T.R.
ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF SOIL SCIENCE AND PLANT NUTRITION

NITROGEN MANAGEMENT IN CROP CULTIVATION USING BIOFERTILIZER CONTAINING FREE-LIVING NITROGEN FIXING BACTERIA

Master's Thesis

Tursynbek KAIYRBEKOV

Supervisor
Prof. Dr. Ridvan KIZILKAYA

II. Supervisor
Prof. Dr. Andon Vassilev

SAMSUN
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.ACCEPTANCE AND APPROVAL OF THE THESIS

The study entitled **"Nitrogen management in crop cultivation using biofertilizer containing free-living nitrogen fixing bacteria"** prepared by **Tursynbek KAIYRBEKOV**, and supervised by **Prof. Dr. Andon VASSILEV and Prof. Dr. Ridvan KIZILKAYA**, was found successful and unanimously accepted by committee members as Master thesis, following the examination on the date 14.8.2023
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Chairman	Prof. Dr. RIDVAN KIZILKAYA Ondokuz Mayıs University Faculty of Agriculture Department of Soil Science and Plant Nutrition	☒ Accept ☐ Reject
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Member	Prof. Dr. ORHAN DENGIZ Ondokuz Mayıs University Faculty of Agriculture Department of Soil Science and Plant Nutrition	☒ Accept ☐ Reject

This thesis has been approved by the committee members that already stated above and determined by the Institute Executive Board.

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Prof. Dr. Ahmet TABAK
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I hereby declare and undertake that I complied with scientific ethics and academic rules in all stages of my Master's Thesis , that I have referred to each quotation that I use directly or indirectly in the study and that the works I have used consist of those shown in the sources, that it was written in accordance with the institute writing guide and that the situations stated in the article 3, section 9 of the Regulation for TÜBİTAK Research and Publication Ethics Board were not violated.

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DECLARATION OF THE THESIS STUDY ORIGINALITY REPORT

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ÖZET

SERBEST AZOT FİKSE EDEN BAKTERİLERİ İÇEREN BİYOGÜBRE KULLANIMININ ÜRÜN YETİŞTİRİCİLİĞİNDE AZOT YÖNETİMİ

Tursynbek KAIYRBEKOV
Ondokuz Mayıs Üniversitesi
Lisansüstü Eğitim Enstitüsü
TOPRAK BİLİMİ VE BİTKİ BESLEME ANA BİLİM DALI
Yüksek Lisans, Haziran/2023
Danışman: Prof. Dr. Andon VASSILEV
Danışman: Prof. Dr. Ridvan KIZILKAYA

Kimyasal aşırı gübreleme, gıda talebinin artmasıyla birlikte çevre ve toprak sağlığı üzerinde zararlı bir etkiye sahiptir. Sürdürülebilir tarımsal uygulamalar, çevre dostu yaklaşımların benimsenmesini gerektirmektedir. Serbest yaşayan azot bağlayıcı mikroorganizmalar içeren biyogübreler bu konuda umut verici bir alternatif sunmaktadır. Biyogübreler, kimyasal gübrelerden bağımsız olarak veya birlikte uygulanabilir, ancak bu yaklaşım optimal dozu, gübre oranlarını, uygun uygulama zamanını ve bitki türlerinin beraber uygulamalara verdiği tepkilerin bilinmesini gerekli kılmaktadır. Bu çalışmanın amacı, biyogübrenin kimyasal gübrelerle birlikte kullanımının toprak ve bitkilerin önemli fiziko-kimyasal, fizyolojik ve biyokimyasal parametreleri üzerindeki etkilerini değerlendirmektir. Araştırma, buğdayla yapılan bir tarla denemesi ile ve kontrollü koşullarda mısır bitkisinde yürütülen saksı denemesi olmak üzere iki denemeden oluşmaktadır. Toprak ve bitkilerde azot içeriği, fotosentetik parametreler, çözünebilir protein düzeyleri ve uygulamaların antiradikal (DPPH) aktivitesi gibi parametreler belirlenmiştir. Buğday'da yürütülen tarla denemesnde, 1 kg/ha dozunda uygulanan Nuptak biyogübresi, kontrol uygulamasına amonyum azot içeriğinde iki kat artışa neden olmuştur. Nuptak biyogübresi ile takviye edilen buğday yapraklarında fotosentetik hızı uyarırken toplam klorofil içeriğini etkilemediği belirlenmiştir. Nuptak biyogübresinin kullanımı, suboptimal gübreleme kaynaklı verim kaybını telafi etmeye yardımcı olmuştur. Örneğin, %50 gübreleme oranında verim sadece %12 azaldığı belirlenmiştir. Mısır'da yürütülen saksı denemesinde, biyogübre uygulaması toprak solunumunu ve amonyum içeriğini artırdığı belirlenmiştir. Ancak, Priestia megaterium CB2001 suşu ile aşılamanın biyogübre etkilerini değerlendirmek için daha fazla araştırmaya ihtiyaç vardır. Genel olarak çalışma sonunda, toprak ve bitki parametrelerini olumlu yönde etkileyerek suboptimal gübreleme uygulamalarının olumsuz sonuçlarını hafifletmeye yardımcı olduğu için biyogübreleri kimyasal gübrelerin tamamlayıcısı olarak kullanmanın potansiyel faydalarının olduğu ortaya konulmuştur.

Anahtar Sözcükler: mikrobiyal aşılayıcılar, serbest yaşayan diazotroflar, azotlu gübreleme, bitki büyümesini teşvik eden bakteriler, Priestia megaterium

ABSTRACT

NITROGEN MANAGEMENT IN CROP CULTIVATION USING BIOFERTILIZER CONTAINING FREE-LIVING NITROGEN FIXING BACTERIA

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Chemical overfertilization, driven by the increasing demand for food, has a detrimental impact on the environment and soil health. Sustainable agricultural practices necessitate the implementation of eco-friendly approaches. Biofertilizers containing free-living nitrogen-fixing microorganisms offer a promising alternative in this regard. Biofertilizers can be applied independently or in conjunction with chemical fertilizers, but this approach requires understanding of optimal dosages, fertilizer ratios, appropriate application timing, and crop-specific responses to combined treatments. The objective of the current study was to evaluate the effects of using biofertilizer as a supplement to chemical fertilizers on key physicochemical, physiological, and biochemical parameters of soil and plants. The research comprised two experiments: a field experiment conducted with wheat and a controlled maize pot experiment. Parameters such as nitrogen content in soil and plants, photosynthetic parameters, soluble protein levels, and antiradical (DPPH) activity of the treatments were observed. In the wheat field experiment, the application of Nuptak biofertilizer at a dose of 1 kg/ha resulted in a twofold increase in ammonium nitrogen content compared to the control treatment. Supplementation with Nuptak biofertilizer stimulated the photosynthetic rate in wheat leaves without affecting total chlorophyll content. The use of Nuptak biofertilizer helped compensate for productivity loss caused by suboptimal fertilization; for instance, at a 50% fertilization rate, the yield only decreased by 12%. In the maize pot experiment, biofertilizer application increased soil respiration and ammonium content. The inoculation with the *Priestia megaterium* CB2001 strain requires further investigation to assess the effects of the biofertilizer. Overall, the study highlights the potential benefits of using biofertilizers as supplements to chemical fertilizers, as they positively influence soil and plant parameters and can help mitigate the negative consequences of suboptimal fertilization practices.

Keywords: microbial inoculants, free-living diazotrophs, nitrogen fertilization, plant-growth-promoting-bacteria (PGPB), Priestia megaterium

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Tursynbek Kaiyrbekov (Kazakhstan)

CONTENTS

SYMBOLS AND ABBREVIATIONS

PGPB	: Plant Growth Promoting Bacteria
RCBD	: Randomized Complete Block Design
FAO	: Food and Agricultural Organization
DPPH	: 2,2-diphenyl-1-picrylhydrazyl
SON	: Soil organic nitrogen
Ndfa	: Nitrogen derived from air
BNF	: Biological nitrogen fixation
NOP	: National Organic Program
GS	: glutamine synthase
WHO	: World Health Organization
ISO	: the International Organization for Standardization

FIGURES LEGENDS

TABLES LEGENDS

1. INTRODUCTION

Application of chemical fertilizers in order to sustain plant growth and yield correspond to the constantly growing world food demand. In present crop production, the problem with the excessive fertilizer use and poor utilization effectiveness is prevalent around the globe. Overuse of chemical fertilizers is linked to increased greenhouse emission, groundwater contamination and decreased economic profits (Peng et al., 2022). Additionally, the overfertilized plants may have weaker defence system and are more vulnerable to pest and disease. The nitrogen, which has not been utilized by crop, can leach into groundwater, can contaminate water sources and drinkable water and thus can endanger human and animal health. The nitrogen cycle can be affected through increased emission of N_2O – a powerful greenhouse gas released by soil organisms that transform NO_3 into N_2O and N_2 in anaerobic waterlogged condition. In addition to the greenhouse impact, the gas destroys the stratospheric ozone layer, which shields from dangerous UV rays (Prather et al., 2012). Overfertilization can affect negatively not only the availability of nitrogen in the soil but also plant growth and nutrient uptake.

Nitrogen (N) participates in all biochemical reactions and is one of the most important chemical elements in plant growth and yield. In soils, nitrogen exist in several different forms, as organic and inorganic nitrogen. The inorganic nitrogen in soil primarily is in ammonium (NH_4^+) or nitrate (NO_3^-) form along with variety of organic molecules. Soil organic matter (SOM) accounts for approximately 92-98% of total N in the soil. However, plant utilizes N mineral forms, but these components constitute only 2-8% of the total soil N content. Organic nitrogen is not available for plant root absorption and must be converted to inorganic forms through microbial processes such as mineralization and nitrification (Wang et al., 2010). The ammonium compounds are typically present in soils with low pH, while nitrate is more prevalent in neutral and slightly alkaline soils. The availability of nitrogen in the soil is influenced by several factors such as: soil pH, temperature, moisture, and the presence of nitrogen-fixing bacteria. Nitrogen assimilation is achieved by glutamine synthase (GS), which is the main catalytic enzyme involved in the transformation of inorganic N into organic components (Yang et al., 2023). The process of biological fixation conducted by symbiotic and non-symbiotic N-fixing microorganisms converts atmospheric N_2 to readily plant-utilizable forms (Feng et al., 2014). In agricultural

practice, nitrogen is provided as a fertilizer in order to improve plant growth, development and yield.

There are two main types of nitrogen fixation: non-biological and biological nitrogen fixation. The non-biological nitrogen fixation refers to the conversion of atmospheric nitrogen into reactive nitrogen compounds through natural or industrial processes that do not involve living organisms. Examples of non-biological nitrogen fixation are fixation by lightning or other electro chemical or photochemical reactions and in the industry by Haber-Bosch process, and cyanamide conversion. During a thunderstorm, lightning energy could break the triple bond between atmospheric nitrogen atoms and convert it into nitrogen oxide. Nitrogen oxide could further interact with water and with the rain fall onto soil surface (Holland et al., 2005). In pre-industrial times, the content of the mineral N forms in the N cycle was almost constant. The great change occurred on a world scale was due to massive human intervention in the agroecosystems after invention and extensive use of chemical fertilizers. The main industrial technique for chemical fertilizer synthesis is based on the Haber-Bosch process. As a result of the process, ammonia (NH_3) has been obtained from nitrogen (N_2) and hydrogen gases (H_2) under high pressure and temperature (Wang et al., 2023). The synthesized ammonia can be used directly as a fertilizer, or they can be processed further to other nitrogen molecules such as urea and ammonium nitrate. However, the production of ammonia through the Haber-Bosch process requires a large amount of fossil fuels, energy and labour expenses which affect the prices of fertilizers. In order to reduce the quantity or necessity for chemical fertilizer and production expenses in agriculture some other options are already available.

In the result of evolution, some organisms such as algae, cyanobacteria, and nodule bacteria have acquired the ability to use atmospheric nitrogen. Bacteria frequently have symbiotic relationships with their hosts, enabling the plant to receive nutrients through the bacteria's activity. The symbiotic relationship exists between rhizobium bacteria and Fabaceae plants such as alfalfa, clover, or vetch and dock onto their roots, providing nitrogen compounds to the plant (Muthusamy et al., 2023). Prior to the invention of artificial fertilizer, people frequently implemented crop rotation and cultivated legumes to replenish the soil fertility. In some cases, a significant portion of N fixed by the leguminous plants is eliminated after the harvest. However, there are some bacteria that live independently and inhabit the plant root zone. Most of the free-

living nitrogen-fixing bacteria belong to the genera *Azotobacter*, *Azospirillum*, and *Bacillus*. Currently, a numerous strains of these genera are applied as biofertilizers and there is a growing interest about their effects on the plant growth, development, and productivity.

Some strains from *Bacillus* genus as well as strain of species *P. megaterium* (formerly known as *Bacillus megaterium*) have shown high N-fixing ability and variety of plant promoting activities. A biofertilizer which active ingredients is carefully selected *P. megaterium* strain is available commercially. Application of biofertilizers is a new eco-friendly approach to fertilization which rely on N-fixing plant growth promoting bacteria (PGPB), for maintaining the crop yield with lower dependency on potentially harmful chemicals (Dinesh et al., 2013). According to Antil et al. (2022) *P. megaterium* and *B. cereus* demonstrated ammonia production (4.7 and 3.2 g mL/1, respectively) as well as other plant development promoting factors such as phytohormone synthesis and production of hydrogen cyanide. Cueva-Yesqu'en et al. (2021) found that inoculation with *Leclercia adecarboxylata* in Cape gooseberry plants (*Physalis peruviana*) demonstrated increased shoot and root lengths (55.4 and 24.5%, respectively), whereas *P. megaterium* have improved shoot and root growth (52.7 and 24.5%, respectively). Another important task is the discovery, successful isolation and characterization of novel strains that are suitable to specific soils and environmental conditions (Backer et al., 2018). The new agricultural approaches, such as switching from intensive application of chemical fertilizers to biofertilizers and organic amendments, have the potential to enhance soil physicochemical characteristics as well as the efficacy of organic and chemical inputs (Chandini et al., 2019).

2. LITERATURE REVIEW

2.1. Importance of soil nitrogen in soil for plant growth and development

The availability of essential macro- and micro-elements is necessary for the efficient metabolism of cells. Nitrogen is one of the essential macro-elements for plant development. The natural biogeochemical processes that supplies nitrogen to living organisms and keeps its recirculation in the biosphere is called nitrogen cycle. The structure of gaseous and other N compounds changes as a result of several processes that contribute to the formation of the N cycle. Any imbalance in the cycle is related to changes in the food chain and ecosystem. The most abundant source of nitrogen is the atmosphere, where it is found in molecular form (N_2). In the soil some trace amounts of gaseous nitrogen are present - molecular nitrogen, ammonia, and nitrogen oxides (NO_2, NO, and N_2O), (Imran *et al.*, 2019). However, none of these nitrogen forms is directly involved in plant metabolism. Different biotic and abiotic factors affect macro and microelements availability and their subsequent metabolism in plant cell. The most important factors are soil moisture, oxygen availability, microbial activity, pH, and other elements. The limited N mobility in dry soil limits nitrate absorption by plants and microbial activity, but solute transport through biopores can increase it (Gauer *et al.*, 1992). The quantity of soil nitrogen, typically present in the form of complex organic materials, is usually inadequate for optimal plant development because plants are directly unable to consume these complex organic materials. The inorganic N is typically liberated from organic wastes due to microbial activity through ammonification which results in production of ammonia nitrogen (NH_4^+) and further conversion of ammonium to nitrate (NO_3-N) by nitrifying bacteria (Williams *et al.*, 2017). In anaerobic conditions the nitrate nitrogen can be utilized by bacteria which produce nitrous oxide. The ammonia can also be produced from atmospheric nitrogen by nitrogen-fixing microorganisms. The consecutive conversion of nitrogen forms affects soil pH: (1) availability of NH_4OH is related to alkaline reaction; (2) a restoration to the initial pH of the soil; and (3) a slightly acidic condition in the soil in the result of HNO_3 formation. The pH of the soil has a significant impact on the nitrogen concentration and cycle. The nitrogen is primarily accessible for plant at pH level from 6.0 to 7.5. In acidic soil, the availability of the nitrogen decreases due to its transformation into NH_4 which is easily attached to the negatively charged

particles and it reduces the soil cation exchange capacity (CEC). On the contrary, in the alkaline soil, the process which decreases N concentration is ammonia (NH_3) volatilization. The macro- or microelement nutritional status of plants is closely related to all these changes related to the nitrogen availability (Murgia and Vigani, 2015).

2.2. The negative effects of overfertilization on ecosystems

The necessity to provide plants with an adequate amount of soil nitrogen justified the broad use of chemical fertilizers. However, the heavy use of fertilizer can lead to several negative effects on soil and surrounding environment. The main disadvantages of the application of N-based fertilizers are nutrient imbalances, soil acidification, increased pest and disease pressure. The ammonium-based fertilizers cause the soil acidification over time, reduce soil pH and nutrient availability, which restrain plant's nutrients absorption. The reduction of some nutrients availability accompanied with the excess of other elements leads to a significant nutrient imbalance, decreases soil fertility and reduces crop yield. The prolonged supply of chemical fertilizers also suppresses symbiosis between plant and soil microorganisms, and as a consequence pest and disease pressure increases. Another explanation for the increased pests and disease pressure is the vulnerability of overfertilized plants which defence mechanisms are very often weakened. Moreover, the nitrogen overfertilization pollutes not only soil, but also groundwater, drinking water and, in general, the water bodies and threatens the living flora and fauna of natural water reservoirs. The observed negative effects include eutrophication, water pollution and other indirect consequences. The eutrophication is caused by leaching of oversupplied nitrogen and phosphorus compounds from agricultural lands towards water basins. As a result, water contamination and disturbing the natural nitrogen cycle in water basins is possible. The problem in the water basins is further worsened by the solar radiation which promotes excessive growth and development of phytoplankton and algae, and the result is loss of animal and vegetal species. The water soluble nitrate may leach into groundwater polluting drinking water sources and endangering human and animal health. An adequate amount of nitrate has an antimicrobial activity on gut pathogens but the high doses of nitrate in water or food are harmful for human health. The excess nitrate is converted into nitrite during digestion and disables erythrocytes causing methemoglobinemia, especially in children. The World Health Organisation (WHO) recommends a limit of 50 ppm for nitrate concentration in the drinking water so the fatal conditions could be prevented.

Nitrogen has a considerable impact on agricultural production since it is a crucial ingredient for plant development as it is necessary for the production of large molecules including amino acids, nucleic acids, and chlorophyll (Razaq *et al.* (2017). Nitrogen is also the sole element that may be absorbed either in anion (NO_3^-) or cation forms. The ammonium is assimilated directly but NO_3^- has to be metabolized in plant cells (He *et al.*, 2018). However, in comparison to nitrate, ammonium is highly harmful for plant cells and must be detoxified immediately through various organic nitrogenous compounds. The fundamental method of NH_4^+ detoxification in higher plants is its conversion to organic molecules via cooperative activities of two enzymes - glutamine synthase (GS) and glutamate synthetase (Pinto *et al.*, 2014). The metabolism of NH_4^+ and NO_3^- in the plant cells goes through two different energy consumption levels. The nitrate utilization happens at higher energy level since its metabolism requires more energy than ammonium. The production of ammonium in soil generally entails the employment of microorganisms and the compound is usually immobilized on the surface of the negatively charged soil particles, hence, it is less vulnerable to leaching but its availability to plants is limited which is an issue in agriculture practice (Bargaz *et al.*, 2018). Mineral elements (mainly N and P) are recognized as nutrients that are frequently lacking in most soils, thus contributing to diminished plant development and agricultural yields (Fricke, 1997)

One of the oldest agricultural practices for nutrient loss prevention and preserving soil fertility is the crop rotation. The non-synchronised application of minerals is the reason of many agricultural and ecological problems. The poorly timed application of the synthetic N causes a mismatch between the flushes of N supply and crop N demand, increasing the susceptibility of the surplus fertilizer N to volatilization, denitrification, and leaching. The legume plant and their association with nitrogen-fixing bacteria may synchronize N supply with demand (Ladha *et al.*, 2020). A legume-fodder/grass is used to accumulate nutrients into the soil N pool and an adequate phosphorus supply and lime may enhance the nitrogen fixation. The farming crop cycle, which begins with ley, can proceed with the most N and other nutrients demanding crops such as wheat, maize, and root crops. Manure (farmyard manure, slurry, compost) is often added in the second part of the crop cycle as additional organic amendments. After cultivation of the high N consuming plants some less nutrient demanding crops such as Brassicas are planted that are excellent nutrient

scavengers. The N-fixing legume crops restart the cycle again by restoring the soil N pool and keep the soil fertility.

The bulk of soil N exists in organic form as part of soil organic matter (SOM). Sources of N can be dead unicellular and multicellular organisms and ammonia from N-fixing microbial activity. The soil microbes may form a symbiotic relationship with legumes to fix atmospheric nitrogen obtaining in return carbohydrates. Faba plants are introduced into the agricultural systems to increase soil fertility through N-fixation and degradation of organic leftovers after harvest (Soares and Rousk, 2019). Soil microorganisms mediate N availability to plants and their activity is critical for decomposing of organic substances and release of inorganic N so the crop roots may absorb N in available forms. The plant available mineral elements, as well as other soil nutrients may be enriched inside pore walls and soil particles through increased microbial activity, which increases CEC (Fuhrmann *et al.* (2019). The most common route for biologically fixed N in legume tissue towards the soil N pool is through legume organic residue decomposition and mineralization. The mineralization can involve also shoot and leaf components, despite that organic N is primarily connected with nodulated roots (Jensen *et al.*, 2020).

However, the biologically fixed N by legumes cannot always provide sufficient amount of N for all crops because the processes of mineralization and decomposition of organic N could be very slow in some cases. This can significantly affect the development of crops with a high N demand. The early-maturing, cool-season heavy feeders, such as broccoli and spinach, require a lot of N for a limited time in spring, when the soil temperature is low. In such conditions, the soil microorganisms may not be able to mineralize enough N to meet crop N requirement. A faster-releasing organic N source, such as feather meal, blood meal, fish meal, non-GMO seed meals, soybean meal and other National Organic Program (NOP) permitted N fertilizers, may be required to supply the necessary nitrogen. A Chilean sodium nitrate is also NOP allowed but due to its restricted use it can satisfy no more than 20% of crop's N requirements. However, most of the organic N amendments are expensive and for that reason are not very often applied in a broad scale. Another drawback of crop rotation technique is that it is a time consuming and complex process. The most consumed crops such as wheat, rice and maize are always in demand but organic farming practices could be very costly in some conditions and are not always suitable for farmers.

The requirement for synthetic N is expected to rise in the future in order to meet the nutritional needs of higher cereal production. The environmental and human health issues associated with N overfertilization are likely to worsen, so the capacity of biological N sources as supplements to the mineral fertilizers should be considered. One of the promising alternatives is to use biofertilizers containing free-living, plant growth promoting bacteria (PGPB) with nitrogen fixing ability.

2.3. Biofertilizers as an eco-friendly approach to fertilization

2.3.1. Biofertilizers and their functions

The biofertilizer is a substance that contains living PGPB that can be applied for better plant development and soil health. Instead of being simply a nutrient supplier for crops, similarly to the synthetic fertilizers, the biofertilizer can provide the necessary minerals through activating the processes in the soil and it is capable to increase the yield in low-input farming systems (Bargaz *et al.*, 2018). According to Katiyar *et al.* (2017) and Di Benedetto *et al.* (2017), soil inoculation with PGPB is a potential method for integrated management systems. PGPB affect plant growth and development through a variety of mechanisms. PGPB can colonize the plant roots by a symbiotic connection (Calvo *et al.*, 2014). Afzal *et al.* (2010) showed the benefit of using N-fixing and P-solubilizing bacteria that provided greater and more sustainable soybean grain output. The use of PGPB also provides a promising green method for boosting the nutritional content of crops and PGPB could be a potential substitute for chemical fertilizers (Gouda *et al.*, 2018).

Recently, PGPB has become a more attractive option due to their agricultural advantages. Their application has been further promoted by the shift towards organic agriculture, demand for less reliance on synthetic products, and the growing need for sustainable agriculture along with environmental protection (Figueiredo *et al.*, 2011). A biological nitrogen fixation, inorganic phosphate solubilisation, organic phosphate mineralization, and the synthesis of plant growth regulators such as cytokines, auxins, and gibberellins are among the most important effects of PGPB (Koskey *et al.*, 2018). PGPB can increase the plant development in two ways: 1) directly by increasing the efficiency of nutrients resource use or 2) indirectly by lowering the inhibitory effects of certain pathogens (Gouda *et al.*, 2018). There are several groups of but not all of them are qualified as biofertilizers. The biopesticides also contain PGPB that enhance plant development but they achieve that effect through control over harmful organisms. Certain PGPB appear to boost plant development by functioning not only

as biofertilizers but also as biopesticides. For example, strains of *Burkholderia cepacia* have been demonstrated to exhibit not only biocontrol properties against *Fusarium* spp., but also accelerate maize growth under iron-deficient conditions via siderophore synthesis (Bevivino *et al.*, 1998).

The majority of PGPB are present in the rhizosphere (Bashan *et al.*, 2008). In comparison to the bulk soil, the thin rhizosphere zone is rich in nutrients which facilitate bacteria growth (Weller and Thomashow, 1994). Additionally, PGPB can improve the concentration of nutrients in the rhizosphere by fixing nutrients and avoiding leaching (Choudhary *et al.*, 2011). N-fixing ability is limited to a small but diversified collection of bacteria and blue-green algae (together known as diazotrophs) from the kingdoms eubacteria and archaebacteria. The bacterial species that fix N represent a diverse range of phylogenetically and physiologically distinct types that occupy various ecological niches. Despite the fact that many PGPR are atmospheric N-fixers the mechanism behind their growth-promoting actions is not always related to the provision of fixed N to the host. However, the N fixing ability of microorganisms makes them well adapted to the rhizosphere because they can supply for themselves the necessary N even at low mineral N levels due to plant absorption. The microorganisms can provide a negligible level of N_2 fixation in terms of agroecosystems demand but this fixation could be sufficient to plants in other natural ecosystems. Due to their diverse metabolic and habitat properties, free-living diazotrophs are of high interest not only ecologically, but also in agronomical aspect

2.3.2. Use of N-fixing biofertilizers in agriculture

The biofertilizers aid plant growth through a variety of mechanisms. Apart from N fixation, several free-living diazotrophs have roles in mineral mobilization and nutrient acquisition, improve the plant resilience to stress, provide protection from pathogen, and participate in soil bioremediation. The direct mechanisms may involve atmospheric nitrogen fixation, production of different phytohormones and enzymes, and mineral solubilisation in the soil. The indirect mechanisms may include phytopathogen inhibition (Figueiredo *et al.*, 2011). In order to make atmospheric N accessible to plants, PGPB may either fix atmospheric N_2 or mobilize mineral or organically bound nutrients from the pedosphere (Di Benedetto *et al.*, 2017). Increased biomass is attributable to the rise in nitrogen intake provided by activity of nitrogen-fixing bacteria (Mahfouz and Sharaf-Eldin, 2007). Cotton inoculation with *Azotobacter chroococcum* strains showed increased plant growth metrics (shoot

length, root length, and shoot dry weight) accompanied with decreased nitrogen fertilization requirements (Romero-Perdomo *et al.*, 2017). Smil (2004) identified biological nitrogen fixation (BNF) as the principal source of reactive N in agriculture. The ability to convert gaseous N_2 into NH_3, which can then be biochemically modified to generate various organic forms of N (Giller, 2001) is possessed only by a few genera of prokaryotic organisms that contain the genetic information necessary for the synthesis of enzyme nitrogenase. Nitrogenase activity further converts NH_3 to ammonium (NH_4^+), which is then used in the biochemical synthesis of amino acids and proteins, which are temporarily stored in microbial or plant biomass.

At the beginning of the twenty-first century, there was increased interest in the development of commercial inoculants of free-living N-fixing bacteria such as *Azoarcus* sp., *Burkholderia* sp., *Gluconacetobacter* sp., *Diazotrophicus* sp., *Herbaspirillum* sp., *Azotobacter* sp., *Bacillus polymyxa*, and *Azospirillum* sp. Unlike rhizobia, these free-living diazotrophs can deliver nitrogen to a broader spectrum of agricultural plants. Commercial *Azospirillum* inoculants developed by small and medium-sized businesses throughout the world have proved the enhance production for variety of cereal crops (Bashan and de-Bashan, 2015). The inoculation with nitrogen-fixing bacteria (*Azospirillum* and *Azobacter*) allowed for half-rate N fertilizer use while increasing sesame seed production and oil quality (Shakeri *et al.*, 2016) and similar results were obtained for *Brassica carinata* cv. Peela raya after inoculation with *Azospirillum vinelandii* (Nosheen *et al.*, 2016). A bacterial consortium (*Bacillus cereus* PX35, *Bacillus subtilis* SM21, and *Serratia* sp XY2) decreased the prevalence of root knot nematode (*Meloidogyne incognito*) in tomato while increasing fruit quality (soluble sugars, vitamin C, and titratable acids) and yield (31.5 to 39%) (Niu *et al.*, 2016).

Bacterial genera such as *Rhizobium*, *Azotobacter*, *Azospirilium*, and blue green algae (BGA) have long been used as biofertilizers. Leguminous crops benefit from *Rhizobium* inoculant and wheat, maize, mustard, cotton, potato, and some vegetable crops can benefit from the application of *Azotobacter*. The genus *Azospirillum* is the most commonly used as inoculant for sorghum, millets, maize, sugarcane, and wheat. *Nostoc* or *Anabaena* or *Tolypothrix* or *Aulosira*, a generic cyanobacteria genus, fix atmospheric nitrogen and are utilized as inoculants for paddy crops cultivated in both upland and lowland settings. Additionally, *Anabaena*, in conjunction with the water fern *Azolla*, could deliver up to 60 kg/ha/season of N and also enriched the soil with

organic matter (Watanabe *et al.*, 1991). In general, the seaweeds are high in mineral elements (potassium, phosphorus, trace elements) and are widely used as manure in coastal areas. A bottom muck from dried up ponds, a substrate rich in blue green algae, is commonly utilized as manure in tropical regions. The combination of seaweeds and BGA is considered as an excellent fertilizer. Due to their properties and effects, the biofertilizers are recognized as eco-friendly fertilizers suitable not only for the conventional agriculture but also for organic farming.

There is a strong connection between the host plant and bacteria when symbiotic relationship is established. Diazotrophic symbionts (e.g., *Rhizobium* spp. and *Anabaena azollae*) dwell within particular structures in their plant hosts, legume creates root nodules and aquatic fern *Azolla* creates specialized cavities on the top surface as an habitat for *A. azollae* (Giller, 2001). Specific low molecular carbon components are released from the host plant favouring certain types of bacteria in rhizosphere and, in return, there is a nutrients supply provided by soil bacteria which correspond to the plant needs.

In environments with balanced levels of organic C and N heterotrophic free-living diazotrophs are often active and able to fix N_2. They are described as non-symbiotic when they collaborate with non-leguminous plants. They live predominantly in the soil-root interspace, but they may also penetrate and thrive inside plant tissues (e.g., roots and/or stems). Diazotrophs may live in a variety of environments, including soil, water, organic material (including agricultural waste), and living plants. Some free-living N-fixing bacteria can survive and function well forming a symbiosis in plant tissues. The majority of free-living soil diazotrophs, including *Herbaspirillum* and *Azoarcus* have been found in the rhizosphere but also as endophytes in wheat, rice, maize, and other plants (Ladha and Reddy, 2019). In the rhizosphere and mucigel of an indigenous Mexican maize, there have also been observed a diverse community of diazotrophs that may fix N_2 (Gonzalez-Ramirez and Ferrera-Cerrato, 1995).

The main entrance points for histospheric or endophytic diazotrophs are cracks on the lateral roots. These endophytic diazotrophs grow largely in the intercellular space in the plant cortex and xylem vessels. The observed intercellular colonization of root cells is most likely to occur in the older parts of roots where cells are generally damaged (James, 2000). In contrast to endosymbiotic relationship with legumes, the presence of endophytes does not cause plant tissue differentiations or formation of

specialized structures, like nodules, which accommodate the microorganism. Some experiments with 16S rRNA gene analysis and shotgun metagenome sequencing revealed that the underground and aerial roots, stems, and aerial root mucin of maize landrace contained a highly diverse array of diazotrophic microorganisms (Van Deynze *et al.*, 2018). A bacterial development appeared to be controlled by the plant's own defensive system (Rosenblueth and Martínez-Romero, 2006).

Some researchers have studied the formation of 'para-nodules' in non-leguminous plants as potential diazotrophic host sites. When the roots of some plants are treated with natural, synthetic, or precursors of the phytohormone auxin, nodular-looking tumours with variable degrees of tissue differentiation emerge, known as a para-nodule. These tumours are most likely the result of proliferation of lateral root meristems and should be regarded as transformed root tissue rather than as an organ with its own ontogenesis (Sprent, 1990). When such treated roots are inoculated with rhizospheric or endophytic diazotrophs the para-nodule may occasionally get colonized and exhibit some nitrogenase activity. Colonization of para-nodules on wheat and various diazotrophs (Gantar and Elhai, 1999), *Azorhizobium caulinodans* and rice (Christiansen-Weniger, 1996), *A. nigricans* and rape (Koval'Skaya *et al.*, 2001) have been studied.

It is common knowledge that most of the plants obtain nutrients primarily through absorption of dissolved soil inorganic components. Nitrogen-fixing microorganism in the rhizosphere can boost N supply for plant and in return carbohydrates, amino acids, vitamins, and organic acids are secreted by plants (Bowsher *et al.*, 2016). According to the research, plants can affect the amount and diversity of microorganisms on root surfaces and in the rhizosphere via secreting certain exudates (Bowsher *et al.*, 2016). Plants are known to increase exudate production in nutrient-limiting soils, which presumably leads to increased microbial activity around roots and 'microbial mining' for nutrients (Bowsher *et al.*, 2016). A research data revealed that microorganisms were attracted to the root exudates and successfully proliferated (Ortíz-Castro *et al.*, 2009; White *et al.*, 2018). The root exudates operated as signal molecules, attracted a population of microorganisms to the root zone and the formation of biofilm around the root tip meristem was observed (Ortíz-Castro *et al.*, 2009). Despite that microorganisms infiltrate root tip meristem cells and settled in the periplasmic space between the cell wall and the plasma membrane they are still exposed to the reactive oxygen (superoxide) during growth of

root cells. Some intracellular microorganisms are degraded by superoxide ions, which cause an electrolyte leakage from damaged cells. The surviving bacteria in the root epidermal cells can stimulate the root hair elongation. Also, these bacteria can escape through the hair tip by reshaping the cell walls and cell morphologies. The microorganisms released through this mechanisms could collect extra nutrients in the rhizosphere. The microbial shift from root intracellular endophytic phase towards free-living state is called rhizophagy cycle. From a certain point of view, plants are 'farming' bacteria through emission of specific root exudates.

The plant rhizosphere stimulates nitrogen fixation through provision of more favourable environment abundant of C substrates and low oxygen (O_2) partial pressure. In rhizospheric symbioses, the PGPB are usually attached to the plant's surface (Andrews and Harris, 2000). A scanning electron microscopy revealed the presence of bacteria on the surface of plant roots. However, the distinction between associative or free-living bacteria and endophytic bacteria is not always clear because the attachment mechanism is not well known. *Azospirillum* sp. is one of the PGPB species which attachment mechanism have been carefully studied (Steenhoudt and Vanderleyden, 2000). It is also known, that PGPB do not colonize evenly the plant root surface. *Kluyvera ascorbata*, for example, may colonize the canola root surface area except the root tips (Wenbo *et al.*, 2001). While free-living diazotrophs have no or little specificity to plant, a symbiotic relationship between a diazotroph and its host typically has a very high degree of specificity. Nonetheless, it is still unclear whether symbiotic N_2-fixing bacteria are facultative or obligate fixers (Sheffer *et al.*, 2015).

2.3.3. Free-living nitrogen fixing bacteria

Diazotrophs can be either heterotrophs or phototrophs. Heterotrophs may thrive in the dark and rely on a source of reduced carbon (C) such as sugars or organic acids that are either discharged into the plant root rhizosphere or are acquired from the plant leftovers (Vives-Peris *et al.*, 2020). The diazotrophic eubacteria may be found in plant symbiotic interactions where they fix N. In nature, both heterotrophs and phototrophs N_2-fixing bacteria exist as free-living organisms, as non-symbiotic or as participants in symbioses (Giller, 2001). A non-symbiotic N_2 fixation occurs in the soil, on the plant-soil interface, and within the plant during cereal cropping. BNF systems, which consist of heterotrophic and phototrophic bacteria and cyanobacteria endemic to soil, plants, and floodwater, can be referred to as autochthonous (indigenous) BNF systems. Researchers have frequently ascribed the integrated estimations of nitrogen fixation to

endophyte fixation (Chalk, 2016). The measurements of BNF of free-living/endophytic diazotrophs combine N_2 fixation in soil whatever it occurs in conjunction with plants or not, because it is difficult to discern the origin of the fixed N.

Some experiments succeeded to demonstrate the N fixation on a molecular level. Uninoculated treatments were employed as "non-fixing" controls in most inoculation experiments. However, a study based on ^{15}N-labelled which aimed to compare crop germplasm, used either the cultivar with the lowest ^{15}N excess or the measured ^{15}N excess of soil were used as reference controls. The majority of N derived from the air (%Ndfa) have been estimated up to 33% for rice, maize and wheat crops. The extraordinarily high %Ndfa values (highest values for rice – 59%, maize – 82%, and wheat – 85%) have been reported by App *et al.* (1986) The free-living N_2 fixation in rice, maize, and wheat systems (as well as other cereals) was studied by using a variety of N_2 fixation measurement methodologies, including $^{15}N_2$ feeding and *nif* (N_2-fixing) gene analysis. However, most of the experiments were typically conducted over a short period of time and on a limited scale (e.g., growth chambers, pots with cultural media and soil), with and without inoculation with diazotrophs. The higher reported values or measured N gains, based on the total N balance, could be considered as indicators for of the upper limit for BNF in these cereal systems. Most of these data was derived from pot experiments and should not be extrapolated directly in the estimation of BNF on a hectare basis. Furthermore, these experimental data did not specify whether the fixed N was translocated from the site of fixation (roots and stem) to above-ground biomass or grain. Despite the fact that $^{15}N_2$ feeding is the only method for direct estimation of N fixation, it has seldom been used to monitor BNF throughout the whole growing season till crop maturity due to its short-term duration, technical requirements, and relatively high expenses for analysis.

Determination of the N balance of long-term field trials is a sensible first step in estimating the contributions of BNF by non-symbiotic diazotrophs. There are few experiments dealing with other crops, although the net N balances have been recorded for wheat and maize with a range from 13 to 35 and from 13 to 26 kg N ha^{-1} $crop^{-1}$, respectively. It is crucial to note that the N balance statistics took in account the total N inputs and outputs but the N losses were either not recorded or were not included in the computations. The BNF estimations done by Ladha *et al.* (2016) in a 50-year cereal N-budgeting experiment included N losses in the computations. The authors projected

non-symbiotic BNF estimations for 2018 by using the correlations and calculated 15.7 Tg of fixed N with values of 3.1, 5.7, and 6.9 Tg N yr^{-1} for wheat, rice, and maize, respectively. It should be mentioned that the estimation of the non-symbiotic N_2 fixation, based on calculations of the soil/plant N balance, should be obtained from numerous independent and unrelated measurements because each measurement, with a different level of accuracy, would inevitably contain some errors (Chalk, 2020). Some of the measurements were based on trials conducted on a limited scale, used crops which were not always brought to maturity, or did not take into account different experimental limitations.

The nutrient fixed by the microorganisms can be deposited in the soil for long period. A nitrogen fixed by the free-living diazotrophs becomes a part of soil organic nitrogen (SON) pool and becomes accessible for crop uptake after death of microorganism cells. (Giller *et al.*, 1984). The most assessment about the effect of diazotrophs was within the range of 4-15 kg N ha^{-1} yr^{-1} (Rousk *et al.*, 2016). However, a five-year maize field experiment which used a combination of controlled environment and multiple measurement techniques showed that in unfertilized N-deficient soil 29-82% of the N appeared to come from BNF. On the contrary, study of Zhang *et al.* (2021) found that application of N fertilizer at rates of 125-250 kg N ha^{-1} significantly reduced N_2 fixation rates by 81-86%. Based on the estimations of %Ndfa and crop N accumulation, it was determined that BNF inputs might have represented up to 122 kg N ha^{-1} yr^{-1} (Van Deynze *et al.*, 2018).

The N-fixing PGPB inoculation has positive influences on the agricultural crops. These mostly applied microorganisms such as strains of *Azotobacter* and *Azospriliium* resulted plant development along with their nutrient synthesis reducing the chemical fertilizer application. However, according to the most of the conducted research, strains of biofertilizers were applied in cluster of microorganisms, applied by seed bacterization technique, and limited with morphological and physiological parameters of the crops. The effects of single N-fixing microorganism through introduction of the PGPB into the agricultural land was not studied in detail. The doses and characteristics of the biofertilizers are not clearly stated as well (Niu *et al.*, 2016; Shakeri *et al.*, 2016; Antil *et al.*, 2022).

Production of wheat and maize is of utmost importance for human and animal nutrition, however, there is a limited research on studies conducted on the application of the *Priestia megaterium* CB2001 strain as a biofertilizer and its impact on the soil's

agrochemical parameters. Additionally, there is a lack of information regarding the effect of biofertilizers on nutritional, physiological and productive characteristics of wheat and maize plants.

The objective of this study was to access the influence of *P. megaterium* CB2001 strain inoculation on important processes within the soil-plant system, utilizing a comprehensive research approach.

The hypothesis was that the application of effective biofertilizers in wheat and maize cultivation could reduce the dependency on chemical fertilizers, thereby lowering production costs and to mitigating the negative environmental effects associated with the agricultural practices.

To achieve the research objectives, several tasks were assigned:

1) Analysing the key microbiological characteristics of the *P. megaterium* CB2001 strain.

2) Determining the agrochemical characteristics of the soil.

3) Establishing the influence of the biofertilizer Nuptak on plant performance, grain yield, and productivity components of wheat at optimal and reduced nitrogen fertilization conditions.

4) Evaluating the influence of the biofertilizer Nuptak on plant performance and growth of maize plants at different level of nitrogen fertilization.

3. MATERIALS AND METHODS

Two different experiments were performed in this study – 1) a field experiment with wheat plants and 2) a lab pot-soil experiment in the controlled environment with maize plants. The field experiment is part of ongoing experiment that started in 2021.

The field experiment was conducted at the Experimental Base of the Agricultural University in Plovdiv (AUP), Bulgaria. The site is located at an altitude of 156 meters above sea level, and coordinates (42.1366921, 24.8046928). The average annual maximum and minimum temperatures range from 5°C to 27.7°C with a relative humidity of 73% and an average annual precipitation of 36.9 mm. The soil at the site is classified as silty clay loam with 2-3% organic matter and high lime content. Prior to the current study, a field fallow method was implemented for one year before planting the wheat plants.

Additionally, a controlled pot-soil experiment was carried out in a climate chamber at the Plant Physiological Laboratory at the AUP using the soil collected from same location as the field experiment.

3.1. Wheat field experiment

3.1.1. Wheat variety

The field experiment used the wheat variety KWS LAZULI is an early vigorous winter wheat variety with strong twining, resistant to cold and lodging. It is suitable for bread making, has high yield potential, and is well adapted to the climate conditions of Bulgaria. The variety is also low susceptible to blight, yellow rust, *Septoriosa spp*. and tolerant to *Fusarium spp*.

3.1.2. Wheat field experimental design

The field experiment employed a randomized complete block design (RCBD). Each plot had a size of 12.5 m × 1.44 m = 18 m^2, and there were four replicates for each treatment (Figure 3.1). The wheat crop was sown on October 25, 2022. The experiment included five treatments: three different doses of N fertilization with or without inoculation of the biofertilizer. One treatment (75NAF) was used for comparison. The control plot was treated with the recommended dose (160 kg N ha^{-1}) of chemical fertilizer, specifically ammonium nitrate, and was abbreviated as 100N. The experimental plots were treated either with the recommended dose of chemical fertilizer in combination with the biofertilizer (100NP) or with reduced doses of 75%

(75NP) and 50% (50NP) of the chemical fertilizers in combination with the biofertilizer. The variant was used for comparison was treated with a 25% reduction in the chemical fertilizer with the addition of a biofertilizer from different manufacturer (75NAF). The specific experimental design is shown in Table 3.1 and Table 3.2

Table 3.1. Description of wheat field experiment treatments.

Treatments abbreviation	**Treatments description**
100N (control)	100% N - 160 kg N ha^{-1}
100NP	100% N + Nuptak *Priestia megaterium CB2001*
75NP	75% N + Nuptak *Priestia megaterium CB2001*
50NP	50% N + Nuptak *Priestia megaterium CB2001*
75NAF	75% N + *Paenibacillus polymyxa*

Table 3.2. A spatial view of field experimental plots according to RCBD

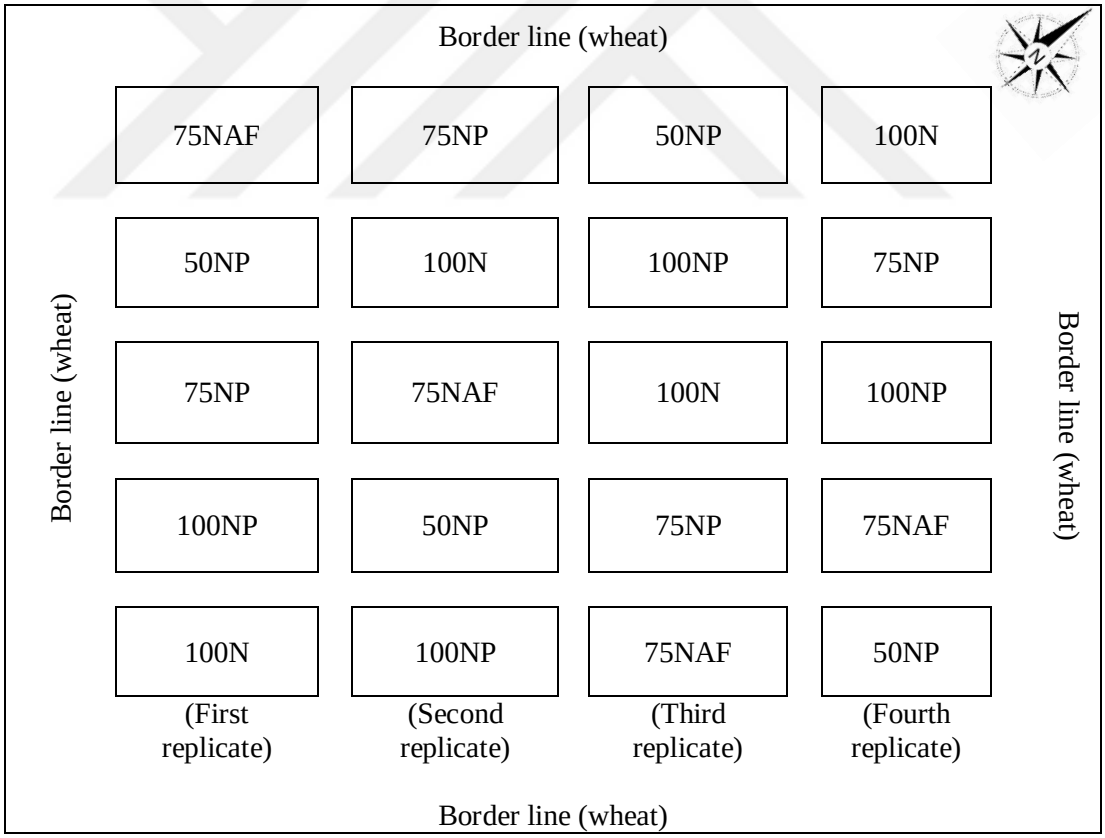

Figure 3.1. General view of the wheat trial at different stages of wheat growth and development

The control plot (abbreviation: 100N) received the recommended dose (160 kg N ha^{-1}) of ammonium nitrate applied in three separate applications: before sowing, and at the early and middle vegetation stages. The other experimental plots were treated with the same (100%) or reduced (75 and 50%) doses of chemical fertilizer, with the addition of the biofertilizer Nuptak (NP) or Azofix (NAF). The specific fertilization types and rates during different phenological phases of the wheat development are shown in Table 3.3.

Table 3.3. Fertilization type and rate during different phenological phases of wheat development

Treatments	**N fertilization (100% N = 160 kg N ha^{-1})**			
	At sowing (kg N/ha)	**At the germination/ Establishment**	**On the early vegetation (February 27) (kg N/ha)**	**At the middle vegetation (April 7) (kg N/ha)**
100N	30	-	50	80
100NP	30	*biofertilizer*	50	80
75NP	30	*Biofertilizer*	50	40
50NP	30	*biofertilizer*	50	-
75NAF	30	*biofertilizer*	50	40

The biofertilizers were applied to the plots on January 31, 2023, at a rate of 1 kg ha^{-1} for Nuptak and 1 L ha^{-1} for Azofix. The application was performed using a backpack electric battery sprayer, Gardenia, at a fixed pressure of 2 atm. The volume of the water solution applied was 300 L ha^{-1}. The treatment accuracy was controlled by a timer, 20 seconds per plot. The untreated (control) plots were sprayed with tap water (Figure 3.2). The broadcast of ammonium nitrate fertilizer was done manually according to the calculated rate for each treatment /plot.

Figure 3.2. Application of biofertilizers in wheat trial

3.2. Maize Pot-Soil Experiment in Controlled Environment

3.2.1. Maize variety

The maize hybrid used in the study was Kneja 307. It was created in the Corn Institute, Kneja, Bulgaria in 2014. The plants reach maturity in 110 days and belong to the intermediate maturity group (300 – 400, GLS 113-129 days/year) according to FAO classification. The plants are medium-tall, strong, and non-decumbent.

3.2.2. Maize Pot-Soil Experimental Design

The pot-soil experiment consisted of four treatments with five replicates (pots) each. Four plants were grown per plot. The descriptions of the treatments are shown in Table 3.4. The soil was sieved and half of it was sprayed with a Nuptak biofertilizer suspension followed by a thorough mixing. The dose of biofertiliser was the same as applied in the field wheat experiment (1 kg/ha). The other half of the bulk soil was

sprayed with tap water. The inoculated soil with biofertilizer and uninoculated soil were further divided into two, and one soil sample of each treatment was fertilized with 30 or 45 mg N per kg soil of ammonium nitrate, respectively. After the application of biofertilizer and ammonium nitrate, all soil samples were mixed with perlite at a 3:1 volume ratio. Each pot was filled with approximately 1 kg of the pre-treated soil.

Table 3.4. Treatments in the pot-soil experiment

Treatments abbreviation	**Treatment description**
30N	30 ppm N
30NP	30 ppm + *Nuptak*
45N	45 ppm N
45NP	45 ppm + *Nuptak*

The maize seeds were soaked in water for 10 hours to speed up their germination and were sown in the pots at an approximate depth of 2 – 2.5 cm. After sowing, the pots were placed in the climate-controlled chamber with the following conditions: photosynthetic photon flux density (PPFD) 200 µmol m^{-2} s^{-1}, an average temperature of 25°C ± 1°C, average relative humidity of 65% and a photoperiod ratio of 13/11 h (day/night) regime (Figure 3.3).

Pot-soil and maize plant samples were collected on March 13, 2023, after one month cultivation. Plants from each pot were cut to obtain suitable above and below ground plant parts for further analyses. The whole plants and the soil samples from the root zone were randomly selected from each plot. The physicochemical analyses were conducted on both soil and plant samples.

Figure 3.3. Maize pot experiment in a climate-controlled chamber (with red and blue light; right)

3.3. Biofertiliser characteristics

Nuptak is a biofertilizer that contains a carefully selected highly active free-living nitrogen fixing bacterium. The unique formulation of biofertilizer aims to maximize efficiency of biological fixation from the atmosphere and provide a biostimulation for the crop, as described by the manufacturer. The biofertilizer formulation is the result of significant research, and the strain (CB2001) was carefully selected among the other strains of *P. megaterium* due to its properties. The strain grows in a wide temperature range between 5°C to 48°C, with an optimum between 20°C and 35°C, and at pH levels from 4.5 to 8. It exhibits high tolerance to salt content and calcium carbonates allowing it to survive in water with high mineral content (hard water). The strain can form spores, enabling its survival in unfavorable environmental conditions, such as drying, exposure to high temperatures and UV radiation. The strain possesses essential genes (*nifH* and *nifDK*) necessary for nitrogen fixation ability and the proper functioning of the nitrogenase enzyme. Additionally, the strain exhibits a wide range of plant growth-promoting properties, including the synthesis of phytohormones (particularly indole-3-acetic acid, IAA), which is associated with plant growth promotion. In *in vitro* conditions, the strain was capable of producing 160 µg/ml of IAA. It also enhances the plant stress resistance through the synthesis of ACC (1-aminocyclopropane-1-carboxylate) deaminase and increases the solubilization of confined nutrients (phosphorus and iron). The biofertilizer Nuptak was kindly provided by the Bulgarian company Agredo EOOD.

Azofix*Plus* was used as a reference product. It contains *Paenibacillus polymyxa* MVY-024, and the producer is the company Bioenergy LT (Lithuania) (Figure 3.4).

Figure 3.4. Biofertilizer package and biofertilizer containing *P. megaterium* CB2001 strain commercialized as "Nuptak" (left) and biofertilizer package containing Paenibacillus polymyxa MVY-024 named Azofix (right)

3.4. Description of soil physicochemical methods

3.4.1. The Mineral Nitrogen

The determination of ammonium and nitrate has been done through extraction with KCL solution according to the modified Kjeldahl method (ISO/TS 14256-1:2003)

3.4.2. The Available Phosphorus

The phosphorus has been extracted with Ca-lactate solution and determined spectrophotometrically according to the Egner-Riem method (DL-method; GOST 26209-91)

3.4.3. Available Potassium

The potassium has been determined through the extraction with 2N HCl and read by Flame Photometry (GOST 26209:1992)

3.4.4. Exchangeable Ca and Mg

The calcium and magnesium have been extracted from soil with 2N HCl and determined by EDTA titration (GOST 27753:9:1989).

3.4.5. Soil Organic Matter (SOM)

The organic carbon content of the soils was determined spectrophotometrically through wet oxidation with $K_2Cr_2O_7$ and H_2SO_4 (BDS EN 13039:2012). To convert the results into organic matter, the values were multiplied by a factor of 1.724.

3.4.6. $CaCO_3$ content

The $(NH_4)_2C_2O_4$ has been used as an extraction solution and determined based on titration with $KMnO_4$ (ISO 10693:2014)

3.4.7. Determination of pH and EC

The pH of the soil (as in ISO 10390:2022) was determined as in the routine determination of pH within the range pH 2 to pH 12 using a glass electrode in a 1:5 ratio treated with water (pH in H_2O) suspension of soil. The suspension is filtered and measured for EC (ISO 11465:2002).

3.5. Identification and microbiology characteristics of *P. megaterium*

The identification of bacteria subjected to ashing on Tryptic Soy Agar (TSA) has been determined using the BIOLOG^{TM} GENIII plate method (Hayward, CA, USA).

3.6. Soil respiration

The released CO_2 from soil samples incubated in Isermayer jars has been determined by titrimetry (Isermayer, 1952).

3.7. Plant physiology and morphology analyses

3.7.1. Plant growth parameters

Morphological parameters such as shoot and root length, fresh and dry weight (biomass) of above and below ground plant parts have been analysed. The seedlings' height was measured from base to apex. The primary root have been measured from base to tip.

3.7.2. Total N content

The plant nitrogen was determined by modified Kheldahl method with catalyser (mixture of K_2SO_4:$CuSO_4$:Se in 100:10:1 ratio) according to BDS ISO 5983-1:2011. Protein content is calculated based on the nitrogen multiplied by 6.25.

3.7.3. Leaf gas exchange

Leaf gas exchange (A – net photosynthetic rate, E – transpiration rate, gs – stomatal conductance) was assessed using the open photosynthetic system LCpro+ (Analytical Development Company Ltd., Hoddesdon, UK) on the first fully developed leaf of plants.

3.7.4. Chlorophyll content

The total chlorophyll concentration was measured with CCM-300 Chlorophyll Content Meter (ADC BioScientific Ltd.) Determination coefficient is greater than r^2=0.95 and is linearly related to chlorophyll concentrations up to 675 mg m^2.

3.7.5. Leaf protein

The determination of total protein was done according to Bradford (1976) method which is based on Coomassie Brilliant Blue G-250 binding to proteins. The sample solution's absorbance at 595 nm is proportional to its concentration. Bovine

serum albumin was used as a standard and the soluble protein content was calculated from a standard curve and expressed as mg per g fresh weight.

3.7.6. Antiradical scavenging activity

The determination of the antiradical activity of plant extracts has been done accoding to Brand-Williams *et al.* (1995) method with minor changes. Absorption was recorded after 10 minutes at 517 nm against absolute alcohol (Singleton and Rossi, 1965). DPPH radical scavenging activity of the sample was expressed as mg Trolox equivalent antioxidant capacity (TEAC) by formula obtained from a standard curve ($y = -0.1954x + 0.708$ $R^2 = 0.9858$).

3.8. Statistical Analysis

The mean value and standard deviation of data which was not subjected to statistical analysis were calculated with Excel 2016. An analysis of variance (ANOVA) and LSD test were performed to determine the significant differences among the treatments. For all the statistical analysis carried out in this study, a comparison was made with SPSS (IBM, ver.26), with Tukey's test post Hoc test ($p < 0.05$).

4. RESULTS AND DISCUSSION

4.1. Microbiological characteristics of biofertilizer

The bacteria isolated from biofertilizer grow well on tryptic soy agar. The colonies were relatively small, round, with smooth edges and yellowish color. There was no morphological difference between the observed colonies on the media. The microscopic observation with 100x objective showed relatively big rod-shaped cell with round edges which with the simple stain technique tend to be arranged in chains or clusters (Figure 4.1).

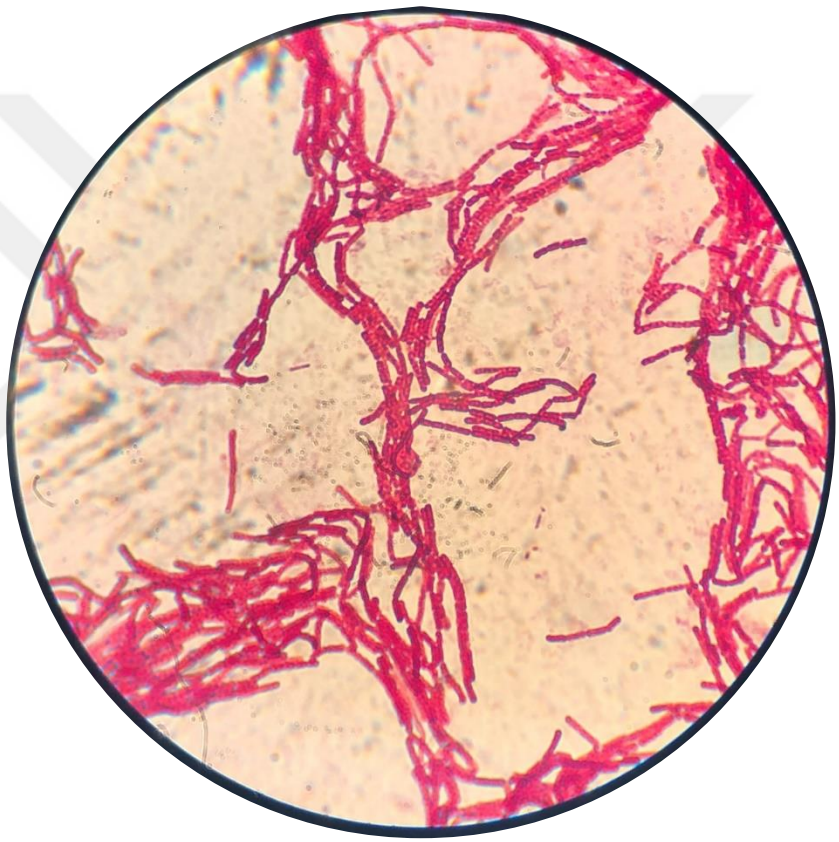

Figure 4.1. View of *P. megaterium* under light microscope (100x magnification)

Identification by BIOLOG^{TM} GENIII assigned *P. megaterium CB2001* as *B. megaterium* after 48h incubation. (Figure 4.2A). Identification is confirmed with the probability of 97.2%. There were some differences between the BIOLOG^{TM} metabolic fingerprint of species *B. megaterium* and Nuptak *P megaterium* strain. *P megaterium* strain showed utilization of sucrose (well A7), L-Alanine (well E3), L-glutamic acid (E6), citric acid (G5) which in the database are denoted as borderline (Fig. 4.2B and 4.2C). On the contrary, strain CB2001 showed a borderline utilization for substrate

dextrin (well A2) and ability to tolerate 4% NaCl (B11), 8% NaCl (B12), L-lactic acid (G4), lithium chloride (well G11) and potassium tellurite (well G12)

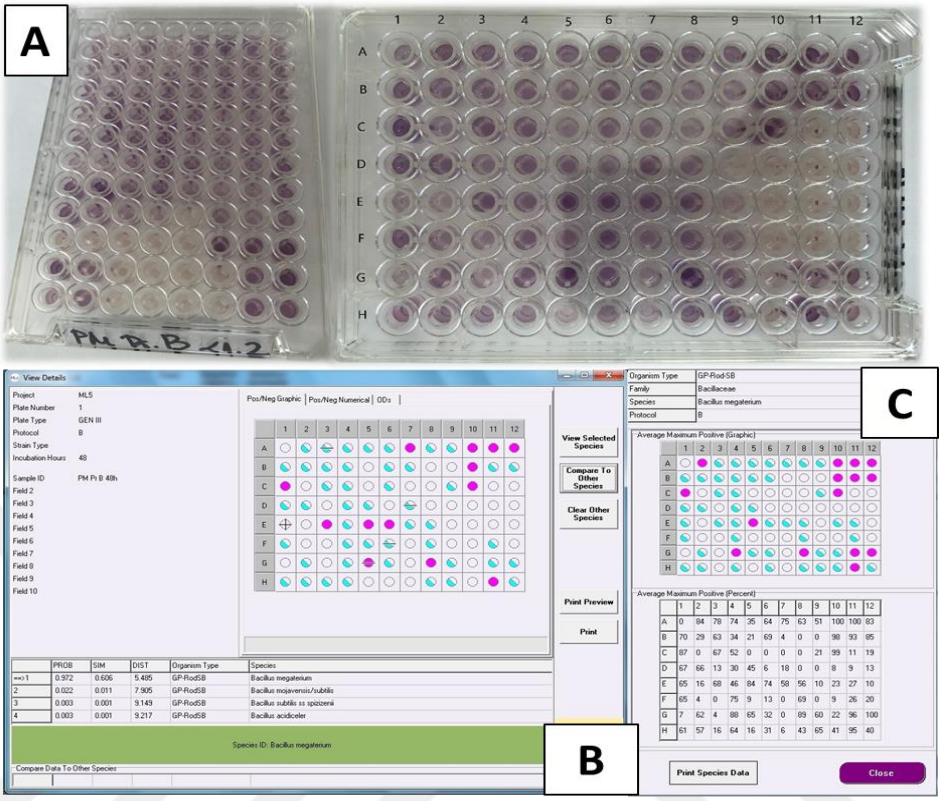

Figure 4.2. *P. megaterium* identification by BIOLOG Gen III technique - A general view of the microplate, B - screenshot of *P. megaterium* identification, C – BIOLOG database metabolic fingerprint of *B. megaterium* (purple circle – positive result, white – negative, half white - borderline)

4.2. Agrochemical characteristics of the soil

The soil of the experiment field was classified as Mollic Fluvisols (Popova *et al.*, 2012). It has a silty-clay composition, average humus content and alkaline reaction. The analysed soil has a high content of soluble phosphorus and potassium and low total nitrogen content (Table 4.1).

Table 4.1. Physicochemical characteristics of experimental field

Soil parameters	Value	Units
Phosphorus (P_2O_5)	21.46	mg / 100 g
Nitrogen	7.94	ppm
Potassium (K_2O)	42.35	mg/100 g

Soil organic matter	2.66	%
pH	8.8	(alkaline)
Electrical conductivity	108.1	µS/cm
Exchangeable cations (Ca+Mg)	16.2	meq/100 g
$CaCO_3$	8.75	g/kg
Soil texture	Silty clay loam Mollic Fluvisols	(19.9% sand, 46.9% silt, 33.2% clay)

4.3. Influence of the biofertilizer Nuptak on plant performance, grain yield and productivity components of wheat, grown at optimal and reduced nitrogen fertilization

Nuptak biofertilizer is expected to have several positive effects on wheat plants, but the main one is related to improved nitrogen nutrition through effective nitrogen fixation. The results presented in Table 4.2 show that the content of ammonium cations in the root zone of the variant with optimal nitrogen fertilization and Nuptak (100NP) was significantly higher (threefold increase) than the control variant (100N). At the same time, the level of nitrate anions was lower, but the content of total mobile nitrogen exceeded the control variant with approximately 22%.

The reduction of ammonium nitrate fertilization with 25 and 50% in combination with *P. megaterium* CB2001 strain decreased ammonium and nitrate ions in the soil (75NP and 50NP). The content of nitrate anions was affected to a greater extent than ammonium cations.

Table 4.2. Content of available N forms (NH_4^+, NO_3^-, ppm) in the root soil after fertilization of wheat plants with ammonium nitrate with or without biofertilizer Nuptak, ppm

Treatment	**Available mineral nitrogen**		
	NH_4^+	NO_3^-	Total
100N (control)	8.07	26.97	35.04 (100)
100NP	27.24	15.39	42.63 (122)
75NP	2.37	3.77	6.14 (18)
50NP	4.36	2.46	6.82 (19)
75NAF	4.33	6.26	10.59 (30)

Table 4.3 presents data on the content of total nitrogen and crude protein in the leaves of wheat plants. The amount of nitrogen varies from 2.45 to 3.89%. According to Bergmann and Wrazidlo (1976) the optimal N content in wheat leaves is in the range of 3-5%. In the current study, the content of total N (and respectively protein) was within optimal limit only in the variants with recommended nitrogen fertilization (100N and 100NP). In the treatments with reduced nitrogen fertilization (75NP and 50NP), the nitrogen content was lower by 20 to 37% compared to the control (100N). Therefore, the Nuptak biofertilizer application was not able to compensate completely the lack of mineral nitrogen. The combination of reduced nitrogen fertilization and Azofix biofertilizer provided similar results (75NAF).

Table 4.3. Content of total nitrogen and protein in the leaves of wheat plants after fertilization with ammonium nitrate with or without biofertilizer Nuptak, %

Treatment	**Total nitrogen and protein content, %**	
	N_{total}	Protein
100N (control)	3.89 (100)	24.3
100NP	3.45 (89)	21.6
75NP	3.10 (80)	19.4
50NP	2.45 (63)	15.3
75NAF	3.00 (77)	18.8

Photosynthetic activity of plants is closely related to nitrogen nutrition. Nitrogen is an essential structural component of proteins, many of which are important photosynthetic enzymes, including the key enzyme Rubisco. Nitrogen is also involved in building chlorophyll molecule (4 atoms per mol).

The results, presented in Table 4.4, showed that net photosynthetic rate (A) of wheat plants cultivated at the recommended N fertilization (100N and 100NP) was between 16.58 and 17.88 μmol $CO_2/m^2/s$. The application of Nuptak increased net photosynthetic rate (A) of these plants by 8%, but did not affect the total chlorophyll content. The reduction of mineral N nutrition by 25 and 50% decreased A with 11 and 16%, respectively. The respective values of chlorophyll content diminished with 5 and 8%. The ANOVA has shown 99% significance with 6.06 factor number for transpiration rate and 95% significance with 3.77 and 3.14 factor for chlorophyll content and stomotal conductance, respectively (Table 8.1–8.6 in Attachments).

The results showed that photosynthetic performance of wheat plants, grown at reduced N fertilization with addition of Nuptak were less affected by the N deficiency than the leaf N content. This result might be explained by the fact that photosynthesis is an integral physiological process, which depends of many other internal and external factors. The relatively stable carbon assimilation was not due to better stomatal regulation, as the gs decreased to the same extend between treatments. Probably, other known effects of the *P. megaterium*, such as auxin production as well as ACC-deaminase activity, might be involved in better photosynthetic adaptation to nitrogen deficiency.

Table 4.4. Photosynthetic performance (leaf gas exchange and chlorophyll content) of wheat plants after fertilization with ammonium nitrate with or without biofertilizer Nuptak. (A – net photosynthetic rate, E – transpiration rate, gs – stomatal conductance).

Treatments	**Photosynthetic parameters**			
	A, µmol CO_2 m^{-2} s^{-1}	E, mmol H_2O m^{-2} s^{-1}	gs, mol m^{-2} s^{-1}	Chlorophyll concentration, mg m^{-2}
100N (control)	16.58±1.78 (100)	1.97±0.47 ^{ab}	0.05±0.01^{bc}	445.4 ± 25.3 (100) ^{ab}
100NP	17.88±2.04 (108)	2.43±0.64 ^{ab}	0.06±0.01^{a}	447.1 ± 29.7 (100) ^{ab}
75NP	14.71±3.39 (89)	1.55±0.50 ^{b}	0.04±0.01^{bc}	424.0 ± 22.1 (95) ^{ab}
50NP	13.91±0.98 (84)	2.00±0.79 ^{ab}	0.04±0.01^{bc}	408.1 ± 26.0 (92) ^{c}
75NAF	15.16±3.48 (91)	2.67±0.41 ^{ab}	0.05±0.00^{ab}	423.1 ± 16.1 (95) ^{ab}

Legend: in brackets is presented % from the control; abc letters indicate LSD test values

Active photosynthesis, especially in the flag leaves of wheat, contributes significantly to yield formation. Therefore, it is very likely that the better preserved photosynthetic performance of Nuptak-treated wheat plants could compensate to some extend the N deficiency and a good yield could be expected. At the time of completion of the presented thesis, the wheat crop from the current experiment has not been harvested yet, so any confirmation of suggested relation was not possible. However, the yield data from the previous field experiment showed that such suggestion could be true.

The yield data from 2022 year varied between 3.77 to 4.28 ton/ha (Table 4.5). In general, the obtained yield was low, which was due to the drought and high temperatures in the second half of June 2022. These combined stress conditions disrupted the grain filling and as a result the seed mass was also low (Table 4.5, G_{1000}).

The application of Nuptak had a weak positive effect on the yield of the control plants (increase of 4% in comparison to variant 100N). The addition of Nuptak in the variants with 25 and 50% reduced nitrogen nutrition had a stronger effect, because the yield decreased only with 8 to 12%, respectively. The data also showed that the increase in yield corresponded to the higher number of seeds in the central class and the bigger mass of 1000 seeds. A similar effect was shown of the variant with the reference product Azofix. The ANOVA has shown 95% significance with 3.94 and 4.31 factor number for the yield and length of ear and, also, 99% significance with 956.7 factor number and 0.48 LSD (0.01) value for the thousand grain mass (Tables 8.7–8.16 in Attachments).

Table 4.5. Effects of Nuptak on the yield and productivity of wheat cv. Lazuli, grown at different nitrogen supply. [2022]

Treatment	**Yield (ton/ha)**	**Wheat plant biometrics parameters**				
		Length of plant (cm)	**Number of tillers per plant**	**Length of ear (cm)**	**Seeds in ear of main tiller**	**G_{1000} seeds**
100N (control)	4.28ab ±0.11 (100)	68.8^{ab}	2.5	8.7 ^{abc}	29.1	35.12 ^{a}
100NP	**4.45^{a} ±0.47 (104)**	69.4^{a}	2.4	8.9 ^{a}	29.0	32.00 ^{d}
75NP	3.92^{bcd} ±0.14 (92)	68^{abc}	2.3	8.7 ^{ab}	28.1	32.86 ^{b}
50NP	**3.77^{cd} ±0.29 (88)**	60^{cd}	2.1	8.2 ^{bcd}	26.8	28.05 ^{e}
75NAF	3.94^{bc} ±0.25 (91)	64^{cd}	2.2	7.9 ^{d}	28.0	32.55 ^{bc}

Legend: in brackets is presented % from the control; abcde letters indicate LSD test values; standard deviation – ±

4.4. Effect of biofertilizer Nuptak on growth and performance of maize plants, cultivated at different nitrogen fertilization levels

The pH of the soil used in the maize pot experiment slightly decreased from 8.8 to 8.45 due to application of chemical fertilizer and biofertilizer (Table 4.6). The potassium level declined from 400 ppm to 345 ppm because it has been utilized by the plant and the used chemical fertilizer does not contain potassium. However, the level of available phosphorus oxide was higher than the level before treatment. The possible explanation for this increase was related to solubilisation of fixed phosphorus due to a presence of phosphorus solubilizing microorganisms in the soil (Mehnaz, 2016). The Kjeldahl method analysis showed a higher level of ammonium in the soil treated with Nuptak in comparison to the non-treated soils. The soil with 30 ppm N had 8% lower ammonium content than 30 NP variant. However, there was not significant difference of available ammonium in the soil of 45N and 45NP treatment. The estimated nitrate level was same at 30N and 30NP variants, however, there was a significant difference between 45N and 45 NP which differed with 4 ppm nitrate content.

Table 4.6. Mineral content of soil in maize pot experiment after application of chemical fertilizer with or without biofertilizer

Variants	**pH**	**Mineral content, ppm**			
		P_2O_5	K_2O	NH_4	NO_3
30N	8.52	296.4	326	2.93	5.65
30NP	8.43	300.1	346	3.19	5.68
45N	8.47	302.6	346.6	3.83	4.56
45NP	8.4	282.8	346	3.92	8.49

A soil respiration was used for microbial activity assessment. A soil oven dry mass, water content at the time of analysis and the average titration volume (including blanks) were measured (Table 4.7). The mean soil respiration value is expressed in mg CO_2 per gram oven dry soil in one day. The inoculated with biofertilizer soils showed higher respiration rate (Table 4.7) in comparison to soils without biofertilizer. The variant 30NP has 67% higher activity than 30N variant. The microbial community in the 30NP and 45NP were more active with 67% and 76% than 30N and 45N variants

with 0.167 and 0.232 mg CO_2/g oven dry soil/24h, respectively. The ANOVA has exhibited insignificance between the treatments due to high variance among replicates of soil respiration test (Table 8.17 in Attachments).

Table 4.7. Soil respiration of different treatments in the maize experiment (mg CO_2/g dry soil / 24 h)

Treatments	Average Titration volume (ml)	Average water content, %	Average oven dry soil mass (g)	Average Value
Blank	17.83	-	-	-
30N	13.96	15.21	42.39	0.1003
30NP	11.41	15.8	42.1	0.1676
45N	12.86	16.85	41.57	0.1314
45NP	9.18	18.03	40.98	0.2321

Legend: ab letters indicate LSD test values

In overall, the soil analyses, in the current experiment, showed low nitrogen content which might be due to pH value of the soil. The alkaline soil affected the experiment in two aspects: the first aspect is related to the biofertilizer effectiveness and the second aspect refers to the behaviour of ammonium in the soil. The high pH of the soil could supress the biofertilizer activity because *P. megaterium* favours neutral and slightly acidic soil. Another reason for low N content could be increased volatilization of ammonium which decreases N in the soil, including the N amount which was provided by microorganisms. Furthermore, the mineral forms of nitrogen are easily available for plants only around neutral pH.

In principle, the NPK content of plant samples presents assimilated soil nutrients either fixed or solubilized by microorganisms. Similar level of phosphorus and potassium between treatments in the leaves and roots are documented except high potassium level in the leaves treated with 30 ppm N and low potassium content in the roots of plants under 30 ppm N + Nuptak. Also, insignificant distinctions in amount of N were exhibited in the plant leaves and roots from all variants. This was defined by that nitrogen available forms haven't been completely assimilated into the plant organs. Summarizing the results in Table 4.6 and Table 4.8, one should note that the low nitrate content has been detected in the 45NP soil and more nitrogen was estimated

in the leaves and roots of plants in the 45N variant in comparison to the other treatments. Moreover, 30 ppm N soil had comparatively lower ammonium level than 30 ppm N supplemented with biofertilizer, however, a higher nitrogen content can be seen in plant samples than in the samples with biofertilizer inoculation. The analysis of plant mineral content showed very low values for nitrogen and phosphorus. In general, the optimal physiological processes in plant require some level of mineral elements. In the case of nitrogen, the content should be at least 3% and for phosphorus - 0.3%. The nitrogen and phosphorus content in the plants in the current study was very close to deficiency levels.

Table 4.8. Leaf and root mineral content (%) of maize plants after application of chemical fertilizer with or without biofertilizer

Plant organ	**Variants**	**Mineral content, (%)**		
		Nitrogen	**Phosphorus**	**Potassium**
Leaves	30N	1.08	0.16	3.93
	30NP	0.92	0.16	3.48
	45N	1.47	0.15	3.36
	45NP	1.41	0.18	3.83
Roots	30N	0.63	0.11	1.82
	30NP	0.46	0.11	1.36
	45N	0.93	0.13	1.7
	45NP	0.61	0.13	1.8

In addition to the soil and plant mineral nutrient concentration, biometrical measurements such as plant's fresh and dry weight and length of shoot and root have been examined (Table 4.9). There was a substantial variation between values of all treatment and measurements demonstrated a marked disparity in relation to the treatments. The 30N plants have better development compared to 30NP maize plants, except root fresh and dry weight, however, more than 1 g of shoot dry weight and the longest (34.5 cm) root length belong to the plants of 30 ppm N treatment. Also, maize treated with 45 ppm N with biofertilizer had increase in biomass in all parameters of biometrics compared to the maize with only 45 ppm N fertilization. Maize under 45 ppm + biofertilizer condition had a noticeable increase in the shoot fresh weight (8.059 g) and shoot length (63.29 cm) among variants. In overall, the maize plants of

treatment 45N had the lowest biomass taking into account both shoot and root measurements. Roots have been developed well under 30 ppm N with biofertilizer condition having the largest mass in the root fresh and dry weight (4.63 g and 1.46 g, respectively).

Table 4.9. Biometrics of maize plants

Treatment	Maize plant biometrics parameters					
	Shoot fresh weight, g	**Shoot dry weight, g**	**Shoot length, cm**	**Root length, cm**	**Root fresh weight, g**	**Root dry weight, g**
30N	6.435	1.007	58.82	34.54	3.97	1.258
30NP	5.482	0.876	55.04	32.68	4.63	1.464
45N	6.705	0.915	59.51	30.46	3.17	1.005
45NP	8.059	0.990	63.29	34.10	3.27	1.035

The results, presented in Table 4.10, showed that net photosynthetic rate (A) of fertilized only with 30 ppm N and 30 ppm N supplemented with Nuptak maize plants was between 7.59 ± 1.33 and 7.77 ± 1.99 μmol $CO_2/m^2/s$. The application of Nuptak insignificantly increased the net photosynthetic rate of these plants by 2.5%, and the total chlorophyll content by 5%. The similar trend is acceptable for the 45 ppm N and 45 ppm N + biofertilizer since A is incremented by 16% and chlorophyll up to 11%. The 45 ppm N fertilization could be greater in only A and chlorophyll content showing 5% and 8% differences but not transpiration rate and stomatal conductance. There was a clear contrast between the transpiration rate and stomatal conductance of maize treated with 30 ppm and biofertilizer and 45 ppm N maize's values showing (50% and 25 % respectively) significant rise.

The results showed that photosynthetic performance of maize plants, grown in combination with biofertilizers was not affected by the N deficiency than leaf N content. This result might be explained by the fact that photosynthesis is an integral physiological process, which depends of many other internal and external factors. The relatively stable carbon assimilation was due to better stomatal regulation, as the gs increased at the same extend between fertilizer and fertilizer with biofertilizer treatments. The ANOVA and LSD test have revealed 99% significance between the treatments with 6.71 factor number and 61.5 LSD (0.01) value for the chlorophyll content of the maize plants (Tables 8.17–8.22 in Attachments).

Table 4.10. Estimation of photosynthetic parameters of maize plants: leaf gas exchange and chlorophyll concentration (A – net photosynthetic rate, E – transpiration rate, gs – stomatal conductance)

Treatments	Photosynthetic parameters			
	A, µmol CO_2 m^{-2} s^{-1}	**E, mmol H_2O m^{-2} s^{-1}**	**gs, mol m^{-2} s^{-1}**	**Chlorophyll concentration, mg m^{-2}**
30N	7.59 ± 1.33 (100)	0.75 ± 0.13	0.052 ± 0.013	377 ± 42 (100) [bcd]
30NP	7.77 ± 1.99 (102)	1.05 ± 0.30	0.054 ± 0.024	394 ± 48 (105) [bc]
45N	8.20 ± 0.90 (100)	0.68 ± 0.14	0.044 ± 0.009	426 ± 49 (100) [ab]
45NP	9.49 ± 1.74 (116)	0.91 ± 0.14	0.058 ± 0.013	474 ± 39 (111) [a]

Legend: in brackets is presented % from the control; ab letters indicate LSD test values

The overfertilization effects plant health negatively. The high nitrogen content disrupts the physiological metabolism and makes the plants susceptible for diseases. An optimum level of nitrogen is necessary the development and disease resistance. The antioxidant activity of plant has been tested by DPPH method and it can also show that biofertilizer could affect nitrogen nutrition. The antioxidant activity can be in a decreasing trend in DPPH inhibition (%) from 38.56% to 31.63% and in an increasing trend expressed as in mg trolox equivalent (TE) per 100 ml from 7.65 to 8.3 between treatments 30N and 45NP (Table 4.11). Also, the soluble protein content was higher in maize leaves treated with biofertilizer (30NP) than plants treated only with chemical fertilizer (30N). According to Table 4.11, the treatment with the least N nutrition maize has greater antiradical activity than plants with higher and N-fixing biofertilized plants. The results are in agreement with other researcher (Kong *et al.*, 2017) (Muhammad *et al.*, 2022) who found that excessive N fertilization resulted in formation of reactive oxygen species and antiradical activity decreased with increasing nitrogen rate until reaching optimal doses. In the present study, DPPH study showed a similar trend which was more pronounced in the variants using the biofertilizer. The presence of N-fixing bacteria, favoring the nitrogen nutrition, is the possible reason for the increase of soluble proteins by 81% in the treatment 30NP compared to 30N.

Table 4.11. Antiradical activity (DPPH) and soluble protein content in the maize leaves

Treatments	Antiradical activity (DPPH)		Soluble protein content of maize leaves, mg/gFW
	%DPPH inhibition	mg TE / 100 ml	
30N	38.56 ± 1.99	7.65 ± 0.14	2.58 ± 0.103
30NP	31.7 ± 0.29	8.3 ± 0.11	4.67 ± 0.078
45N	32.71 ± 1.33	8.23 ± 0.15	7.14 ± 0.065
45NP	31.63 ± 1.9	8.26 ± 0.22	5.68 ± 0.09

In general, the significant N content neither in soil nor in plants has been observed in maize pot-soil experiment. However, biofertilizer has shown a high N-fixing potential summarizing the results of the analyses despite the fact that the soil was alkaline. The pot-soil experiment should be repeated at least twice so reliable data on the effectiveness of the biofertilizer could be obtained. In the current study, the pot-soil experiment has been done once due to the time limitation. The length of the experiment has been restrained to one month unlike the wheat field experiment. This could be a reason for the weak effect of N-fixation on the estimated plant parameters. The next factor was the soil pH. The site location was the only available land to conduct the experiment and it was not possible to allocate the experiment to more fertile soil. The effectiveness of biofertilizer should be tested in neutral soil as well because the pH of the soil has a huge influence on the nutrient availability. In alkaline soils, the fixed N by N-fixing bacteria ammonium can volatilize into the atmosphere and in turn the productivity of the N-fixing bacteria could be low at such unfavourable environmental condition.

5. CONCLUSİONS

The conclusions which are presented have been drawn mainly based on the data from the wheat field experiment. In comparison to the maize pot experiment, the field experiment has duration of two years and yield data was available.

- Identification with BIOLOG system confirmed the species identification as *Priestia megaterium* (formerly *Bacillus megaterium*) based on its metabolic fingerprint.
- Application of Nuptak biofertilizer at a dose of 1 kg/ha resulted in two-fold increase the level of ammonium nitrogen content in comparison to the control treatment in the wheat fields experiment.
- Combination of Nuptak biofertilizer and reduced (25 and 50%) doses of chemical fertilizer was not capable to compensate the plants' need for nitrogen and the corresponding indicators in the wheat leaves were the lower total content of nitrogen, crude and soluble protein.
- Supplementation of Nuptak biofertilizer to optimal (160 kg ha-1) and reduced (120 kg ha-1) doses of chemical fertilizer stimulated the photosynthetic rate without affecting the total chlorophyll content in the wheat leaves. The further reduction of nitrogen fertilization by 25 and 50% decreased the photosynthetic rate, but has a significantly lower effect on the leaf nitrogen content.
- Nuptak biofertilizer increased the grain yield of wheat up to 4% when was used as a supplement to the chemical fertilizer with optimal (160 kg ha-1) and reduced (120 kg ha-1) doses. Nuptak biofertilizer was able to compensate the loss of productivity due to suboptimal fertilization e.g. at 50% fertilization rate the yield decreased only by 12%.

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7. CURRICULUM VITEA

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2. Comparison of anatomical characteristics and phytochemical components between two species of *Hedysarum* (Fabaceae). Published in 2023
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Won Awards, Incentives and Scholarships

1. International Soil Science Symposium Participation Certificate (2022)
2.

8. ATTACHMENTS

Statistical analyses for wheat field experiment

Table 8.1. ANOVA for net photosynthetic rate of wheat at the field experiment (F critical value (0.05) = 3.0069)

Source of variation	**df**	**SS**	**MS**	**F**
Blocks	4	7.34132	1.83533	
Treatments	4	67.59356	16.89839	2.88954228
Error	16	93.56992	5.84812	
Total	24	168.5048		

Note. Not significant

Table 8.2. ANOVA for transpiration rate of wheat at the field experiment (F critical value (0.01) = 4.7726)

Source of variation	**df**	**SS**	**MS**	**F**
Blocks	4	3.092136	0.773034	
Treatments	4	3.967336	0.991834	6.06704225
Error	16	2.615664	0.163479	
Total	24	9.675136		

Note. Significance code: '**' 0.01

Table 8.3. ANOVA for stomotal conductance of wheat at field experiment (F critical value (0.05) = 3.0069)

Source of variation	**df**	**SS**	**MS**	**F**
Blocks	4	0.000136	3.4E-05	
Treatments	4	0.000616	0.000154	3.14285714*
Error	16	0.000784	4.9E-05	
Total	24	0.001536		

Note. Code for significance: '*' 0.05

Table 8.4. LSD test for stomotal conductance of wheat at field experiment

t-value (0.05)	Variance	LSD (0.05)	Average of treatments	Non-significant ranges
2.12	4.9E-05	0.105	T1 – 0.05 T2 – 0.06 T3 – 0.04 T4 – 0.04 T5 – 0.05	bc a bc bc ab

Table 8.5. ANOVA for chlorophyll content of wheat at field experiment (F critical value (0.05) = 2.6335)

Source of variation	**df**	**SS**	**MS**	**F**
Blocks	9	3409.22	378.8022	
Treatments	4	10917.32	2729.33	3.77560456*
Error	36	26023.88	722.8856	
Total	49	40350.42		

Note. Code for significance: '*' 0.05

Table 8.6. LSD test for chlorophyll content of wheat at field experiment

t-value (0.05)	Variance	LSD (0.05)	Average of treatments	Non-significant ranges
2.042	722.8855556	25.88119079	T1 – 445.4 T2 – 447.1 T3 – 424 T4 – 408.1 T5 – 423.1	ab ab ab c ab

Table 8.7. ANOVA for yield of wheat (2022) at field experiment (F critical value (0.05) = 3.2592)

Source of variation	**df**	**SS**	**MS**	**F**
Blocks	3	0.229495	0.076498	
Treatments	4	1.28255	0.320638	3.94093186*
Error	12	0.97633	0.081361	
Total	19	2.488375		

Note. Code for significance: '*' 0.05

Table 8.8. LSD test for yield of wheat (2022) at field

t-value (0.05)	Variance	LSD (0.05)	Average of treatments	Non-significant ranges
2.179	0.081361	0.507480425	T1 – 4.28 T2 – 4.4525 T3 – 3.92 T4 – 3.7675 T5 – 3.9425	ab a bcd cd bc

Table 8.9. ANOVA for height of wheat (2022) plants at the field experiment (F critical (0.01) = 4.7726)

Source of variation	**df**	**SS**	**MS**	**F**
Blocks	4	6.8	1.7	
Treatments	4	314	78.5	13.4763948**
Error	16	93.2	5.825	
Total	24	414		

Note. Significance code: '**' 0.01

Table 8.10. LSD test for height of wheat (2022) plants at the field experiment

t-value (0.05)	Variance	LSD (0.05)	Average of treatments	Non-significant ranges
2.921	5.825	4.984992669	T1 – 68.8 T2 – 69.4 T3 – 68 T4 – 60.2 T5 – 63.6	ab a abc cd cd

Table 8.11. ANOVA for number of wheat tillers (2022) at the field experiment

Source of variation	**df**	**SS**	**MS**	**F**
Blocks	4	0.96	0.24	
Treatments	4	0.56	0.14	0.52830189
Error	16	4.24	0.265	
Total	24	5.76		

Note. Not significant

Table 8.12. ANOVA for length of main ear of wheat plants (2022) at the field experiment (F critical value (0.05) = 3.0069)

Source of variation	**df**	**SS**	**MS**	**F**
Blocks	4	0.2584	0.0646	
Treatments	4	3.2984	0.8246	4.31501832*
Error	16	3.0576	0.1911	
Total	24	6.6144		

Note. Code for significance: '*' 0.05

Table 8.13. LSD test for length of main ear of wheat plant at the field experiment

t-value (0.05)	Variance	LSD (0.05)	Average of treatments	Non-significant ranges
2.12	0.1911	0.655316656	T1 – 8.66 T2 – 8.86 T3 – 8.74 T4 – 8.16 T5 – 7.92	abc a ab bcd d

Table 8.14. ANOVA for seeds in the ear of main wheat tiller (2022) at the field experiment

Source of variation	**df**	**SS**	**MS**	**F**
Blocks	4	10.16	2.54	
Treatments	4	23.76	5.94	0.77495108
Error	16	122.64	7.665	
Total	24	156.56		

Note. Not significant

Table 8.15. ANOVA for mass of one-thousand grain of wheat plants (2022) at the field experiment (F critical value (0.01) = 7.0061)

Source of variation	**df**	**SS**	**MS**	**F**
Blocks	2	0.05872	0.02936	
Treatments	4	78.49536	19.62384	956.793759**
Error	8	0.16408	0.02051	
Total	14	78.71816		

Note. Significance code: '**' 0.01

Table 8.16. LSD for mass of one-thousand grain wheat plants (2022) at the field experiment

t-value (0.01)	Variance	LSD (0.01)	Average of treatments	Non-significant ranges
3.355	0.02051	0.480480044	T1 – 35.12 T2 – 32.003 T3 – 32.86 T4 – 28.07 T5 – 32.563	a d b e bc

Statistical analyses of maize pot-soil experiment

Table 8.17. ANOVA for soil respiration of maize pot-soil experiment (F critical value (0.01) = 5.417)

Source of variation	df	Sum of squares	Mean squares	F
Treatments	3	0.057772282	0.0192574	2.16379173
Error	20	0.177997052	0.0088999	
Total	23	0.235769335		

Note. Insignificant

Table 8.18. ANOVA for net photosynthetic rate of maize at pot-soil condition (F critical value (0.05) = 3.2389)

Source of variation	df	Sum of squares	Mean squares	F
Treatments	3	11.02508	3.6750267	1.53813787
Error	16	38.22832	2.38927	
Total	19	49.2534		

Note. Not significant

Table 8.19. ANOVA for transpiration of maize leaves at pot-soil condition (F critical value (0.05) = 3.2389)

Source of variation	df	Sum of squares	Mean squares	F
Treatments	3	0.14658	0.04886	1.32223801
Error	16	0.59124	0.0369525	
Total	19	0.73782		

Note. Not significant

Table 8.20. ANOVA for stomotal conductance of maize leaves at the pot-soil condition (F critical value (0.05) = 3.2389)

Source of variation	df	Sum of squares	Mean squares	F
Treatments	3	0.00052	0.0001733	0.69333333
Error	16	0.004	0.00025	
Total	19	0.00452		

Note. Not significant

Table 8.21. ANOVA for chlorophyll content of maize leaves at the pot-soil condition (F critical value (0.01) = 4.4594)

Source of variation	df	Sum of squares	Mean squares	F
Treatments	3	48838.77778	16279.59259	8.1245909
Error	32	64119.77778	2003.743056	
Total	35	112958.5556		

Note. Significance code: '**' 0.01

Table 8.22. LSD test for chlorophyll content of maize leaves at the pot-soil condition

t-value (0.01)	Variance	LSD (0.01)	Average of treatments	Non-significant ranges
2.750	2003.74	61.54938	T1 – 376.89 T2 – 394 T3 – 425.89 T4 – 473.67	bcd bc ab a