metabolism and energy transferring activities (HAFEEZ *et al.*, 2013; ULLAH *et al.*, 2020). To deal this challenging, a prompt and sustainable solution is required to increase Zn concentration in plant, grains and productivity of wheat.

Zinc deficiency has been addressed by adapting different techniques, including soil and foliar Zn application, seeds treatment and synthetic nutrient solution (HAIDER *et al.*, 2019). High phosphorous fertilization into tropical soils and bicarbonates and oxides can increase Zn adsorption and deficiency for cereals production (PENN; CAMBERATO, 2019; AMANULLAH *et al.*, 2020). Therefore, foliar Zn application is often considered less expensive and rapid strategy in comparison of soil Zn fertilization for better agronomic biofortification (ZHOU *et al.*, 2012). Foliar Zn application is an effective and prompt technique that could improve grains Zn accumulation and cope with malnutrition (AZIZ *et al.*, 2019). Agronomic biofortification is better defined by the time of foliar Zn application, which improved grain Zn uptake either at later or early crop growth stages (CAKMAK *et al.*, 2010; AFSHAR *et al.*, 2020). Foliar Zn fertilization counters Zn scarcity in standing crops, Zn spray at tasseling and milking growth stages improve grain development and grain Zn concentration in wheat (JALAL *et al.*, 2022). In recent years, nano-Zn

is adopted in agriculture system for its multiple impacts on plants and environment. Nano-Zn foliar application can increase Zn mobility in phloem, up-surging its bioavailability in endosperm and contributing to protein synthesis and other biochemical traits of crop plants (ROSSI *et al.*, 2019; PALACIO-MÁRQUEZ *et al.*, 2021). However, foliar fertilization is limited by source, particulate size and formulation (FERNÁNDEZ; BROWN, 2013), which restrict its application in field crops production. Therefore, it is unprecedented to adopt sustainable agriculture practices to deal with challenge of food and nutritional security under low environmental risks.

Plant growth-promoting bacteria (PGPBs) multiply in rhizosphere of plants to promote soil fertility, crop productivity, nutrients bioavailability and food security, thus contributing to sustainable agriculture (SINGH *et al.*, 2022). Among PGPBs, *Azospirillum brasilense*, *Bacillus subtilis* and *Pseudomonas fluorescens* are the most studied inoculants in Brazil. These PGPBs could increase Zn bioavailability and uptake through solubilization, nitrogen fixation, production of organic and inorganic acids, phyto-hormones, and chelators (IDAYU *et al.*, 2017; KHOSHROU *et al.*, 2020). Inoculation of these PGPBs by seeds is a promoting plant growth and development through higher use efficiency of nutrients like phosphorous (P), potassium (K), improving water retention and synthesis of secondary metabolites, which consequently support plants against biotic and abiotic stresses (HUNGRIA *et al.*, 2018; KUMAR *et al.*, 2019; JALAL *et al.*, 2021). Inoculation of *A. brasilense* under Zn fertilization can increase Zn use efficiency and uptake, and yield of cereal crops under tropical environment (GALINDO *et al.*, 2021). In addition, *B. subtilis* and *P. fluorescens* are being recognized the most efficient inoculants that solubilize Zn and P thus, improving plant growth and development under different climatic conditions (ROSA *et al.*, 2020; AHMED *et al.*, 2021; JALAL *et al.*, 2021; JALAL *et al.*, 2022a, b).

Wheat has been reported Zn sensitive cereal crop due to inherently low Zn content and also has great agronomic relevance for food and nutritional security (ZOU *et al.*, 2012; FAO, 2019). Wheat cultivation in tropical savannah can be deal a potential crop of food security and sustainability. Wheat cultivation in tropical and marginal regions can be a diversified source of food to feed increasing global population with sufficient and nutritious food. There is literature lacking on the investigations of inoculation with PGPBs and nano-Zn foliar application on nutritional status, yield and Zn use efficiency in tropical savannah. Hence, hypothesis of the present study was that inoculation with PGPBs and nano-Zn foliar can improve nutrition, productivity, Zn use efficiencies and biofortification of wheat. In this context, the objective was to

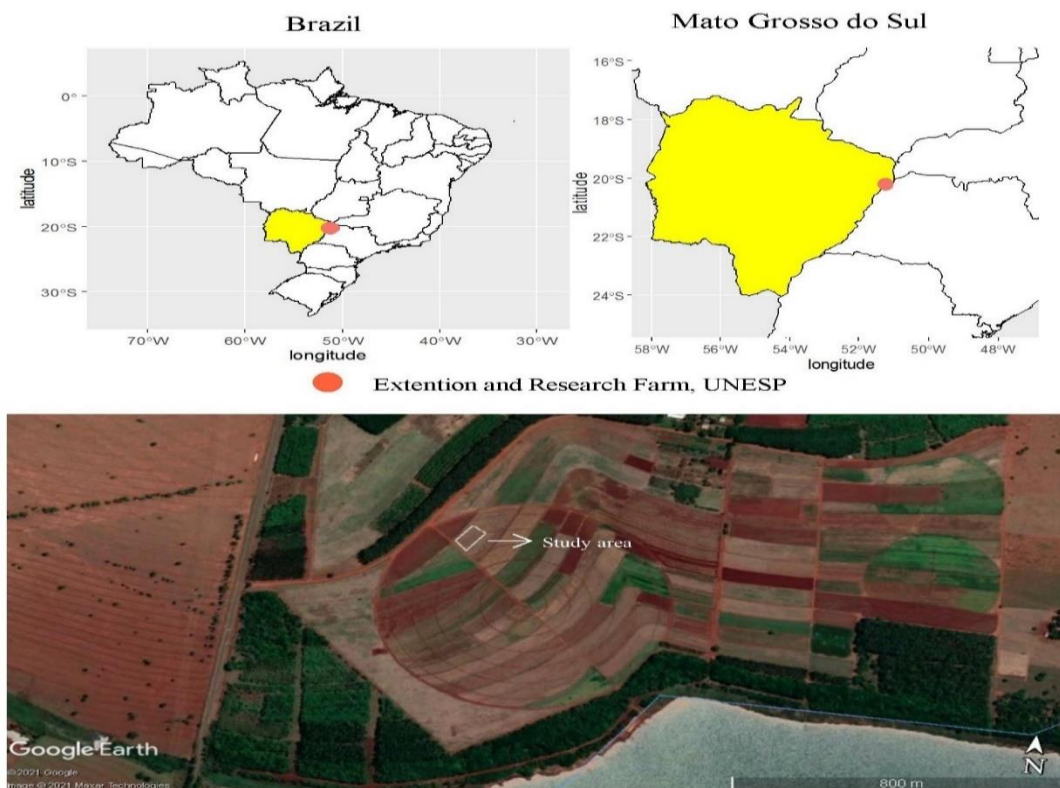
determine the best performing PGPBs inoculant with nano-Zn foliar application on growth, yield, concentration of Zn in shoot and grains, Zn use efficiencies and estimated Zn intake under cultivation of wheat in tropical savannah of Brazil.

## 6.2 Materials and Methods

### 6.2.1 Experimental Area and Location

Wheat field experiment were performed for two consecutive cropping seasons (2019 - 2020) at the Extension and Research Farm of School of Engineering, São Paulo State University (UNESP) at Selvíria, Mato Grosso do Sul, Brazil. The field is located at geographical coordinates of 20°22' S latitude, 51°22' W longitude, and an altitude of 335 m (Figure 6.1). The soil was classified as Rhodic Haplustox with clay texture (SOIL SURVEY STAFF, 2014). The experimental site is used for the cultivation of annual crop (cereals and legumes) for more than 28 years, the last 13 being under no tillage system (SANTOS *et al.*, 2018).

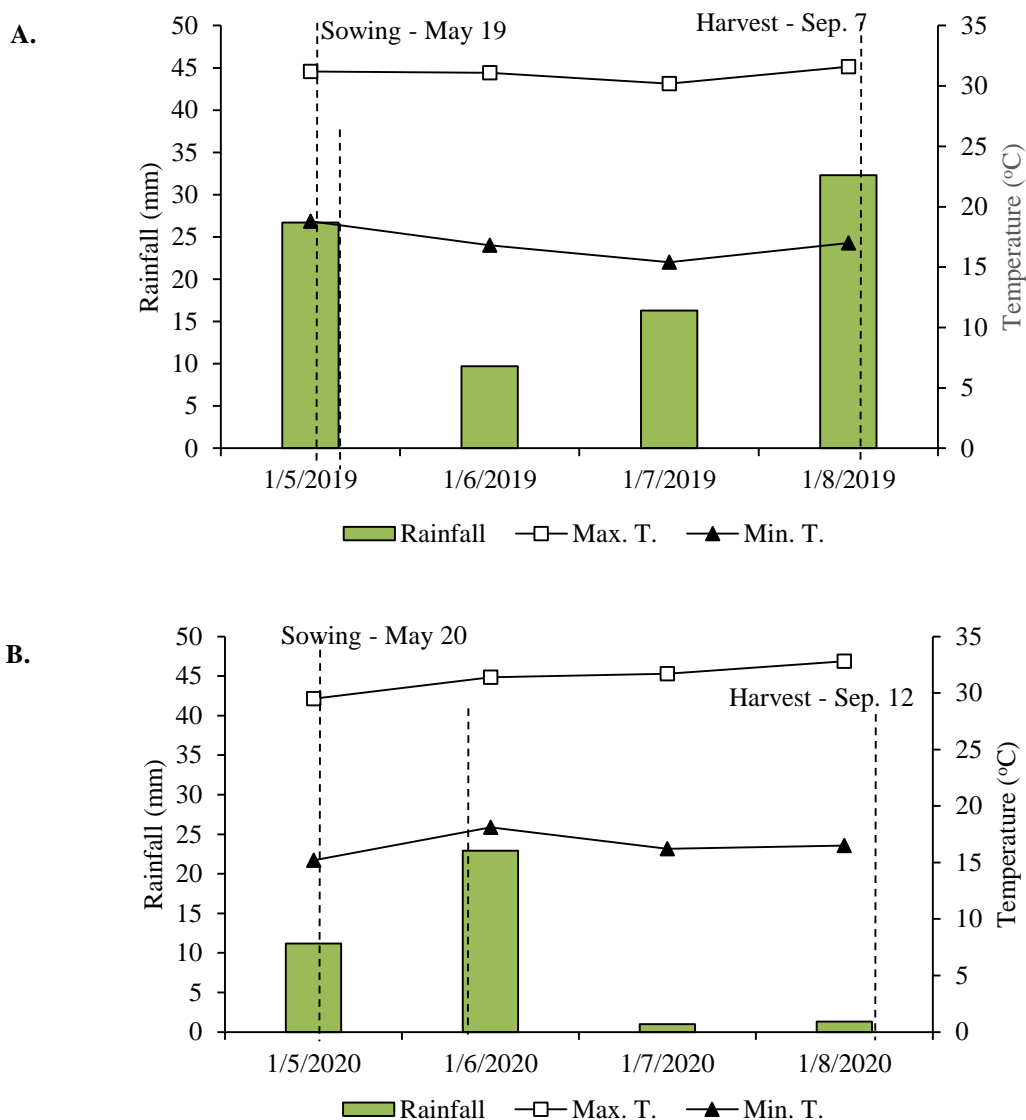
**Figure 6.1.** Location of experimental area at the Research and Extension Farm at Selvíria - Mato Grosso do Sul state, Brazil (20°22'S, 51°22'W, altitude of 335 m) in 2019 and 2020 crop seasons.



**Source:** Author. The map was created using pacot, geobr and ggplot with R software (R software (R DEVELOPMENT CORE TEAM, 2015). Projection System WGS 84/UTM 200DC [EPSG: 4326]. This image was taken from Google Earth program, Google Company (2021). Map data: Google, Maxar Technologies.

The soil is acidic in nature, analyzed for chemical properties according to van Raij *et al.* (2001): pH (CaCl<sub>2</sub>) = 5.2; P (resin) = 37 mg dm<sup>-3</sup>; S-SO<sub>4</sub><sup>-2</sup> = 5 mg dm<sup>-3</sup>, K<sup>+</sup> = 2.3 mmol<sub>c</sub> dm<sup>-3</sup>; Ca<sup>2+</sup> = 27 mmol<sub>c</sub> dm<sup>-3</sup>; Mg<sup>2+</sup> = 17 mmol<sub>c</sub> dm<sup>-3</sup>; Al<sup>3+</sup> = 0.01 mmol<sub>c</sub> dm<sup>-3</sup>; H+Al = 35 mmol<sub>c</sub> dm<sup>-3</sup>; Cation exchange capacity = 69.1 mmol<sub>c</sub> dm<sup>-3</sup>; Base saturation = 52%; organic matter = 19 g kg<sup>-1</sup>; B (Hot water) 0.15 mg dm<sup>-3</sup>. The pre-experiment soil Diethylenetriaminepentaacetic acid (DTPA) extractable Zn content (0.7 mg dm<sup>-3</sup>) was medium low, Fe = 29 mg dm<sup>-3</sup>, Mn = 41 mg dm<sup>-3</sup> and Cu = 4 mg dm<sup>-3</sup>. The granulometric attributes followed the methodology of Teixeira *et al.* (2017) with clay (439 g kg<sup>-1</sup>), sand (471 g kg<sup>-1</sup>), and slit (90 g kg<sup>-1</sup>). The meteorological data of both cropping seasons is summarized in Figure 6.2.

**Figure 6.2.** Rainfall maximum, average and minimum temperatures and air relative humidity are acquired from the weather station of Education and Research Farm during wheat cultivation period from May to September, 2019 (A) and May to September, 2020 (B).



## 6.2.2 Experimental design and treatments

The experiment was designed in randomized complete block having 4 x 5 factorial scheme, with four replicates. The treatments consisted of four kind of seeds inoculations with PGPBs (no inoculation, *Azospirillum brasilense*, *Bacillus subtilis*, and *Pseudomonas fluorescens*) and five

zinc foliar rates (0, 0.75, 1.5, 3.0 and 6.0 kg ha<sup>-1</sup>), applied 50% at tillering and 50% at grain filling stage of wheat (DHALIWAL *et al.*, 2019).

Seeds were manually inoculated by mixing seeds and respective inoculant in a plastic bag an hour before to sowing. Inoculation with *A. brasilense* strains Ab-V5 (CNPSO 2083) and Ab-V6 (CNPSO 2084) was performed at a dose of 200 mL liquid inoculant per 24 kg seeds with guarantee of  $2 \times 10^8$  CFU mL<sup>-1</sup> while *B. subtilis* strain (CCTB04) with guarantee of  $1 \times 10^8$  CFU mL<sup>-1</sup> and *P. fluorescens* strain (CCTB03) with guarantee of  $2 \times 10^8$  CFU mL<sup>-1</sup> at liquid inoculants dose of 150 mL ha<sup>-1</sup> per 24 kg seeds were performed by following the recommendation of inoculants providing company (Biotrop®), Curitiba, Brazil. These inoculants are commercially used in Brazil with strains of *A. brasilense* (AzoTotal™), *B. subtilis* (Vult™) and *P. fluorescens* (Audax™) to promote growth and productivity.

The foliar Zn application was performed from liquid source of Zn (Nano R1 zinco™), obtained from Allplant® fertilizers industry, São Paulo, Brazil. The company is already registered with Ministry of Agriculture, Brazil. Nano R1 zinc is characterized as fluid suspension with 50% p/p Zn, 1000 g L<sup>-1</sup> solubility and 2.0 density and being successfully used in previous studies (Nakao *et al.*, 2018; JALAL *et al.*, 2022c). The application was performed through manual sprayer pump of 6.0 liters water capacity (300 L ha<sup>-1</sup> of volume application). The field was visited soon after foliar spray but no leaf damage was observed.

### 6.2.3 Plant materials

The experimental site was thoroughly sprayed with herbicides (glyphosate, 1800 g ha<sup>-1</sup>; carfentrazone, 40 g ha<sup>-1</sup> and cletodim, 240 g ha<sup>-1</sup> of a.i.) for controlling all narrow and broad leaves weeds 15 days before the experiment implementation. The seeds were chemically treated with Standak Top® [co-formulation of fungicides {carbendazim + thiram (45 g + 105 g of active ingredient (a.i.))} and insecticides {imidacloprid + thiodicarb (45 g + 135 g of a.i.)}] per 100 kg seed before inoculation and plantation. This chemical seed treatment is a common agriculture practice in Brazil before growing cereal crops.

The wheat cultivar (TBIO SOSSEGO) with potential quality and production was sown on 11<sup>th</sup> May, 2019 and 3<sup>rd</sup> May, 2020 respectively on a no-till with drill sowing method. A basal dose of 270 kg ha<sup>-1</sup> of 08-28-16 (N-P<sub>2</sub>O<sub>5</sub>-K<sub>2</sub>O) was applied at sowing according to soil analysis results with the recommendation of Cantarella *et al.* (1997). Each plot comprised of 13 rows, 5m long and 0.17 m apart, totalizing 12.15 m<sup>2</sup> with a useful area of 9.35 m<sup>2</sup>. The recommended dose of 120 N

kg ha<sup>-1</sup> was applied from ammonium sulphate fertilizer after 30 days of emergence. The experimental area was applied with boron 1 kg ha<sup>-1</sup> as boric acid (18% of B) through tractor sprayer machine on the basis of soil analysis with the interpretation of Campinas Agronomic Institute (IAC) (RAIJ *et al.*, 2001). The crop was irrigated with sprinkler central-pivot irrigation system (14 mm in average) according to crop need. The crop was manually harvested on 12<sup>th</sup> September, 2019 (with 125 days cycle) and 7<sup>th</sup> September, 2020 (with 128 days cycle).

## **6.2.4 Evaluations and Analysis**

### **6.2.4.1 Nutritional analysis**

The plant materials (shoot and grain) were collected in proper labeled paper bags and was dried in an air-tight oven at  $60 \pm 5$  °C for 72 hours until uniform humidity attained. The material was then grounded in a stainless-steel Willey knife mill by passing through a 10-mesh sieve and placed in labeled plastic. Each sample was weighed (0.25 g), digested with nitroperchloric (HNO<sub>3</sub>:HClO<sub>4</sub> solution) and quantified by atomic absorption spectrophotometry. The analysis was developed by following methodology of Malavolta *et al.* (1997). Zinc shoot and grains accumulation (g ha<sup>-1</sup>) were calculated from respective Zn concentration in shoot and grains and dry matter and yield per hectare.

### **6.2.4.2 Growth and productivity attributes**

Plant height at maturity was determined with ruler meter from the ground to the upper apex. Shoot dry matter was determined after harvest of useful four central lines. The wheat was harvested and packed in jute bags and then dried in the shade for approximately 1 week. Each plot sample was threshed with electric thresher to attain weight of processed grains for calculating yield per hectare (productivity at 13% moisture content). After drying, the grains were ground in a Willey mill for Zn concentration analysis.

### **6.2.4.3 Zinc partitioning index, intake and use efficiencies**

Zinc partitioning index (ZPI) toward grains was calculated from the ratio of grains Zn concentration to shoot Zn concentration into percent following the methodology of Rengel and Graham (1996). Estimated Zn intake in Brazil was calculated from the fortified wheat grains of the present study (Eq. 1) following Lessa *et al.* (2019). The world consumption of wheat/Foreign Agricultural Service - United States Department of Agriculture (USDA, 2020) reported the

consumption of wheat in Brazil is 56.86 kg person<sup>-1</sup> year<sup>-1</sup> (156 g person<sup>-1</sup> day<sup>-1</sup>). Based on this information, the intake of fortified wheat grains was calculated below in Eq. 1.

$$\text{Zn intake} = [\text{Zn grain}] \times C \quad (\text{Eq. 1})$$

Where: Zn intake (g person<sup>-1</sup> day<sup>-1</sup>) is daily Zn consumption estimation per person, [Zn grain] (g kg<sup>-1</sup>) is Zn concentration in biofortified grains from the current study results and C (g person<sup>-1</sup> day<sup>-1</sup>) is the mean consumption of wheat grains per person in Brazil (USDA 2020).

Zinc use efficiency (ZnUE) and recovery applied Zn (RAZn) were calculated from Zn concentration and accumulation in shoot and grains, and dry matter and grain yield, following methodology of Fageria (2009); Fageria *et al.* (2011); Jalal *et al.* (2021).

$$\text{ZnUE} = (\text{GY ZnF} - \text{GY ZnWF}) \div \text{Zn applied dose (foliar)} \quad (\text{Eq. 2})$$

$$\text{RAZn (\%)} = (\text{ZnAF} - \text{ZnAWF}) \div \text{Zn applied dose (foliar)} \quad (\text{Eq. 4})$$

Where GY ZnF = Grain yield in Zn applied treatments, GY ZnWF = Grain yield in without Zn applied treatments, ZnAF = Zinc accumulation in shoot + grain with fertilized plots, ZnAWF = Zinc accumulation in shoot + grain without fertilized plots.

### 6.2.5 Statistical analysis

All data were initially tested for normality using Shapiro and Wilk test which showed that data is normally distributed ( $W \geq 0.90$ ). The data were submitted to analysis of variance (F test). The Zn soil application, Zn foliar rates and their interactions were considered fixed effects in the model. When a main effect or interaction was observed significant by F test ( $p \leq 0.05$ ) then Tukey test ( $p \leq 0.05$ ) was used for comparison of means of soil Zn application. In addition, regression analysis was performed for Zn foliar rates using R software (R Development Core Team, 2015).

## 6.3 Results

### 6.3.1 Zinc, Nitrogen, and Phosphorous concentration in wheat plant and grains

Inoculation with plant growth-promoting bacteria (PGPBs) and nano-Zn spray had positively influence nutrients (N, P and Zn) concentration in shoot and grains wheat crop. Inoculation with PGPBs and nano-Zn significantly increased shoot Zn concentration of wheat in 2019 and 2020 cropping seasons (Table 6.1). The interaction for shoot Zn concentration of wheat in 2019 was significant (Table 6.1).

**Table 6.1** Shoot Zn, N, and P concentration of wheat as influenced by plant growth-promoting bacteria and foliar applied nano-Zn doses.

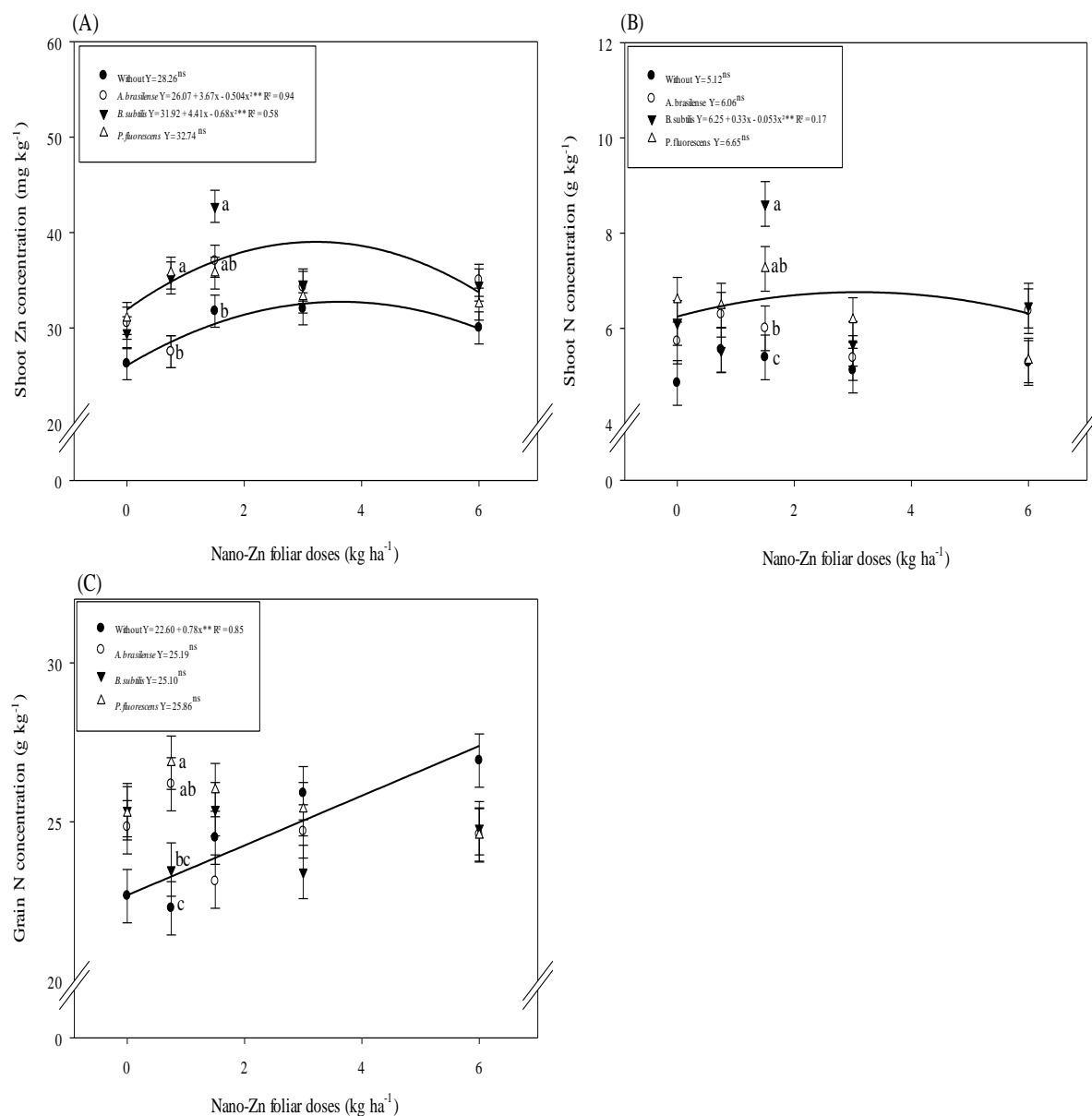
Treatments	Shoot Zn concentration		Shoot N concentration		Shoot P concentration	
	----- g kg <sup>-1</sup> -----					
	2019	2020	2019	2020	2019	2020
<b>Inoculations</b>						
Without	29.5	32.9 b	5.4 b	5.2 b	1.17 b	1.15 b
<i>A. brasilense</i>	32.8	35.9 ab	5.5 b	5.9 ab	1.27 ab	1.27 ab
<i>B. subtilis</i>	35.3	37.1 a	6.7 a	6.4 a	1.26 ab	1.40 ab
<i>P. fluorescens</i>	33.6	39.0 a	6.3 ab	6.3 a	1.48 a	1.52 a
<b>Foliar Zn application (kg ha<sup>-1</sup>)</b>						
0	29.3	32.4	5.7	5.8	1.12	1.12
0.75	31.5	35.5	6.7	5.9	1.18	1.20
1.5	36.8	40.1	5.9	6.8	1.49	1.42
3	33.5	37.0	5.7	5.5	1.40	1.44
6	33.0	35.9	5.7	5.8	1.28	1.48
<b>F values</b>						
Inoculation (I)	10.5**	8.9**	6.8**	7.2**	3.5*	5.5**
Foliar Zn (FZn)	10.8**	8.5**	2.9*	4.0**	3.7*	4.5**
I x FZn	2.06*	0.8 ns	0.9 ns	2.1*	1.7 ns	1.0 ns
<b>CV (%)</b>	10.23	10.5	16.7	15.6	23.9	22.7

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ );

\*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; ns—non-significant, by F-test.

Graphical trend indicated that foliar increasing foliar nano-Zn up to 3.6 and 3.2 kg ha<sup>-1</sup> in combination with inoculation of *A. brasilense* and *B. subtilis* increased shoot Zn concentration respectively, while combination of inoculation with *P. fluorescens* and foliar nano-Zn application was observed non-significant (Figure 6.3A). Further increase in foliar Zn fertilization led to the reduction of shoot Zn concentration in 2019.

**Figure 6.3.** Influence of inoculation with PGPBs and nano-Zn foliar application on; shoot Zn concentration in 2019 (A), shoot N concentration in 2020 (B) and grain N concentration in 2020 (C) of wheat.



**Source:** Author. The different letters correspond to a significant difference at 5% probability level ( $p \leq 0.05$ ). The identical alphabetic letters do not differ from each other as analyzed by Tukey test (PGPBs inoculations;  $p \leq 0.05$ ) and Regression (Foliar Zn rates;  $p \leq 0.05$ ) tests for wheat cropping year 2019 and 2020 respectively. Error bars indicate the standard error of the mean ( $n = 4$  replications). Selvíria, 2020. \*\*: significant at  $p \leq 0.01$ .

In 2020 wheat cropping season, shoot Zn concentration was increased by 18.5% with inoculation of *P. fluorescens*, which was statistically similar with inoculation of *B. subtilis* and *A. brasilense* as compared to without inoculation treatments. The quadratic equation of foliar nano-Zn application was adjusted to 3.38 kg ha<sup>-1</sup> for increasing shoot Zn concentration in 2020 cropping season (Table 6.2).

**Table 6.2.** Concentration of Zn, N, P in shoot and grains, shoot dry matter, Zn partitioning index and estimated daily Zn intake as a function of foliar nano-Zn application regardless of the inoculations in 2019 and 2020 cropping seasons of wheat.

Sources	Equations
Shoot Zn concentration 2020	$y = 33.44 + 3.38x - 0.50x^2$ ( $R^2 = 0.58^{ns}$ )
Shoot N concentration 2019	$y = 6.11 - 0.02x - 0.0078x^2$ ( $R^2 = 0.15^{ns}$ )
Shoot P concentration 2019	$y = 1.12 + 0.20x - 0.02x^2$ ( $R^2 = 0.70^{**}$ )
Shoot P concentration 2020	$y = 1.12 + 0.17x - 0.01x^2$ ( $R^2 = 0.90^{**}$ )
Grain Zn concentration 2019	$y = 40.77 + 2.56x - 0.36x^2$ ( $R^2 = 0.60^{**}$ )
Grain Zn concentration 2020	$y = 45.95 + 3.74x - 0.55x^2$ ( $R^2 = 0.51^{**}$ )
Grain N concentration 2019	$y = 23.72 + 1.22x - 0.17x^2$ ( $R^2 = 0.67^{**}$ )
Grain P concentration 2019	$y = 2.72 + 1.12x - 0.02x^2$ ( $R^2 = 0.17^{ns}$ )
Grain P concentration 2020	$y = 2.70 + 0.23x - 0.03x^2$ ( $R^2 = 0.82^{**}$ )
Shoot dry matter 2019	$y = 4952 + 81.86x - 13.64x^2$ ( $R^2 = 0.78^*$ )
Shoot dry matter 2020	$y = 4979 + 104.1x - 15.9x^2$ ( $R^2 = 0.48^*$ )
Zinc partitioning index 2019	$y = 75.48 + 2.73x - 0.45x^2$ ( $R^2 = 0.50^{ns}$ )
Zinc intake (Brazil) 2019	$y = 6.36 + 0.39x - 0.05x^2$ ( $R^2 = 0.61^{**}$ )
Zinc intake (Brazil) 2020	$y = 7.16 + 0.58x - 0.08x^2$ ( $R^2 = 0.51^{**}$ )

\*\* Significant and <sup>ns</sup> = non-significant at  $p \leq 0.01$

Shoot N concentration of wheat was positively increased with inoculation and foliar nano-Zn application in both cropping seasons (Table 6.1). Interaction of shoot Zn concentration was not significant in 2019 (Table 6.2) and significant in 2020 wheat cropping season (Figure 6.3B). Shoot N concentration was increased by 24% with inoculation of *B. subtilis* as compared to control treatments in first cropping season. In addition, increasing foliar nano-Zn fertilization up to 3.11

kg ha<sup>-1</sup> under the only inoculation with *B. subtilis* increased shoot N concentration, further increase in foliar nano-Zn doses lead to the reduction of shoot N concentration of wheat (Figure 6.3B).

The interactions of inoculation with PGPBs and foliar nano-Zn fertilization for shoot P concentration were not significant (Table 6.1). Both inoculation and foliar nano-Zn fertilization significantly increased shoot P concentration of wheat in 2019 and 2020. Inoculation with *B. subtilis* was observed the best treatment that increased shoot P concentration by 26.5 and 32.2% in first and second cropping season respectively, when compared to without inoculation treatments. The quadratic adjustment indicated that shoot P concentration of wheat was increased with a maximum estimated foliar nano-Zn dose of 5 and 4.4 kg ha<sup>-1</sup> in 2019 and 2020, respectively (Table 6.2). Further increase in foliar nano-Zn fertilization led to the reduction of shoot P concentration.

Inoculation and foliar nano-Zn fertilization positively increased grain Zn concentration while the interactions were not significant in both studied years (Table 6.3). Inoculation with *P. fluorescens* increased grain Zn concentration by 23.7 and 16.5%, which was statistically at par with the treatments inoculated with *B. subtilis*, when compared to without inoculation treatments in 2019 and 2020 respectively. The quadratic equation of nano-Zn foliar application was adjusted to a maximum estimated dose of 3.5 and 3.4 kg ha<sup>-1</sup> to increase grain Zn concentration of wheat in 2019 and 2020 cropping seasons (Table 6.2). These calculations indicated that further increasing of foliar nano-Zn fertilization cause reduction in the grain Zn concentration of wheat.

**Table 6.3** Grain Zn, N, and P concentration of wheat as influenced by plant growth-promoting bacteria and foliar applied nano-Zn doses.

Treatments	Grain Zn concentration		Grain N concentration		Grain P concentration	
	----- mg kg <sup>-1</sup> -----		----- kg ha <sup>-1</sup> -----			
	2019	2020	2019	2020	2019	2020
<b>Inoculations</b>						
Without	37.6 c	44.2 b	24.5	24.4	2.49 b	2.53 c
<i>A. brasilense</i>	42.6 b	49.6 a	24.6	24.7	2.79 b	2.82 cb
<i>B. subtilis</i>	45.6 ab	50.8 a	25.3	24.5	3.16 a	3.15 a
<i>P. fluorescens</i>	46.5 a	51.5 a	24.8	25.6	2.79 b	2.91 ab
<b>Foliar Zn application (kg ha<sup>-1</sup>)</b>						
0	40.3	45.5	23.5	24.5	2.68	2.63
0.75	41.8	47.0	24.3	24.7	2.69	2.91
1.5	45.9	54.0	26.1	24.7	3.16	3.05
3	43.8	50.0	25.3	24.8	2.74	2.96
6	43.3	48.6	24.9	25.2	2.77	2.71
<b>F values</b>						
Inoculation (I)	16**	7.2**	0.7 ns	2.1 ns	9.1**	8.8**
Foliar Zn (FZn)	3.6*	5.7**	3.8*	0.3 ns	3.7*	3.3*
I x FZn	1.3 ns	0.9 ns	1.1 ns	3.1**	1.6 ns	1.0 ns
<b>CV (%)</b>	10.2	11.2	7.87	6.7	14.4	13.5

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ ); \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; ns—non-significant, by F-test.

Grain N concentration was not significant either under inoculation or interaction of foliar nano-Zn  $\times$  inoculation in 2019, while only interaction was significant in 2020 cropping season (Table 6.3). The quadratic equation of foliar nano-Zn application was adjusted to a maximum estimated dose of 3.5 kg ha<sup>-1</sup> for increasing grain N concentration in 2019 cropping season (Table 6.2). The graph trend indicated grain N concentration was linearly increased with increasing foliar nano-Zn fertilization regardless of the applied inoculants (Figure 6.3C).

Inoculation and foliar nano-Zn fertilization increased grain P concentration, while their interactions were not significant in 2019 and 2020 cropping seasons (Table 6.3). Inoculation with *B. subtilis* increased grain P concentration by 26.9 and 24.5% as compared to without inoculation in first and second cropping season respectively. The quadratic adjustment of foliar nano-Zn indicated that increasing Zn fertilization up to 3.0 and 3.4 kg ha<sup>-1</sup> increased grain P concentration while further increase cause reduction in grain P concentration of wheat in 2019 and 2020 respectively (Table 6.2).

### 6.3.2 Shoot dry matter, yield and zinc partitioning

Inoculation with PGPBs and foliar nano-Zn application positively increased shoot dry matter of wheat whereas their interactions were not significant in both 2019 and 2020 cropping seasons (Table 6.4). Inoculation with *P. fluorescens* increased shoot dry matter of wheat by 5.3% in both studied years as compared to without inoculation. The quadratic trend of nano-Zn foliar application was adjusted to maximum Zn rate of 3 and 3.2 kg ha<sup>-1</sup> in 2019 and 2020 cropping seasons (Table 6.2). Any further increase in Zn-foliar application led to the reduction of wheat shoot dry matter.

**Table 6.4** Shoot dry matter, grain yield, Zn partitioning index and estimated daily Zn intake of wheat as influenced by plant growth-promoting bacteria and foliar applied nano-Zn doses.

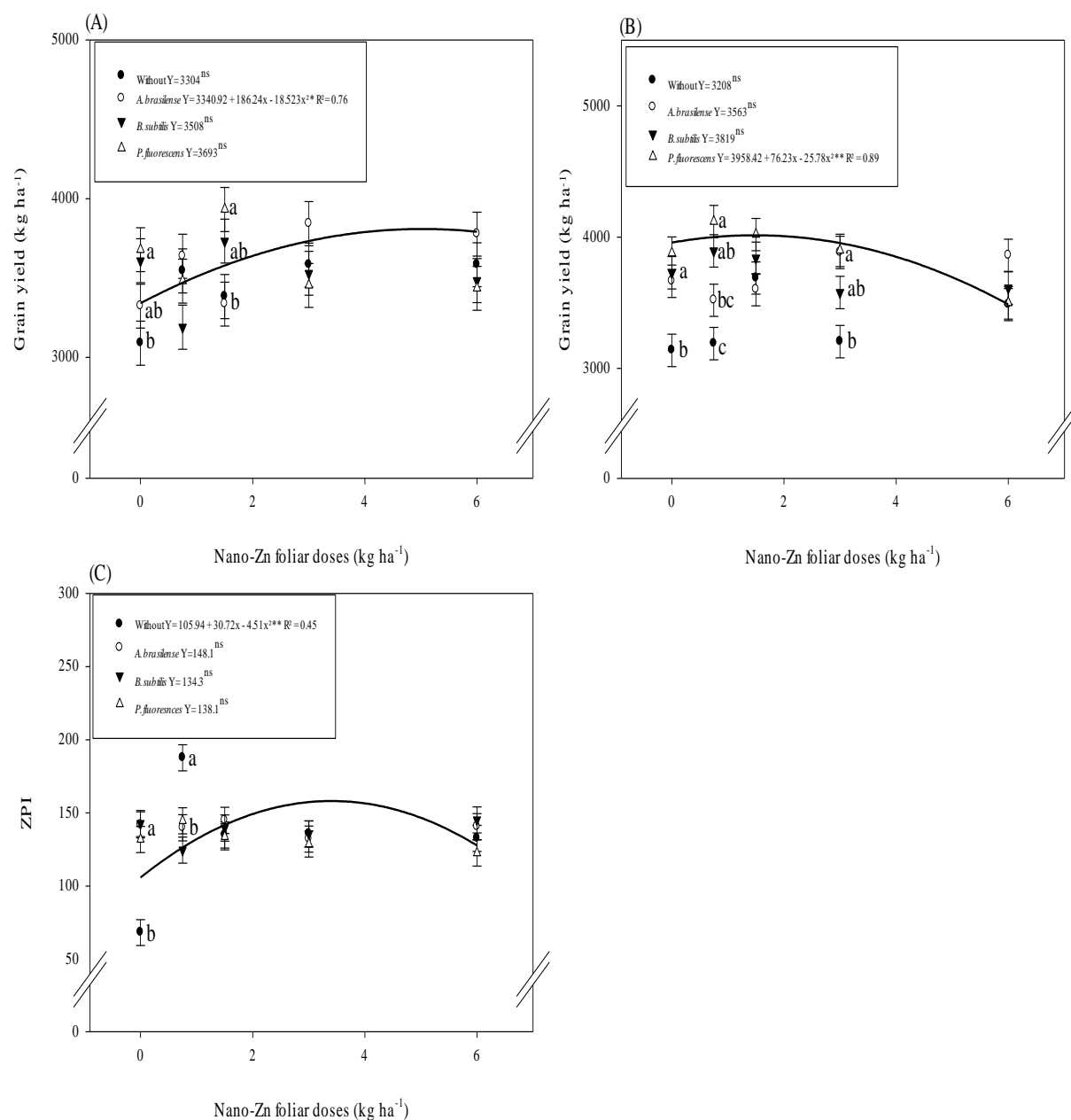
Treatments	Shoot dry matter		Grain yield		Zn partitioning index	
	----- kg ha <sup>-1</sup> -----		-----		----- % -----	
	2019	2020	2019	2020	2019	2020
<b>Inoculations</b>						
Without	4843 b	4909 b	3436	3341	70 b	131
<i>A. brasilense</i>	5009 ab	5095 a	3508	3705	75 b	139
<i>B. subtilis</i>	5072 a	5066 ab	3582	3731	78 ab	137
<i>P. fluorescens</i>	5099 a	5172 a	3595	3883	84 a	132
<b>Foliar Zn application (kg ha<sup>-1</sup>)</b>						
0	4941	4940	3424	3602	73	121
0.75	4998	5049	3462	3680	78	149
1.5	5090	5203	3595	3787	80	138
3	5044	5070	3601	3641	77	133
6	4957	5041	3569	3615	75	135
<b>F values</b>						
Inoculation (I)	6.5**	6.4**	1.4 ns	17**	6.2**	0.9 ns
Foliar Zn (FZ)	1.5 ns	3.7**	1.3 ns	1.4 ns	1.0 ns	5.1**
I x FZ	1.5 ns	0.8 ns	2.8**	2.9 **	0.8 ns	6.4**
<b>CV (%)</b>	4	3.8	7.8	6.7	13.2	13.2

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ ); \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; ns—non-significant, by F-test.

The interactions of inoculation with PGPBs and foliar nano-Zn application positively enhanced grain yield of wheat in 2019 and 2020 cropping seasons (Table 6.4). The treatments with Zn- foliar spray were set to quadratic function (Figure 6.4A, B). Increasing nano-Zn foliar spray up to 5 kg ha<sup>-1</sup> increased grain yield under inoculation of *A. brasilense* in 2019 (Figure 6.4A) while foliar nano-Zn up to a dose of 1.5 kg ha<sup>-1</sup> in combination with inoculation of *P. fluorescens*

increased grain yield of wheat in 2020 cropping season (Figure 6.4B). Further increase in Zn-foliar spray caused reduction in grain yield of wheat in both cropping seasons.

**Figure 6.4** Influence of inoculation with PGPBs and nano-Zn foliar application on; (A and B) grain yield in 2019 and 2020 respectively, and (C) Zn partitioning index (ZPI) in 2020 of wheat.



**Source:** Author. The different letters correspond to a significant difference at 5% probability level ( $p \leq 0.05$ ). The identical alphabetic letters do not differ from each other as analyzed by Tukey test

(PGPBs inoculations;  $p \leq 0.05$ ) and Regression (Nano-Zn foliar doses;  $p \leq 0.05$ ) tests for wheat cropping year 2019 and 2020 respectively. Error bars indicate the standard error of the mean ( $n = 4$  replications). Selvíria, 2020. \*\*: significant at  $p \leq 0.01$ .

Zinc partitioning index (ZPI) was positively improved by inoculation with PGPBs and foliar Zn application (Table 6.4). The effects of foliar Zn application and interaction for ZPI were not significant in 2019, while the effect of inoculation with PGPBs was not significant in 2020. Inoculation with *P. fluorescens* increased ZPI by 20% (Table 6.4), while quadratic adjustment indicated that increasing Zn-foliar up to 3 kg ha<sup>-1</sup> increased Zn partitioning index in wheat grain (Table 6.2). In 2020, graph trend of foliar nano-Zn was adjusted to quadratic function up to 3.4 kg ha<sup>-1</sup> regardless of the inoculation (Figure 6.4D).

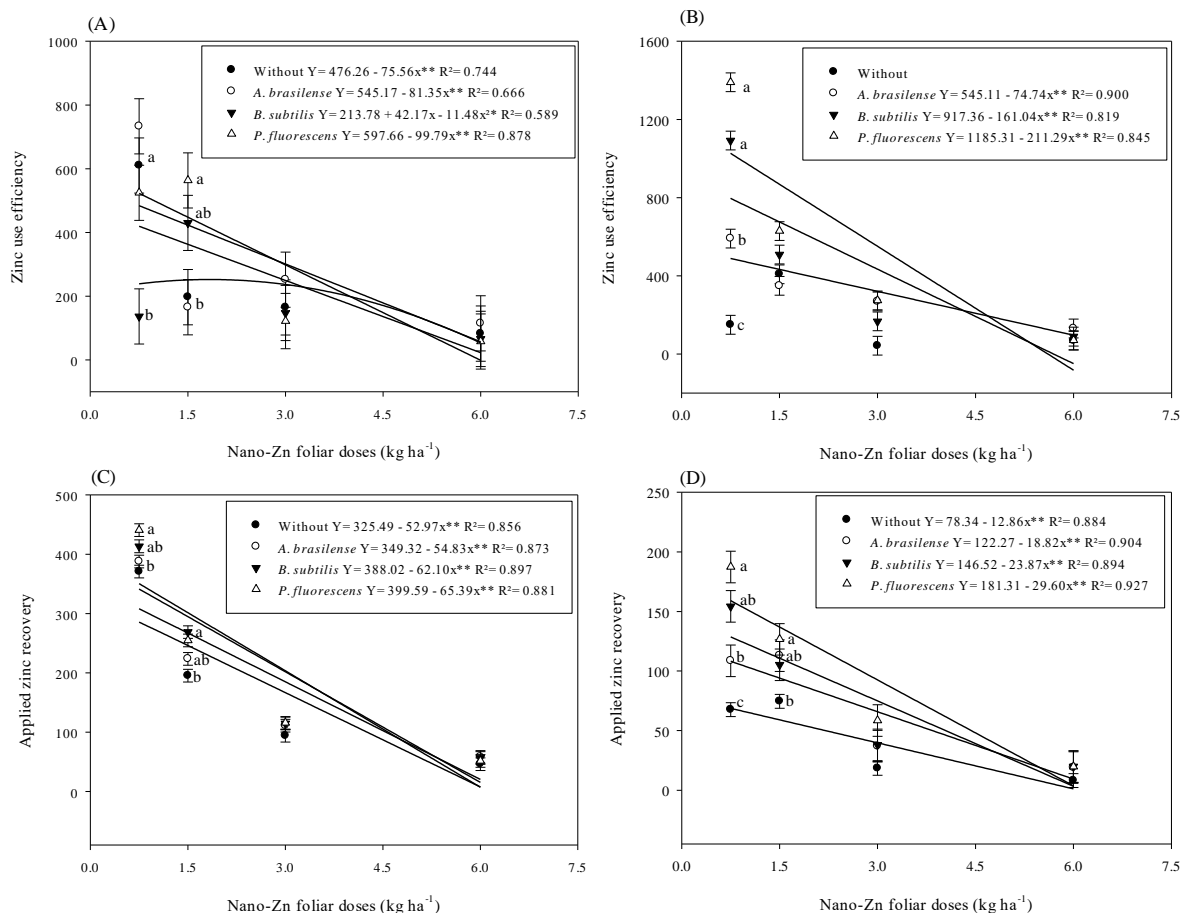
### 6.3.3 Zinc intake and Zn use efficiencies

The interactions of inoculation and nano-Zn foliar fertilization were not significant for estimated daily Zn intake in both cropping seasons (Figure 6.5). Inoculation with *P. fluorescens* was observed the best treatment that improved estimated daily Zn intake in Brazil by 24 and 16% as compared to without inoculation. The quadratic function for Zn intake described that increasing foliar nano-Zn application up to 3.9 and 3.6 kg ha<sup>-1</sup> improved estimated Zn intake in 2019 and 2020 respectively (Table 6.2). In both cases, further increase in Zn-foliar doses reduced estimated daily Zn intake in wheat grains.

The interaction of inoculation with PGPBs and foliar nano-Zn application were significant for both Zn use efficiency (ZnUE) and applied Zn recovery (AZnR) (Appendix A; Table 7). Treatments with inoculation of *P. fluorescens* increased ZnUE by 20.5 and 254% in 2019 and 2020 cropping season respectively. In addition, inoculation with *P. fluorescence* was observed with higher AZnR (22.7 and 133%) in 2020 and 2021 cropping season as compared to without inoculation treatments (Appendix A; Table 7). Graph trend indicated that increasing nano-Zn foliar application up to 3.3, 1.8 and 3.0 kg ha<sup>-1</sup> in combination with inoculation of *A. brasilense*, *B. subtilis* and *P. fluorescens* respectively increased Zn use efficiency in the first wheat growing season (Figure 6.5A). In 2020, calculated doses of 3.6, 2.8 and 2.8 kg ha<sup>-1</sup> of nano-Zn foliar application along with inoculation of *A. brasilense*, *B. subtilis* and *P. fluorescens* respectively, increased Zn use efficiency (Figure 6.5B). Applied Zn recovery was increased with increasing doses of nano-Zn foliar up to 3.18, 3.12 and 3.05 in 2019 (Figure 6.5C) and 3.25, 3.06 and 3.06 in

2020 (Figure 6.5D) along with inoculation of *A. brasilense*, *B. subtilis* and *P. fluorescens*, respectively.

**Figure 6.5** Influence of inoculation with PGPBs and nano-Zn foliar application on; grain yield in 2019 (A) and 2020 (B), and Zn partitioning index (ZPI) in 2019 (C) 2020 (D) of wheat.



**Source:** Arshad. The different letters correspond to a significant difference at 5% probability level ( $p \leq 0.05$ ). The identical alphabetic letters do not differ from each other as analyzed by Tukey test (PGPBs inoculations;  $p \leq 0.05$ ) and Regression (Foliar Zn rates;  $p \leq 0.05$ ) tests for wheat cropping year 2019 and 2020 respectively. Error bars indicate the standard error of the mean ( $n = 4$  replications). \*\*: significant at  $p \leq 0.01$ .

## 6.4 Discussion

Wheat grains are inherently low in Zn concentration and may not deal with human Zn requirement in Zn deficient regions therefore, a potential and sustainable strategy is needed to

improve grain Zn concentration from inadequate to adequate concentration for better human health (HUSSAIN *et al.*, 2013). Integrated use of Zn and PGPBs is one the sustainable and easily adoptable technology that can effectively eliminate human and plant Zn scarcity by improving nutrition and productivity of wheat (JALAL *et al.*, 2022). In this sense, the current experiment exhibited that nano-Zn foliar application in combination with inoculation of PGPBs increased nutrients (N, P and Zn) concentration in stem and grains of wheat under field conditions (Table 6.1; Table 6.3). This might be due to the involvement of PGPBs in different soil below and above mechanisms such as production of phyto-hormones and enzymes, carboxylation, and biological fixation of nitrogen that could contribute the solubilization and availability of nutrients to plants for better absorption (MITTER *et al.*, 2013; REHMAN *et al.*, 2019b). Inoculation with *Pseudomonas* sp. increased roots architecture, branching and proliferation, which could increase bioavailability of nutrients to keep plants healthy and fit in harsh environmental conditions (Abadi *et al.*, 2021). In addition, Zn is an important constituent and co-factor of several enzymes, cell division and elongation that help the plants to maintain different biochemical activities, lead to better crop growth, physiology, fortified grains and greater yield (ULLAH *et al.*, 2019; JALAL *et al.*, 2020a,b). Therefore, the present study indicated that inoculation with *B. subtilis* and *P. fluorescens* in combination with foliar nano Zn fertilization increased N, P and Zn concentration shoot and grains of wheat (Table 6.1, 3; Figure 6.3). Some previous studies support our results that inoculants like *A. brasilense*, *B. subtilis* and *P. fluorescens* promote nutrient uptake and plant growth (PEREIRA *et al.*, 2020; ROSA *et al.*, 2020).

The present study indicated that inoculation with *P. fluorescens* and *B. subtilis* along with nano-Zn foliar fertilization were observed with greater shoot dry matter and yield of wheat (Table 6.4; Figure 6.4). It is possible due to the role of these PGPBs in developing root system that are acting as gateway for better nutrients absorption, leading to better plant performance and productivity (CALZAVARA *et al.*, 2018; MORETTI *et al.*, 2020). Previously, inoculation with *Bacillus* sp. along zinc oxide is considered the most effective strategy for improving different physiological and biochemical traits of maize thus, enhancing growth and yield with high nutritional values under field conditions (HUSSAIN *et al.*, 2019). Co-application of PGPBs and Zn increased Zn use efficiency under tropical soils that had promoted plant growth and yield of maize-wheat cropping system (GALINDO *et al.*, 2021). In addition, zinc is one of the important nutrients of plant growth regulation, cell multiplication and biochemical mechanisms, all these

functions together lead to higher dry matter and yield production (DOOLETTE *et al.*, 2018). It has also reported that co-application of Zn and PGPBs could modulate plant defensive system by improving photosynthetic pigments and primary metabolites, leading to better plant performance and yield (TANVEER *et al.*, 2022).

Wheat expansion to tropical and marginal region is a best option to execute food security however, its inherent makeup of high phytic acid ratio to Zn concentration in grains can cause malnutrition under Zn deficient regions (CAKMAK *et al.*, 2010; CHATTA *et al.*, 2017). Therefore, the current study indicated that foliar application of nano-Zn with *P. fluorescens* increased Zn partitioning and dietary intake of wheat grains (Table 6.4; Figure 6.4C). It had also reported that Zn solubilizing bacteria like *Pseudomonas* and *Bacillus* sp. strains activate different soil-plant Zn mechanisms to convert insoluble soil into plant available to improve accumulation, grain biofortification and greater yield (HAFEEZ *ET AL.*, 2013; RAMESH *et al.*, 2014; SINGH *et al.*, 2017a; SINGH *et al.*, 2017b; JALAL *et al.*, 2021). Zinc solubilizing bacteria had potentially increased Zn bioavailability, assimilation in plant and grains tissues and consumption by human beings through reduction in phytic-P concentration (RAMESH *et al.*, 2011; MUMTAZ *et al.*, 2017). Some previous studies reported that application of foliar Zn is highly mobile in phloem where it is quickly assimilated and remobilized into new generating grains of wheat (DOOLETTE *et al.*, 2020; LIU *et al.*, 2019; REHMAN *et al.*, 2021). The remobilization and localization in wheat grain could better deal with Zn malnutrition in humans (FIRDOUS *et al.*, 2020).

Zinc efficiencies are better defined by the ratio of grain Zn concentration to Zn deficient soils, where increasing single Zn sources can decrease Zn use efficiencies. Therefore, the present study exhibited that inoculation of *P. fluorescens* in combination with nano-Zn foliar application increased Zn use efficiency and applied Zn recovery under field cultivation of wheat (Figure 6.5). This might be due to the effectiveness of PGPBs in dissolution of oxides, sulphides and carbonates, which increase bioavailability of Zn for better use of plants (SARAVANAN *et al.*, 2004). Previous studies performed by Ullah *et al.* (2020) and Jalal *et al.* (2020a, b) reported that inoculation of PGPBs along with Zn fertilization increased Zn use efficiencies under cultivation of different crops on low Zn content soils.

## 6.5 Conclusion

The combined use of foliar nano-Zn and plant growth-promoting bacteria (PGPBs) is one of the quick and sustainable alternative strategies for cereal production. It was verified from our results that inoculation of PGPBs along with nano-Zn foliar application increased plant and grain N, P and Zn concentrations, growth and yield of wheat. The seed inoculation with *B. subtilis* and *P. fluorescens* increased shoot and grain N, P and Zn concentrations in shoot and grains, dry matter, grain yield and Zn partitioning index in wheat. The combined inoculation of *P. fluorescens* and *B. subtilis* along with optimal calculated dose of nano-Zn foliar application ranging from 3 to 3.5 kg ha<sup>-1</sup> increased most of the evaluated traits of wheat. Zinc intake with daily wheat consumption in Brazil, Zn use efficiency and applied Zn recovery were also increased with inoculation of *P. fluorescens* in combination with nano-Zn foliar application under field conditions. In this context, combined use of inoculation with *P. fluorescens* and foliar nano-Zn under tropical savannah regions could be an effective mechanism to improve plant nutrient acquisition and use efficiencies particularly Zn, leading to sustainable production and biofortification of wheat. Prospective research aiming to improve Zn use efficiency and recovery and know the hormonal or regulator effects of inoculation and co-inoculation with PGPBs and their influence on cereal performance, biofortification, and physiological processes in different edaphic conditions to better understand Zn solubilization, assimilation, and partitioning under field conditions.

## CHAPTER-7

### 7. PLANT GROWTH-PROMOTING BACTERIA IS AN ALTERNATIVE STRATEGY FOR INCREASING GRAIN BIOFORTIFICATION, YIELD AND ZINC USE EFFICIENCY OF MAIZE

#### 7.1 Introduction

Maize (*Zea mays* L.) is a crop of social and economic importance, feed more than 65% of global population with sustainable intake of proteins and calories (XUE *et al.*, 2019). It is a versatile cereal cultivated in diversified environments due to its changing food habits and increasing non-vegetarians' consumption (AYYAR *et al.*, 209). Brazil is the 3<sup>rd</sup> largest producer of maize around the world with a production of 95 million tons from 17.5 million hectares (CONAB, 2020) but still low in average yield as compared to American and European regions (USDA, 2020). Maize is inherently poor in minerals concentration which is usually plagued by a widespread zinc (Zn) deficiency in tropical regions and ultimately confront plant nutrients acquisition, productivity and food quality as well as human nutrition and health (OBAID *et al.*, 2022).

Zinc deficiency is a global threat, affecting one third of agricultural soils and leading to poor production and nutritional quality of cereal crops (CAKMAK *et al.*, 2017). Zinc soil deficiency is mainly caused by its abundant soil silicate, oxide, phosphate, and carbonates in soil as well as extensive farming and chemical fertilization, and inadequate irrigation (MASOOD *et al.*, 2022). In addition, Zn is the most transition nutrient for plant physiological processes, protein synthesis, energy production, genes expression, photosynthesis and enzymatic activities, pollen fertility, hormonal and carbohydrate metabolism while discouraging pathogens infestation in cereal crops (SUGANYA *et al.*, 2020). Besides this, cereals based low Zn nutritional security is mainly contributing to human Zn deficiency and has become challenge of the day, especially in developing countries (DHALIWAL *et al.*, 2022) and tropical soils (JALAL *et al.*, 2021). This introduced the requirement of ultra-nourished strategy like agronomic biofortification to alleviate malnutrition with effective improvement of nutrition and dietary consumption for targeted population (AHSIN *et al.*, 2020). However, biofortification of crops with single nutrient in soil application may not be enough for better growth, nutrition, and productivity under harsh tropical conditions. Thus, microbes mediated biofortification of field crops is an ecofriendly and sustainable strategy to better

understand transport of nutrients to grains with greater productivity and nutrient use efficiency (JALAL *et al.*, 2021).

Several diazotrophic bacteria are being involved in stimulation of different direct (nutrients acquisition and growth stimulation) and indirect mechanisms (stress diminution and bio-control resistance) that can improve nutrients cycling, maintaining homeostasis, decomposition of organic material with greater crop production under sustainable and ecofriendly environment (HAKIM *et al.*, 2021). These rhizosphere bacteria unlocked Zn in the soil by establishing association with plant roots for better accessibility to support plant growth and production (MASOOD *et al.*, 2022). These microbes synthesis chelating compounds in the roots rhizosphere where they form complexes with Zn and increase its availability and consequently biofortification (BASHIR *et al.*, 2021). Several genera of beneficial bacteria such as *Rhizobium*, *Pseudomonas*, *Azospirillum*, and *Bacillus* are being quoted as Zn solubilizer that facilitate translocation of Zn from soil to different plant tissues, promoting productivity and enriching grains, thus supporting ecofriendly agronomic biofortification (MASOOD *et al.*, 2022; MUMTAZ *et al.*, 2017; REHMAN *et al.*, 2018). Inclusion of Zn solubilizing bacteria is the most competent, feasible and low expensive strategy for Zn biofortification of edible grains (especially maize) with admirable results on sustainable agriculture (UPADHAYAY *et al.*, 2022).

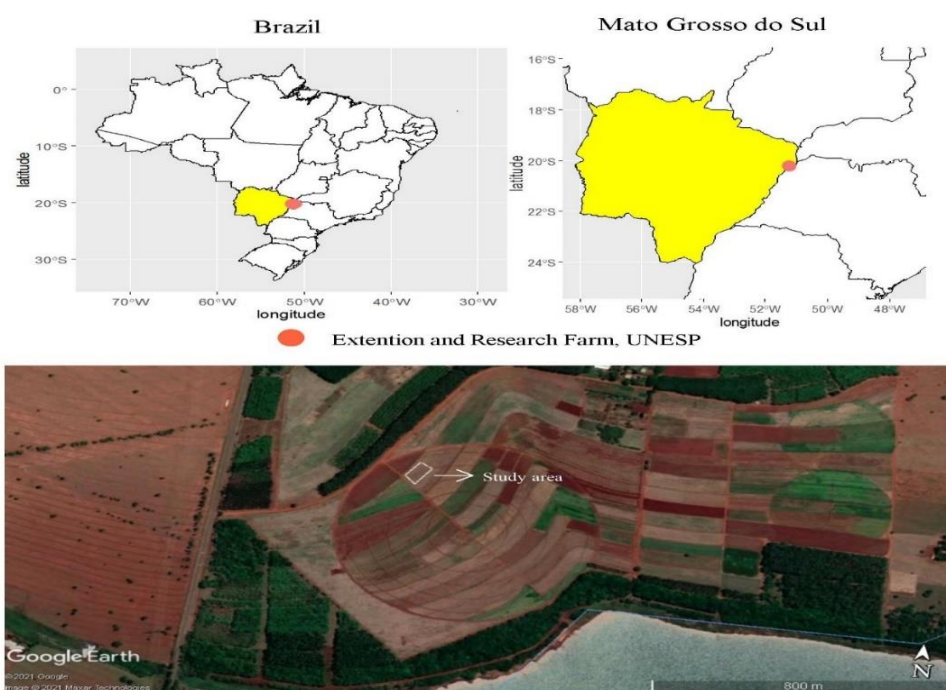
Maize is currently the largest cereal source in world therefore, it is required to determine sources and dissemination of Zn uptake in maize grains for better understanding its performance on global Zn cycling. The literature is lacking with Zn biofortification of maize under the interaction of diazotrophic bacteria and soil applied Zn in tropical Savannah. There is also a research gap on the association of diazotrophic bacteria and residual Zn fertilization on Zn nutrition, Zn use efficiency (ZnUE) and yield of maize crop. The integrated use of diazotrophic bacteria and chemical fertilizer is an emerging alternative in agricultural world. Therefore, it was hypothesized that inoculations of diazotrophic bacteria may have synergetic relation with residual Zn application on plant and grain concentrations, growth, yield, ZnUE and daily intake of biofortified maize grains in tropical Savannah of Brazil. Therefore, the specific objectives of this study were to evaluate better performing diazotrophic inoculant in presence and absence of residual soil Zn fertilization on maize growth, leaf and grains Zn concentration and accumulation, yield and Zn use efficiencies in two consecutive growing seasons.

## 7.2 Materials and Methods

### 7.2.1 Experimental site and climate description

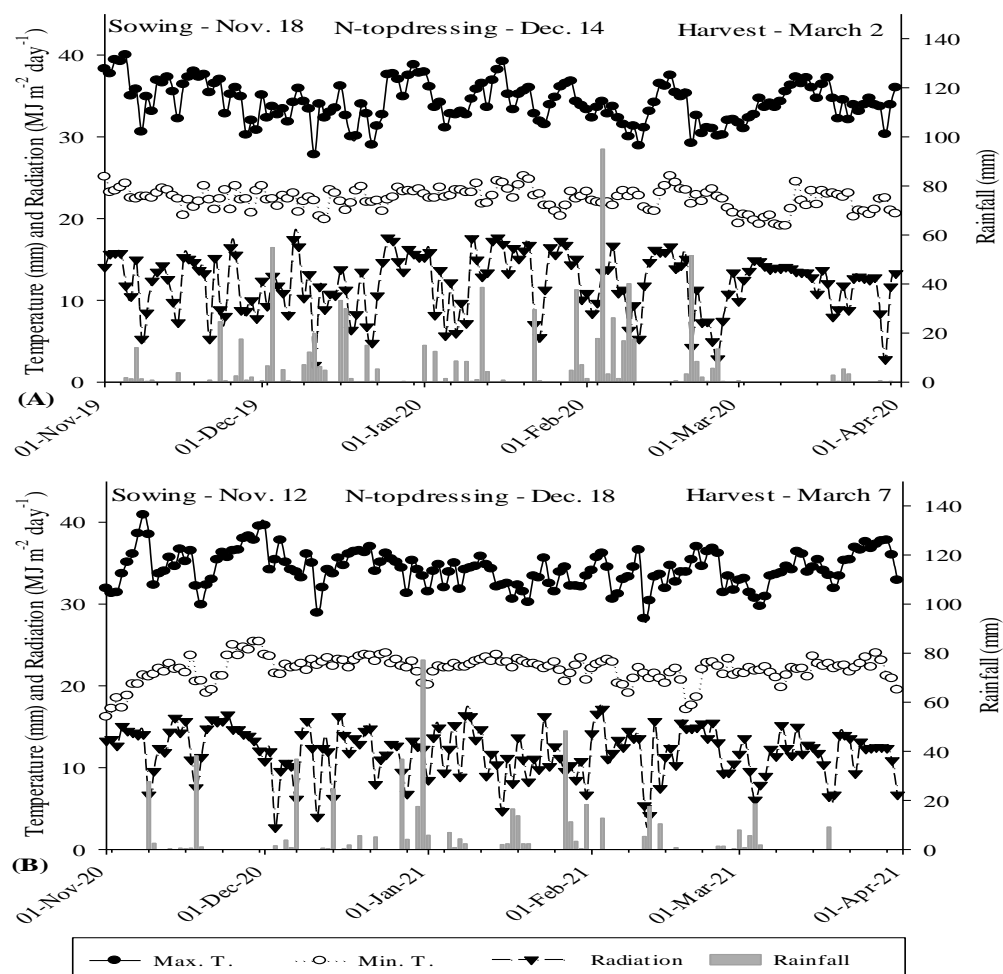
Two years maize (*Zea mays* L.) field experiment were conducted at Research and Extension Farm of School of Engineering, São Paulo State University (UNESP) in Selvíria, state of Mato Grosso do Sul, Brazil at geographical coordinates of 20° 22' S latitude, 51° 22' W longitude and an altitude of 335 m above sea level (Figure 7.1). The site has been cultivated with cereals-legumes cropping system for more than 28 years, previously cultivated with wheat, wherein last 13 years under no-tillage system. The soil of experimental site is classified as Rhodic Haplustox (SOIL SURVEY STAFF, 2014) and Red Dystrophic with a clayey texture (SANTOS *et al.*, 2018). The climate of the site is classified as humid tropical of Aw type, rainy in summer and dry in winter according to Koppen climate classification (ALVARES *et al.*, 2013). The rainfall, minimum, maximum and average temperatures and air humidity of maize cultivation period is summarized in Figure 5.2.

**Figure 7.1.** Location of the experimental area at Extension and Research Farm, UNESP -Ilha Solteira Campus, at Selvíria, state of Mato Grosso do Sul, Brazil (20°22' S, 51°22' W, altitude of 335 m) in 2019-2020 and 2020-2021 crop seasons.



**Source:** Author. The map was created by using geographic information system (QGIS) software and the Google Earth program. The QGIS Development Team (2021). Open-Source Geospatial Foundation project. <http://qgis.osgeo.org>. Accessed on: 27 February, 2022. Projection System WGS 84/UTM 200DC [EPSG: 4326]. This image was taken from the Google Earth program, Google Company (2021). Map data: Google, Maxar Technologies.

**Figure 7.2.** Rainfall, maximum and minimum temperatures, and light radiation were acquired from the weather station of Extension and Research Farm of School of Engineering—UNESP during maize cultivation period from November to March 2019-2020 and 2020-2021.



### 7.2.2 Soil analysis

Twenty random soil samples were collected before experiment installation with cup auger from 0.00-0.20 m soil layer. The samples were mixed to make a homogeneous/composite sample

for the determination of chemical and granulometric characterization (RAIJ *et al.*, 2001). The chemical and physical characterization of the site are summarized in Table 5 (Appendix A).

### 7.2.3 Experimental design and treatments

The experiments were designed in randomized complete block in 4 x 2 factorial scheme with four replications. The treatments were consisted of four seed inoculations with diazotrophic bacteria (No inoculation, *Azospirillum brasilense*, *Bacillus subtilis*, and *Pseudomonas fluorescens*) and two residual zinc (Zn) applications (without- 0 kg ha<sup>-1</sup> of Zn and with- 8 kg ha<sup>-1</sup> of Zn), applied from zinc sulphate (21% Zn and 10% S).

The inoculation of *A. brasilense* strains Ab-V5 and Ab-V6 (Ab-V5 = CNPSo 2083 and Ab-V6 = CNPSo 2084 with guarantee of 2x10<sup>8</sup> CFU ml<sup>-1</sup>) was conducted at a dose of 200 mL ha<sup>-1</sup> (liquid inoculant) added in small quantity of water to uniformly mix in around 24 kg of maize seeds sown ha<sup>-1</sup>. The *B. subtilis* (strain CCTB04 with guarantee of 1x10<sup>8</sup> CFU ml<sup>-1</sup>) and *P. fluorescens* (strain CCTB03 with guarantee of 2x10<sup>8</sup> CFU ml<sup>-1</sup>) were performed at a dose of 150 ml ha<sup>-1</sup> (liquid) according to the recommendation of inoculants providing company (Total Biotechnology®), Curitiba, Brazil. The inoculations were performed an hour before plantation of the crop, followed in both cropping seasons.

Zinc fertilization (0 and 8 kg ha<sup>-1</sup>) was performed only in 2019 and 2020 (May to September both years) of wheat cropping seasons. Zinc sulphate was manually applied to soil surface between rows of wheat at V1/V2 stage (one to two completely unfolded leaves) and followed by 14 mm irrigation to incorporate into soil. Thus, zinc was not directly applied in maize cultivation season, residual effect of Zn applied in wheat was evaluated in current experiment.

### 7.2.4 Plant materials

The experimental site was applied with herbicides glyphosate (1800 g ha<sup>-1</sup> of a.i.) and 2,4-D (670 g ha<sup>-1</sup> of a.i.) 15 days before plantation. Seeds were chemically treated with Standak Top®, a mix formulation of insecticide- imidacloprid + thiodicarb (45 g + 135 g of a.i. per 100 kg seeds) and fungicide- carbendazim + thiram (45 g + 105 g of a.i. per 100 kg seeds) a day before inoculations and plantation. A simple maize hybrid (2B810 PW-DOW) was sown on 18<sup>th</sup> November, 2019 and 12<sup>th</sup> November, 2020 in a no-tillage system at 3.3 seeds m<sup>-1</sup>. All treatments were applied with 350 kg ha<sup>-1</sup> of NPK (08-28-16, urea) on the basis of soil analysis. Each experimental unit was 6 m long with 6 rows, 0.45 m apart with total plot size of 16.2 m<sup>2</sup>. Post-

emergence herbicides atrazine + tembotrione (1000 + 84 g a.i. ha<sup>-1</sup> respectively) were applied at V3 stage to control weeds. The topdressing fertilization of nitrogen (120 kg N ha<sup>-1</sup> from ammonium sulphate) at V6 stage was performed in all treatments to uniformly distribute on soil surface. The crop was irrigated with pivot-irrigation system at 14 mm water volume according to the need of the crop. The crop was manually harvested on 2<sup>nd</sup> March, 2020 and 7<sup>th</sup> March, 2021.

## **7.2.5 Evaluations and analysis**

### **7.2.5.1 Growth and yield attributes**

Plant height was measured with meter-rod from ground to upper apex. Shoot dry matter was determined by harvesting four central lines, sun dried and weighed. Ten random ears were collected at harvest to count number of rows and grains ear<sup>-1</sup>plot<sup>-1</sup>. Hundred grains mass was measured with a precise scale on 13% humidity (wet basis). Ears were collected from central lines of each plot, threshed with electric thresher and processed to calculate yield in kg ha<sup>-1</sup> (productivity at 13% moisture content). The dried grains were then ground in a Willey mill for nutritional analysis.

### **7.2.5.2 Nutritional analysis**

Twenty random leaves were collected from ear insertion at flowering stage in each plot. The plant material (shoot and grain) was collected at the time harvest. The samples were dried in an air-tight oven at 60 ± 5 °C for 72 hours to attain uniform humidity. The material for each attribute was then individually grounded in a stainless-steel Willey knife mill by passing through a 10-mesh sieve in labeled plastic bags. Each sample was weighed (0.25 g), digested with nitroperchloric digestion (HNO<sub>3</sub>:HClO<sub>4</sub> solution) and quantified by atomic absorption spectrophotometry following procedure of Malavolta *et al.* (1997) Zinc shoot and grains accumulation (g ha<sup>-1</sup>) were calculated from respective Zn concentration in shoot and grains and dry matter yield ha<sup>-1</sup> respectively.

### **7.2.5.3 Zinc partitioning index, intake and use efficiencies**

Zinc partitioning index (ZPI) was calculated from the ratio of shoot Zn concentration to total (shoot + grains) Zn concentration in percent following Rengel and Graham (Teixeira *et al.*, 2017). Estimated Zn intake in Brazil (Eq. 1) was calculated from Zn biofortified grains of present study (RENGEL; GRAHAM, 1996). Brazil per capita maize consumption is around 24.69 kg

person<sup>-1</sup> year<sup>-1</sup> (67.6 g person<sup>-1</sup> day<sup>-1</sup>). Based on this information, estimated Zn intake of biofortified grains was calculated below in Eq. 1.

$$\text{Zn intake} = [\text{Zn grain}] \times C \quad (1)$$

Where Zn intake (g person<sup>-1</sup> day<sup>-1</sup>) is daily estimated Zn consumption person<sup>-1</sup>, [Zn grain] (g kg<sup>-1</sup>) is Zn concentration in biofortified grains and C (g person<sup>-1</sup> day<sup>-1</sup>) is average maize consumption per person in Brazil (LESSA *et al.*, 2019)

Zinc use efficiency (ZnUE), agro-physiological efficiency (APE), recovery applied Zn (RAZn) and utilization efficiency (UE) were derived from the fractions of Zn concentration and accumulation in shoot and grains, dry matter and grain yield following procedures of (FEGERIA *et al.*, 2011; JALAL *et al.*, 2021).

$$\text{ZnUE} = (\text{GYF} - \text{GYWF}) \div \text{Zn applied dose} \quad (2)$$

$$\text{APE} = (\text{GYF} - \text{GYWF}) \div (\text{ZnAF} - \text{ZnAWF}) \quad (3)$$

$$\text{RAZn} (\%) = (\text{ZnAF} - \text{ZnAWF}) \div \text{Zn applied dose} \quad (4)$$

$$\text{UE} = \text{PE} \times \text{RAZn} \quad (5)$$

Where GYF = grain yield in Zn fertilized treatments, GYWF = grain yield in without Zn fertilized treatments, ZnAF = zinc accumulation in shoot + grain within fertilized treatments, ZnAWF = zinc accumulation in shoot + grain without fertilized treatments, PE = physiological efficiency.

### 7.2.6 Statistical analysis

The data was tested for normality with Shapiro and Wilk test which showed that data is normally distributed ( $W \geq 0.90$ ). The data were submitted to analysis of variance (F test). Zn soil application, diazotrophic bacterial inoculations and their interactions were considered fixed effects in the model. When a main effect or interaction was observed significant by F test ( $p \leq 0.05$ ) then Tukey test ( $p \leq 0.05$ ) was used for comparison of means of residual soil Zn fertilization and diazotrophic bacterial inoculations.

The Pearson correlation analysis ( $p \leq 0.05$ ) was performed using R software. To create a heatmap, the corrplot package was used, using the "cor" and "cor.mtest" functions to calculate the coefficients and p-value matrices.

## 7.3 Results

### 7.3.1 Plant height, dry matter and grain yield

The insertion of first productive cob, plant height, shoot dry matter and grain yield of maize were significantly increased with diazotrophic bacterial inoculation in residual Zn applied treatments as compared to without Zn residual treatments (Table 7.1). The residual Zn applied treatments produced taller plants with elevated insertion of productive cob (first cob insertion) as compared to control. The plant height was increased by 1.9 and 2.2% in 2019-2020 and 2020-2021 cropping seasons, respectively. Seed inoculation with *A. brasilense* increased plant height by 4.5% while height of productive cob was increased by 5.7 and 6.4% with seed inoculation of *P. fluorescens* as compared to control in both cropping seasons respectively.

**Table 7.1.** First productive cob insertion, plant height, shoot dry matter, 100-grain mass and grain yield of maize as influenced by diazotrophic bacteria and residual zinc doses in 2019-2020 and 2020-2021 cropping season.

Treatments	Plant height		First cob insertion		Shoot dry matter		100 grains mass		Grain yield	
	----- m -----		-----		--- kg ha <sup>-1</sup> ---		----- g -----		--- kg ha <sup>-1</sup> ---	
	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
<b>Inoculations (I)</b>										
Without	2.66 b	2.67 b	1.22 b	1.25 b	11945 b	11832	30.6 b	31.7	7379	7307
<i>A. brasilense</i>	2.78 a	2.79 a	1.29 ab	1.31 a	12642 a	12654	33.5 a	34.4	8109	8233
<i>B. subtilis</i>	2.67 ab	2.72 ab	1.27 ab	1.31 a	12381 a	12381	32.7 ab	35.4	8449	8555
<i>P. fluorescens</i>	2.72 ab	2.77 a	1.29 a	1.33 a	12355 a	12243	31.8 ab	33.5	7911	7952
<b>Residual Zinc (Zn) doses (kg ha<sup>-1</sup>)</b>										
0	2.67 b	2.71 b	1.25 a	1.28 b	12102 b	12040	31.3 b	32.7	7709	7806
8	2.72 a	2.77 a	1.28 a	1.32 a	12559 a	12515	32.9 a	34.8	8215	8218
<b>F-values</b>										
I	0.004 **	0.00 **	0.03 *	0.00 **	0.00 **	0.00 **	0.01 *	0.00 **	0.00 **	0.00 **
Zn	0.01 **	0.00 **	0.11 <sup>ns</sup>	0.008 **	0.00 **	0.00 **	0.01 *	0.00 **	0.00 **	0.00 **
I x Zn	0.63 <sup>ns</sup>	0.19 <sup>ns</sup>	0.99 <sup>ns</sup>	0.86 <sup>ns</sup>	0.36 <sup>ns</sup>	0.04 *	0.94 <sup>ns</sup>	0.02 *	0.03 *	0.02 **
<b>CV (%)</b>	2.1	1.7	3.9	2.4	2.3	1.8	5.2	2.8	4.0	3.6

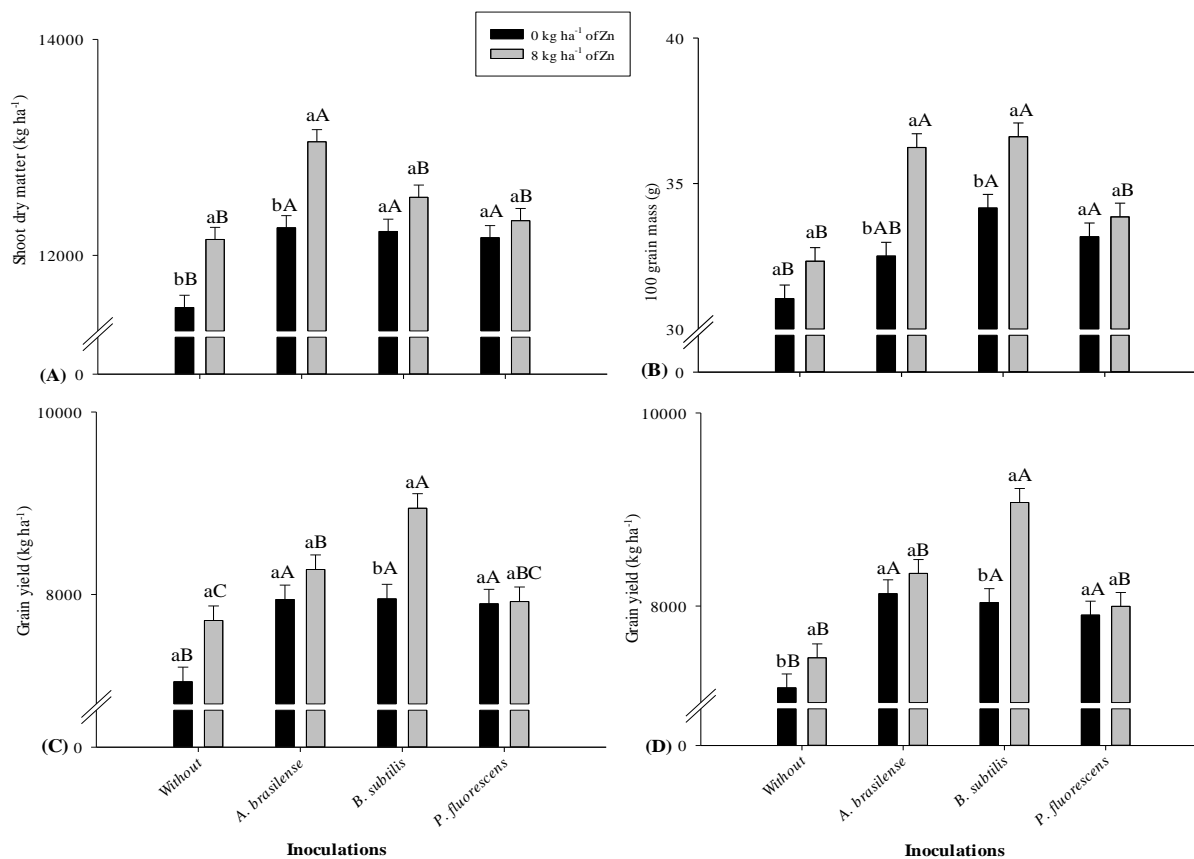
Means in the column followed by different letters are statistically different by Tukey test,  $p \leq 0.05$ .

\*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$  respectively while <sup>ns</sup>—non-significant by F-test.

Shoot dry matter was significantly greater with residual Zn application and bacterial inoculation in 2019-2020 and 2020-2021 cropping seasons (Table 7.1). Residual Zn applied treatments were observed with greater shoot dry matter (3.7 and 3.9%) as compared to control treatments in both cropping seasons. The treatments with inoculation of *A. brasilense* were noted with greater dry matter (5.8 and 6.9%), which were statistically at par with treatments of *B. subtilis* and *P. fluorescens* in both studied cropping seasons. The interaction of residual Zn doses and bacterial inoculations for shoot dry matter was significant only in the second cropping season (Figure 7.3A).

Hundred grains mass and grain yield of maize were significantly increased with residual Zn application and diazotrophic bacteria inoculations in 2019-2020 and 2020-2021 cropping seasons (Table 7.1). The single effect of residual Zn ( $8 \text{ kg ha}^{-1}$ ) increased mass of 100 grains by 5.9 and 6.4% in relation to control. The inoculation with *A. brasilense* produced heavy 100 grains (9.5%), which was statistically similar to treatments of *B. subtilis* and *P. fluorescens* in 2019-2020 maize harvest in comparison to control. The treatments inoculated with *B. subtilis* increased 100 grains mass by 11.7% in 2020-2021 maize harvest which was statistically at par with treatments of *A. brasilense* as compared to control. The interaction for 100 grain mass was significant only in second harvest (Figure 7.3B). In addition, residual Zn applied treatments increased grain yield by 6.6 and 5.3% while inoculation with *B. subtilis* increased by 14.5 and 17.1% in 2019-2020 and 2020-2021 cropping seasons as compared to control (Table 7.1). The interactions for grain yield were significant in both cropping seasons (Figure 7.3C, D). The seed inoculation with *B. subtilis* was observed with greater grain yield under residual Zn application while *A. brasilense* treatments were noted with greater grain yield in the treatments without Zn fertilization (control) in both maize harvests. The treatments with *P. fluorescens* and the control were observed with lower grain yield regardless of the Zn fertilization in both years of crop harvest (Figure 7.3C, D).

**Figure 7.3.** Maize shoot dry matter (A) and 100 grains mass (B) in 2020-2021 respectively, and grain yield in 2019-2020 (C) and 2020-2021 (D) as function of residual Zn doses and diazotrophic bacteria.



**Source:** Author. Without = control (no inoculation). The uppercase letters are used for inoculation interactions within each level of soil applied residual Zn whereas lowercase letters are used for the interactions of Zn doses (presence and absence) within each inoculation treatment. The identical alphabetic letters do not differ from each other by Tukey test ( $p \leq 0.05$ ) for Zn doses and inoculations in 2019-2020 and 2020-2021. Error bars indicate standard error of the mean ( $n = 4$  replications).

### 7.3.2 Zinc concentration in leaf, shoot and grains

Residual Zn application and bacteria inoculation significantly increased leaf, shoot and grain Zn concentrations of maize in 2019-2020 and 2020-2021 cropping seasons (Table 7.2). Leaf Zn concentration was increased by 15.2 and 17.6% under soil applied Zn doses in relation to

control in first and second cropping seasons respectively. Inoculation with *P. fluorescens* was observed with higher leaf Zn concentration (23.8 and 34.1%), which were statistically at par with the treatments inoculated with *B. subtilis* and *A. brasilense* in both cropping seasons.

**Table 7.2.** Leaf, shoot and grain zinc (Zn) concentrations of maize as function of residual Zn doses and diazotrophic bacteria in 2019-2020 and 2020-2021 cropping season.

Treatments	Leaf Zn concentration		Shoot Zn concentration		Grain Zn concentration	
	----- mg kg <sup>-1</sup> -----					
	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
<b>Inoculations (I)</b>						
Without (control)	20.6 b	21.7	29.1 b	29.5 b	28.2 b	32.5
<i>A. brasilense</i>	23.5 ab	27.4	35.8 ab	35.7 a	35.4 a	41.0
<i>B. subtilis</i>	23.9 ab	28.0	33.4 ab	33.9 ab	32.9 a	36.9
<i>P. fluorescens</i>	25.5 a	29.1	37.7 a	38.5 a	34.6 a	38.5
<b>Residual Zinc (Zn) dose (kg ha<sup>-1</sup>)</b>						
0	21.7 b	24.4	31.6 b	31.9 b	30.8 b	34.1
8	25.0 a	28.7	36.4 a	36.9 a	34.7 a	40.3
<b>F-values</b>						
I	0.03 *	0.00 *	0.01 *	0.002 **	0.00 **	0.00 **
Zn	0.008 **	0.00 **	0.01 *	0.002 **	0.00 **	0.00 **
I x Zn	0.43 ns	0.04 *	0.76 ns	0.78 ns	0.33 ns	0.03 *
<b>CV (%)</b>	13.5	6.2	14.2	12.0	8.6	6.7

Means in the column followed by different letters are statistically different by Tukey test,  $p \leq 0.05$ .

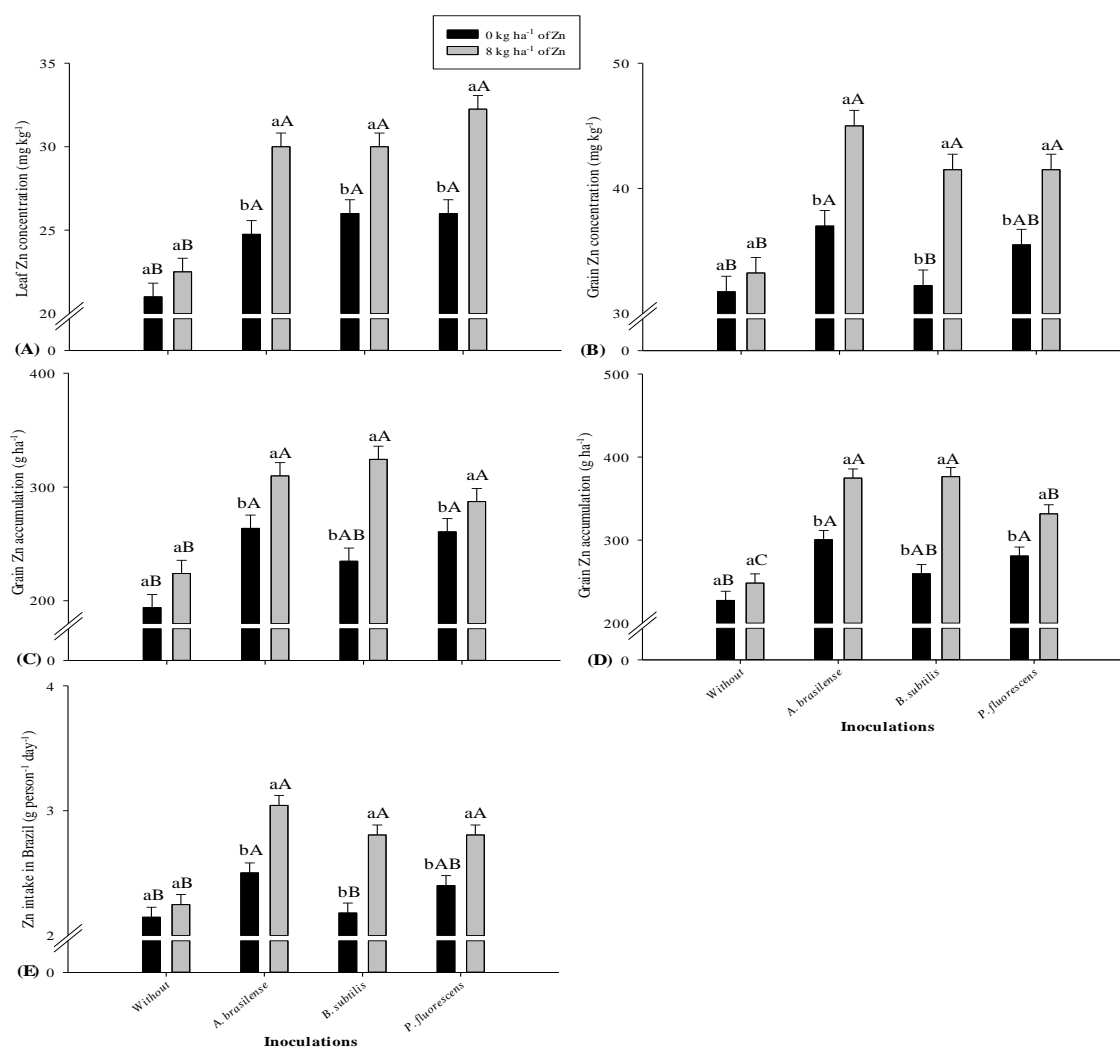
\*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$  respectively while ns—non-significant by F-test.

The interaction of residual Zn and bacterial inoculations for leaf Zn concentration was significant only in 2020-2021 cropping season (Figure 7.4A). The inoculations of all studied bacteria increased leaf Zn concentration in the presence of residual Zn fertilization. However, inoculation with *P. fluorescens* was observed for higher leaf Zn concentration in the presence of residual Zn fertilization while *A. brasilense* with lower in the absence of Zn fertilization (Figure 7.4A).

Maize shoot Zn concentration was increased by 15.2 and 15.7% as a function of residual Zn fertilization in 2019-2020 and 2020-2021 harvest as compared without Zn fertilization treatments (Table 7.2). The inoculation with *P. fluorescens* increased shoot Zn concentration by 29.5 and 30.5% in first and second harvest as compared to control, which were statistically similar to the treatments inoculated with *B. subtilis* and *A. brasilense* (Table 7.2).

Grain Zn concentration in 2019-2020 and 2020-2021 maize harvest were significantly increased by 12.7 and 18.2% under 8 kg ha<sup>-1</sup> soil residual Zn fertilization while inoculation with *A. brasilense* increased grain Zn concentration by 25.5 and 26.2% as compared to control (Table 7.2). The interaction for grain Zn concentration was significant only the second maize harvest (Figure 7.4B). The inoculation with *A. brasilense* was noted for higher grain Zn concentration regardless of the residual Zn fertilization while treatments with *B. subtilis* was observed with low grain Zn concentration in the absence of Zn fertilization (Figure 7.4B).

**Figure 7.4.** Leaf zinc concentration (A) and grains zinc concentration (B) in 2020-2021 respectively, and grain zinc accumulation in 2019-2020 (C) and 2020-2021 (D), and estimated daily zinc intake in Brazil in 2020-2021 cropping maize season (E) as function of residual Zn doses and diazotrophic bacteria.



**Source:** Author. The uppercase letters are used for inoculation interactions within each level of soil applied residual Zn whereas lowercase letters are used for the interactions of Zn doses (presence and absence) within each inoculation treatment. The identical alphabetic letters do not differ from each other by Tukey test ( $p \leq 0.05$ ) for Zn doses and inoculations in 2019-2020 and 2020-2021. Error bars indicate standard error of the mean ( $n = 4$  replications).

### 7.3.3 Zinc shoot and grain accumulation, partitioning and intake in maize

Zinc accumulation in shoot and grain, partitioning index, and estimated Zn intake in maize consumption were significantly influenced by residual Zn doses and diazotrophic bacteria inoculations in maize cropping seasons of 2019-2020 and 2020-2021 (Table 7.3). Shoot Zn accumulation in maize was improved by 19.2 and 20.5% with residual Zn fertilization in first and second maize harvest respectively. Treatments with inoculation of *P. fluorescens* were observed with better accumulation of Zn in shoot (33.8 and 35%) in relation to control, which were statistically similar to the values obtained in treatments with *A. brasilense* in both maize harvest seasons (Table 7.3).

Residual Zn fertilization in 2019-2020 and 2020-2021 maize cropping season improved grain Zn accumulation by 20.2 and 24.6% (Table 7.3). Inoculation with *A. brasilense* was noted with higher grain Zn accumulation (37 and 42%) in first and second maize cropping seasons, which was statistically at par with inoculated treatments of *B. subtilis* and *P. fluorescens* in 2019-2020 maize harvest and with *B. subtilis* in 2020-2021 maize harvest. The interactions of the study factors for grain Zn accumulation were significant in both cropping seasons (Figures 7.4C, D). Inoculation with *A. brasilense* and *B. subtilis* tended to perform better for grain Zn accumulation under residual Zn fertilization while *B. subtilis* in the absence of residual Zn fertilization was observed with low grain Zn accumulation in both crop harvests (Figures 7.4C, D).

Zinc partitioning index was not significantly influenced by residual Zn fertilization and bacteria inoculation (Table 7.3). The treatments with residual Zn fertilization and bacteria inoculations were not statistically different however, inoculation with *P. fluorescens* performed better in partitioning Zn to grains from low Zn in soil, which statistically similar to non-inoculated treatments.

The estimated daily Zn intake in maize consumption in Brazil was significantly increased with residual Zn fertilization and diazotrophic bacteria inoculation in 2019-2020 and 2020-2021

maize harvests seasons (Table 7.3). The residual Zn fertilization increased daily Zn intake by 14.3 and 17.4% in first and second cropping seasons as compared to control.

**Table 7.3.** Shoot and grain zinc accumulation, zinc partitioning index and estimated daily zinc intake by maize in Brazil as function of residual zinc fertilization and diazotrophic bacteria inoculations in 2019-2020 and 2020-2021 cropping seasons.

Treatments	Shoot Zn accumulation		Grain Zn accumulation		Zn partitioning index		Zn intake (Brazil)	
	g ha <sup>-1</sup>		g ha <sup>-1</sup>		%		g person <sup>-1</sup> day <sup>-1</sup>	
	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
<b>Inoculations (I)</b>								
Without I	348.5 b	349.3 b	208.9	237.7	51.8 a	47.5 a	1.9 b	2.2
<i>A. brasilense</i>	452.8 a	453.4 a	286.7	337.6	50.2 a	46.6 a	2.4 a	2.7
<i>B. subtilis</i>	413.5 ab	420.0 ab	279.5	317.9	50.1 a	47.8 a	2.2 a	2.5
<i>P. fluorescens</i>	466.3 a	471.6 a	273.9	306.2	51.8 a	49.8 a	2.3 a	2.6
<b>Residual Zinc (Zn) dose (kg ha<sup>-1</sup>)</b>								
0	383.4 b	384.2 b	238.2	266.9	50.5 a	48.1 a	2.1 b	2.3
8	457.2 a	463.0 a	286.3	332.7	51.1 a	47.8 a	2.4 a	2.7
<b>F-values</b>								
I	0.002 **	0.00 **	0.00 **	0.00 **	0.86 ns	0.31 ns	0.00 **	0.00 **
Zn	0.001 **	0.00 **	0.00 **	0.00 **	0.71 ns	0.77 ns	0.00 **	0.00 **
I x Zn	0.63 ns	0.80 ns	0.04 *	0.00 **	0.99 ns	0.78 ns	0.33 ns	0.02 **
<b>CV (%)</b>	13.7	12.5	8.9	7.3	8.6	6.8	8.6	6.6

Means in the column followed by different letters are statistically different by Tukey test,  $p \leq 0.05$ . \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$  respectively while ns—non-significant by F-test.

The treatments with inoculation of *A. brasilense* tended to increase Zn intake by 26.3 and 22.7% in 2019-2020 and 2020-2021 maize harvest, which were statistically similar to the treatments with *B. subtilis* and *P. fluorescens* in first and with *P. fluorescens* in second maize cropping season. The interaction for daily Zn intake was significant only in the second cropping season (Figure 7.4E). Inoculation with *A. brasilense* tended to increase Zn intake in daily maize consumption regardless of the Zn fertilization, while *B. subtilis* was observed with lower daily Zn intake in the absence of residual Zn fertilization (Figure 7.4E).

### 7.3.4 Zinc efficiencies

Zinc efficiencies such as Zn use efficiency, agro-physiological efficiency, utilization efficiency and applied Zn recovery were positively increased by diazotrophic bacteria inoculations in residual Zn fertilization (Table 7.4). Zinc use efficiency (ZnUE) of maize was increased with inoculation of *B. subtilis* in the treatments applied with residual Zn fertilizations in first and second maize cropping seasons (Table 7.4). The lower ZnUE was observed in the treatments without inoculations.

**Table 7.4.** Zinc efficiencies of maize as function of residual zinc fertilization and diazotrophic bacteria inoculations in 2019-2020 and 2020-2021 cropping seasons.

Treatments	ZnUE		APE				UE		AZnR	
	----- kg kg <sup>-1</sup> -----		----- % -----							
	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21		
<b>Inoculations (I)</b>										
Without	164 c	68 c	19 a	4.5 b	297 b	131 b	12 b	15 b		
<i>A. brasilense</i>	233 b	178 b	7 a	3.8 b	483 a	353 a	35 a	47 a		
<i>B. subtilis</i>	317 a	270 a	10 a	6.5 a	509 a	381 a	32 a	42 a		
<i>P. fluorescens</i>	190 c	135 b	7 a	3.2 b	379 b	219 b	33 a	43 a		
F-values										
I	0.00**	0.00**	0.05*	0.003*	0.00**	0.00**	0.008**	0.00**		
<b>CV (%)</b>	8.3	13	54	20	10	17	29	21		

ZnUE = Zinc use efficiency, APE = Agro-physiological efficiency, UE = Utilization efficiency, and AZnR = Applied zinc recovery. Means in the column followed by different letters are statistically different by Tukey test,  $p \leq 0.05$ . \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$  respectively while <sup>ns</sup>—non-significant by F-test.

Agro-physiological efficiency (APE) was statistically not significant 2019-2020 maize cropping season. Interestingly, APE of maize was significantly increased by with inoculation of *B. subtilis* under residual Zn fertilization in 2020-2021 cropping seasons as compared to control

(Table 7.4). The highest APE was observed in the treatments of *B. subtilis* while the lowest was recorded in treatments of *P. fluorescens* inoculation (Table 7.4).

Zinc utilization efficiency (UE) was increased by 77.4 and 190.8% with seed inoculation of *B. subtilis* in residual Zn fertilization in relation to non-inoculated treatments, which were statistically similar to the treatments of *A. brasilense* in first and second maize cropping seasons respectively (Table 7.4). The highest Zn utilization efficiency was observed with *B. subtilis* while the lowest was noted in control treatments (Table 7.4).

Inoculation with *A. brasilense* under residual Zn fertilization performed better in recovery of applied Zn fertilization in 2019-2020 and 2020-2021 maize cropping seasons. Applied Zn recovery was increased by 191.6 and 213.3% in the treatments with residual Zn fertilization and *A. brasilense* inoculation, which were statistically similar to the treatments inoculated with *B. subtilis* and *P. fluorescens* in both crop harvests (Table 7.4). The lowest applied Zn recovery was observed in control (without inoculation) treatments.

### **7.3.5 Pearson's correlation among evaluated attributes of maize**

There were overall positive and significant correlations among zinc concentrations in maize plants (leaf, shoot and grains) and insertion of first productive cob, plant height, shoot dry matter shoot and grain Zn accumulation, and negative correlation with agro-physiological efficiency while non-significant correlations with zinc partitioning index, zinc use efficiency, applied zinc recovery and utilization efficiency (Appendix B; Figure 3A). A positive correlation was observed between leaf, shoot and grain concentration and shoot and grain Zn accumulation, daily Zn intake, applied Zn recovery, plant height, shoot dry matter, insertion of productive cob and grain yield. A negative correlation was noted between Zn partitioning index and plant height, shoot dry matter, insertion of first productive cob, leaf, shoot and grain concentration and shoot and grain Zn accumulation, and daily Zn intake. A non-significant correlation was noted between zinc utilization efficiency and Zn partitioning index, zinc use efficiency, and 100 grains mass (Appendix B; Figure 3B).

## **7.4 Discussion**

Agronomic biofortification has been recognized the most feasible and effective mechanism for correcting zinc (Zn) deficiency in soil and plant along with better quality yield to improve human health. Single Zn fertilization could not enough facilitate Zn soil, plant, human and

environment at the same time, especially in tropical regions (JALAL *et al.*, 2021; GALINDO *et al.*, 2021). Therefore, integrated use of bio- and mineral fertilizers is an emerging strategy that can mediate nutrients acquisition for soil-plant-human health to facilitate millions of population in a sustainable and ecofriendly manner. Diazotrophic bacteria are colonializing root rhizosphere to soluble mineral nutrients, stimulating plant growth with greater yield as well as improving acquisition of nutrient to edible grains (CHANDRA *et al.*, 2020). The positive correlation between zinc concentrations in maize plants (leaf, shoot and grains) and insertion of first productive cob, plant height, shoot dry matter, grain yield, and shoot and grain Zn accumulation validated hypothesis of the current study (Appendix B; Figure 3B).

Zinc is an essential element of cell development and multiplication and pollen fertility for better plant establishment, growth and reproduction which its deficiency plagued growth and yield (NOULAS *et al.*, 2020; JALAL *et al.*, 2020). However, the integrated application of diazotrophic bacteria such as Zn solubilizing bacteria and Zn fertilization is one of the best alternative and sustainable strategy to improve Zn nutrition with greater growth and productivity (JALAL *et al.*, 2021; UPADHAYAY *et al.*, 2022). Therefore, the current results verified that residual Zn fertilization and inoculation of *A. brasilense* and *P. fluorescens* has increased plant height, height of insertion of first productive cob, dry matter and hundred grains and grain yield of maize (Table 7.1; Figure 7.3). Several previous studies reported that Zn solubilizing bacteria can rapidly colonize in root rhizosphere, where they could increase Zn solubilization by producing siderophores, chelators and several plant growth hormones such as indole acetic acid (IAA), gibberellins and cytokinins that are immensely linked to better plant health, growth and production (UPADHAYAY *et al.*, 2022; MARAG *et al.*, 2018). Zinc solubilizing bacteria such as *A. brasilense* (GALINDO *et al.*, 2021) and *P. fluorescens* (JALAL *et al.*, 2022) together with Zn fertilization are being reported with greater growth and yield of cereal crops.

Maize grains are inherently low in Zn concentration which can further hinder nutrients acquisition and yield (OBAID *et al.*, 2022) in Zn deficient soils. The adequate Zn concentration in maize leaf is ranging from 15-50 mg kg<sup>-1</sup>, below this is considered adequate (MALAVOLTA *et al.*, 1997). Plants-microbes interactions in root rhizosphere stimulate nutrients cycling by solubilization, mineralization and carboxylation and hormones synthesis (REHMAN *et al.*, 2018; MITTER *et al.*, 2017), that could empower Zn concentration and uptake in cereals to support biofortification (JALAL *et al.*, 2022). Thus, our results verified that residual Zn fertilization and

inoculation with *P. fluorescens* and *A. brasilense* has increased concentration in leaf, shoot and grains (Table 7.2; Figures 7.4A, 2B) and Zn accumulation in shoot and grains of maize (Table 7.3; Figures 7.4C, 2D). The reason might be the presence of microbes in root rhizosphere which could interact with applied inoculants to stimulate transportation of nutrients (especially Zn) to leaf, shoot and grains by modifying root architecture, secreting phenolic acids and reducing phytic acid supply to grains (SINGH *et al.*, 2020). Several other studies exhibited that different strains of *Azospirillum*, *Bacillus* and *Pseudomonas sp.* promote availability and solubilization of nutrients by synthesis of different plant hormones and enzymes as well as biological nitrogen fixation (JALAL *et al.*, 2020; ABADI *et al.*, 2021). According to Abadi *et al.* (2021) exhibited that inoculants of *Pseudomonas sp.* could alleviate Zn deficiency by increasing roots branching and proliferation for greater Zn accumulation and better plant health under harsh environmental circumstances.

Zinc is an important nutrient of several biological and anabolic processes of human while its deficiency could lead to several disorders and hidden hunger (KHAN *et al.*, 2022). Zinc is an indispensable element for plant and human to perform their functions and increase productivity (JALAL *et al.*, 2020). In addition, most of the population are consuming cereals to meet their daily food requirements and therefore, an urgent based approach like microbes-mediated Zn biofortification of staple crops can be the most authentic strategy to increase Zn concentration in edible crops under Zn-deficient soils (JALAL *et al.*, 2022). In this context, the current research indicated that residual Zn fertilization and bacterial inoculation has increased estimated daily Zn intake in Brazil while Zn partitioning was not statistically different (Table 7.3; Figure 7.2E). The fact may be the activation of different mechanisms such as acidification, exchange reactions, chelation and release of organic matter by soil microbes to solubilize nutrients (especially Zn) for better uptake in edible parts (KAUR *et al.*, 2020). The strains of *Azospirillum*, *Pseudomonas* and *Bacillus sp.* are being observed with increasing daily intake and partitioning of Zn from soil to grains of different cereal crops (JALAL *et al.*, 2022; SHAKEEL *et al.*, 2015). It has also described that inoculation of wheat Zn solubilizing bacteria could increase root volume, diameter, length and surface area that has ultimately increased Zn uptake by two folds in edible grains (SINGH *et al.*, 2017). These microbes had increased bioavailability and transportation of Zn to edible grains by reducing phytic acids, which could substantially increase human consumption in more greener and sustainable manner (JALAL *et al.*, 2022; MUMTAZ *et al.*, 2017).

Zinc efficiencies such Zn use, agro-physiological and utilization efficiency, and applied Zn recovery has differently responded to inoculation and residual Zn fertilization (Table 7.4). These efficiencies are derived from shoot and grains Zn concentration in Zn deficient soils, where only Zn fertilization is fraction of Zn use efficiency while increasing fertilizer dose could decrease Zn efficiency (SHIVAY *et al.*, 2008). Most of the studied efficiencies were increased with inoculation of *B. subtilis* under residual Zn fertilization (Table 7.4). This increase might be due to greater growth, yield and Zn uptake in current experiment (Table 7.2; Table 7.3). Roots and soil Zn interaction is severally decreased due to low soil moisture and organic matter that can limit Zn absorption however, greater root dry matter can scavenge and intercept nutrients into plants (GALINDO *et al.*, 2020), which is the main access point to increase nutrients uptake and assimilation with higher Zn use efficiency. Several studies reported that seed inoculation with strains of *Bacillus*, *Pseudomonas* and *Azospirillum* enhanced Zn translocation to grains with higher Zn use, agro-physiological and utilization efficiency, and applied Zn recovery in cereal crops (GALINDO *et al.*, 2021; JALAL *et al.*, 2022).

## 7.5 Conclusions

Microbes-mediated Zn biofortification is one the most accessible, easy and authentic strategy to increase Zn concentration and accumulation in edible part of maize crop. Our results indicated that residual Zn fertilization is a feasible and sustainable technique which has increased plant growth, yield and Zn nutrition in both cropping seasons. The inoculation of diazotrophic bacteria along residual Zn fertilization performed better than without Zn fertilized treatments. Seed Inoculation of *A. brasilense* and *B. subtilis* has increased height of insertion of first productive cob, plant height, shoot dry matter and grain yield of maize under residual Zn fertilization. Most of the growth and yield attributes performed better with inoculation of *A. brasilense* in the absence of residual Zn fertilization. Zinc concentration in leaf, and accumulation in shoot and grains of maize were increased with *A. brasilense* and *P. fluorescens* under residual Zn fertilization. The highest Zn partitioning and daily Zn intake were also increased with inoculation of *P. fluorescens* and *A. brasilense* with residual Zn fertilization. All Zn efficiencies were increased with inoculation of *B. subtilis* except applied Zn recovery, which was greater with inoculation of *A. brasilense* when analyzed in residual Zn fertilized treatments. Therefore, inoculation of maize seeds with *B. subtilis* and *P. fluorescens* together with residual Zn fertilization could be an efficient alternative

mechanism to improve Zn acquisition and use efficiencies and productivity of maize in a sustainable manner in tropical savannah. Prospective research aiming to improve Zn use efficiency and recovery with inoculation and co-inoculation of diazotrophic bacteria and their influence cereal biofortification, physiological and molecular aspects is required to carry out in different edaphic conditions to better understand Zn solubilizing bacteria under field conditions.

## CHAPTER-8

### 8. NANO-ZINC AND PLANT GROWTH-PROMOTING BACTERIA IMPROVE BIOCHEMICAL AND METABOLIC ATTRIBUTES OF MAIZE IN TROPICAL CERRADO

#### 8.1 Introduction

Environmental disaster, food and nutritional insecurities are the foremost devastating challenges to agricultural sector. Malnutrition is a global dietary concern and one of the most a serious threat to agriculture crop production system, affecting over half of the global population (RAMZAN *et al.*, 2020). Zinc (Zn) is one of the dietary nutrients and its malnutrition has affected over one third of agricultural soils due to the presence of excessive soil carbonates, oxides, silicates and phosphates as well as extensive farming system and practices (MASOOD *et al.*, 2022). Zinc is an essential micronutrient for normal growth, development and physiological activities of each living organisms (STANTON *et al.*, 2022). In addition, Zn is involved in numerous metabolic and biochemical functions of plants, such as protein and chlorophyll synthesis, lipids and carbohydrates metabolism, enzymatic activities and photosystem, pollen fertility and energy production (SUGANYA *et al.*, 2020; ZAFAR *et al.*, 2022). Zinc is responsible for stabilization and catalyzation of ~10% of human body proteins and help in the mitigation of reactive oxygen species (ROS) through antioxidant metabolism and lipid peroxidation of cell membrane (OJEDA-BARRIOS *et al.*, 2021; LI *et al.*, 2022). Plants are the major source of Zn entrance into human body. Therefore, a quick and inexpensive alternative strategy is needed to improve Zn bioavailability in edible tissues and crop productivity to combat with malnutrition and food security.

Nanotechnology is an ecofriendly alternative that increase targeted nutrient concentration and metabolism as well as photosynthetic machinery of the chosen crop (KAPOOR *et al.*, 2022). Nano-fertilizer with zinc oxide (ZnO) is being recognized an important and effective alternative to increase growth and productivity by regulating primary photosynthetic activities and carbohydrate metabolism to satisfy nutritional quality of plants (SINGH *et al.*, 2021; JALAL *et al.*, 2022c). Nano-fertilizer reduces the use of synthetic fertilizers while increasing targeted nutrient availability for plants uptake and its intake by human in edible grains (PRASAD *et al.*, 2017). Foliar application of nano-fertilizer has widely adapted for enhancing plant nutrition and

productivity as it enters the cell member more effectively and contributing in the metabolism of proteins, sugars, amino acids and photosynthesis of plants to increase nutrient use efficiency and reducing environmental constraints (WEISANY *et al.*, 2021; Kandil *et al.*, 2022). Foliar spray of ZnO is more viable and prompt strategy than root/soil Zn application due to large surface area and direct absorption through stomata and cuticles, translocation via phloem into chloroplast (SU *et al.*, 2019; ZHU *et al.*, 2020). The delivery of nano-Zn enhances plant growth, productivity and Zn concentration in the edible tissues (DIMKPA *et al.*, 2022). However, these benefits are still far to be adapted at field scale due to the nature and size of particulate (Sudhakaran *et al.*, 2020). Hence, introduction of plant growth-promoting bacteria (PGPBs) in combination with nano-Zn fertilizer could be the best integrated alternative to improve agricultural productivity in more sustainable and ecofriendly environment.

Plant growth-promoting bacteria are applied via seeds, soil, and leaf to enhance efficiency of plant growth manage abiotic stresses through root morphological alterations (GOSWAMI; SURESH, 2020). Seed inoculation with PGPBs is a promising strategy to promote plant growth and development by facilitating nutrient use efficiency, modulating hormonal activities and inhibiting pathogenic infestation (Di Benedetto *et al.*, 2017; KUMAR *et al.*, 2019). In addition, PGPBs contribute to the synthesis of secondary metabolites, water absorption, nutrients (phosphorus (P), Zn, and potassium (K)) solubilization and tolerance to biotic and abiotic stresses (HUNGRIA *et al.*, 2018; JALAL *et al.*, 2021; LOPES *et al.*, 2021). The inoculants of genus *Azospirillum* are being recognized in the biosynthesis of auxin synthesis, nutrient cycling and availability, and biological nitrogen (N) fixation by reducing N<sub>2</sub> into ammonia (NH<sub>3</sub>) (BHAT *et al.*, 2019; CARRILLO-FLORES *et al.*, 2022; GALINDO *et al.*, 2022). *Bacillus subtilis* has the ability to promote plant growth through P solubilization, increasing Zn use efficiency, bioremediation of heavy metal and controlling phyto-pathogenic infestation, leading to higher root-shoot development and productivity (LOBO *et al.*, 2019; DOS SANTOS *et al.*, 2021; JALAL *et al.*, 2022a). In addition, *Pseudomonas fluorescens* is considered one the most effective inoculant to synthesis antibiotics, metabolites and volatile organic compounds to combat soil pathogens (DAVID *et al.*, 2018), improving Zn and P concentrations (JALAL *et al.*, 2022b; ROSA *et al.*, 2022) and also helping in N-fixing activities for sustainable crop production (Jing *et al.*, 2020; AGBODJATO *et al.*, 2021).

Plant growth-promoting bacteria could increase Zn solubility and uptake through production of organic and inorganic acids, and several chelators (IDAYU *et al.* 2017; KHOSHRU *et al.* 2020). Green synthesized ZnO increases morphological and biochemical attributes that lead to sustainable crop production system (NATARAJAN *et al.*, 2021). Zinc fertilization in combination with inoculation of *A. brasilense* increase Zn use efficiency and accumulation, and yield of cereal crops under tropical environment (GALINDO *et al.*, 2021). In addition, *B. subtilis* and *P. fluorescens* are being recognized the most effective inoculants to solubilize Zn and P, improve plant growth and development under different climatic conditions (ROSA *et al.*, 2020; AHMED *et al.*, 2021; JALAL *et al.*, 2021; JALAL *et al.*, 2022a, b).

Maize is recognized as “Queen of cereals” due to its extensive use and flexibility. It is the most frequently cultivated grain crop and serving as a major nutritional source in many developing countries (KUMAWAT *et al.*, 2020). Therefore, it is important to adapt new biotechnology like use of nano-fertilizer and PGPBs inoculation for improving physiochemical and yield traits of maize under changing environmental conditions. The literature is lacking on the combined effect of PGPBs and nano-Zn on growth and development, and nutritional status of maize in tropical savannah of Brazil. There exists a research gap on the effect of PGPBs and nano-Zn on primary metabolic and biochemical attributes, and yield of maize crop in tropical savannah of Brazil. In this context, it was hypothesized that inoculation with PGPBs and foliar nano-Zn fertilization would be interesting strategy to improve primary metabolic and biochemical attributes, and yield of maize crop. Therefore, the objective of the study was to evaluate the effect of inoculation with PGPBs in association with or without foliar nano-Zn application, on the chlorophyll a, b and total chlorophyll, concentrations of amino acid, sucrose and total sugar of maize. Also, to know the effect of PGPBs and foliar nano-Zn on uptake of Zn in shoot and grains, and grain yield of maize in tropical savannah of Brazil.

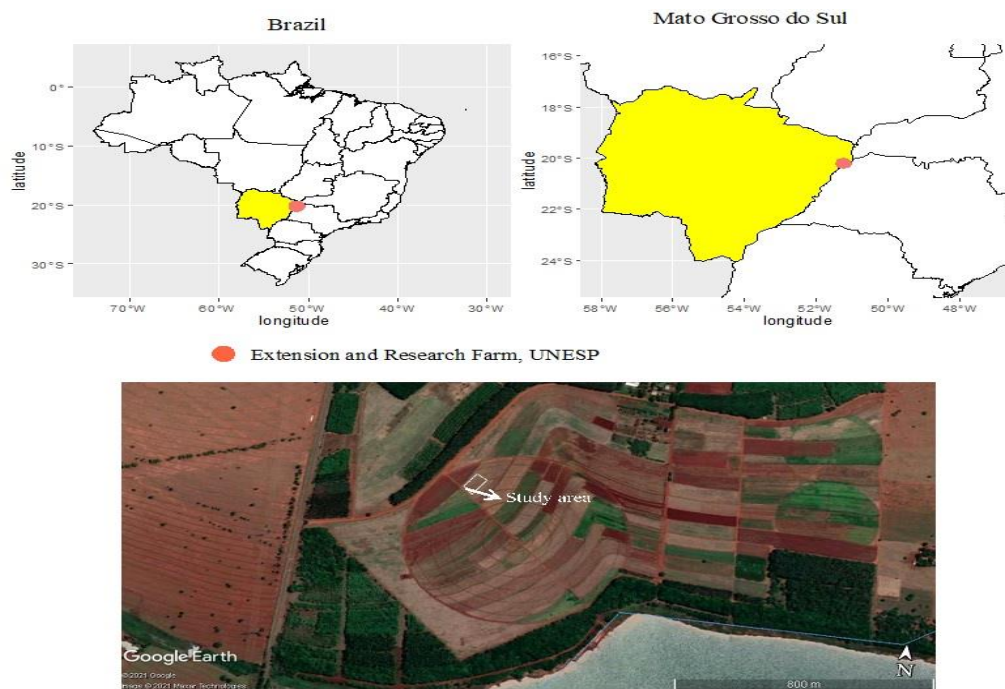
## **8.2 Material and Methods**

### **8.2.1 Description of experimental site**

Field experiment with maize were performed during summer (October-March) of 2019-2020 and 2020-2021 cropping season at Extension and Research Farm of School of Engineering, São Paulo State University (UNESP) at Selvíria, Mato Grosso do Sul, Brazil. The site is located

at geographical coordinates of 20°22' S latitude, 51°22' W longitude, and an altitude of 335 m (Figure 8.1).

**Figure 8.1.** Geographical location of experimental site at Extension and Research Farm, UNESP—Ilha Solteira, at Selvíria, state of Mato Grosso do Sul, Brazil (20°22' S, 51°22' W, altitude of 335 m) during 2019-2020 and 2020-2021 crop seasons.



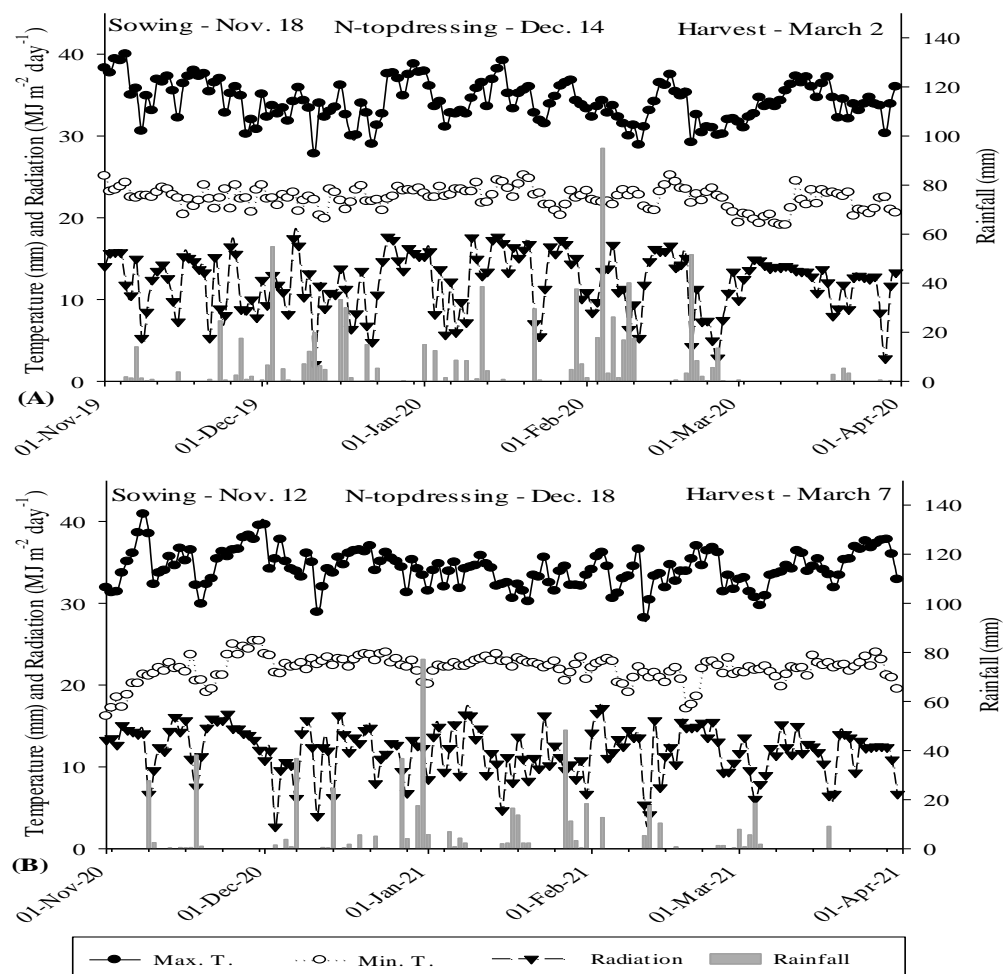
**Source:** Author. The map was created using `pacot`, `geobr` and `ggplot` within R software (R software (R Development Core Team, 2015). Accessed on: 27 February, 2022. Projection System WGS 84/UTM 200DC [EPSG: 4326]. This image was taken from the Google Earth program, Google Company (2021). Map data: Google, Maxar Technologies.

The soil is clayey oxisol defined as Rhodic Haplustox (SOIL SURVEY STAFF, 2014) and Red Latosol Dystrophic (Santos, 2018), with granulometric characterization of 777, 98, 125 g kg<sup>-1</sup> of sand, silt and clay at 0.00-0.25 m soil depth (TEIXEIRA *et al.*, 2017). The experimental site has a history of more than 30 years cultivation with annual cereal-legume crop rotation. In addition, the site was under no-tillage system for the last 13 years while wheat was cultivated prior to the current maize experiments in both years.

The experimental region is characterized as Aw- Köppen with rainy summer (an average rainfall of 1370 mm and 23.5 °C) and humid tropical with relative humidity 70-80% (ALVARES

*et al.*, 2013). Different climatic factors (rainfall, temperature and light radiation) during current experiments in both cropping seasons were carefully monitored (Figure 8.2).

**Figure 8.2.** Rainfall, maximum and minimum temperatures, and light radiation were acquired from the weather station of Extension and Research Farm of School of Engineering—UNESP during maize cultivation period from November to March 2019-2020 and 2020-2021.



### 8.2.2 Soil analysis

Twenty random soil samples were collected before experiment initiation from a soil layer of 0.00–0.20 m in both cropping seasons. The collected samples were properly mixed to attain a composite sample, air-dried, sieved (2 mm) and determined for chemical characterizations (RAIJ *et al.*, 2001). The soil chemical characterizations are summarized in Appendix-A; Table 8.

### 8.2.3 Experimental Design and Treatments

The experiments were conducted in a randomized complete block design with four replications in 4 x 2 factorial scheme. There were four kind of seeds inoculations with PGPBs (no inoculation, *Azospirillum brasilense*, *Bacillus subtilis*, and *Pseudomonas fluorescens*) and two foliar nano-zinc oxide applications (without or with 3 kg Zn ha<sup>-1</sup>), applied 50% at tasseling and grain setting/filling stage of maize.

The maize seeds were chemically treated with Standak Top™, a co-formulation of fungicide [arbendazim + thiram (45 g + 105 g of active ingredient (a.i.) 100 kg<sup>-1</sup> seeds) and insecticide [imidacloprid + thiodicarb (45 g + 135 g of a.i. 100 kg<sup>-1</sup> seeds)] 24 hours prior to inoculations. Treating cereal seeds with Standak Top™ is a common practice in Brazilian tropical savannah to prevent soil pathogenic attack without any harmful effect on the bacterial inoculation (MUNARETO *et al.*, 2018; CARDILLO *et al.*, 2019).

Seeds were manually inoculated by mixing seeds and respective inoculant in a plastic bag an hour before to sowing. Inoculation with *A. brasilense* strains Ab-V5 (CNPSO 2083) and Ab-V6 (CNPSO 2084) was performed at a dose of 200 mL liquid inoculant per 24 kg seeds with guarantee of 2 × 10<sup>8</sup> CFU mL<sup>-1</sup> while *B. subtilis* strain (CCTB04) with guarantee of 1 × 10<sup>8</sup> CFU mL<sup>-1</sup> and *P. fluorescens* strain (CCTB03) with guarantee of 2 × 10<sup>8</sup> CFU mL<sup>-1</sup> at liquid inoculants dose of 150 mL ha<sup>-1</sup> per 24 kg seeds were performed by following the recommendation of inoculants providing company (Biotrop®), Curitiba, Brazil. These inoculants are commercially used in Brazil with strains of *A. brasilense* (AzoTotal™), *B. subtilis* (Vult™) and *P. fluorescens* (Audax™) to promote growth and productivity. The gene sequencing of *A. brasilense* highlighted that strains Ab-V5 and Ab-V6 are carrying *fix* and *nif* genes, which promote nutrients cycling and availability, biological nitrogen fixation, auxin production and induce plant tolerance against biotic and abiotic stresses (FUKAMI *et al.*, 2017; FUKAMI *et al.*, 2018b; GALINDO *et al.*, 2021). *B. subtilis* is first gram positive bacterium which is carrying non-ribosomal peptide synthetases and beta-glucanase that resist phyto-pathogens attack and retain heavy metals accumulation while *zntR* as Zn transporter induces plant growth-promotion (CHAOPRASID *et al.*, 2015; Rekha *et al.*, 2017; MUÑOZ-MORENO *et al.*, 2018). *P. fluorescens* is considered one the efficient bio-control agent with the synthesis of antibiotics and volatile organic compounds to resist soil pathogens, produce gluconic acid, solubilize nutrients and fixing N biologically (DAVID *et al.*, 2018; JING *et al.*, 2020).

The foliar Zn application was performed from liquid source of Zn (Nano R1 zinco™) that was obtained from Allplant® fertilizers industry, São Paulo, Brazil. The company is already registered with Ministry of Agriculture, Brazil. Nano R1 zinc is characterized as fluid suspension with 50% p/p Zn, 1000 g/L solubility and 2.0 density and being successfully used in previous studies (Nakao *et al.*, 2018; JALAL *et al.*, 2022c). A total of 3 kg ha<sup>-1</sup> of ZnO was applied in two splits, 50% Zn at V8/V10 and 50% at R1 stage of maize (Stewart *et al.*, 2020). The application was performed through manual sprayer pump of 6.0 L water capacity (300 L/ha of volume application). The field was visited soon after foliar spray and no leaf damage was observed.

#### **8.2.4 Field Management**

The field site was sprayed with glyphosate (Round up™) + 2,4-D (1800 + 670 g ha<sup>-1</sup> of a.i.) 15 days prior to experiment implantation to control pre-emerged weeds. A simple maize hybrid FS500PWU-Forseed (registered with National Technical Commission on Biosafety of Brazil under reference n°. 1596/2008 for tropical and sub-tropical regions) was planted on 18<sup>th</sup> November, 2019 and 12<sup>th</sup> November, 2020 in a no-tillage system at 3.3 seeds m<sup>-1</sup>. All the treatments were uniformly fertilized with 350 kg ha<sup>-1</sup> NPK (08-28-16, urea) on the basis of soil analysis and expected yield. Seedlings were emerged after 5 days of plantation in both experimental years. Each experimental plot was consisted of 6 m long 6 maize rows with 0.45 m row to row space, a total plot size of 16.2 m<sup>2</sup>. The data were collected from four central rows with a useful area of 10.8 m<sup>2</sup>. The post emergence weeds were controlled by spraying herbicides atrazine + tembotrione (1000 + 84 g a.i. ha<sup>-1</sup>) at V3 growth stage of maize. Nitrogen side dressing (120 kg ha<sup>-1</sup>, applied from ammonium sulphate, 21% N) at V6 growth stage (30 and 31 days after emergence in 2019-2020 and 2020-2021 maize cropping seasons) was performed in all treatments to uniformly distribute on soil surface and incorporated by central pivot irrigation on the same day. Irrigation was performed with central pivot sprinkler irrigation system at 14 mm water volume on a shift of 72 hours or as per crop requirement. The crop was manually harvested on 2<sup>nd</sup> March, 2020 and 7<sup>th</sup> March, 2021.

#### **8.2.5 Assessments and Evaluations**

##### **8.2.5.1 Growth and productivity attributes**

Plant height was determined by measuring plant length from ground surface to the upper apex of tassel. The plants from four central lines were harvested, dried and weighed with analytical

balance for shoot dry matter. Ten random ears were collected at harvest to count number of rows and grains ear<sup>-1</sup>. Hundred grains mass was measured with a precise scale on 13% humidity (wet basis). The ears from central lines of each plot were manually harvested, threshed mechanically and grains weight was transferred into kg ha<sup>-1</sup> at 13% humidity to quantify yield.

### 8.2.5.2 Zinc nutrition and use efficiency

Zinc accumulation in shoot and grains were estimated from the ratio of Zn concentration in shoot and grains, and shoot dry matter and grain yield respectively. Shoot and grain Zn concentration were determined by nitroperchloric digestion and quantified with atomic absorption spectrophotometry, following the protocols of Malavolta *et al.* (1997). Zinc use efficiency (ZnUE) via Eq. 1 and applied Zn recovery (AZnR) via Eq. 2 were calculated according to the methodology of Fageria *et al.* (2011).

$$\mathbf{ZnUE} = \frac{\mathbf{GYF-GYC}}{\mathbf{applied\ Zn\ dose}} \quad (1)$$

$$\mathbf{AZnR} = \frac{\mathbf{GSZnAF-GSZnAC}}{\mathbf{applied\ Zn\ dose}} \quad (2)$$

Where GYF = grain yield with nano-Zn foliar fertilization, GYC = grain yield in control treatments, GSZnAF = grain + shoot Zn accumulation in nano-Zn applied treatments and GSZnAC = grain + shoot Zn accumulation in control treatments.

### 8.2.5.3 Photosynthetic pigments

The photosynthetic pigments (chlorophyll a, b, and total, and carotenoid) were extracted and analyzed by the procedure of Lichtenthaler (1987). Fresh leaves were collected at flowering stage. The samples of 0.5 g were macerated in liquid nitrogen and 50 ml of 80% acetone, stored in refrigerator and then centrifuged at 10,000 × g for 10 min. The acetone extracts absorbance were quantified at 663, 645, and 470 nm using a UV-160 A UV–via spectrometer for chlorophyll a, and total, chlorophyll b and carotenoids concentration respectively.

### 8.2.5.4 Primary Metabolism assay

#### 8.2.5.4.1 Extraction for total soluble sugar and amino acids

Total soluble sugar (TSS) and amino acids were extracted from lyophilized leaves (~0.5 g) in 10 mL MCW solution (60% methanol, 25% chloroform, and 15% water) according to the procedure of Bielecky and Turner (1966). The material solution was homogenized in a 15 mL polystyrene tube by vortexing, placed in refrigerator for 48 h and centrifuged at 10,000 rpm for 10

min at 4 °C. A 5 mL of MCW extract supernatant was collected in a tube and added with 1 mL of chloroform and 1.5 mL of distilled water. After 24 h, the separation phase of aliquots from hydrophilic was used for the determination of total soluble sugar and amino acids concentration.

#### **8.2.5.4.2 Determination of total soluble sugar**

Total soluble sugar in maize leaves was quantified according the procedure of Dubois *et al.* (1956). A 20 µL of MCW extract was mixed with 500 µL of 5% phenol (w/v) and 2 mL of concentrated H<sub>2</sub>SO<sub>4</sub> in a glass tube. After homogenization by a vortex mixer, the tube was heated at 100 °C for 10 min and then cooled down to the room temperature. Afterward, the readings were performed at an absorbance of 490 nm in spectrophotometer (SP-220, biospectro™). The standard sucrose curve was used to quantify total sugar and expressed as mg g<sup>-1</sup> fresh weight (FW).

#### **8.2.5.4.3 Determination of total amino acids**

The protocols of Cocking and Yemm (1954) were used to quantify variation in total free amino acids concentration in maize leaves. An aliquot of 300 µL of MCW extract was mixed with 500 µL of 0.2 M sodium citrate, 200 µL of 5% ninhydrin in ethylene glycol and 1 mL of 0.0002M KCN solution in a glass tube. The tubes were homogenized by vortexing and heated at 100 °C for 20 min and then cooled with tap water for ~10 min. After cooling to room temperature, 1 mL of 60% ethanol was added into the glass tube and homogenized by vortexing. The readings were obtained at 570 nm absorbance using spectrophotometer (SP-220, biospectro™). Methionine standard curve was used to calculate free amino acids concentration and expressed in mg g<sup>-1</sup> FW.

#### **8.2.5.4.4 Determination of storage proteins**

The concentrations of grains storage proteins (albumin, globulin, prolamin, and glutelin) were determined according the protocols of Bradford (1976). Dried and grinded grain sample of 0.25 g was extracted with 5 mL of deionized water in 15 mL falcon tubes. The material was homogenized by vortexing for 1 min and then centrifuged at 10,000 rpm for 20 min at 4 °C. A 20 µL supernatant was extracted with 1 mL Bradford solution in 2 mL micro-tubes, homogenized and read at 595 nm absorbance in spectrophotometer (SP-220, biospectro™) for the sequential extraction of albumin concentration. The same sample was used for the quantification of globulin by replacing water with 5 mL of 5% NaCl then replaced NaCl by 5 mL of 60% ethanol to determine prolamin. Finally, glutelin fraction was quantified with 5 mL of 0.4% NaOH. Bovine serum albumin (BSA) was used as a standard and expressed in mg g<sup>-1</sup> DM (dry mass).

### 8.2.6 Statistical analysis

The entire data were tested for normality with Shapiro–Wilk test and Levene’s homoscedasticity test ( $p \leq 0.05$ ) which showed that data were to be normally distributed ( $W \geq 0.90$ ). The data were subjected to analysis of variance (F test) where foliar nano-Zn spray, PGPBs inoculations and their interactions were considered fixed variables and replication was considered random variable in the model. When a main effect or interaction was observed significant by F test ( $p \leq 0.05$ ), then Tukey test ( $p \leq 0.05$ ) was used for means comparison of nano-Zn spray and PGPBs inoculations using R software (R CORE TEAM, 2015).

The Pearson correlation analysis ( $p \leq 0.05$ ) was calculated and heatmap was created using corrplot package of "cor" and "cor.mtest" functions to calculate coefficients and evaluate relationship among growth, yield, nutritional, biochemical and metabolic attributes of maize using R software (R CORE TEAM, 2015).

The principal component analysis (PCA) was used to evaluate maize growth, grain yield and components, nutritional, biochemical and metabolic attributes in both studied years. The PCA was performed using factoextra and FactoMineR packages in R software (R CORE TEAM, 2015). The number of PCs were selected on the basis of eigenvalues. The biplot graphs representing PC1 at axis-x and PC2 at axis-y in the plot.

## 8.3 Results

### 8.3.1 Growth, yield components and yield of maize

The current study addressed the impact of PGPBs and foliar nano-Zn application on the growth performance and nutrient metabolism of maize crop in tropical savannah region. Inoculation with PGPBs increased plant height in both studied years while foliar nano-Zn and interaction of foliar nano-Zn and PGPBs did not influence plant height in 2020-2021 maize cropping season (Table 8.1).

**Table 8.1.** Plant height, shoot dry matter, number of rows per cob of maize as function of PGPB inoculations together with or without nano zinc oxide spray in 2019-2020 and 2020-2021 crop seasons.

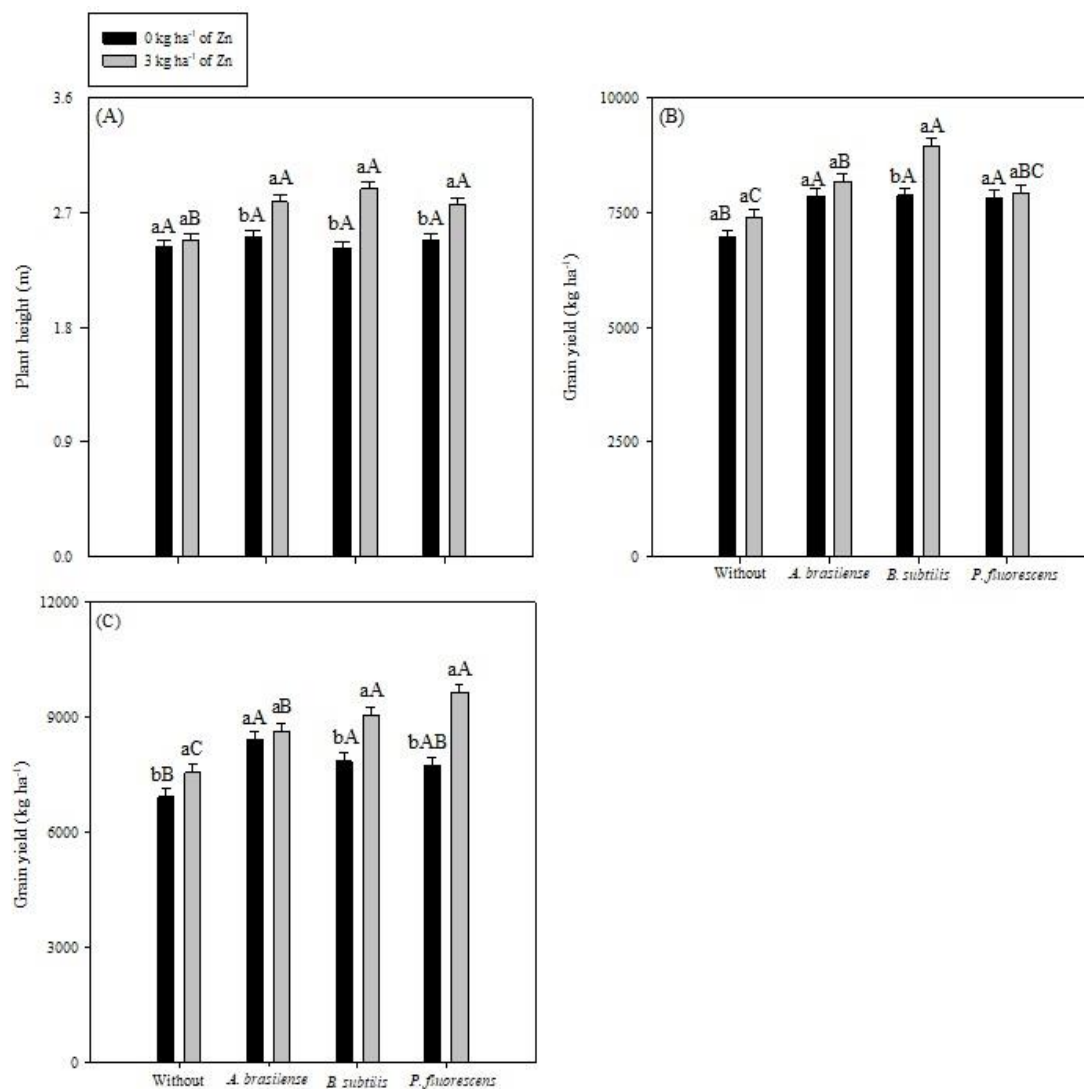
Treatments	Plant height		Shoot dry matter		Number of rows cob <sup>-1</sup>	
	----- m -----		----- kg ha <sup>-1</sup> -----		-----	
	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
<b>Inoculation (I)</b>						
Without	2.45	2.75 a	11526 b	11359	16.93 b	16.03 b
<i>A. brasilense</i>	2.64	2.74 ab	12157 a	12196	17.62 ab	16.91 ab
<i>B. subtilis</i>	2.65	2.64 b	12368 a	12478	17.81 a	16.80 ab
<i>P. fluorescens</i>	2.62	2.74 ab	12119 a	12623	17.25 ab	17.17 a
<b>Foliar zinc (ZnF) spray (kg ha<sup>-1</sup>)</b>						
0	2.45	2.69	11595 b	11521 b	17.22	16.40 a
3	2.73	2.74	12490 a	12807 a	17.59	17.05 b
<b>F-test</b>						
I	6.6 *	4.2 *	9.6 **	2.0 <sup>ns</sup>	4.0 *	3.8 *
ZnF	54.6 **	3.2 <sup>ns</sup>	59.3 **	10.6 **	3.7 <sup>ns</sup>	6.5 *
I x ZnF	5.3 *	2.0 <sup>ns</sup>	1.7 <sup>ns</sup>	0.13 <sup>ns</sup>	0.5 <sup>ns</sup>	0.24 <sup>ns</sup>
<b>CV (%)</b>	3.9	2.6	2.7	9.2	3.2	4.3

Means in the column followed by different letters are statistically different by Tukey test,  $p \leq 0.05$ .

\*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively, while <sup>ns</sup>—non-significant by F-test.

The interaction foliar nano-Zn and PGPBs for plant height in 2019-2020 was significant (Figure 8.3A). Foliar nano-Zn at a dose of 3 kg ha<sup>-1</sup> along with inoculation of *A. brasilense*, *B. subtilis* and *P. fluorescens* produced taller maize plants. All inoculations treatments were observed with taller plant under foliar nano-Zn application as compared to control treatments. There was no significant difference among inoculation treatments in the absence of foliar nano-Zn application (Figure 8.3A).

**Figure 8.3.** Plant height in 2020-2021 (A), and grain yield in 2019-2020 (B) and 2020-2021 (C) of maize as function of plant growth-promoting bacteria in combination with or without foliar nano zinc oxide application.



**Source:** Author. The uppercase letters compare interactions of inoculations within each dose of foliar nano ZnO application and lowercase letters are used to compare interactions of foliar Zn doses (presence and absence) within each inoculation treatment. The identical alphabetic letters do not differ from each other by Tukey test ( $p \leq 0.05$ ) for foliar ZnO doses and inoculations in 2019-2020 and 2020-2021 cropping seasons. Error bars indicate standard error of the mean ( $n = 4$  replications).

The interaction of PGPBs and nano-Zn foliar spray was not significant for shoot dry matter of maize. However, the effect of foliar nano-Zn spray was significant in both crop seasons (Table 8.1). Nano-Zn foliar spray increased shoot dry matter of maize by 7.7 and 11.2% in 2019-2020

and 2020-2021 cropping seasons respectively. In addition, inoculation with *B. subtilis* was observed with greater shoot dry matter ( $12368 \text{ kg ha}^{-1}$ ), which was statistically at par with other inoculations as compared to control while there were no statistical differences among treatments regardless of inoculations in 2020-2021 cropping season (Table 8.1).

Inoculation with PGPBs and foliar nano-Zn application positively increased number of rows  $\text{cob}^{-1}$  of maize (Table 8.1). The maximum number of rows  $\text{cob}^{-1}$  were noted with inoculation of *B. subtilis* and *P. fluorescens* in 2019-2020 and 2020-2021 cropping seasons respectively, which were statistically similar with other inoculation treatments in relation to control. In addition, foliar nano-Zn application increased number of rows  $\text{cob}^{-1}$  of maize only in the second cropping season. The interactions of inoculation with PGPBs and foliar nano-Zn application for number of rows  $\text{cob}^{-1}$  were not significant in both studied years (Table 8.1).

In addition, number of grains  $\text{cob}^{-1}$  of maize was significantly influenced by inoculation with PGPBs and foliar nano-Zn application whereas their interactions were not significant in 2019-2020 and 2020-2021 cropping seasons (Table 8.2). The number of grains  $\text{cob}^{-1}$  were increased by 11.9 and 15% with the inoculation of *B. subtilis* and *P. fluorescens* in first and second maize crop seasons respectively in comparison of without inoculation treatments. The foliar nano-Zn application increased number of grains  $\text{cob}^{-1}$  by 10.4 and 16.6% as compared to control (absence of foliar Zn spray).

**Table 8.2.** Number of grains cob<sup>-1</sup>, 100-grains weight and grain yield as function of PGPB inoculations together with or without nano zinc oxide in 2019-2020 and 2020-2021 crop seasons.

Treatments	Number of grains cob <sup>-1</sup>		100 grains weight		Grain yield	
	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
<b>Inoculation (I)</b>						
Without	614 b	557 b	30.6 b	27.5 b	7172	7241
<i>A. brasilense</i>	675 a	623 a	33.5 a	30.1 a	8015	8521
<i>B. subtilis</i>	687 a	560 ab	32.7 ab	29.7 a	8405	8447
<i>P. fluorescens</i>	654 ab	642 a	31.8 ab	30.0 a	7866	8693
<b>Foliar zinc (ZnF) spray (kg ha<sup>-1</sup>)</b>						
0	625 b	559 b	31.3 b	28.5 b	7620	7730
3	690 a	652 a	32.9 a	30.2 a	8109	8720
<b>F-test</b>						
I	8.1 **	11.3 **	4.4 *	10.5 **	20.1 **	19.9 **
ZnF	32.7 **	72.8 **	7.9 *	19.4 **	18.1 **	44.2 **
I x ZnF	0.5 <sup>ns</sup>	1.8 <sup>ns</sup>	0.12 <sup>ns</sup>	1.9 <sup>ns</sup>	3.3 *	6.1 **
<b>CV (%)</b>	4.9	5.1	5.2	3.6	4.1	5.1

Means in the column followed by different letters are statistically different by Tukey test,  $p \leq 0.05$ .

\*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively, while <sup>ns</sup>—non-significant by F-test.

The interaction of inoculations with PGPBs and nano foliar Zn application was not significant for 100-grains weight of maize (Table 8.2). Inoculation with *A. brasilense* increased 100-grains weight of maize 9.47 and 9.45% in 2019-2020 and 2020-2021 cropping seasons, which were statistically similar with inoculation of *B. subtilis* and *P. fluorescens* as compared to without inoculation treatments. Foliar application of nano-Zn at the dose of 3 kg ha<sup>-1</sup> increased 100-grains weight by 5.1 and 5.9% in 2019-2020 and 2020-2021 maize crop season, respectively (Table 8.2).

The effect of inoculation with PGPBs and foliar nano-Zn application, and their interactions were significant for maize grain yield in 2019-2020 and 2020-2021 growing seasons (Table 8.2). The treatments with inoculations of *B. subtilis* and *P. fluorescens* increased maize grain yield by 17.2 and 20.1% in 2019-2020 and 2020-2021 cropping seasons respectively in relation to without inoculation treatments. In addition, foliar applied nano-Zn also increased grain yield of maize by 6.4 and 12.8% in comparison of control treatments. In case of interactions, the treatments with inoculation of *B. subtilis* performed better with nano-Zn foliar spray in the first maize crop season (Figure 8.3B). In addition, inoculation with *P. fluorescens* was observed with greater grain yield in the presence of nano-Zn foliar application, which was statistically at per with treatments

inoculated with *B. subtilis* and foliar nano-Zn application in 2020-2021 cropping season (Figure 8.3C). In general, the treatments with inoculation of PGPBs produced greater grain yield regardless of foliar nano-Zn application in both cropping seasons. The lowest grain yield of maize was noted in control treatments in both cropping seasons (Figure 8.3B, C).

### 8.3.2 Shoot and grain Zn accumulation and use efficiencies

There was positive influence of the treatments on shoot Zn accumulation of maize, however their interactions were not significant in 2019-2020 and 2020-2021 cropping seasons (Table 8.3). Inoculation with *P. fluorescens* improved shoot Zn accumulation by 30 and 51% in first and second maize cropping seasons respectively as compared to without inoculation treatments. In addition, foliar nano-Zn application improved shoot Zn accumulation by 35 and 36% in first and second cropping seasons respectively in comparison with control treatments.

**Table 8.3.** Shoot Zn accumulation (SZnA) and grains Zn accumulation (GZnA), Zn use efficiency (ZnUE) and applied Zn recover (AZnR) as a function of PGPB inoculations together with or without nano zinc oxide in 2019-2020 and 2020-2021 crop seasons.

Treatments	SZnA		GZnA		ZnUE		AZnR	
	----- g ha <sup>-1</sup> -----				---- kg kg <sup>-1</sup> ----		----- % -----	
	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
<b>Inoculation (I)</b>								
Without	333 b	306 b	216	211	357 c	321 b	76	53 b
<i>A. brasilense</i>	330 b	379 ab	288	287	619 b	676 ab	95	92 ab
<i>B. subtilis</i>	379 ab	372 ab	322	287	876 a	816 a	147	111 ab
<i>P. fluorescens</i>	433 a	462 a	285	318	535 b	1016 a	136	160 a
<b>Foliar zinc (ZnF) spray (kg ha<sup>-1</sup>)</b>								
0	314 b	322 b	236	243	----	---	----	----
3	423 a	438 a	320	309	----	---	----	----
<b>F-test</b>								
I	3.7 *	6.23 **	28 **	13.6 **	78.3 **	11.1 **	3.9 ns	7.1 *
ZnF	18.9 **	20.7 **	102 **	28.0 **	----	----	----	----
I x ZnF	0.5 ns	0.37 ns	10.8 *	4.3 *	---	---	----	----
<b>CV (%)</b>	19.2	19.0	8.5	12.7	8.2	24.9	30.2	32.2

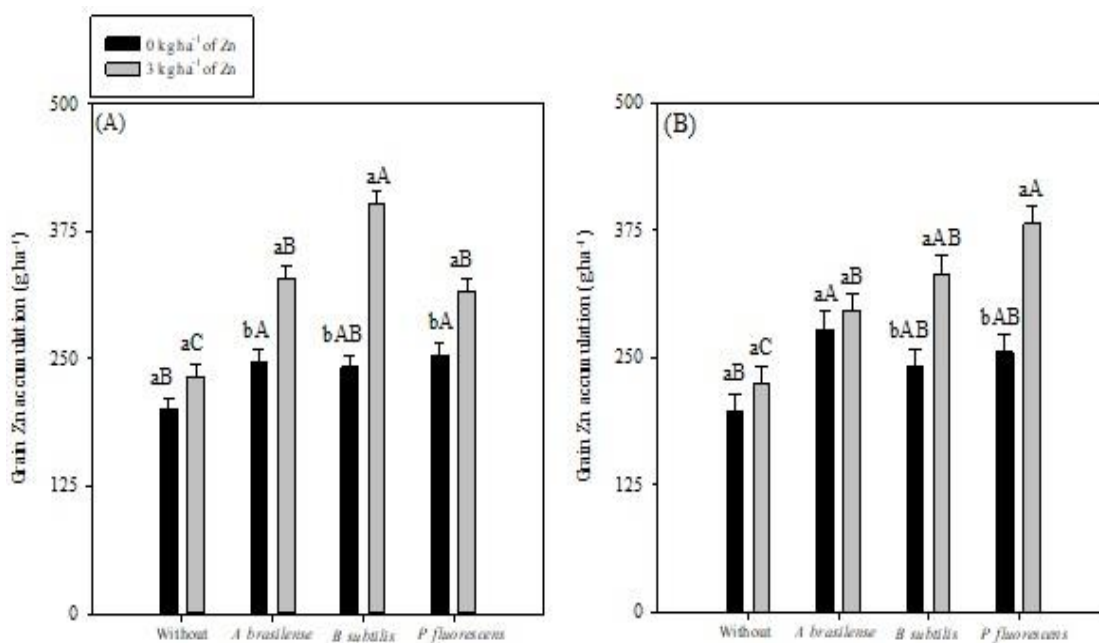
Means in the column followed by different letters are statistically different by Tukey test,  $p \leq 0.05$ .

\*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively, while ns—non-significant by F-test.

Inoculation with PGPBs and foliar nano-Zn application had positive influence on grain Zn accumulation of maize in 2019-2020 and 2020-2021 crop seasons (Table 8.3). The interactions of

PGPBs and foliar nano-Zn application for grain Zn accumulation were also significant (Figure 8.4A). Inoculation with *B. subtilis* and *P. fluorescens* in combination with foliar nano-Zn application improved grain Zn accumulation by 49 and 51% in first and second maize cropping seasons (Figure 8.4A, B). The treatments with inoculation of *P. fluorescens* and *A. brasilense* performed better regardless of foliar nano-Zn application in both cropping seasons. In addition, the lowest grain Zn accumulation was observed in the treatments without inoculation of PGPBs and nano-Zn application in both maize cropping seasons (Figure 8.4A, B).

**Figure 8.4** Maize grain zinc (Zn) accumulation in 2019-2020 (A) and 2020-2021 (B) as function of plant growth-promoting bacteria with or without foliar zinc oxide application.



**Source:** Author. The uppercase letters compare interactions of inoculations within each dose of foliar nano ZnO application and lowercase letters are used to compare interactions of foliar Zn doses (presence and absence) within each inoculation treatment. The identical alphabetic letters do not differ from each other by Tukey test ( $p \leq 0.05$ ) for foliar ZnO doses and inoculations in 2019-2020 and 2020-2021 cropping seasons. Error bars indicate standard error of the mean (n = 4 replications).

Zinc use efficiency (ZnUE) and applied Zn recovery (AZnR) were increased in the treatments with inoculation of PGPBs and foliar nano-Zn application (Table 8.3). The treatments with inoculation of *B. subtilis* increased ZnUE by 145% in 2019-2020 cropping season.

Interestingly, inoculation with *P. fluorescence* increased ZnUE by 216% in the second season, which was statistically at par with the treatments of inoculation with *B. subtilis* and *A. brasilense* as compared to without inoculation (Table 8.3). In addition, the treatments with inoculation of PGPBs positively influenced AZnR only in the second maize cropping season (Table 8.3). Inoculation with *P. fluorescence* was observed with higher AZnR (160%), which was statistically similar with inoculation of *B. subtilis* (111%) and *A. brasilense* (92%) as compared without inoculation in 2020-2021 cropping season (Table 8.3).

### 8.3.3 Photosynthetic pigments

There was positive impact of PGPBs inoculations and foliar nano-Zn spray on the photosynthetic pigments of maize leaves at flowering stage (Table 6.4). The interaction and seeds inoculation with PGPBs didn't affect chlorophyll a content in 2019-2020 maize cropping season. However, the effect of treatments and their interaction for chlorophyll a content in maize leaves was significant in 2020-2021 cropping season (Table 8.4; Figure 8.5A).

**Table 8.4** Photosynthetic pigment of maize leaves as a function of PGPB inoculations together with or without nano zinc oxide, in 2019-2020 and 2020-2021 crop seasons.

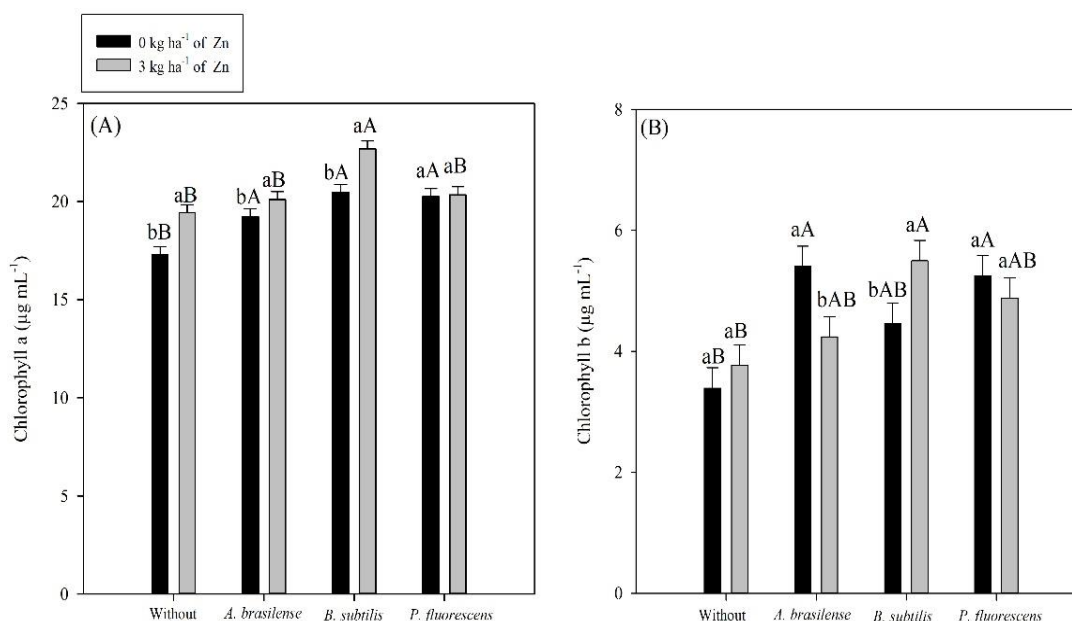
Treatments	Chlorophyll a		Chlorophyll b		Total chlorophyll		Carotenoids	
	----- $\mu\text{g mL}^{-1}$ -----							
	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
<b>Inoculation (I)</b>								
Without	19.3	18.4	3.57	2.99 b	22.6 b	23.7 b	2.15 a	1.78 b
<i>A. brasilense</i>	20.4	19.7	4.82	4.47 a	24.8 ab	26.6 a	2.48 a	2.69 a
<i>B. subtilis</i>	20.1	21.6	4.97	4.8 a	26.0 a	27.7 a	2.61 a	3.25 a
<i>P. fluorescens</i>	19.9	20.3	5.10	3.98 ab	24.9 ab	26.2 a	2.68 a	2.85 a
<b>Foliar zinc (ZnF) spray (<math>\text{kg ha}^{-1}</math>)</b>								
0	19.4 b	19.3	4.6	3.34 b	22.3 b	24.7 b	2.29 b	2.12 b
3	20.5 a	20.6	4.5	4.78 a	26.8 a	27.4 a	2.67 a	3.17 a
<b>F-test</b>								
I	2.3 <sup>ns</sup>	21.4 <sup>**</sup>	8.4 <sup>**</sup>	6.6 <sup>**</sup>	3.8 <sup>*</sup>	7.8 <sup>**</sup>	1.7 <sup>ns</sup>	11.8 <sup>**</sup>
ZnF	14.9 <sup>*</sup>	21.4 <sup>**</sup>	0.02 <sup>ns</sup>	22.3 <sup>**</sup>	38.1 <sup>**</sup>	19 <sup>**</sup>	4.36 <sup>*</sup>	33.6 <sup>**</sup>
I x ZnF	0.3 <sup>ns</sup>	3.2 <sup>*</sup>	3.9 <sup>*</sup>	0.34 <sup>ns</sup>	0.31 <sup>ns</sup>	1.3 <sup>ns</sup>	0.47 <sup>ns</sup>	0.65 <sup>ns</sup>
<b>CV (%)</b>	4.1	4.1	14.7	21.2	8.5	6.6	20.9	19.4

Means in the column followed by different letters are statistically different by Tukey test,  $p \leq 0.05$ .

\*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively, while <sup>ns</sup>—non-significant by F-test.

Inoculation with *B. subtilis* and foliar nano-Zn spray was observed with highest chlorophyll a content as compared to other inoculations and without inoculation treatments (Figure 8.5A). In addition, the treatment with inoculation of *P. fluorescence* was observed with higher chlorophyll a content in the absence foliar nano-Zn application as compared to other treatments (Figure 8.5A). The lowest leaf chlorophyll a content was observed in control treatments (Figure 8.5A). Foliar nano-Zn spray at the dose of 3 kg ha<sup>-1</sup> increased chlorophyll a content by 5.7 and 6.7% in the first and second crop seasons in relation to without nano-Zn foliar spray (Table 8.4).

**Figure 8.5.** Concentrations of chlorophyll a in 2020-2021 (A) and chlorophyll b in 2019-2020 (B) of maize leaf as function of plant growth-promoting bacteria with or without foliar zinc oxide application.



**Source:** Author. Without = control (no inoculation). The uppercase letters compare interactions of inoculations within each dose of foliar nano ZnO application and lowercase letters are used to compare interactions of foliar Zn doses (presence and absence) within each inoculation treatment. The identical alphabetic letters do not differ from each other by Tukey test ( $p \leq 0.05$ ) for foliar ZnO doses and inoculations in 2019-2020 and 2020-2021 cropping seasons. Error bars indicate standard error of the mean ( $n = 4$  replications).

The interaction of PGPBs inoculations and foliar nano-Zn spray for chlorophyll b was significant in 2019-2020 while non-significant in 2020-2021 maize cropping season (Table 8.4). Inoculation with *B. subtilis* in combination with foliar nano-Zn foliar spray was observed with

highest chlorophyll b content, which was statistically similar with inoculation of *A. brasilense* and foliar nano-Zn spray in the first maize cropping season (Figure 8.5B). The lowest chlorophyll b content was noted in the treatments without inoculation and foliar nano-Zn spray (Figure 8.5B). In addition, foliar nano-Zn spray didn't influence leaf chlorophyll b content in the first season. Interestingly, foliar nano-Zn spray increased leaf chlorophyll b content by 43% in 2020-2021 cropping season as compared to control (Table 8.4).

The interactions of PGPBs and foliar nano-Zn spray for total chlorophyll contents were not significant in both maize studied seasons (Table 8.4). Although, leaf total chlorophyll content was positively influenced by the treatment effects. Seeds inoculation with *B. subtilis* increased total chlorophyll content by 15 and 16.8% in 2019-2020 and 2020-2021 cropping seasons respectively, which was statistically similar with inoculation of *P. fluorescens* and *A. brasilense*, when compared to without inoculation treatments (Table 8.4). In addition, foliar nano-Zn spray at the dose of 3 kg ha<sup>-1</sup> increased total chlorophyll content of maize leaves by 20.2 and 10.9% in the first and second cropping seasons respectively.

Leaf carotenoids content of maize was only significantly influenced by inoculations in 2020-2021 while foliar nano-Zn was observed with positive impact carotenoids content in both cropping seasons (Table 8.4). Inoculation with *B. subtilis* increased leaf carotenoids content by 82.6%, which was statistically at par with other inoculations treatments during second maize crop season, when compared to without inoculations treatments (Table 8.4). In addition, the treatment with foliar nano-Zn spray increased leaf carotenoids content by 16.6 and 49.5% as compared to control in the first and second cropping seasons respectively (Table 8.4).

#### **8.3.4 Total soluble sugar, amino acids and storage proteins**

Total soluble sugar content in maize leaves was significantly influenced by inoculation and foliar nano-Zn spray in both cropping seasons (Table 8.5). Inoculation with *A. brasilense* increased total soluble sugar content in leaves by 33 and 40% in 2019-2020 and 2020-2021 cropping seasons respectively, when compared to without inoculation treatments (Table 8.5). In addition, foliar nano-Zn spray increased total soluble sugar content by 35 and 56% in the first and second cropping seasons of maize respectively as compared to control. The interaction of inoculations and foliar nano-Zn spray was significant only in 2019-2020 cropping season (Table 8.5).

**Table 8.5.** Total soluble sugar, free amino acids and albumin concentration as a function of PGPB inoculations together with or without nano zinc oxide, in 2019-2020 and 2020-2021 crop seasons.

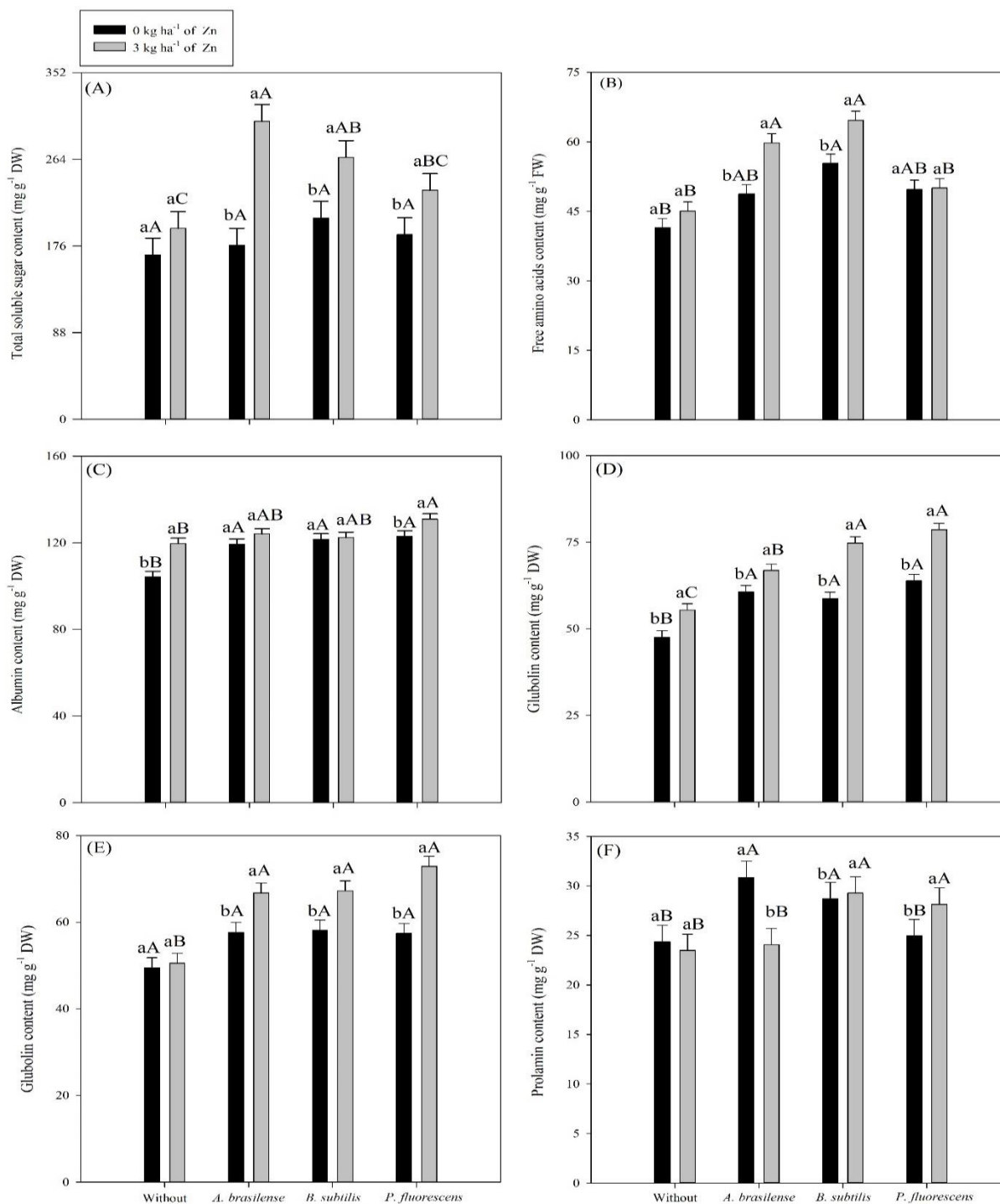
Treatments	Total soluble sugar		Free amino acids		Albumin	
	----- mg g <sup>-1</sup> DW -----					
	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
<b>Inoculation (I)</b>						
Without	180	173 b	42.3 c	43.2	109 c	112
<i>A. brasilense</i>	240	242 a	51.2 b	54.3	119 bc	122
<i>B. subtilis</i>	235	224 ab	58.5 a	60.0	125 ab	122
<i>P. fluorescens</i>	210	204 ab	51.9 b	49.9	131 a	127
<b>Foliar zinc (ZnF) spray (kg ha<sup>-1</sup>)</b>						
0	184	165 b	47.8 b	48.8	116 b	117
3	249	257 a	54.1 a	54.9	126 a	124
<b>F-test</b>						
I	5.2 *	4.15 *	24.6 **	25.5 **	12.7 **	12.8 **
ZnF	29.1 **	39.7 **	22.6 **	18.6 **	12.8 *	16.8 **
I x ZnF	3.2 *	1.8 <sup>ns</sup>	1.12 <sup>ns</sup>	3.12 *	0.19 <sup>ns</sup>	3.1 *
<b>CV (%)</b>	15.7	19.5	7.4	7.6	6.0	4.1

Means in the column followed by different letters are statistically different by Tukey test,  $p \leq 0.05$ .

\*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively, while <sup>ns</sup>—non-significant by F-test.

The treatments co-application of *A. brasilense* and foliar nano-Zn spray at a dose of 3 kg ha<sup>-1</sup> were observed with highest total soluble sugar contents in maize leaves (Figure 8.6A). The treatments with foliar nano-Zn application and without PGPBs inoculation were observed with the lowest total soluble sugar content in leaves of maize. However, treatments without foliar nano-Zn application and inoculation with *B. subtilis* were observed with higher total soluble sugar content, which was statistically at par with other inoculation treatments (Figure 8.6A).

**Figure 8.6.** Concentrations of total soluble sugar in 2019-2020 (A), free amino acids in 2020-2021 (B), albumin in 2020-2021 (C), globulin in 2019 and 2020 (D and E) and prolamins in 2020-2021 maize crop season (F) as function of plant growth-promoting bacteria with or without foliar zinc oxide application.



**Source:** Author. Without = control (no inoculation). The uppercase letters compare interactions of inoculations within each dose of foliar nano ZnO application and lowercase letters are used to compare interactions of foliar Zn doses (presence and absence) within each inoculation treatment. The identical alphabetic letters do not differ from each other by Tukey test ( $p \leq 0.05$ ) for foliar ZnO doses and inoculations in 2019-2020 and 2020-2021 cropping seasons. Error bars indicate standard error of the mean ( $n = 4$  replications).

The content of free amino acids in maize leaves was positively influenced by inoculation and foliar nano-Zn spray in 2019-2020 and 2020-2021. The interaction was only significant in the second crop season of maize (Table 8.5). Inoculation with *B. subtilis* increased amino acids contents by 38.3 and 38.9% in first and second cropping seasons respectively as compared to without inoculation. Foliar nano-Zn spray also enhanced free amino acids content by 13.2 and 12.5% in 2019-2020 and 2020-2021 maize cropping seasons respectively in comparison to control. The interaction exhibited that the treatments with foliar Zn spray at a dose of 3 kg ha<sup>-1</sup> under inoculations of *B. subtilis* and *A. brasilense* increased free amino acids content in maize leaves as compared without inoculation (Figure 8.6 B). Among PGPBs inoculations, the treatments with *B. subtilis* were observed with higher amino acids content in the absence of foliar nano-Zn application. The lowest amino acids content was observed in control treatments (Figure 8.6 B).

Grain storage proteins of maize were significantly influenced by inoculation with PGPBs and nano-Zn foliar spray (Table 8.5, 8.6). Inoculation with *P. fluorescens* enhanced grain albumin concentration by 20.2 and 13.4% in first and second crop seasons respectively as compared without inoculation treatments (Table 8.5). Foliar nano-Zn spray also improved grains albumin concentration by 8.6 and 5.9% in first and second crop seasons as compared to control. The interaction was only significant in the second crop season, where the highest grain albumin concentration was observed with combined application of *P. fluorescens* inoculation and foliar nano-Zn spray as compared to the rest of the treatments (Figure 8.6 C). All treatments with inoculation of PGPBs improved grains albumin concentration regardless of foliar nano-Zn application. The lowest albumin concentration was observed in the treatments without inoculation and nano-Zn application (Figure 8.6 C).

The interactive effect of inoculation  $\times$  foliar nano-Zn spray was significant for grains globulin concentration in both crop seasons (Table 8.6; Figure 8.6 D, E). The highest grain globulin concentration was observed with foliar nano-Zn fertilization under inoculation with *P. fluorescens*,

which was statistically similar with inoculation of *B. subtilis* in 2019-2020 (Figure 8.6 D) and with *B. subtilis* and *A. brasilense* in 2020-2021 (Figure 8.6 E) maize cropping seasons. The treatments with inoculation of PGPBs were observed with higher globulin concentration maize grains even in the absence of foliar nano-Zn application, when compared to without inoculation treatments. The lowest globulin concentration in both studies was noted in the treatments without inoculation and nano-Zn application (Figure 8.6 D, E).

**Table 8.6.** Globulin, glutelin and prolamin concentration of maize grains as a function of PGPB inoculations together with or without nano zinc oxide in 2019-2020 and 2020-2021 crop seasons.

Treatments	Globulin		Glutelin		Prolamin	
	----- mg g <sup>-1</sup> DW -----					
	2019-20	2020-21	2019-20	2020-21	2019-20	2020-21
<b>Inoculation (I)</b>						
Without	51	50	190 b	205 b	24.8 a	23.9
<i>A. brasilense</i>	64	62	215 a	222 a	26.5 a	27.5
<i>B. subtilis</i>	67	63	219 a	223 a	28.5 a	28.9
<i>P. fluorescens</i>	71	65	216 a	219 a	25.1 a	26.5
<b>Foliar zinc (ZnF) spray (kg ha<sup>-1</sup>)</b>						
0	58	56	203 b	214 b	24.2 b	26.2
3	69	64	216 a	220 a	28.2 a	27.2
<b>F-test</b>						
I	41.8 **	17.3 **	15.6 **	14.2 **	1.4 <sup>ns</sup>	3.3 *
ZnF	73.4 **	28.3 **	16.2 **	7.0 *	8.5 *	0.71 <sup>ns</sup>
I x ZnF	3.6 *	3.27 *	0.12 <sup>ns</sup>	0.52 <sup>ns</sup>	0.55 <sup>ns</sup>	3.3 *
<b>CV (%)</b>	5.8	7.7	4.5	2.9	15	12.3

Means in the column followed by different letters are statistically different by Tukey test,  $p \leq 0.05$ .

\*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively, while <sup>ns</sup>—non-significant by F-test.

The interaction of inoculations  $\times$  foliar nano-Zn for glutelin concentration was not significant in both studied seasons (Table 8.6). Grains glutelin concentration was improved by 15.3 and 8.8% with inoculation of *B. subtilis* in first and second cropping seasons respectively, which was statistically at par with other inoculation, when compared to without inoculation treatments. Foliar nano-Zn spray improved grain glutelin concentration by 6.4 and 2.8% in 2019-2020 and 2020-2021 cropping seasons in comparison to control (Table 8.6).

Grain prolamin concentration of maize was not significantly influenced by inoculation and interaction of inoculation  $\times$  nano-Zn spray in 2019-2020, while the effect of only foliar nano-Zn was not significant in 2020-2021 cropping seasons (Table 8.6). Foliar nano-Zn spray improved

grain prolamin concentration of maize by 16.5% in the second crop season. In 2020-2021, inoculations with *B. subtilis* and *P. fluorescens* along with foliar nano-Zn were observed with higher prolamin concentration in the second crop season (Figure 8.6 F). In addition, inoculation with *A. brasilense* was observed with highest grain prolamin concentration in the absence of foliar nano-Zn application. The treatments without inoculation were observed with the lowest grain prolamin concentration regardless of the nano-Zn application (Figure 8.6 F).

### 8.3.5 Pearson's correlation among evaluated attributes of maize

There were positive and significant correlations between zinc use efficiency and plant height, shoot dry matter, yield components, grain yield, grain Zn accumulation, chlorophyll a, amino acids, total soluble sugar, glutelin and prolamin concentration of maize, regardless the treatments applied in 2019-2020 crop season (Appendix B: Figure 4A). A positive correlation was observed between Zn use efficiency and shoot and grain Zn accumulation, applied Zn recovery, shoot dry matter, grain yield and photosynthetic and biochemical attributes of maize in 2020-2021 crop season (Appendix B: Figure 4B).

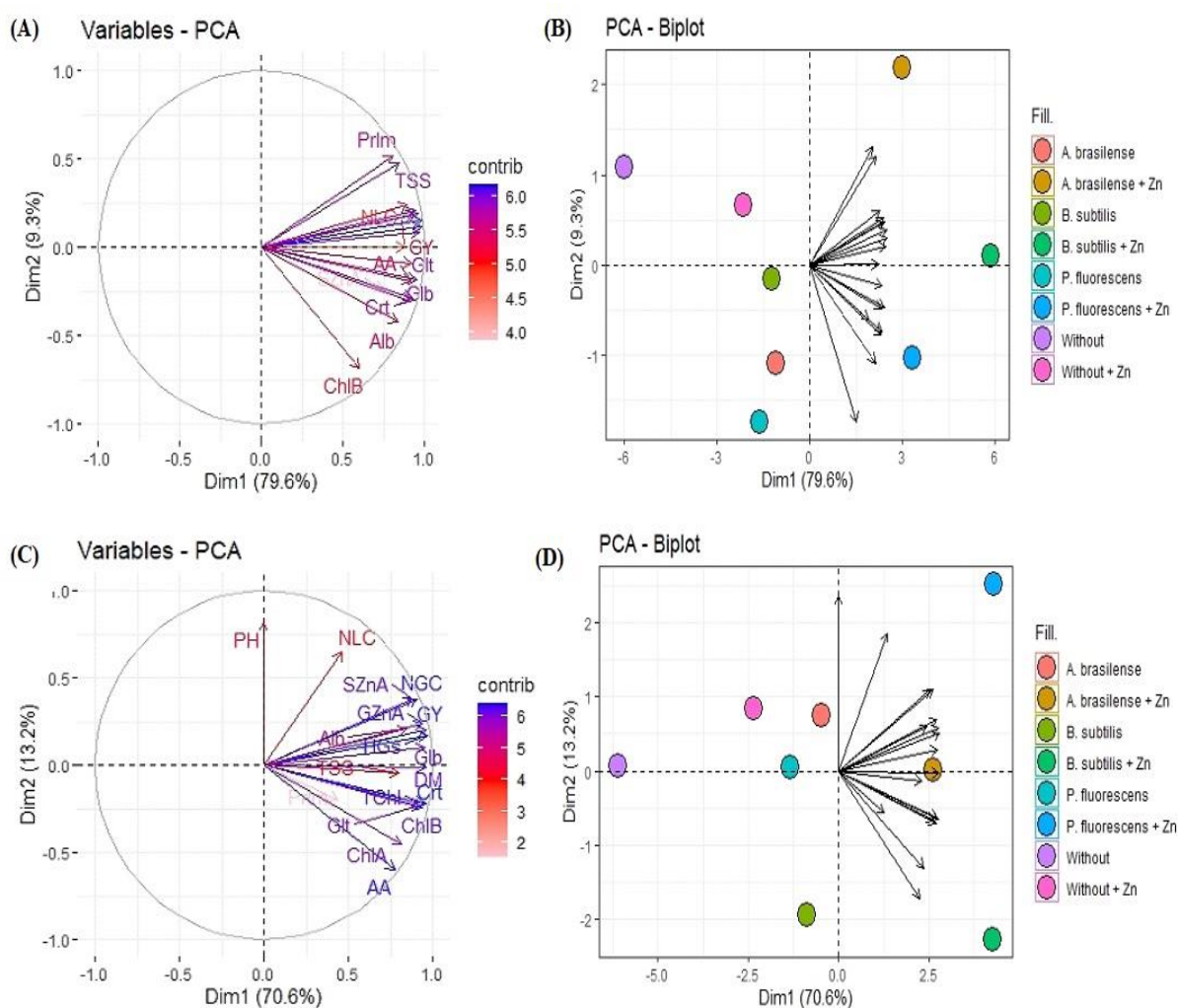
In addition, a positive and significant correlations between grain yield and zinc use efficiency, shoot dry matter, yield components, grain Zn accumulation, chlorophyll a, amino acids, total chlorophyll and prolamin concentration of maize, regardless the treatments applied in 2019-2020 crop season (Appendix B: Figure 4A). A positive correlation was observed between grain yield and all the growth, yield components, as well as photosynthetic and biochemical attributes of maize in 2020-2021 crop season (Appendix B: Figure 4B).

### 8.3.6 Principal component analysis among evaluated attributes of maize

Principal component analysis (PCA) were performed to investigate changes in the yield, nutritional and biochemical attributes of maize in 2019-2020 and 2020-2021 cropping seasons (Figure 8.7). The eigenvalues of all eight principal components were greater than 1 and account for 100% of the data variation in both maize cropping seasons (Appendix B; Table 7, 8). The PC1 explained 79.6 and 70.6% of the data cumulative variation, while PC2 represented 88.9 and 82.7% in 2019-2020 and 2020-2021 cropping seasons of maize respectively. The biplot graphs of PC1 and PC2 indicated that group formed by inoculation with *Azospirillum brasilense*, *Bacillus subtilis*, and *Pseudomonas fluorescens* with foliar nano-Zn at a dose of 3 kg ha<sup>-1</sup> obtained positive

correlation for all analyzed maize parameters in first cropping season (Figure 8.7A, B). While, analyzing the biplot graph of grouped PC1 and PC2 in 2020-2021 cropping season, all analyzed parameters showed positive correlation with group of inoculation with plant growth-promoting bacteria, except plant height (Figure 8.7C, D).

**Figure 8.7** Loadings and biplot graphics of principal component analysis among the relationship of plant growth-promoting bacteria and foliar nano ZnO applications for growth, nutritional, yield and biochemical attributes of maize in 2019-2020 (A, B) and 2020-2021 (C, D) cropping seasons.



**Source:** Author. Abbreviations: PH= plant height, DM= shoot dry matter, NLC= number of row per cob, NGC= number of grains per cob, HGs= 100-grains weight, GY= grain yield, SZnA= shoot Zn accumulation, GZnA= grain Zn accumulation, ChlA= chlorophyll a, ChlB= chlorophyll b,

TChl= total chlorophyll, Crt= carotenoids, AA= free amino acids, TSS= total soluble sugar, Alb= albumin, Glb= globulin, Glt= glutelin, and Prlm= prolamin.

## 8.4 Discussion

Plants adaptation and responses to nutrients deficiency are being satisfied by ensuring a minimal requirements and carbon tradeoff cost. Several strategies are being adapted to protect plants from the damages of harsh environmental conditions, especially in tropical rainfed regions (GALINDO *et al.*, 2021). In this scenario, limited literature is available on the use of PGPBs in combination with foliar nano zinc oxide on the growth and performance of maize. To address this incessant problem, the current study used Zn improving PGPBs such as *A. brasilense*, *B. subtilis* and *P. fluorescens* in combination with foliar nano-Zn application to assist biochemical, primary metabolisms and yield of maize crop in tropical savannah.

The positive correlation between maize growth and yield, shoot-grain accumulation and photosynthetic pigments and primary metabolism endorsed the hypothesis of the current study (Appendix B: Figure 4; Figure 8.7). It is possible due to the synergetic effect of PGPBs with Zn enrichment and their role in different metabolic processes, nutrient use and acquisition, and synthesis of phyto-hormones (MITTER *et al.*, 2017; KUDOYAROVA *et al.*, 2019; Housh *et al.*, 2021). The current study indicated that inoculations with *B. subtilis* and *P. fluorescence* along with foliar nano-Zn application were observed with taller plants (Figure 8.3A), greater shoot dry matter, higher number of rows, grains cobs<sup>-1</sup>, heavier 100-grains weight (Table 8.1, 8.2), and grain yield (Figure 8.3B, C) in two maize crop seasons. It might be due to the role of PGPBs in nutrient solubilization and phyto-hormones production that stimulate nutrients availability and absorption through roots as well Zn role in cell multiplication and protein synthesis (ROSSI *et al.*, 2019; SWARNALAKSHMI *et al.*, 2020). The individual or combined use of PGPBs with Zn could modulate enzymatic activities of soil (phosphatase and invertase) and plant (proline) (TANVEER *et al.*, 2022), thus contributing to higher plant growth and yield as an end product (JALAL *et al.*, 2022a, b). It has also reported that inoculation with PGPBs promote maize growth by regulating phyto-hormones and growth regulators that could improve nutrient solubilization and uptake for better plant growth and production (OLEŃSKA *et al.*, 2020; RIBEIRO *et al.*, 2022). It has reported that PGPBs are associated with greater root-shoot biomass and dry matter, which can lead to the

promotion of vegetative growth at early stage and greater productivity at maturity (SOUSA *et al.*, 2021; JALAL *et al.*, 2022b). In addition, foliar spray of nano-fertilizer improves growth and yield of maize by enhancing to plant biochemical processes and resistance against reactive oxygen species (BABAEI *et al.*, 2017). Zinc regulates different biochemical attributes of plants through cell elongation and multiplication (as a result of auxin synthesis), thus leading to greater biomass and productivity of cereal crops (DOOLETTE *et al.*, 2018; JALAL *et al.*, 2022c). Despite of all this, the non-significant effect of foliar nano-Zn application for plant height and number of row cobs<sup>-1</sup> in the first and second maize cropping season respectively (Table 8.2) may be due plant nutrition with Zn was adequate while these parameters are more influenced by genetic factors and tropical climatic conditions (Figure 8.2). In addition, low foliar Zn supply is another factor which cause physiological and leaf anatomical alterations that can consequently affect nutrient penetration and accumulation depending on the deficiency of target nutrient (BRAIN, 2008).

Results of the current study indicated that inoculation with PGPBs such as *B. subtilis* and *P. fluorescens* in combination with foliar nano-Zn application improved Zn accumulation in shoot (Table 8.3) and grains (Figure 8.4A, B), as well as increased Zn use efficiency (ZnUE) and applied Zn recovery (AZnR) in maize crop cultivation (Table 8.3). It may be due to the positive interception of PGPBs in scavenging roots to produce growth-promoting hormones, and increase water and nutrients uptake to shoot and grains tissues (MUMTAZ *et al.*, 2017). Previous studies indicated that applied inoculants interact with already existing microbes in root rhizosphere, modifying root architecture, reducing phytic acids assimilation and stimulating nutrients transportation to shoot and edible tissues (SINGH; PRASANNA, 2020). It has also reported that foliar Zn application could enhance translocation of Zn to shoot and edible tissues (JALAL *et al.*, 2020; JALAL *et al.*, 2022c) by developing coordination with amino acid (cysteine) and protein synthesis (GUPTA *et al.*, 2016). Foliar Zn application is effective strategy to overcome the edaphic deficiency by improving bioavailability in edible tissue, leading to better biofortification (MISHRA, 2022). In this context, results of the present study are a progressive step to understand the integrated use of PGPBs and foliar ZnO application for greater growth, yield and biofortification of maize grains with higher Zn use efficiency (Table 8.3). It has reported in another study that plant growth-promoting microbes are being identified as natural bio-fortifiers, synthesize organic acids, chelating agents and siderophores that ultimately result in biofortification and higher crop yield in a sustainable manner (RAMESH *et al.*, 2014; UPADHAYAY *et al.*, 2022).

Maize is one of the most important cereal crops for food and nutritional security with high phytic acid and low Zn concentration that may be the origin cause of malnutrition, especially in the regions under Zn deficiency like tropical savannah of Brazil (CAKMAK *et al.*, 2010; FAGERIA *et al.*, 2011). In this context, the present study exhibited that inoculation with *B. subtilis* and *P. fluorescens* along with foliar nano-Zn improved grain Zn accumulation (Figure 8.4) and Zn use efficiency in maize cultivation (Table 8.3). It has reported that inoculation with PGPBs in combination with foliar or soil Zn application contributing to the reduction of phytic acid which consequently increase Zn concentration in embryo, aleurone, endosperm and whole grains of cereal crops (REHMAN *et al.*, 2018; JALAL *et al.*, 2022b). In addition, foliar Zn spray is considerably high mobilized in the phloem as compared to conventional soil Zn fertilization and deal with malnutrition due to its rapid remobilization and localization into the grains (Firdous *et al.*, 2020; REHMAN *et al.*, 2021; JALAL *et al.*, 2022c).

In the present study, the considerable increase in maize growth and Zn nutrition is due to the improvement of photosynthetic pigments (chlorophyll a, b, total chlorophyll and carotenoids content) under inoculation with PGPBs and foliar nano-Zn application (Table 8.4). This increase in photosynthetic pigments might be due to the role of PGPBs in stability of biochemical and physiological functions of plants, which can be attributable to stomatal conductance, transpiration and intercellular gas exchange processes to increase photosynthetic rate of the plants (PEREIRA *et al.*, 2020). The present results indicated that chlorophyll a, b, total and carotenoids concentrations were improved with inoculation of *B. subtilis* and foliar Zn spray in both maize cultivated seasons (Table 8.4; Figure 8.5A, B). It might be due to the critical role of PGPBs and Zn in the production of phyto-hormones, nitrogen fixation and improving photosystem II efficiency (SEYED SHARIFI *et al.*, 2020). It has reported that combined application of PGPBs and Zn oxide revealed as a promising technique to increase chlorophyll concentrations and performance of wheat (AZMAT *et al.*, 2022). The application of nano-Zn with PGPBs including *Bacillus* and *Pseudomonas* sp. regulate defensive enzymes and intercellular homeostasis of plants to create optimal cellular conditions, which may lead to higher concentration of photosynthetic pigments (Yasmin *et al.*, 2020a). Batool *et al.* (2020) reported that inoculation with *B. subtilis* is an effective strategy that regulates chlorophyll a, b and carotenoids content as well as other biochemical process, thus leading to sustainable growth and production of the plants under harsh environmental conditions. In addition, the non-significant effect of inoculation with PGPBs on the

leaf concentration of chlorophyll a and carotenoids (Table 8.4) might be due plant nutrition with Zn was adequate and tropical climatic conditions (Figure 8.2). Despite this, inoculation of PGPBs attribute to competition of already existing microbial consortium in rhizosphere, which can ultimately affect nutrient transportation and growth performance of maize (TANG *et al.*, 2020).

There was a remarkable increase in the concentration of total soluble sugar (TSS), free amino acids and grain storage proteins (albumin, globulin, glutalin and prolamin) of maize, when treated with PGPBs and foliar nano-Zn spray (Table 8.5; Table 8.6). It may be possible due to the role of foliar nano-Zn in up-regulation of antioxidant system and primary metabolites of plant that contribute to enzymes activation and proteins synthesis (GHANI *et al.*, 2022). Previous studies claimed that PGPBs regulate the production of photo-assimilates and interlinking the outcomes of foliar nano-Zn application with other physiological and biochemical functions that could ultimately improve primary metabolites in leaves and storage proteins in grains of different crops (BATOOL *et al.*, 2020; AZMAT *et al.*, 2022). The present results exhibited that combined application of PGPBs and foliar nano-Zn improved concentration of TSS (Figure 8.6A), free amino acids (Figure 8.6B), albumin (Figure 8.6C), globulin (Figure 8.6D, E) and prolamin (Figure 8.6F) in maize leaves and grains. The reason might be due to the rapid absorption and transportation of foliar nano-Zn with the involvement of several factors (thickness, density and chemical composition of cuticle, trichomes and stomata conductance), which are responsible for the operation of entire plant machinery and thus improving metabolic and biochemical processes of the plants (YUMEI *et al.*, 2014; XIE *et al.*, 2020). Zinc fertilization increases grain reserve proteins due to its involvement in nitrate reductase activities and nitrogen assimilation pathways (LIU *et al.*, 2015; SILVA *et al.*, 2021). It has also reported that co-application of Zn and PGPBs could modulate plant defensive system by improving photosynthetic pigments and primary metabolites, leading to better plant performance and yield (TANVEER *et al.*, 2022). Plant growth-promoting bacteria induce multiple physiological functions by absorbing available nutrients through roots that may stimulate plant nutrition and primary metabolism in sustainable manner (YAHAGHI *et al.*, 2019). The co-application of PGPBs and Zn improved total soluble sugar, amino acids and protein content, leading to better performance and biofortification of maize (UPADHYAY *et al.*, 2021). Hence, inoculation with PGPBs and foliar nano-Zn application improved performance, primary metabolism, and yield of maize and proved as a sustainable management practice for higher productivity and Zn biofortification of maize in tropical savannah conditions.

## 8.5 Conclusions

To satisfy the food and nutritious demands of exponentially growing human population; use of plant growth-promoting bacteria (PGPBs) is one of the sustainable and ecofriendly strategy that can increase nutrition, performance, productivity and nutrient assimilation into the edible tissues of maize crop. In addition, foliar nano zinc (Zn) also proved a feasible and environmental safe technique for improving Zn accumulation, growth and biochemical attributes of maize. Therefore, it was verified from the current field findings that co-application of *Bacillus subtilis* and foliar nano zinc at a dose of 3 kg Zn ha<sup>-1</sup>, applied in two splits, increased plant height, shoot dry matter, yield components and yield of maize in tropical savannah. Zinc accumulation in shoot and grains as well as Zn use efficiency and applied Zn recovery were also improved with inoculation of *B. subtilis* and *Pseudomonas fluorescens* along with foliar nano-Zn application. Chlorophyll a, b, total and carotenoids, total soluble sugar, free amino acids in the leaves, and storage proteins (albumin, globulin, glutelin and prolamin) in grains of maize were improved with inoculation of *B. subtilis* in combination with foliar nano-Zn application.

Therefore, seed inoculation with *B. subtilis* and *P. fluorescens* in combination with foliar nano-Zn application is considered relatively high effective and low-cost alternative strategy to improve Zn acquisition and Zn use efficiency, biochemical and primary metabolism with higher productivity of maize in tropical savannahs. The present study gives an insight on the interaction of PGPBs and foliar nano-Zn application about various morphological and biochemical aspects of maize. Using these information, prospective research aiming to know the molecular and laboratory mechanisms (translocation, localization, loading, transporter proteins etc.) behind the higher accumulation and improved biochemical and physiological attributes of maize to better understand the responses of plant growth-promoting bacteria in different edapho-climatic conditions.

## 9. Final considerations and conclusions

The low available content of micronutrients in the soil can lead to hidden hunger, therefore, proper nutrient management especially zinc (Zn) under sustainable agricultural practices is considered one of the important strategies to overcome food and nutritional security crop plants. In this context, the current experiments were performed with integrated use of Zn and plant growth-promoting bacteria (PGPBs), one of the feasible and sustainable alternative techniques, to improve growth, productivity, Zn use efficiency and agronomic biofortification of common beans, wheat and maize. A total of six experiments were performed on these crops in a no-till Rhodic Haplustox soil under pivot irrigation system.

Soil Zn fertilization at the rate of 8 kg ha<sup>-1</sup> in combination with PGPBs improved growth, yield and nutritional status of common bean, wheat and maize under tropical savannah of Brazil. Co-inoculation with *R. tropici* + *B. subtilis* along with soil Zn application improved Zn concentration and accumulation in shoots and grains, with a promising effect on grain yield and estimated Zn intake of common bean. In addition, Zn use efficiency, applied Zn recovery, and Zn utilization efficiency were increased with co-inoculation of *R. tropici* + *B. subtilis* irrespective of soil Zn fertilization.

Seed inoculation with *P. fluorescens* under soil applied Zn increased leaf, shoot and grain Zn concentrations and accumulations as well as Zn partitioning index. Inoculation with *P. fluorescens* and *B. subtilis* in the presence of soil applied Zn increased plant height, shoot dry matter and grain yield of wheat. Nitrogen and phosphorous concentrations in shoot and grain were increased with inoculation of *B. subtilis* under soil Zn application. Zn use efficiencies were interestingly greater with inoculation of *P. fluorescens* and *B. subtilis* under soil applied Zn treatments.

Seed inoculation with *A. brasilense* and *B. subtilis* increased insertion of productive cob, plant height, shoot dry matter and grain yield of maize under residual Zn fertilization. Zinc concentration in leaf, and accumulation in shoot and grains of maize were increased with *A. brasilense* and *P. fluorescens* under residual Zn fertilization. Zinc partitioning and daily Zn intake were also increased with inoculation of *P. fluorescens* and *A. brasilense* with residual Zn fertilization. Zinc efficiencies including Zn use efficiency and applied Zn recovery were increased with inoculation of *B. subtilis* except applied Zn recovery, which was greater with inoculation of *A. brasilense* under residual Zn fertilized treatments.

Foliar nano Zn application at a dose of  $1.5 \text{ ha}^{-1}$  along with co-inoculation of *R. tropici* + *B. subtilis* was observed the most effective strategy for improving performance and yield, Zn nutrition and its efficiencies, leading to biofortification of common bean grains in tropical savannah. Nitrogen and phosphorous shoot and grain concentrations were increased with co-inoculation of *R. tropici* + *P. fluorescens* and *R. tropici* + *B. subtilis* under nano-Zn foliar fertilization.

Seed inoculation with *B. subtilis* and *P. fluorescens* in combination with foliar nano-Zn spray increased shoot and grain N, P and Zn concentrations in shoot and grains, dry matter, grain yield and Zn partitioning index in wheat. Zinc intake with daily wheat consumption in Brazil, Zn use efficiency and applied Zn recovery were also increased with inoculation of *P. fluorescens* in combination with nano-Zn foliar application under field conditions.

Co-application of *Bacillus subtilis* and foliar nano zinc at a dose of  $3 \text{ kg Zn ha}^{-1}$  increased plant height, shoot dry matter, yield components and yield of maize in tropical savannah. Zinc accumulation in shoot and grains as well as Zn use efficiency and applied Zn recovery were also improved with inoculation of *B. subtilis* and *Pseudomonas fluorescens* along with foliar nano-Zn application. Chlorophyll a, b, total and carotenoids, total soluble sugar, free amino acids in the leaves, and storage proteins (albumin, globulin, glutelin and prolamin) in grains of maize were improved with inoculation of *B. subtilis* in combination with foliar nano-Zn application.

Soil Zn application is an inexpensive and easy way of agronomic biofortification and greater grains productivity however, sole Zn application is not enough to meet the goal. Foliar nano zinc (Zn) also proved a feasible and environmentally safe technique for improving Zn accumulation, growth and biochemical attributes of cereal and legume crops. Microbes-mediated Zn biofortification is one the most accessible, easy and authentic strategy to increase Zn concentration and accumulation in edible part of crops. Co-inoculation of *R. tropici* and *B. subtilis* in association with soil Zn application and foliar nano Zn application at a dose of  $1.5 \text{ ha}^{-1}$  improved nutrients acquisition and Zn use efficiencies for the better biofortification of common bean grains in tropical regions. Seed inoculation with *B. subtilis* and *P. fluorescens* in association with soil Zn fertilization and foliar Zn fertilization at a dose of  $3\text{-}3.5 \text{ kg ha}^{-1}$  increased N, P and Zn concentrations in shoot and grains, dry matter, grain yield and Zn partitioning index in wheat. Inoculation of maize seeds with *B. subtilis* and *P. fluorescens* in combination with residual Zn fertilization and foliar nano-Zn application are considered relatively high effective and low-cost

alternative strategy that efficiently improved Zn acquisition, use efficiencies, productivity and primary metabolism of maize in a sustainable manner in tropical savannah. Hence, it is recommended that seed inoculation with *B. subtilis* and *P. fluorescens* along with Zn fertilization via soil at a dose of 4.5 3 kg ha<sup>-1</sup> or foliar nano-Zn at a dose 3 kg ha<sup>-1</sup> could be an effective mechanism to improve plant nutrients acquisition and use efficiencies particularly Zn, leading to sustainable production and biofortification of common beans, maize and wheat under tropical savannah regions.

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## APPENDICES

## APPENDIX A: TABLES

**Table 1.** Analyzed results of soil sample (0–0.20 m) from experimental site before implementation of common bean experiment in 2019.

Properties	Units	Values
Clay	g kg <sup>-1</sup>	433
Sand	g kg <sup>-1</sup>	471
Silt	g kg <sup>-1</sup>	90
pH (CaCl <sub>2</sub> )	----	5.2
Organic matter	mg dm <sup>-3</sup>	18
P (resin)	mg dm <sup>-3</sup>	38
K	mmol <sub>c</sub> dm <sup>-3</sup>	1.7
Ca	mmol <sub>c</sub> dm <sup>-3</sup>	21
Mg	mmol <sub>c</sub> dm <sup>-3</sup>	15
B (hot water)	mg dm <sup>-3</sup>	0.14
Cu (DTPA)	mg dm <sup>-3</sup>	3.4
Fe (DTPA)	mg dm <sup>-3</sup>	25
Zn (DTPA)	mg dm <sup>-3</sup>	0.9
Mn (DTPA)	mg dm <sup>-3</sup>	38.1
S-SO <sub>4</sub>	mg dm <sup>-3</sup>	4.0
H + Al	mmol <sub>c</sub> dm <sup>-3</sup>	34
CEC (pH 7.0)	mmol <sub>c</sub> dm <sup>-3</sup>	75.7
V	%	50

CEC: cation exchange capacity; V: base saturation; DTPA: diethylenetriaminepentaacetic acid.

**Table 2.** Soil physio-chemical analysis of field site before experiment initiation during 2019 and 2020 common bean growing seasons.

Properties	Units	Values	
		2019	2020
Clay <sup>†</sup>	g kg <sup>-1</sup>	----- 433 -----	-----
Sand <sup>†</sup>	g kg <sup>-1</sup>	----- 471 -----	-----
Silt <sup>†</sup>	g kg <sup>-1</sup>	----- 90 -----	-----
pH (CaCl <sub>2</sub> )	----	5.2	5.1
Organic matter	mg dm <sup>-3</sup>	18	21
P	mg dm <sup>-3</sup>	38	42
K	mmol <sub>c</sub> dm <sup>-3</sup>	1.7	1.9
Ca	mmol <sub>c</sub> dm <sup>-3</sup>	21	22
Mg	mmol <sub>c</sub> dm <sup>-3</sup>	15	13
B	mg dm <sup>-3</sup>	0.14	0.35
Cu	mg dm <sup>-3</sup>	3.4	3.6
Fe	mg dm <sup>-3</sup>	25	29
Zn	mg dm <sup>-3</sup>	0.9	1.1
Mn	mg dm <sup>-3</sup>	38.1	37.1
S-SO <sub>4</sub>	mg dm <sup>-3</sup>	4.0	9.2
H+Al	mmol <sub>c</sub> dm <sup>-3</sup>	34	30
CEC	mmol <sub>c</sub> dm <sup>-3</sup>	75.7	67.9
V	%	50	52

\* CEC: cation exchange capacity, V: base saturation, <sup>†</sup>: Teixeira *et al.* (2017).

**Table 3.** Zinc partitioning index (ZPI) and estimated Zn intake in Brazil in common bean grains as a function of plant growth-promoting bacteria and nano Zn foliar doses in 2019 and 2020 cropping seasons.

Treatments	Grain Zn uptake		ZPI		Zn intake	
	----- g ha <sup>-1</sup> -----		----- % -----		g person <sup>-1</sup> day <sup>-1</sup>	
	2019	2020	2019	2020	2019	2020
<b>Inoculations (I)</b>						
Without (Control)	154	211	122 d	149	6.2	7.3
<i>R. tropici</i>	169	300	128 c	155	6.4	7.7
<i>R. tropici</i> + <i>A. brasilense</i>	185	245	132 b	158	6.7	8.0
<i>R. tropici</i> + <i>B. subtilis</i>	219	279	139 a	166	7.4	8.6
<i>R. tropici</i> + <i>P. fluorescens</i>	193	259	134 b	162	6.8	8.3
<i>R. tropici</i> + <i>A. brasilense</i> + <i>B. subtilis</i>	176	239	129 c	156	6.5	7.8
<i>R. tropici</i> + <i>A. brasilense</i> + <i>P. fluorescens</i>	164	235	127 c	154	6.3	7.7
<b>Nano Zn foliar spray (kg ha<sup>-1</sup>)</b>						
0.0	156	226	127 b	150	6.3	7.7
1.5	204	262	136 a	167	7.0	8.2
3.0	180	240	126 b	157	6.5	7.9
<b>F- values</b>						
I	81 **	43 **	42 **	37 **	43 **	44 **
Nano Zn	229 **	73 **	81 **	138 **	89 **	38 **
I × nano Zn	9 **	3 **	1.4 <sup>ns</sup>	1.6 <sup>ns</sup>	5 **	2.3 *
<b>CV (%)</b>	5	4.7	2.3	2	3.3	2.6

Means in the column followed by similar letters are statistically not different by Tukey test ( $p \leq 0.05$ ) for foliar nano Zn dose and Scott Knott test for PGPBs ( $p \leq 0.05$ ). \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$  respectively while <sup>ns</sup>—non-significant by F-test.

**Table 4.** Zinc use efficiencies (Zinc use efficiency, agro-physiological efficiency, physiological efficiency, utilization efficiency and applied zinc recovery) of common beans as a function of plant growth-promoting bacteria and nano Zn foliar doses in 2019 and 2020 cropping seasons.

Treatments	ZnUE		APE		AZnR		UT	
	----- kg kg <sup>-1</sup> -----		----- % -----		--- kg kg <sup>-1</sup> ---			
	2019	2020	2019	2020	2019	2020	2019	2020
<b>Inoculations (I)</b>								
Without (Control)	274	65	7.3 a	2.8	38	23	345	141
<i>R. tropici</i>	363	132	7.5 a	2.6	52	39	484	247
<i>R. tropici</i> + <i>A. brasilense</i>	434	255	6.8 a	4.1	69	56	595	417
<i>R. tropici</i> + <i>B. subtilis</i>	647	422	5.7 b	4.4	118	96	951	694
<i>R. tropici</i> + <i>P. fluorescens</i>	513	329	6.3 b	4.3	86	70	716	519
<i>R. tropici</i> + <i>A. brasilense</i> + <i>B. subtilis</i>	405	248	7.3 a	5.2	58	49	533	395
<i>R. tropici</i> + <i>A. brasilense</i> + <i>P. fluorescens</i>	327	219	7.1 a	5.0	46	42	426	355
<b>Nano Zn foliar spray (kg ha<sup>-1</sup>)</b>								
1.5	604	369	6.3 b	4.1	100	81	828	595
3.0	243	108	7.5 a	4.0	34	26	329	196
<b>F- values</b>								
I	24 **	12 **	2.4 *	1.9 <sup>ns</sup>	85 **	46 **	74 **	16 **
		102			862			
Nano Zn	348 **	**	13 **	0.01 <sup>ns</sup>	**	441 **	784 **	138 **
I × nano Zn	7 **	4 **	0.9 <sup>ns</sup>	0.8 <sup>ns</sup>	27 **	10 **	18 **	4.8 **
<b>CV (%)</b>	17	40	16	51	13	18	11	32

Means in the column followed by similar letters are statistically not different by Tukey test ( $p \leq 0.05$ ) for foliar nano Zn dose and Scott Knott test for PGPBs ( $p \leq 0.05$ ). \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$  respectively while <sup>ns</sup>—non-significant by F-test.

ZnUE = Zinc use efficiency, APE = Agro-physiological efficiency, AZnR = Applied zinc recovery, and UE = Utilization efficiency.

**Table 5.** Zinc (Zn) concentration in leaf, shoot and, grain and accumulation in shoot and grains of wheat under the influence of diazotrophic bacteria and soil zinc application. Selvíria - MS, Brazil, 2019 and 2020

Treatments	Leaf Zn concentration		Shoot Zn concentration		Grain Zn concentration		Shoot Zn accumulation		Grain Zn accumulation	
	----- mg kg <sup>-1</sup> -----									
	----- g ha <sup>-1</sup> -----									
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Zinc (Zn) application (kg/ha)										
0	40 b	43 b	30 b	32 b	43 b	47 b	148 b	161 b	146 b	163 b
8	46 a	48 a	35 a	37 a	48 a	55 a	177 a	195 a	183 a	215 a
Diazotrophic bacterial inoculations (I)										
Without (Control)	36 c	37 c	28 b	29 c	38 d	42 d	131 b	143 c	119 c	138 c
<i>A. brasilense</i>	43 b	46 b	34 a	35 b	49 b	54 b	171 a	183 b	175 b	196 b
<i>B. subtilis</i>	43 b	46 b	33 a	34 b	46 c	51 c	169 a	179 b	169 b	197 b
<i>P. fluorescens</i>	50 a	54 a	35 a	38 a	51 a	56 a	180 a	206 a	195 a	224 a
F- values										
Zn	74 **	87 *	19 *	32 *	67 **	177 **	30 **	70 **	101 **	125 **
I	79 **	132 **	9 *	22 **	89 **	112 **	16 *	41 **	72 **	62 **
Zn x I	2.4 ns	8 *	1.9 ns	3 ns	5 *	10 **	4 *	6 *	12 *	14 **
CV (%)	4.26	3.6	9.0	6.5	3.7	3.3	9.5	6.4	6.5	6.9

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ ); \*\* and \* Significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; and ns, non-significant by F-test.

**Table 6.** Plant height, dry matter, grain yield, Zn partitioning index (ZPI), Zn intake in world and Brazil with consumption of wheat grains as influenced by diazotrophic bacteria and soil zinc application.

Treatments	Plant height		Dry matter		Grain yield		ZPI		Zn intake (world)		Zn intake (Brazil)	
	----- cm -----		----- kg ha <sup>-1</sup> -----				----- % -----		----- g person <sup>-1</sup> day <sup>-1</sup> -----			
	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020	2019	2020
Zinc (Zn) application (kg/ha)												
0	73 b	76 b	4887 b	5001 b	3339 b	3450 b	69 b	70 b	11.9 b	12.6 b	6.9 b	7.3 b
8	77 a	80 a	5112 a	5299 a	3755 a	3877 a	73 a	75 a	13.1 a	14.7 a	7.6 a	8.6 a
Diazotrophic bacterial inoculations (I)												
Without (control)	71 b	73 c	4680 b	4839 b	3160 b	3269 c	67 b	69 c	10.5 c	11.3 c	6.1 c	6.6 d
<i>A. brasilense</i>	73 b	76 b	5090 a	5217 a	3591 a	3634 b	72 a	73 b	12.9 b	14.5 b	7.5 b	8.4 b
<i>B. subtilis</i>	77 a	80 a	5109 a	5227 a	3637 a	3807 a	71 a	71 b	12.7 b	13.8 c	7.4 b	8.1 c
<i>P. fluorescens</i>	79 a	83 a	5120 a	5317 a	3800 a	3943 a	75 a	76 a	14.1 a	15.1 a	8.2 a	8.8 a
F-values												
Zn	13 *	22 *	27 **	67 **	35 **	39 **	8 *	50 **	39 **	177 **	39 **	176 **
I	12 **	22 **	25 **	34 **	15 **	18 **	6 **	23 **	69 **	112 **	69 **	111 **
Zn x I	0.6 ns	0.05 ns	4 *	5 *	5 *	6 *	0.4 ns	3 *	4 *	10 *	4 *	10 *
CV (%)	3.8	3.2	2.4	2.0	5.5	5.3	5.5	2	4.0	3.3	4.0	3.3

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ ); \*\* and \* Significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; and ns, non-significant by F-test.

**Table 7.** Zinc intake in Brazil, Zn use efficiency (ZnUE) and applied Zn recovery (AZnR) in wheat as influenced by plant growth-promoting bacteria and foliar applied nano-Zn doses.

Treatments	Zn intake (Brazil)		ZnUE		AZnR	
	g person <sup>-1</sup> day <sup>-1</sup>		kg kg <sup>-1</sup>		%	
	2019	2020	2019	2020	2019	2020
<b>Inoculations</b>						
Without	5.8 c	6.9 b	263	167 c	176 c	42 c
<i>A. brasilense</i>	6.6 b	7.7 a	316	335 bc	195 cb	69 b
<i>B. subtilis</i>	7.1 ab	7.9 a	195	464 ab	213 ab	79 ab
<i>P. fluorescens</i>	7.2 a	8.0 a	317	591 a	216 a	98 a
<b>Foliar Zn application (kg ha<sup>-1</sup>)</b>						
0	6.3	7.0	-----	----	-----	-----
0.75	6.5	7.3	501	805	403	129
1.5	7.2	8.4	339	474	235	105
3	6.8	7.8	171	188	109	38
6	6.7	7.5	80	90	53	17
<b>F values</b>						
Inoculation (I)	16**	7.2**	1.77ns	13.5**	11.6**	12.4**
Foliar Zn (FZn)	3.6*	5.7**	18.4**	42.6**	844**	65**
I x FZn	1.3 ns	0.9 ns	4.13**	6.7**	2.38*	2.58*
<b>CV (%)</b>	10.2	11.2	63.45	50.6	10.7	36.6

Means in the column followed by different letters are significantly different ( $p$ -value  $\leq 0.05$ ); \*\* and \*—significant at  $p \leq 0.01$  and  $p \leq 0.05$ , respectively; ns—non-significant, by F-test.

**Table 8.** Pre-maize experiments soil analysis of composite sample in a soil layer (0 – 0.20 m) in 2019-20 and 2020-21 cropping seasons.

Properties	Units	Status	
		2019-2020	2020-2021
pH (CaCl <sub>2</sub> )	----	5.2	5.3
Organic matter	mg dm <sup>-3</sup>	18	23
P (resin)	mg dm <sup>-3</sup>	38	40
K	mmol <sub>c</sub> dm <sup>-3</sup>	1.7	1.9
Ca	mmol <sub>c</sub> dm <sup>-3</sup>	21	22
Mg	mmol <sub>c</sub> dm <sup>-3</sup>	15	12
B (Hot water)	mg dm <sup>-3</sup>	0.14	0.39
Cu (DTPA)*	mg dm <sup>-3</sup>	3.4	3.7
Fe (DTPA)*	mg dm <sup>-3</sup>	25	28
Mn (DTPA)*	mg dm <sup>-3</sup>	38.1	37.3
S-SO <sub>4</sub>	mg dm <sup>-3</sup>	4.0	22
H+Al	mmol <sub>c</sub> dm <sup>-3</sup>	34	31
CEC (pH7)*	mmol <sub>c</sub> dm <sup>-3</sup>	75.7	66.9
V*	%	50	54
Zn content (DTPA)			
Without Zn fertilization	mg dm <sup>-3</sup>	0.9	1.1
Residual Zn fertilization	mg dm <sup>-3</sup>	2.2	3.0

\* CEC: cation exchange capacity, V: base saturation, DTPA: Diethylenetriaminepentaacetic acid

**Table 9.** Eigenvalue and percentage of variance and factor loadings generated by principal component analysis for maize in 2019-2020 cropping season.

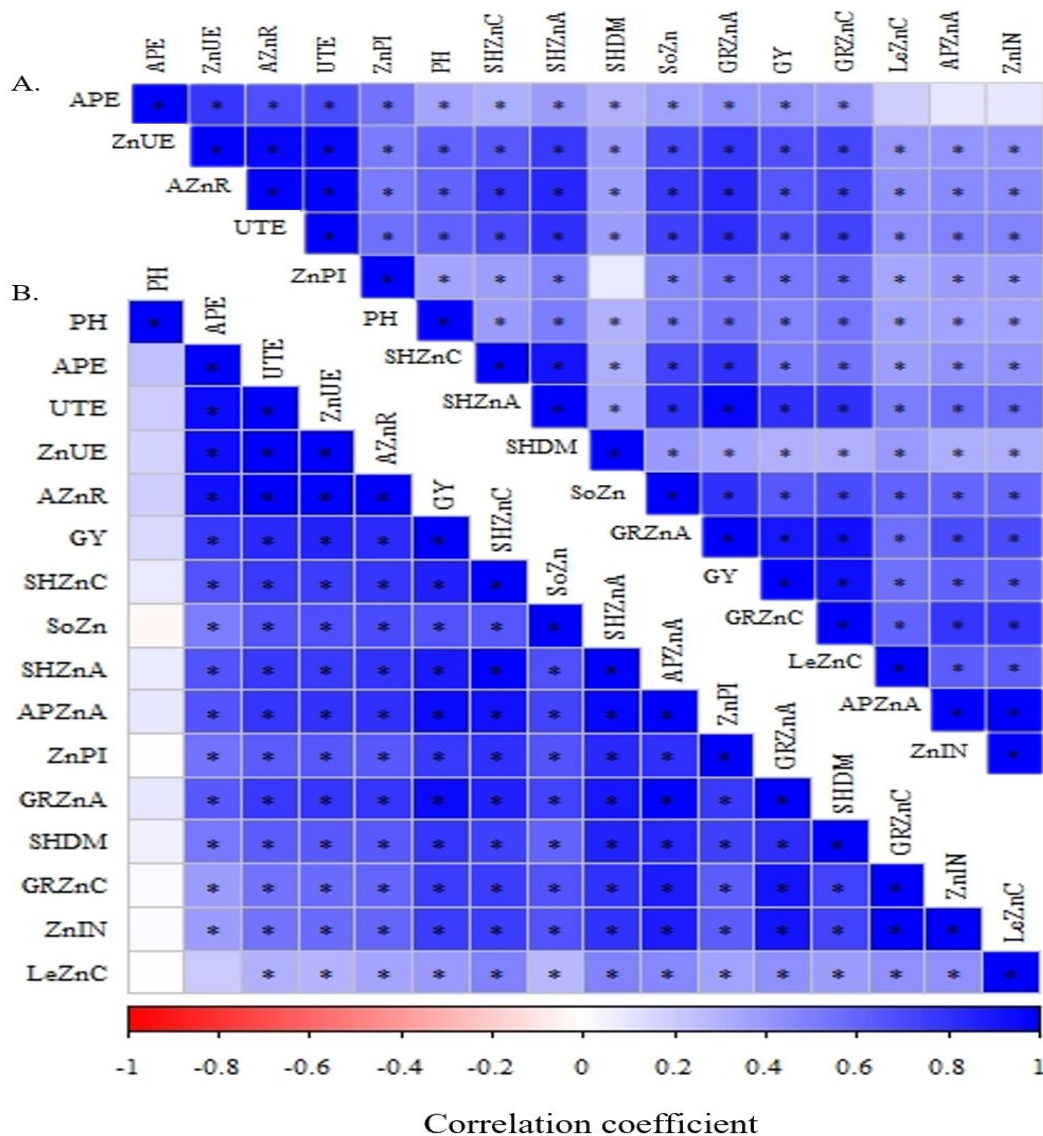
	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Eigenvalues	1.43	1.67	1.09	3.67	2.56	1.93	8.71	2.93
Variability (%)	7.96	9.28	6.07	2.04	1.42	1.07	4.84	1.63
Cumulative variance (%)	79.6	88.90	94.97	97.02	98.44	99.5	100	100

**Table 10.** Eigenvalue and percentage of variance and factor loadings generated by principal component analysis for maize in 2020-2021 cropping season.

	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
Eigenvalues	1.27	2.37	1.36	7.75	4.60	2.35	8.82	5.84
Variability (%)	7.06	1.32	7.56	4.31	2.55	1.31	4.90	3.24
Cumulative variance (%)	70.62	83.78	91.34	95.64	98.20	99.51	100	100

## APPENDIX B – Figures

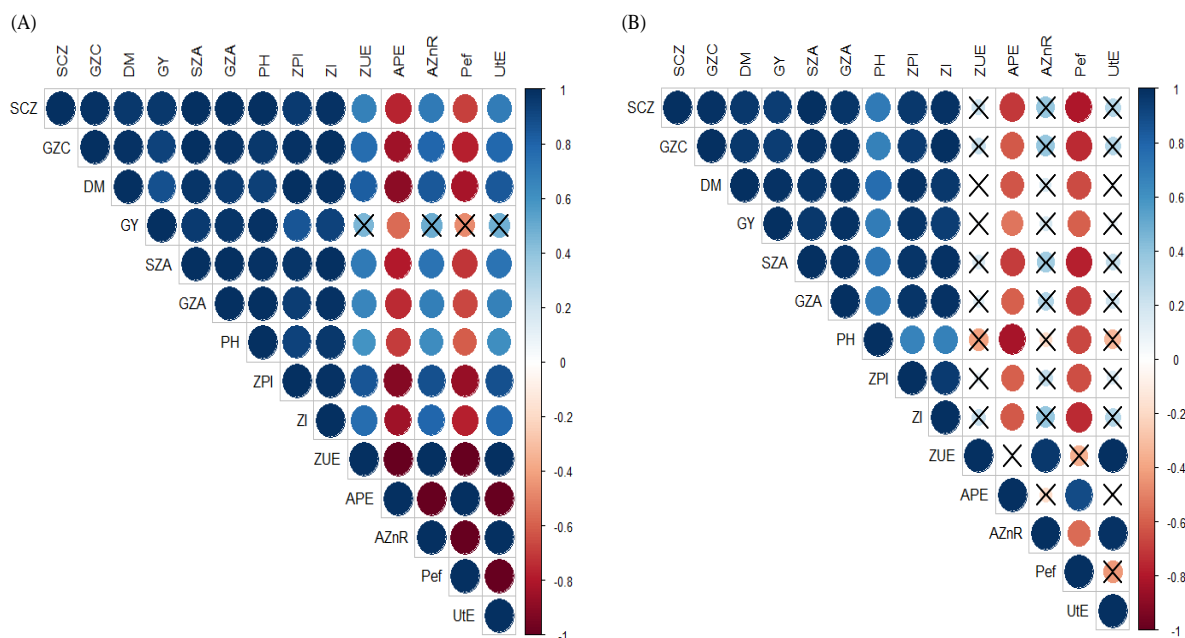
**Figure 1.** Heatmap showing Pearson's correlation among the analyzed parameters from soil and common bean plants in response to different soil Zn applications and diazotrophic bacteria inoculations in the 2019 (A) and 2020 (B) crop seasons.



**Source:** Author. \* indicates a significant relationship ( $p \leq 0.05$ ). Abbreviations: SoZn = Zn content in soil, LeZnC = Zn concentration in leaf tissue, SHZnC = Zn concentration in shoot, SHZnA = Zn accumulated in shoot, APZnA = Zn accumulated in aerial part (shoot + grain), GRZnC = Zn concentration in grain, GRZnA = Zn accumulated in grain, SHDM = shoot dry matter, GY = grain yield, PH = plant height, ZnPI = Zn partitioning index, ZnIN = Zn intake, ZnUE = Zn use efficiency,

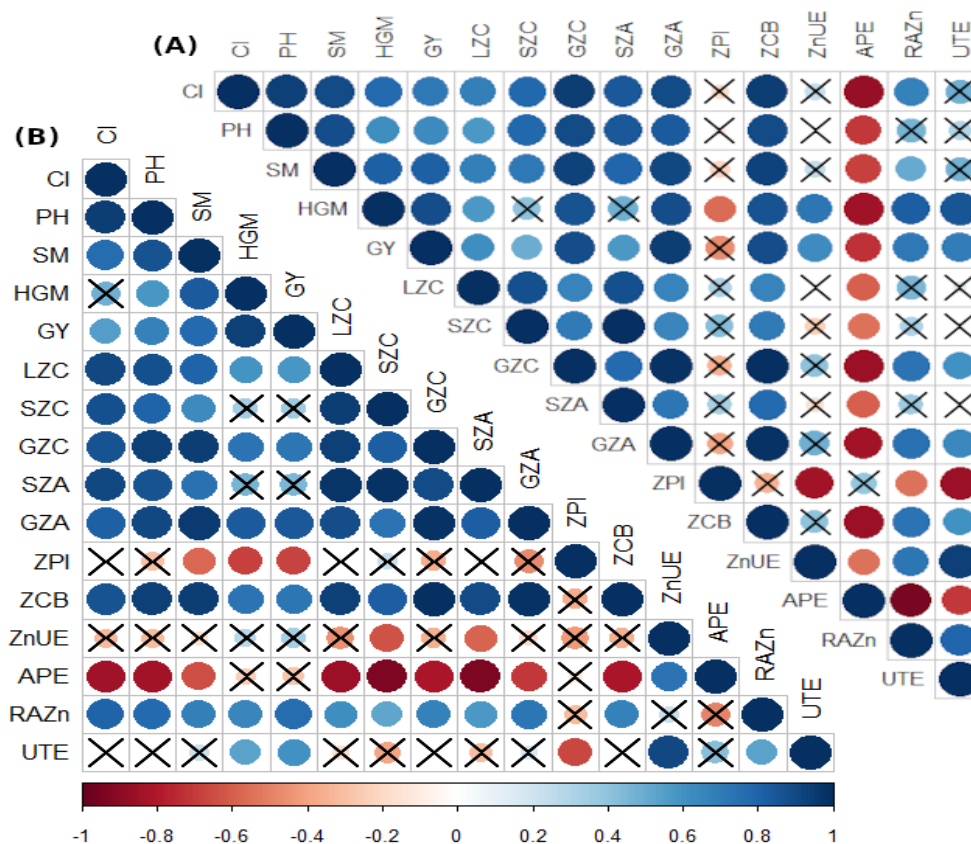
APE = Agro-physiological efficiency, AZnR = Applied Zn recovery, and UTE = Utilization efficiency.

**Figure 2.** Heat-map color scale indicating Pearson's correlation among evaluated attributes of common beans in response to plant growth-promoting bacteria and foliar nano-Zn doses in 2019 (A) and 2020 (B) cropping seasons.



**Source:** Author. × = indicates a non-significant relationship ( $p \leq 0.05$ ). Abbreviations: PH= plant height, DM= shoot dry matter, NLC= number of row per cob, NGC= number of grains per cob, HGs= 100-grains weight, GY= grain yield, SZnA= shoot Zn accumulation, GZnA= grain Zn accumulation, ZnUE= Zn use efficiency, AZnR = applied Zn recovery, APE = agro-physiological efficiency and UE = utilization efficiency.

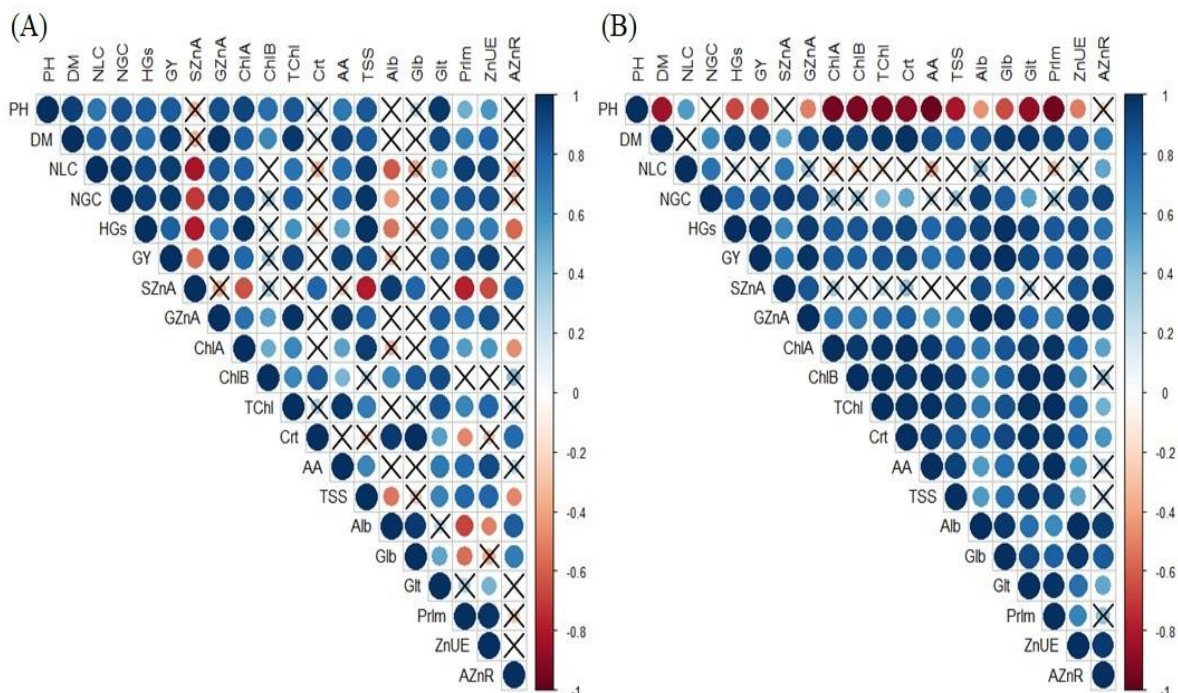
**Figure 3.** Heat-map indicating Pearson's correlation among evaluated parameters of maize plants in response to residual soil Zn applications and diazotrophic bacteria inoculations in 2019-2020 (A) and 2020-2021 (B) cropping seasons.



**Source:** Author.

X = indicates a non-significant relationship ( $p \leq 0.05$ ). Abbreviations: CI = Insertion of first productive cob, PH = plant height, SM = shoot dry matter, GY = grain yield, LZC = Leaf Zn concentration, SZC = Shoot Zn concentration, GZC = Grain Zn concentration, SZA = Shoot Zn accumulation, GZA = Grain Zn accumulation, ZPI = Zn partitioning index, ZCB = Zn intake in Brazil, ZnUE = Zn use efficiency, APE = Agro-physiological efficiency, RAZn = Applied Zn recovery, and UTE = Utilization efficiency.

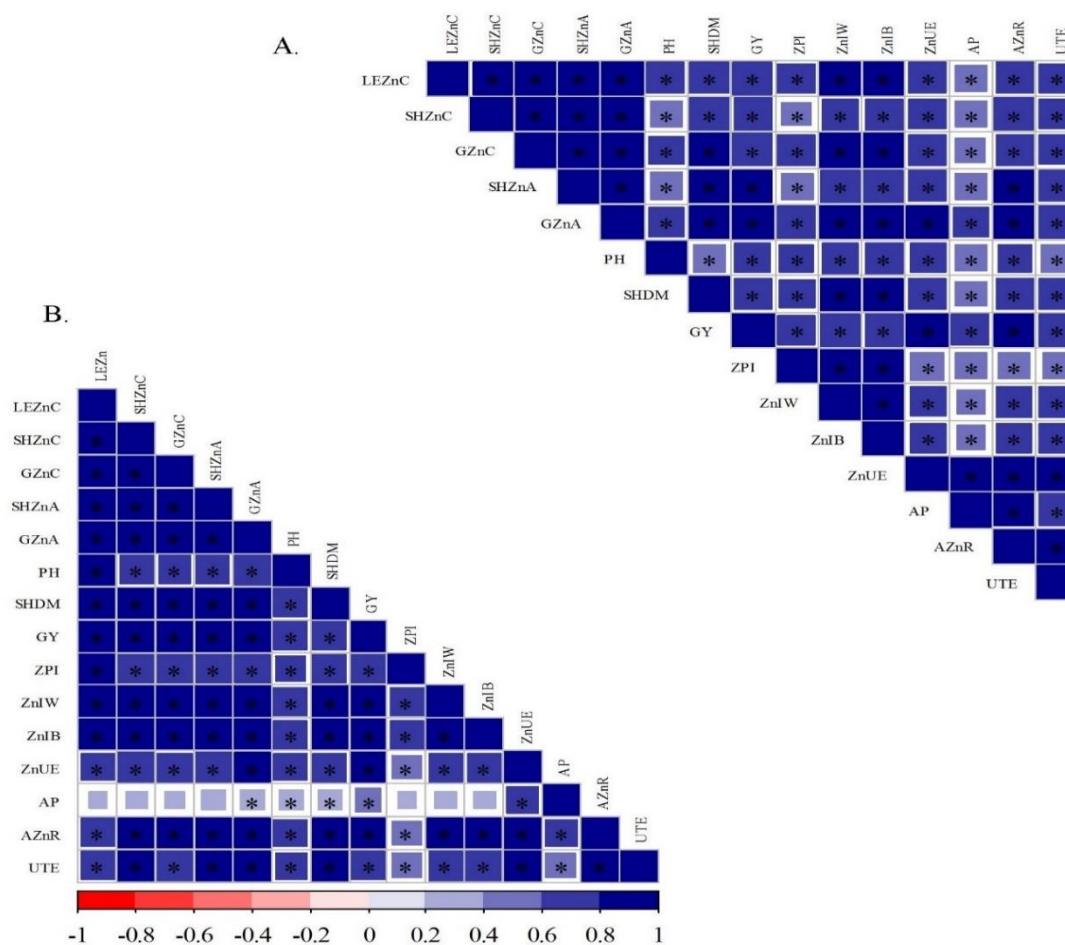
**Figure 4.** Heat-map color scale indicating Pearson's correlation among evaluated attributes of maize in response to plant growth-promoting bacteria and foliar nano ZnO applications in 2019-2020 (A) and 2020-2021 (B) cropping seasons.



**Source:** Author.

× = indicates a non-significant relationship ( $p > 0.05$ ). Abbreviations: PH= plant height, DM= shoot dry matter, NLC= number of row per cob, NGC= number of grains per cob, HGs= 100-grains weight, GY= grain yield, SZnA= shoot Zn accumulation, GZnA= grain Zn accumulation, ChIA= chlorophyll a, ChIB= chlorophyll b, TChl= total chlorophyll, Crt= carotenoids, AA= free amino acids, TSS= total soluble sugar, Alb= albumin, Glb= globulin, Glt= glutelin, Prlm= prolamin, ZnUE= Zn use efficiency, and AZnR = applied Zn recovery.

**Figure 5.** Heat-map indicating Pearson's correlation among the evaluated parameters of wheat plants in response to soil Zn applications and diazotrophic bacteria inoculations in 2019 (A) and 2020 (B) crop seasons.



**Source:** Author.

\* indicates a significant relationship ( $p \leq 0.05$ ). Abbreviations: LEZnC = Leaf Zn concentration, SHZnC = Shoot Zn concentration, GZnC = Grain Zn concentration, SHZnA = Zn accumulated in shoot, GZnA = Zn accumulated in grain, PH = plant height, SHDM = shoot dry matter, GY = grain yield, ZnPI = Zn partitioning index, ZnWI = Zn intake in world, ZnBI = Zn intake in Brazil, ZnUE = Zn use efficiency, AP = Agro-physiological efficiency, AZnR = Applied Zn recovery, and UTE = Utilization efficiency.

## APPENDIX-C

## Common Bean Production Process

Overview of the study area (A), seeds inoculation (B), Sowing (C).

**A****B****C**

Soil Zn application (D), examining effect of soil applied Zn (E), general inspection of crop (F).



Grain filling and dough growth stages of the crop (G) and (H), disease and pest attack inspection (I).

**G****H****I**

Crop harvest and drying (J), threshing process (K), final crop production of common beans.



## Cereal Crop: Maize Production and Biofortification Process

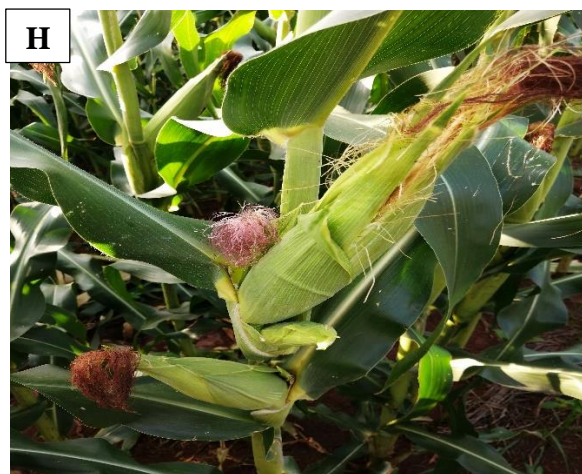
Overview of the study area (A), seeds chemical treatments and inoculation (B), sowing (C).



Soil Zn application (D), examining effect of soil Zn application (E), A general inspection of crop with supervisor (F).



Maize at full flowering (G), maize crop in the end of grain filling (H) and following the end of corn grain filling (I).



Maize crop at harvest time and grains for final evaluations (J) and (K), Biofortified cobs (L).



### Cereal Crop: wheat Production Proces

Overview of the study area (A), Wheat planting (B), area overview after planting (B), Wheat early development stage and plot partitioning (C).

**A****B****C**

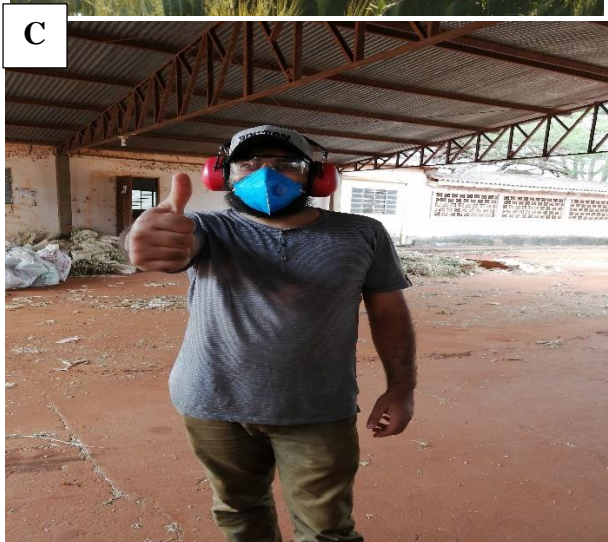
Wheat crop at grain formation (D), wheat crop at grain filling (E), wheat crop at physiological maturity and grain filling (F).



Wheat crop inspection with supervisor (G), wheat crop at harvest time (H), wheat grain harvested for final evaluations and analysis (I).



Random research activities at different stages of crop: inoculation effect on root growth (A), foliar spray application (B), and threshing crops (C).



Research activities: Soil Collection (D), Soil Analysis (E) and Seeds chemical treatment (E).



Research activities: Nutritional and biochemical analysis at laboratory.



Plant Nutrition Group: UNESP – Campus of Ilha Solteira,

