Nitrogen fixation in wheat (*Triticum aestivum*)

Effect of *Azotobacter salinestris CECT 9690* on N-accumulation

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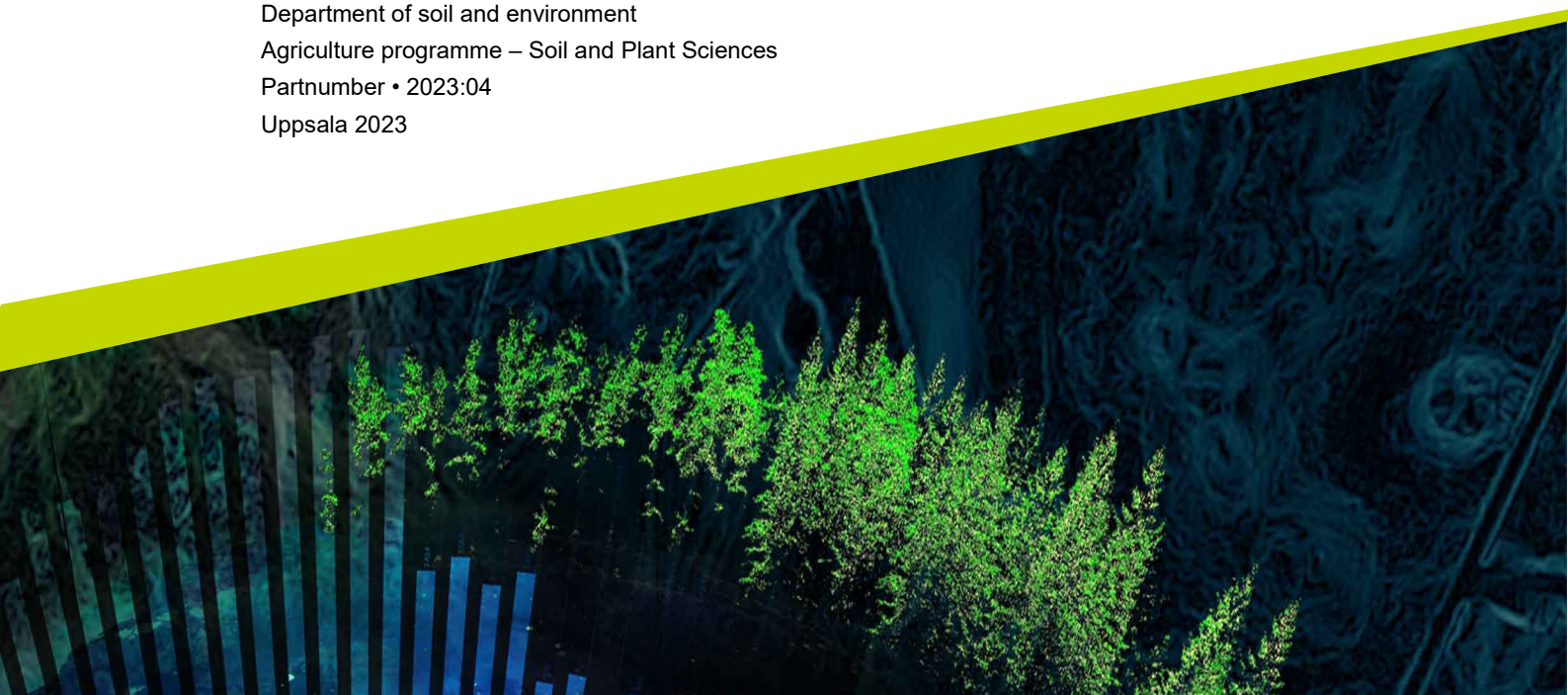
Master thesis • 30 credits
Swedish University of Agricultural Sciences, SLU
Department of soil and environment
Agriculture programme – Soil and Plant Sciences
Partnumber • 2023:04
Uppsala 2023



Nitrogen fixation in wheat (Triticum aestivum). Effect of Azotobacter salinestris CECT 9690 on N- accumulation

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Credits: 30 credits
Level: Second cycle, (A2E)
Course title: Master thesis in Biology, A2E–Agriculture Programme–Soil and Plant Sciences

Course code: EX0898
Programme/education: Agriculture Programme, Soil and Plant Sciences
Course coordinating dept: Soil and Environment

Place of publication: Uppsala, Sweden
Year of publication: 2023

Keywords: Biostimulants, Vixeran, N-uptake, N-concentration, yield, cereals

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Department of Soil and Environment

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Abstract

Biostimulants are products that can improve nutrient availability, nutrient uptake, nutrient use efficiency and tolerance to abiotic stress with the aim to increase plant growth and yield. During the last decades, the interest in, and market for biostimulants has grown and is expected to have a large compound annual growth rate. The number of products on the market increase every year and the sales agencies promote them as yield rising and profitable. This thesis aimed to investigate the effect of the product Vixeran (*Azotobacter salinestris CECT 9690*) on N-accumulation in spring wheat under different temperatures and N-levels. The study was conducted as a pot experiment where spring wheat was cultivated, with and without supply of Vixeran, in climate chambers in BioCentrum at Ultuna. The experiment had a three-factorial split-plot design with temperature regimes as main plots and N-rates and Vixeran treatment as sub plots. The effect of Vixeran treatment varied between the two temperature regimes. In the high temperature, there was significant higher N-accumulation corresponding to 4,09 kgN/ha in Vixeran treated plants compared to the untreated control. On the other hand in the low temperature there was significantly 5,90 kgN/ha higher N-accumulation in the untreated control compared to the Vixeran treated wheat. There was no significant interaction effect between N-level and Vixeran treatment in any of the temperatures. It is essential to conduct research on how the bacteria works under different environmental conditions. Parameters such as soil type, pH, temperature and cultivar can influence the effect of the bacteria and it is important to understand how the interaction between these parameters and the bacteria works. The result of this study shows that more research is needed to guarantee N-fixation from *Azotobacter salinestris CECT 9690.*

Keywords: Biostimulants, Vixeran, N-uptake, N-concentration, yield, cereals

Sammanfattning

Biostimulanter är produkter som kan förbättra näringstillgänglighet, näringsupptag, näringseffektivitet och tolerans mot abiotisk stress med syfte att öka växternas tillväxt och skörd. Under de senaste decennierna har intresset och marknaden för biostimulanter växt och förväntas ha en hög sammansatt årlig tillväxttakt. Antalet produkter på marknaden ökar varje år och försäljningsbyråerna lyfter fram dem som avkastningshöjande och lönsamma. Detta examensarbete syftade till att undersöka effekten av produkten Vixeran (*Azotobacter salinestris CECT 9690*) på N-ackumulering i vårvete under olika temperaturer och N-nivåer. Studien genomfördes som ett kruk-experiment där vårvete odlades, med och utan tillförsel av Vixeran, i klimatkammare i BioCentrum vid Ultuna. Försöket var upplagt med en trefaktoriell split-plot-design med temperatur i storrutor och Vixeran-behandling och N-nivå i smårutor. Effekten av behandling med Vixeran varierade mellan de två temperaturerna. I den höga temperaturen var det signifikant högre N-ackumulering motsvarande 4,09 kgN/ha i Vixeran-behandlade plantor jämfört med den obehandlade kontrollen. I den låga temperaturen var det tvärtom signifikant 5,90 kgN/ha högre N-ackumulering i den obehandlade kontrollen jämfört med det Vixeran-behandlade vetet. Det fanns ingen signifikant interaktionseffekt mellan N-nivå och Vixeran-behandling i någon av temperaturerna. Det är väsentligt att forska om hur bakterierna fungerar under olika miljöförhållanden. Parametrar som jordtyp, pH, temperatur och sort kan påverka bakteriernas effekt och det är viktigt att förstå hur samspelet mellan dessa parametrar och bakterierna fungerar. Resultatet av denna studie visar att mer forskning behövs för att garantera N-fixering från *Azotobacter salinestris CECT 9690.*

Nyckelord: Biostimulanter, Vixeran, N-upptag, N-koncentration, avkastning, spannmål

Populärvetenskaplig sammanfattning

Biostimulanter är produkter som kan förbättra tillgängligheten av näringsämnen, näringsupptag, näringseffektivitet och toleransen mot abiotisk stress med syfte att öka växternas tillväxt och avkastning. Dessa produkter består inte i sig själva av näringsämnen utan istället av aminosyror, hormoner och mikrober, vilka kan påverka naturliga processer som kan leda till ökad tillväxt och avkastning. Jordbrukssektorn står inför betydande utmaningar när det gäller att öka produktiviteten med lägre insatser och mindre miljöpåverkan. Detta, i kombination med klimatförändringar som leder till mer extrema väderhändelser, kan öka abiotiska stressfaktorer som extrema temperaturer, översvämmningar, torka och begränsad tillgång på näringsämnen i marken. Biostimulater har potentialen att användas som komplement till mineralgödsel för att göra odlingssystemen mer hållbara under stressade förhållanden. Under de senaste decennierna har intresset och marknaden för biostimulanter växt där marknaden förväntas ha en sammansatt årlig tillväxttakt på 12,1 %. Med en växande marknad kommer en rad av nya biostimulanter, producerade av företag som ser en lönsam marknad. Effekten av alla dessa biostimulanter är oklar och det finns begränsat med underlag, men där effekten kan variera beroende på miljöförhållanden.

Detta examensarbete syftade till att undersöka effekten av produkten Vixeran (*Azotobacter salinestris CECT 9690*) på N-upptag i vårvete under olika temperaturer och N-nivåer. Vete är idag en av de viktigaste grödorna i världen och tillhandahåller mat till cirka 4 miljarder människor. Studien genomfördes som ett krukexperiment där vårvete odlades, med och utan tillförsel av Vixeran, i två olika temperaturer i klimatkammare i BioCentrum vid Ultuna.

Resultaten från behandlingen med Vixeran varierade mellan de två temperaturerna. Det I den höga temperaturen var det ett signifikant högre N-upptag i Vixeran-behandlade plantor jämfört med den obehandlade kontrollen som motsvarande 4,09 kgN/ha. I den låga temperaturen var det istället 5,90 kgN/ha högre N-upptag i den obehandlade kontrollen jämfört med det Vixeran-behandlade vetet. Effekten av Vixeran behandlingen påverkades inte av N-nivå i någon av temperaturerna. Mer forskning behövs under fältförhållanden för att förstå hur bakterien påverkas av oilka miljöförhållanden. Parametrar som jordtyp, pH, temperatur och sort kan påverka bakteriernas effekt och det är viktigt att förstå hur samspelet mellan dessa parametrar och bakterierna fungerar. Denna studie visar att mer forskning behövs för att garantera N-fixering från *Azotobacter salinestris CECT 9690*.

Table of contents

List of tables

List of figures

1. Introduction

The agriculture sector faces significant challenges in rising productivity with lower inputs and less environmental impact. This, combined with climate change leading to more extreme weather events, can increase abiotic stress factors such as extreme temperatures, waterlodging, drought, and limited nutrient availability in the soil. To produce more with less external inputs and more prevalent abiotic stress factors can sound like an impossible equation, but with the influence of biostimulants, this may become a reality. Biostimulants for crop production have the potential to enhance nutrient use efficiency, tolerance to abiotic stress, quality traits or increasing the availability of confined nutrients.

Wheat is one of the most important crops in the world, providing calories for billions of people. The wheat yields have increased significantly due to plant breeding leading to varieties with very high yield potential. However, these high yields can only be achieved with external inputs from humans. One of the most yield-raising inputs is nitrogen (N) fertilization. Fertilizer prices are rising, and N management is more critical than ever. Both from an economic perspective for the farmer and from an environmental perspective to reduce the N losses and increase the nitrogen use efficiency. The biostimulant Vixeran, a nitrogen-fixing bacteria, has considerable potential to be a good complement to synthetic fertilizers and thereby increase the resilience and nitrogen use efficiency in agricultural cropping systems.

Nitrogen-fixing biostimulants in wheat are new, and there is a need for more agronomic knowledge how these products perform under different field conditions. It also needs to be mapped precisely how the biostimulant works and what factors that affect the crop response of the product. The work aims to investigate the effect of the biostimulant *Vixeran* on N-accumualtion under different growing conditions. As a result, the following research question have been formulated:

How does different temperatures and nitrogen fertilization rates influence the effect of Vixeran (*Azotobacter salinestris CECT 9690*) on N accumulation in spring wheat?

2. Background

2.1 Nitrogen

Nitrogen input is essential in crop production and is the primary reason why yields have been able to keep pace with the human population growth. Nitrogen plays a crucial role in plants and is involved in various critical processes. Some of these are growth, leaf area expansion, and biomass yield production (Anas et al. 2020). Around 109 million tonnes of nitrogen fertilizer are consumed by agriculture each year, but less than 50 % of this nitrogen are assimilated into the above-ground biomass of crops (Peoples et al. 2019). As a result, the nitrogen use efficiency varies from approximately 30-50 % in agriculture (Anas et al. 2020). This means there is an excessive and inefficient use of nitrogen with a great possibility for improvement.

2.1.1 Nitrogen in soil

Nitrogen exists in different forms in the soil and it can quickly transform from one form to another (Robertson & Groffman 2007). The nitrogen inputs in crop production come from mineral fertilizers, soil organic matter, crop residues, animal manures, and nitrogen fixation. Nitrogen is the most yield-raising nutrient, and a majority is applied through mineral fertilizers. The nitrogen in the mineral fertilizers is derived from the atmospheric N-pool, where N_2 is combined with hydrogen H_2 under high temperatures and pressure to create ammonia (NH_3). This is called the Haber-Bosch process and is not a naturally occurring process, but instead requires a lot of energy input. Ammonia is then the starting point for manufacturing different nitrogen fertilizers. The most common forms of nitrogen in mineral fertilizers are ammonium (NH_4^+), nitrate (NO_3^-), and urea ($CO(NH_2)_2$). Plant available forms of nitrogen are NH_4^+ and NO_3^-. Nitrogen in organic form, such as in crop residues and animal manure, is also an important nitrogen source for the crop. Organic nitrogen has to undergo mineralization to become inorganic and be available for the crops. This is done by microorganisms which digest organic material and thereby release ammonium. Ammonium can also convert into nitrate via nitrification in the soil which is a process that proceeds rapidly in warm, moist,

and well-aerated soils. The ammonia ions are positively charged and bind easily to the mineral particles and humus in the soil, which are negatively charged. The nitrate ions are negatively charged and are therefore bound to a lower extent to the soil particles. These different properties has practical importance for N management due to that ammonium ions move slowly downward in soils, meanwhile nitrate can easily move below the root zone if not taken up by crops and leach out in either groundwater or surface water. Nitrogen can also be lost through denitrification and immobilization. In the denitrification process, bacteria convert nitrate into N gases that are lost to the atmosphere. Immobilization is the process where bacteria consume nitrogen from the soil solution to maintain the proper nutrient balance for their metabolism (Robertson & Groffman 2007). This process occur when organic matter with a high carbon and low nitrogen content is decomposed.

2.1.2 Nitrogen fixation

Biological nitrogen fixation is a process of high importance in agriculture (Mahmud et al. 2020). The biggest challenge in fixing nitrogen from the atmosphere is to break the strong triple covalent bond in the N_2 molecule. This is very energy-demanding and is accomplished by the catalyst nitrogenase and the addition of three hydrogen atoms to each nitrogen atom, thereby forming ammonia. This reaction requires 16 moles of ATP to reduce each mole of nitrogen. Associative and symbiotic nitrogen microorganism gets this energy from their host plant. On the other hand, non-photosynthetic free-living microorganisms must obtain this energy from other sources. Examples of this include *Azotobacter*, which has to oxidize organic molecules released by other organisms or from decomposition. The Haber-Bosch process uses the same principles as in biological nitrogen fixation, except that the energy source is fossil fuels (Mahmud et al. 2020).

2.1.3 Nitrogen in plants

Plants contains around 1-6 % nitrogen and absorb nitrogen as nitrate and ammonium (Havlin 2013). In the plant, a reduction from nitrate to ammonium occurs, which is energy-demanding. The plant metabolizes nitrate to ammonia and then to amino acids and proteins. Nitrogen has many essential functions in the plant. It is a critical component in the chlorophyll molecule, which convert light into chemical energy via photosynthesis. It is an essential part of energy transfer compounds such as ADP and ATP. An optimal nitrogen amount in the plant is associated with heavy vegetative growth, dark green color, and high photosynthetic activity. Too much nitrogen in relation to other nutrients, such as phosphorus (P), potassium (K), and sulfur (S), can lead to a delay in maturity.

2.1.4 Nitrogen use efficiency

Nitrogen use efficiency (NUE) is the relationship between the total nitrogen input and the total nitrogen output (*Yara UK* 2019). In the most simplified way, NUE can be described as the grain yield per unit of total available nitrogen, which is both applied nitrogen and soil mineral nitrogen (*Yara UK* 2019). The NUE varies with crop species and with varieties within species (Fageria & Baligar 2005). One way to increase the NUE is to breed N-efficient genotypes, which is good for reducing costs, improving yields, and keeping a healthy environment. The efficiency could also increase through improved crop management practices. For example, precision application of N with help from sensors that sense variations in chlorophyll content, accurate N rates to yield expectations, and apply N in periods when the crop can utilize it and has a rapid N uptake. Further, the NUE could also increase by introducing biological N fixation systems in crops that are not naturally nitrogen fixating, for example, in cereals (Fageria & Baligar 2005).

2.2 Wheat

Wheat was cultivated for the first time around 10 000 years ago as diploid einkorn wheat and tetraploid emmer wheat (Shewry 2009). Landraces were the earliest forms of cultivated wheat and were selected by the farmer because of their superior characteristics and yield associated with the place of cultivation. During domestication, breeding has given wheat a very high yield potential. However, this high yield potential can only be achieved with external inputs from humans, and these wheat varieties do not survive in the wild. Today around 95% of the cultivated wheat is hexaploid bread and fodder wheat, and 5% is tetraploid durum wheat.

Wheat is today one of the most important crops in the world, providing calories for around 4 billion people. In Sweden, wheat was cultivated on 481 600 hectares of land in 2021, which corresponds to approximately 19% of the total farmland (Jordbruksverket, 2021). Globally, wheat was cultivated on around 219 million hectares in 2020 (Statista 2020). The mean yield for winter wheat in Sweden 2021 was 6 590 kg ha $^{-1}$ and 3 540 kg ha $^{-1}$ for spring wheat, with a total production of 3 028 300 tonnes (Jordbruksverket, 2021). Wheat production in 2020 was 761 million tons globally (Our World in Data, 2022).

2.2.1 Nitrogen fertilization in wheat

In Sweden, the recommendations for nitrogen fertilization in wheat range from 105 to 245 kg/ha depending on the yield potential, the soil's mineralization, and where the crop is grown (Andersson et al. 2022). It is essential to have a high N concentration in the plant tissue during the development phases where a reduction

of formed yield components otherwise can occur. Yield components in wheat are ears per square meter, grains per ear, and grain weight (Engström & Bergkvist 2009). A study by Darwinkel (1983) show that the maximum effect of nitrogen on tiller and spikelet formation was when N was supplied at the beginning of tillering (DC 21). Best effect on the number of shoots with ears was achieved when N was given at the beginning of stem elongation (DC 30). On the number of fertile flowers per spikelet, grains per fertile spikelet, and grains per ear the optimal effect was achieved when N was applied during stem elongation until flag leaf emergence (DC 30-39). The maximum effect on grain weight was achieved when N was given at ear emergence (DC 47) (Darwinkel 1983). One of the most critical development stages in wheat is stem elongation (Engström & Bergkvist 2009). This is where the reduction of tillers can occur, which is strongly correlated with plant-available nitrogen. If the wheat plant has high access to nitrogen during stem elongation, less tillers get reduced. High access to nitrogen also increases the chance that the remaining tillers become fertile and produce ears.

2.2.2 Abiotic stress factors in wheat production

Abiotic stress refers to non-biological factors such as high temperatures, drought, flood, and nutrient deficiency. Climate change will affect global wheat production through different abiotic factors (Liu et al. 2019). Global warming will lead to higher temperatures, more extreme weather, and high variability in weather conditions from year to year. The impact on wheat production will vary spatially, where temperate high rainfall regions are projected to have a more significant increase in yield compared to hot low rainfall and irrigated regions. Grain yields are projected to increase in cool regions and decrease in warm regions due to global warming with a higher frequency of low yields, and the annual yield variability will increase. Due to extreme weather in the future, there is a possibility that the amount of nitrogen that is suppose to benefit the crop will not be accessible at different critical development stages. Arid conditions will decrease the nitrogen accessability for the crop, and at extreme rainfall conditions, there is an increased risk of high nitrogen leaching (Liu et al. 2019). If the conditions are unfavorable, the crop will not have enough nitrogen at critical development stages to achieve the highest possible yield, and the NUE will decrease.

2.3 Biostimulants

During the last decades, the interest in, and market for biostimulants has grown. The global market value for biostimulants was estimated to be USD 3.2 billion in 2021 and is expected to reach USD 5.6 billion by 2026, with a compound annual growth rate of 12.1 % (Marketsandmarkets 2022). The largest biostimulant market

is Europe, where around 6.2 million hectares were treated with biostimulants in 2012 (Calvo et al. 2014). To promote and develop the definition of biostimulants, the industry is a key player. A couple of associations have been created, like the European Biostimulant Industry Council (EBIC) in Europe and the Biostimulant Coalition in the USA. There are several definitions of biostimulants, but no legal or regulatory definition exists (du Jardin 2015). The U.S Department of Agriculture (USDA) defines biostimulants as " *Substance(s), microorganism(s), or mixtures thereof, that, when applied to seeds, plants, the rhizosphere, soil or other growth media, act to support a plant's natural nutrition processes independently of the biostimulant's nutrient content. The plant biostimulant thereby improves nutrient availability, uptake or use efficiency, tolerance to abiotic stress, and consequent growth, development, quality or yield"* (Agricen n.d.). Meanwhile, EBIC define biostimulants as "*substance(s) and/or microorganisms whose function, when applied to plants or the rhizosphere, is to stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality, independent of its nutrient content*" (*EBIC* 2019). In the EU Fertilizing Products Regulation (FPR), biostimulants are defined as *" Certain substances, mixtures and micro-organisms, referred to as plant biostimulants, are not as such inputs of nutrients, but nevertheless stimulate plants' natural nutrition processes. Where such products aim solely at improving the plants' nutrient use efficiency, tolerance to abiotic stress, quality traits or increasing the availability of confined nutrients in the soil or rhizosphere, they are by nature more similar to fertilizing products than to most categories of plant protection products. They act in addition to fertilizers, with the aim of optimizing the efficiency of those fertilizers and reducing the nutrient application rates*" (*EUR-Lex* 2019).
The definition of biostimulants is still evolving because of the diversity of what biostimulants can be. However, the most common categories of biostimulants are microbial inoculants, humic and fluvic acids, protein hydrolysates, amino acids, and seaweed extracts (Calvo et al. 2014).

2.3.1 Microbial inoculants

Microbial inoculants are living microorganisms such as free-living bacteria, fungi, or arbuscular mycorrhizal fungi. There are three major groups included in microbial inoculants. These groups are plant growth-promoting rhizobacteria (PGPR), arbuscular mycorrhiza, and nitrogen-fixing rhizobia. The microbial inoculants can be applied to seed, plant surface, or soil to increase nutrient supply, nutrient uptake capacity, and root biomass (Calvo et al. 2014). Different terminologies represent microbial inoculants, such as biostimulants, bioinoculants, biofertilizers, and biopesticides. The tested product Vixeran is categorized as a microbial inoculant and a PGPR (see chapter 2.4).

Microbial inoculants are not nutrients, but the different microorganisms can implement different biophysical and biochemical activities in the soil, which increase the availability of certain nutrients (Alori et al. 2017). There are several mechanisms that microbial inoculants use to stimulate plant growth and nutrient uptake. The main mechanisms are non-symbiotic nitrogen fixation, solubilization of nutrients, sequestering of iron by the production of siderophores, and production of volatile organic compounds (Calvo et al. 2014). Studies have shown that application of PGPR has increased the uptake of N, P, and K, leading to a higher root and shoot dry weight in wheat (Shaharoona et al. 2008). The development of these microbial inoculants can be complicated because many factors must be considered (Calvo et al. 2014). Plant species and cultivars must be considered because they can produce different kinds of rot exudates, affecting the impact of the inoculated microorganisms. Different soil types and environmental conditions also have to be tested because they can affect the reproduction of the inoculated microorganisms. Finally, when the inoculated microorganisms are used in conventional agriculture, they must be compatible with fertilizers and crop protection chemicals.

2.3.2 Humic and fulvic acids

Humic and fluvic acids, also called humic substances, are natural components of soil organic matter. Humic and fluvic acids are heterogenous compounds categorized according to their weights and solubility (du Jardin 2015). Humic acids that mainly consist of hydrophobic compounds are soluble in alkaline media, and if it is exposed to acid media, it starts to precipitate. Humic acids also have a high molecular weight. On the other hand, fluvic acid is soluble in both alkali and acid media and has a low molecular weight (Calvo et al. 2014). In a greenhouse study, under controlled conditions, wheat showed increased root and shoot growth when treated with humic acids (Tahir et al. 2011). Also fluvic acids have been shown to positively impact root and shoot growth in wheat and maize in growth chambers, hydroponic conditions, greenhouses, and field conditions (Eyheraguibel et al. 2008; Anjum et al. 2011). There are also studies showing increased uptake of N in maize (Eyheraguibel et al. 2008) and phosphate uptake in wheat (Xudan 1986). Humic and fulvic acids interact with soil nutrients and induce physiological processes that lead to increased plant growth and increased resistance to abiotic stresses (Calvo et al. 2014).

2.3.3 Protein hydrolysates and amino acids

The application of various protein-based products can increase the nitrogen use efficiency and increase the resilience of agriculture production systems (Ertani et al. 2009). The products can be divided into two main categories: 1) Protein

hydrolysates, a mixture of peptides and amino acids originating from animals or plants, and 2) individual amino acids such as glutamate, glutamine, proline, and glycine betaine. For example, glutamine can act as a signaling molecule regulating nitrate uptake. In contrast, glycine and proline are amino acids that can function as osmolytes and protect plant cells from high temperatures and high salt concentrations (Calvo et al. 2014).

2.3.4 Seaweeds extracts

Seaweed has been used for a very long time by man as a product to enhance soil fertility and crop productivity (Craigie 2011; Khan et al. 2012). Seaweed extracts contain a mixture of polysaccharides, micronutrients, and plant growth hormones. Their modes of action have yet to be well understood, but recent studies show enhanced growth and yield in wheat (Kumar & Sahoo 2011). Seaweed extracts have also improved resistance to abiotic and biotic stresses (Zhang & Ervin 2004). The most used seaweed extract comes from brown seaweeds such as *Ascophyllum nodosum*, *Fucus*, *Laminaria*, *Sargassum*, and *Turbinaria* spp (Calvo et al. 2014).

2.3.5 Potential of biostimulants in agriculture

Biostimulants have great potential to increase the resilience of the agriculture cropping system by reducing the dependence on external inputs. They can potentially improve the tolerance to abiotic stresses and, through that, improve crop production (Calvo et al. 2014). However, it is very difficult to understand the function of the different types of biostimulants due to the different origins and complexity of these products. More studies must be conducted to understand why, when, and how the different biostimulants work. In addition, we must understand their modes of action and how they respond to different biotic and abiotic factors (Brown & Saa 2015).

2.4 The biostimulant Vixeran

Vixeran is a biostimulant within the plant growth-promoting bacteria group that supplies nitrogen to the crop through nitrogen fixation of the atmospheric nitrogen. Vixeran contains an endophytic (root-leaf) strain of Azotobacter salinestris *CECT 9690*, which colonize the rhizosphere, but also penetrates the plant through the root and the leaf (MandoAdmin n.d.).

2.4.1 Plant growth promoting rhizobacteria

Plant growth promoting rhizobacteria (PGPR) are bacteria that can promote plant growth and crop yield through several mechanisms. Based on their effect on plant

growth and development, these bacteria can be grouped as biofertilizers, phytostimulants, and biopesticides, depending on their different metabolic activities. Bacteria that work as biofertilizers make inaccessible nutrients accessible for plants due to atmospheric N fixation and P or K solubilizing (Abod et al. 2019). Phytostimulants produce phytohormones such as auxins, gibberellins, cytokinins, and ethylene, which influence plant growth promotion (Shukla 2019). Phytostimulants can also improve plant tolerance to abiotoc stress (Gupta et al. 2015). Biopesticides can control the growth of harmful microorganisms for the crop through different secondary metabolites or extracellular cell wall decomposing enzymes (Akram et al. 2017). The application of plant growth-promoting bacteria can make crop production more sustainable since it allows growers to use less amount of fertilizers but still get the same yields (Sheirdil et al. 2019).

2.4.2 Azotobacter

Azotobacter is oval and quite large compared to other bacteria. They are gram-negative and can be found in neutral to alkaline soils and in the rhizosphere of plants (Das 2019). They have an optimal growth at around 35 ˚C, when temperatures become higher or lower than 35 ˚C the growth of *Azotobacter* starts to decrease (Chennappa et al. 2017). The bacteria can grow in pH values ranging from 6-9 but has its optimal growth at pH ranging from 7,2-7,5 (Page & Shivprasad 1991). The atmospheric nitrogen fixation done by *Azotobacter* is carried out without the formation of nodules and is called non-symbiotic nitrogen fixation (Akram et al. 2017). *Azotobacter* is an aerob bacteria and needs oxygen for its biological activity (Robson & Postgate 1980). Inoculating seeds with *Azotobacter* has been reported to increase yields in cereals like corn, wheat, barley, rice, pearl millet, and sorghum (Mrkovac & Milic 2001). The most crucial property of *Azotobacter* is the ability to convert nitrogen gas into ammonia, which qualifies the bacteria as a biostimulant. This conversion is made possible by the enzyme nitrogenase. (Das 2019).
The nitrogen contribution from biological nitrogen fixation of *Azotobacter* varies depending on environmental conditions management and cropping practices, and genotypic differences. Studies show that *Azotobacter* can fix 0,3-60 kg N/ha (Aasfar et al. 2021). The nitrogen fixation from *Azotobacter* will not be able to cover 100 % of the nitrogen requirement in wheat, but has the possibility to be a supplement to mineral fertilizers (Das 2019). Potentially, the addition of biostimulants can enable farmers to decrease the rate of mineral fertilizers and still get the same yield.

2.4.3 Azotobacter salinestris

Azotobacter salinestris is an endophytic bacteria that can live inside plants and improve plant growth under both normal and challenging conditions (Afzal et al.

2019). They benefit the host plant directly by improving nutrient uptake through, for example, nitrogen fixation. The endophytic bacteria are considered a subclass of rhizospheric bacteria and belong to the PGPR group (Afzal et al. 2019). Endophytic bacteria represent a class of specialized rhizobacteria that can invade roots after establishing a rhizospheric population. After entering the root, the bacteria can spread and colonize above-ground tissue (Reinhold-Hurek & Hurek 1998). The colonization of the plant by the endophytic bacteria also involves complex communication between the plant and the bacteria. The process usually starts at the roots, where the bacteria require recognition of different root exudates from the plant (Rosenblueth & Martínez-Romero 2006). The plant produces these root exudates to interact with beneficial bacteria for their own advantage (Compant et al. 2005). Endophytic bacteria have a broad range, can be used in many crops, and have great potential as a biofertilizer and biopesticide (Afzal et al. 2019). Both genotypes of the crop and the bacteria play a crucial role in the performance of the symbiosis where the bacteria gets its energy from natural leaf and root excudates, mainly carbon sources like sugars.

2.4.4 Azotobacter salinestris CECT 9690

Rodriguez[1] says that *Azotobacter salinestris CECT 9690* is an endophytic bacteria that can penetrate through the leaves and roots but can also stay in the rhizosphere. When the bacteria arrives at the leaves, it will start to fix N and feed from natural leaf exudates, mainly carbon sources like sugars. The bacteria colonizes the leaf surface by producing a biofilm and will penetrate inside internal tissues via stomata, tiny pores, or fratures. During this process, the bacteria will fix N by the nitrogenase enzyme that reduces the gaseous N to ammonia that can be taken up by the plant. When the plant needs N, it will take up the fixed ammonia produced by the bacteria via glutamine synthetase required for plant protein biosynthesis. When the plant does not need more N, it will block it via glutamine synthetase, and the ammonia fixed by the bacteria will accumulate in the surrounding area, inactivating the nitrogenase of the bacteria. When the plant again starts to to use the accumulated N, the nitrogenase of the bacteria will fix more N to supply it to the plant. There is a communication between the plant and the bacteria to supply the necessary ammonia that the plant requires for growth and development. The same will happen in the root. The root will attract the community of the bacteria to the rhizosphere by releasing exudates (carbon sources), and the bacteria will move to the root via flagella, motile appendixes that allow the bacteria to move. Later, part of the population can penetrate the root via pores and fractures. If part of the population remains in the rhizosphere, it will also fix N, and the fixed ammonia will be transferred from the soil to the plant tissues through the active transport of the plant.

[1] Esteban Rodriguez, Product Manager Microbiologist & Probiotics specialist, Ceres Biotics, mail 2023-01-11

3. Method

The study was conducted as a pot experiment where spring wheat (cv. Diskett) was cultivated, with and without supply of Vixeran in different temperatures and N-levels, in climate chambers in BioCentrum at Ultuna.

3.1 Experimental design

The experiment had a three-factorial split-plot design with temperature regimes as main plots and N-rates and Vixeran treatment as sub plots. All treatments had four replicates and are shown in Table 1. The wheat was cultivated in two different climate chambers with different temperatures (low and high). The temperature was changed at two occations in the low temperature chamber and at one occasion in the high temperature chamber to promote growth and development (Table 2). Periods with light were around 16 hours per day. The factor nitrogen fertilization rates had five different nitrogen levels (Table 1). The factor Vixeran treatment consisted of application of the Vixeran product and a control treatment with no application.

Table 1 The design of the trial. For each treatment there were four replicates. The amount of nitrogen for each N-rate were (100%=154 kg N/ha, 80%= 123 kg N/ha, 60%= 92 kg N/ha, 40%= 62 kg N/ha, 0%= 0 kg N/ha).

Treatment	Temperature	N-rates
Control	High	100%
Control	High	80%
Control	High	60%
Control	High	40%
Control	High	0%
Control	Low	100%
Control	Low	80%
Control	Low	60%
Control	Low	40%
Control	Low	0%
Vixeran	High	100%
Vixeran	High	80%
Vixeran	High	60%
Vixeran	High	40%
Vixeran	High	0%
Vixeran	Low	100%
Vixeran	Low	80%
Vixeran	Low	60%
Vixeran	Low	40%
Vixeran	Low	0%

Two weeks after emergence the temperature was raised in the low temperature chamber (Table 2). The temperature was changed from the initial mean temperature 10 ˚C to 12,3 ˚C in mean temperature, 14 ˚C during the day and 9 ˚C during the night. Futher three weeks after the first increase in temperature a second increase in temperature was done. The remperature was changed to 15,3 ˚C in mean temperature, 17 ˚C during the day 12 ˚C during the night. 6 weeks after emergence the temperature was raised in the high temperature chamber. The temperature was changed from the initial 16 ˚C to 20 ˚C in mean temperature, 22 ˚C during the day and 16 ˚C during the night (Table 2).

Table 2 Temperature regimes in the two climate chambers for different time periods of the experiment.

Chamber	Temperature day/night (˚C)	Mean temp (˚C)	Period (weeks)
Low	12/7	10	1-2
Low	14/9	12,3	3 -5
Low	17/12	15,3	6-9
High	18/12	16	1-6
High	22/16	20	7-8

3.2 Practical work and preperations

Soil collection and preparation

Approximately one week before the start of the pot experiment, a sandy soil with low nutrient content were collected from a field at Krusenberg south of Uppsala (Table 3). The field was cultivated with ley and the upper 3 cm with grass and roots were discarded and then around 20 cm of the topsoil were collected. In total, around 340 liters of soil were collected. The soil was sieved to get rid of stones and roots etc. After sieving, the soil was limed with $CaCO_3$ with a rate of 0,5 g/l soil, in order to rise the pH value with approximately one unit, from 5,5 to 6,5. The CaCO3 was thouroghly mixed into the soil.

Table 3 Nutrient values and soil texture for the soil used in the experiment (topsoil from Krusenberg). SOM stands for soil organic matter.

Location	Clay (%)	SOM (%)	pH	P-AL (mg/100g)	Kl	K-AL (mg/100g)	Kl	Mg-AL (mg/100g)	K/Mg-AL
Krusenberg	9,1	2,9	5,5	8,1	IVA	7,4	II	5,1	2,5

Nitrogen solutions

The NH_4NO_3 were mixed in 200 ml solutions with a dose of 7 ml to each pot. Four different solutions with different nitrogen concentration were prepared. There were five different N-rates in total (Table 4).

Table 4 Nitrogen addition for the different fertilization rates used in the trial. Amount of ammonium nitrate and nitrogen per pot for each treatment. For each N solution, 7 ml was added to each pot.

N rate (%)	Amount Nitrogen (kg/ha)	Amount Nitrogen (g/pot)	Amount NH_4NO_3 (g/pot)
100	154	0,385	1,099
80	123	0,308	0,882
60	92	0,231	0,658
40	62	0,154	0,441
0	0	0	0

All of this was done in the lab using a scale to weigh the different nutrient salts and then mixing them in a measuring piston to get the exact volume of the mixtures. The nutrient solutions were then stored in labeled glass bottles.

Solutions of other nutrients

All essential plant nutrients were applied in optimal amounts to each pot to make sure no nutrient except nitrogen would be limiting to plant growth. All nutrient except phosphorus (P) and calcium (Ca) was added as nutrient solutions, one for macro- and one for micronutrients. The doses for each nutrient are presented in Table 5 and 6. The solution for macronutrients included potassium (K), sulphur (S), and magnesium (Mg). The nutrient salt containing P and Ca was not soluble in water and were therefore applied as a powder to the soil. The P and Ca were prepared by weighing 0,629g of $CaHPO_4$ into small plastic cups corresponding to the dose for each pot.

Table 5 Amount of nutrients (P, K, S, Mg and Ca) and corresponding nutrient salts added per pot in the trial. For each solution, 10 ml was added to each pot.

Nutrient	Nutrient salt	Amount nutrient (g/pot)	Amount nutrient salt (g/pot)
P	$CaHPO_4$	0,112	0,629
K	K_2SO_4	0,196	0,437
S	Via K_2SO_4	0,080	0,437
Mg	$MgCl_2$	0,084	0,329
Ca	Via $CaHPO_4$	0,146	0,629

The micronutrient solution contained zink (Zn), iron (Fe), manganese (Mn), copper (Cu), boron (B), and molybdenum (Mo) was also added as nutrient solutions.

Table 6 Amounts of micronutrients (Zn, Fe, Mn, Cu, B and Mo) and corresponding nutrientsalt added per pot in the trail. For each solution 10 ml was added to each pot.

Nutrient	Nutrient salt	Amount nutrient salt (g/pot)	Amount nutrient (g/pot)
Zn	$ZnCl_2$	1,2	0,778
Fe	$FeCl_3$	3,3	0,682
Mn	$MnCl_2$	2,6	0,722
Cu	$CuCl_2$	0,5	0,186
B	NaB_4O_7	0,8	0,023
Mo	Na_2MoO_4	0,3	0,119

Pot preparation and sowing

Pots with a volume of 4 liter (16 cm in diameter) were prepared with around 3 kg of soil and put into the cultivation chambers, 40 pots in each chamber. After 2 days, the different nutrients were applied and mixed into the soil. A seedbed were created using a spoon to make a stable seed bottom. The seedbed was then watered to create favourable conditions for the seeds and an optimal seedbed. The sowing was done by putting 15 kernels per pot on the seedbed. The seeds were then covered with around 3cm (500g) of soil and then watered. The wheat emerged 6 and 10 days after sowing in the high and low temperature chamber, respectively. The germination and emergence was high and around 15 kernelas sprouted per pot in both the cold and the warm chamber. The pots were thinned out to 10 plants per pot 2 days after emergence in the high temperature chamber and 3 days after emrgence in the low temperature chamber.

Application of Vixeran

Vixeran was applied at the development stage of DC 12-13 with a dose of 20mg/pot according to recommendations from Syngenta. The application was made with a hand sprayer around 20 cm above the wheat plant to cover as much leaf area as possible. The applications were done 13 days after emergence in the high temperature chamber and 18 days after emergence in the low temperature chamber.

3.3 Data collection

Harvest of biomass

At DC 41-43, 8 weeks after emergence in the high temperature chamber and 9 weeks after emergence in the low temperature chamber, the biomass was harvested. The biomass was cut as close as possible to the soil surface and put into bags. The bagswere then dried for 24 hours in 50 ˚C and then weighed. The samples were then grinded, put into plastic cans and sent to the lab for analysis of total nitrogen content and dry matter content.

3.4 Statistical analysis

The data from the trail were analysed with the statistical software JMP Pro (version 16.0.0). ANOVA-analysis was done with the factors temperature, Vixeran treatment and N-level for the response variables N accumulation, N concentration and dry matter yield. A p-value below 0.05 was considered significant. The ANOVA test was also done for each temperature separate to see the effect of each treatment in each temperature. Tukey´s test was used to detect differences between specific treatments and was run when the ANOVA showed a significant result.

4. Results

The results of the analysis of variance (ANOVA) for the whole data set and the three included factors, temperature, N-level and Vixeran treatment and the three response variables dry matter yield (kg/ha), N-concentration (%) and N-accumulation (kgN/ha) is shown in Table 7. There was significant differences between the temperatures and different N-level for all three response variables while Vixeran treatement differed significantly compared to the untreated control only for dry matter yield and N-concentration. There was a significant interaction effect between temperature and N-level for all three reponse variables while there was no interaction between N-level and Vixeran treatment for any response variables. The was also a significant interaction effect between temperature and Vixeran treatment for dry matter yield and N-accumulation. Due to the strong interaction effect between temperature and Vixeran treatment, the data was futher evaluated for the two tempertures separately (see chapter 4.1 and 4.2).

Table 7 ANOVA-analysis for the whole data set where effects on dry matter yield (kg/ha), N-concentration (%) and N-accumulation (kgN/ha) were tested by the factors temperature, N-level and Vixeran treatment. Prob> F is the significance level where a value below 0,05 indicate significance.

		Dry matter yield (kg/ha)		**N-concentration (%)**		**N-accumulation (kg N/ha)**	
Source	**DF**	**F ratio**	**Prob >F**	**F ratio**	**Prob >F**	**F ratio**	**Prob >F**
Temperature	1	89,9397	<0,0001	128,1802	<0,0001	6,1584	0,0157
N-level	4	246,2078	<0,0001	201,6223	<0,0001	665,9494	<0,0001
Vixeran treatment	1	11,6402	0,0011	8,0360	0,0061	0,3828	0,5383

Temperature*N-level	4	5,9342	0,0004	5,5005	0,0007	4,4894	0,0029
N-level* Vixeran treatment	4	1,7650	0,1468	0,4243	0,7906	1,1067	0,3611
Temperature*Vixeran treatment	1	13,3888	0,0005	0,0570	0,8121	11,5365	0,0012

4.1 High temperature treatment

Analysis of variance for the high temperature showed that there were significant differences for both N- level and Vixeran treatment on all three response variables (Table 8). However, there was no significant interaction effect between N-level and Vixeran treatment for any response variable.

Table 8 ANOVA-analysis for the high temperature where effects on dry matter yield (kg/ha), N-concentration (%) and N-accumulation (kgN/ha) were tested by the factors N-level and Vixeran treatment. Prob> F is the significance level where a value below 0,05 indicate significance.

		Dry matter yield (kg/ha)		**N-concentration (%)**		**N-accumulation (kg N/ha)**	
Source	**DF**	**F ratio**	**Prob > F**	**F ratio**	**Prob >F**	**F ratio**	**Prob >F**
N-level	4	117,2843	<0,0001	122,3588	<0,0001	433,9951	<0,0001
Vixeran treatment	1	20,1743	<0,0001	5,5236	0,0255	4,9615	0,0336
N-level*Vixeran treatment	4	0,8313	0,5160	0,2595	0,2399	0,2595	0,9015

4.1.1 Dry matter yield

In the high temperature treatment, the results showed significant differences in dry matter yield between Vixeran treated plants and the untreated control ($p<0,0001$). The Vixeran treated wheat had on average 620 kg higher dry matter yield per hectare than the untreated wheat (Figure 1).

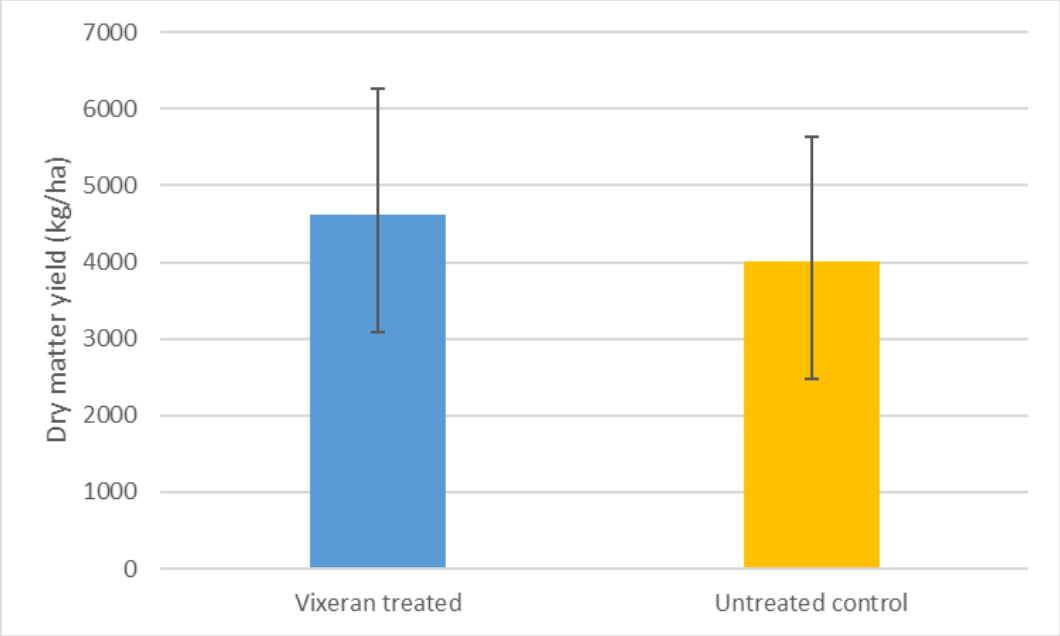

Figure 1 Dry matter yield (kg/ha) of wheat for Vixeran treated and untreated control in the high temperature chamber. Bars show an average yield for all included N-levels (n=40) and error bars indicate standard deviation.

Since there was no significant interaction effect between N-level and Vixeran treatment (p=0,5160), no separate analysis for the different N-levels has been conducted. However, there was a tendency that Vixeran treated wheat had a more pronounced positive effect at the 60% N-level compared to the other (Figure 2).

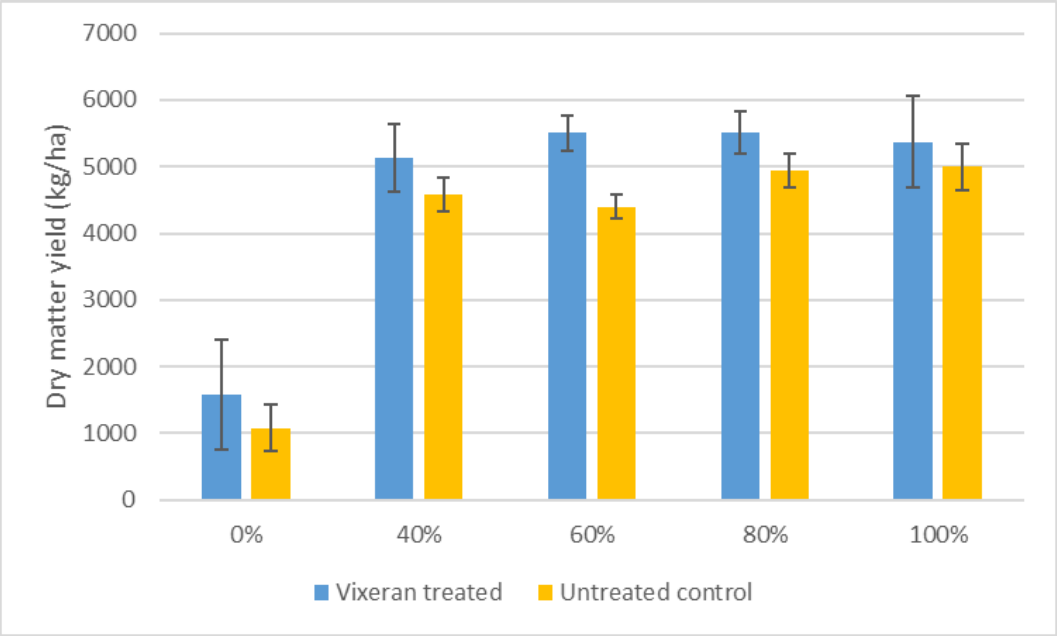

Figure 2 Dry matter yield (kg/ha) of wheat for Vixeran treated and untreated control for different N-levels in the high temperature chamber (n=40). The different N-levels are 100%=154 kg N/ha, 80%= 123 kg N/ha, 60%= 92 kg N/ha, 40%= 62 kg N/ha, 0%= 0 kg N/ha. Bars show the average yield and error bars indicate standard deviation.

4.1.2 N-concentrations in wheat

The N-concentration in Vixeran treated and the untreated control differed significantly in the high temperature treatment (p=0,0255). The untreated control show on average 0,11 percentage points higher N-concentration than the Vixeran treated wheat (Figure 3).

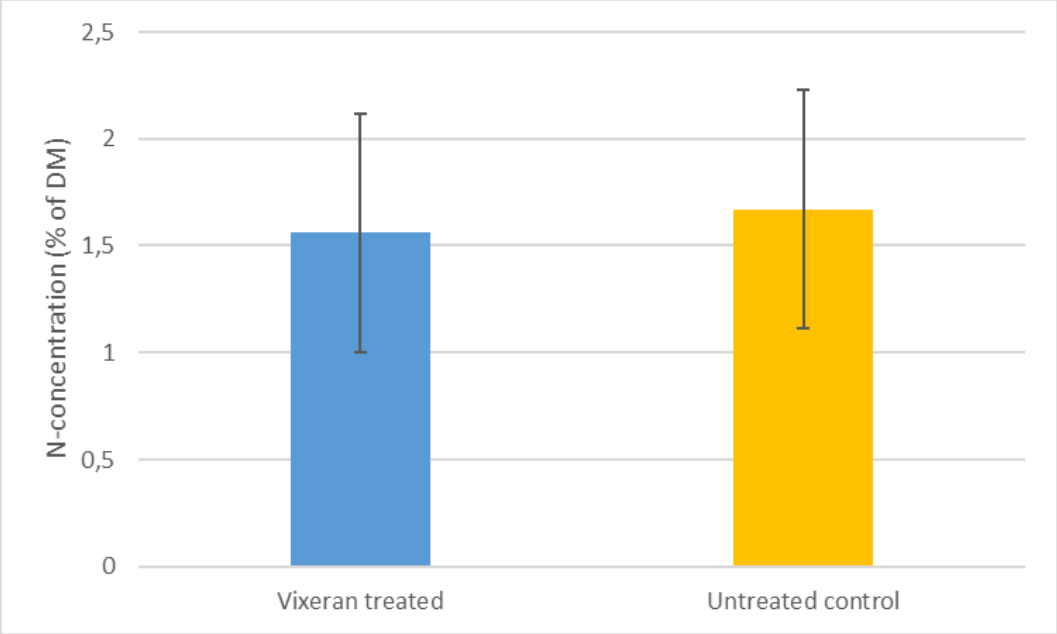

Figure 3 N- concentration (% of DM) of wheat for Vixeran treated and untreated control in the high temperature chamber. Bars show an average N-concentration for all included N-levels (n=40) and error bars indicate standard deviation.

Since there was no significant interaction effect between N-level and Vixeran treatment (p=0,2399), no separate analysis for the different N-levels has been conducted. However, the untreated control wheat had a slightly higher N-concentration than the Vixeran treated wheat in all N-levels except at 100% N-rate (Figure 4).

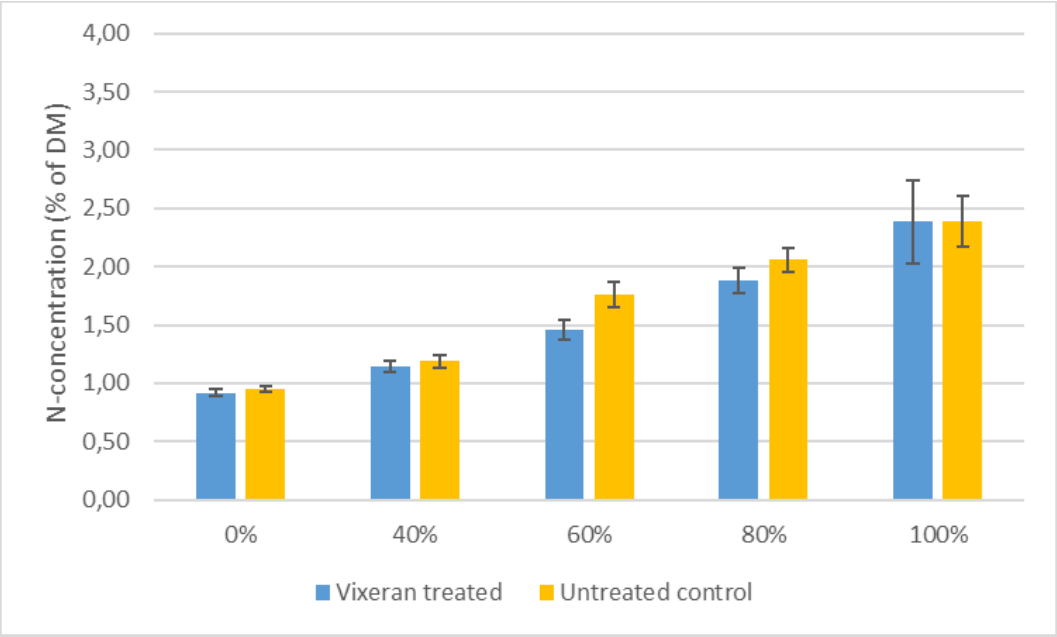

Figure 4 N- concentration (% of DM) in wheat for Vixeran treated and untreated control for different N-levels in the high temperature chamber. The different N-levels are 100%=154 kg N/ha, 80%= 123 kg N/ha, 60%= 92 kg N/ha, 40%= 62 kg N/ha, 0%= 0 kg N/ha. Bars show the average N-concentration and error bars indicate standard deviation.

4.1.3 N-accumulation in wheat

In the high temperature treatment, the results showed significant differences in N-accumulation between Vixeran and the untreated control (p=0,0336). The Vixeran treated wheat had on average 4,09 kg higher N-uptake per hectare then the untreated wheat (Figure 5).

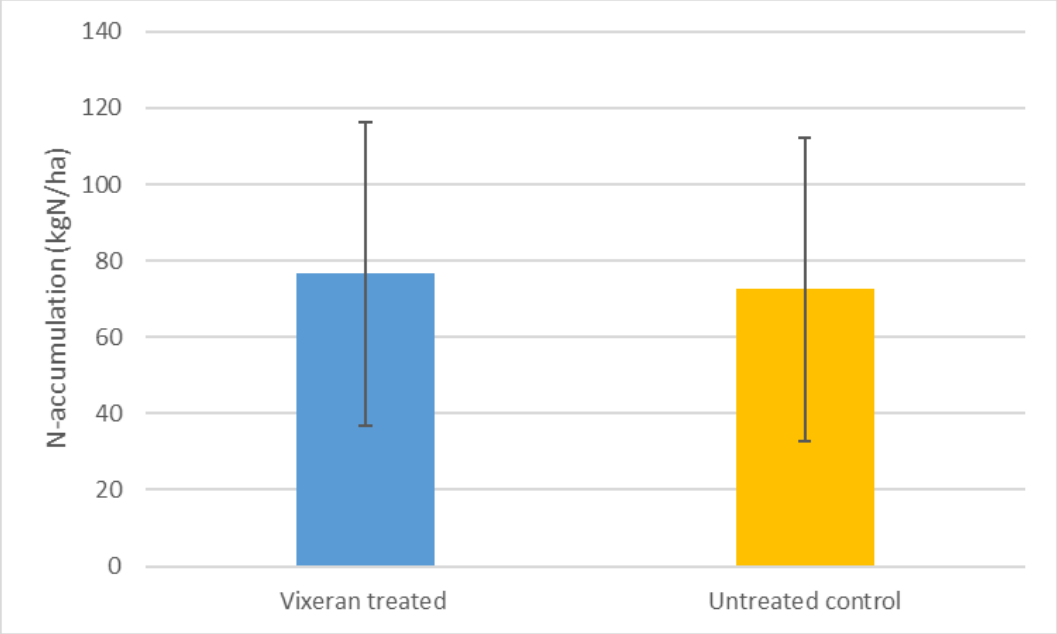

Figure 5 N- accumulation (kgN/ha) of wheat for Vixeran treated and untreated control in the high temperature chamber. Bars show an average N-uptake for all included N-levels (n=40) and error bars indicate standard deviation.

Since there was no significant interaction effect between N-level and Vixeran treatment (p=0,9015), no separate analysis for the different N-levels has been conducted. However, the Vixeran treated wheat had a slightly higher N- uptake than the control treated wheat in all N-levels (Figure 6).

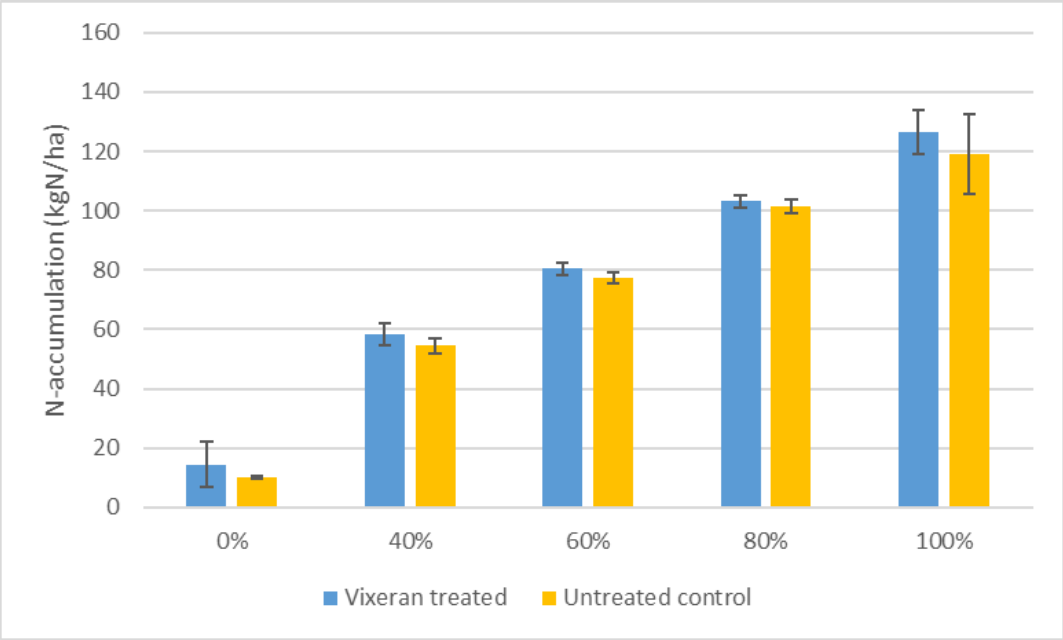

Figure 6 N- accumulation (kgN/ha) of wheat for Vixeran treated and untreated control for different N-levels in the high temperature chamber. The different N-levels are 100%=154 kg N/ha, 80%= 123 kg N/ha, 60%= 92 kg N/ha, 40%= 62 kg N/ha, 0%= 0 kg N/ha. Bars show the average N-accumulation and error bars indicate standard deviation.

4.2 Low temperature treatment

Analysis of variance for the low temperature showed that there were significant differences for N- level for all three response variables (Table 9). Vixeran treatment only showed significant effect on N-accumulation and there was no significant interaction effect between N-level and Vixeran treatment.

Table 9 ANOVA-analysis for the low temperature where effects on dry matter yield (kg/ha), N-concentration (%) and N-accumulation (kgN/ha) were tested for the factors N-level and Vixeran treatment. Prob> F is the significance level where a value below 0,05 indicate significance.

		Dry matter yield (kg/ha)		**N-concentration (%)**		**N-accumulation (kg N/ha)**	
Source	**DF**	**F ratio**	**Prob > F**	**F ratio**	**Prob >F**	**F ratio**	**Prob >F**
N-level	4	127,7790	<0,0001	94,9877	<0,0001	294,6266	<0,0001
Vixeran treatment	1	0,0366	0,8496	3,3866	0,0756	7,1332	0,0121
N-level*Vixeran treatment	4	1,4023	0,2571	0,3585	0,8360	2,2982	0,0820

4.2.1 Dry matter yield

In the low temperature treatment, the results did not show any significant difference in dry matter yield between Vixeran treated plants and the untreated control (p=0,8496). The untreated control had on average 22 kg higher dry matter yield per hectare then the Vixeran treated wheat (Figure 7).

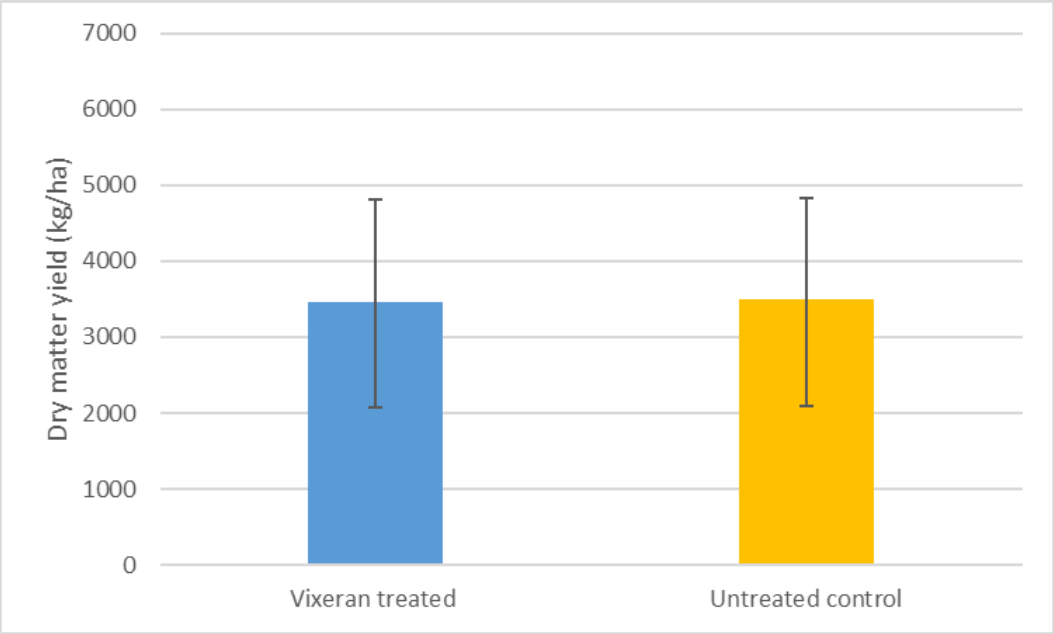

Figure 7 Dry matter yield (kg/ha) of wheat for Vixeran treated and untreated control in the high temperature chamber. Bars show an average yield for all included N-levels (n=40) and error bars indicate standard deviation.

Since there was no significant interaction effect between N-level and Vixeran treatment (p=0,2571), no separate analysis for the different N-levels has been conducted. However, there was a tendency that Vixeran treated wheat had a slightly higher dry matter yield than the control treated wheat for N-levels 0%, 40% and 60%. For the N-levels 80 % and 100% the untreated control showed slightly higher dry matter yield than the Vixeran treated wheat (Figure 8).

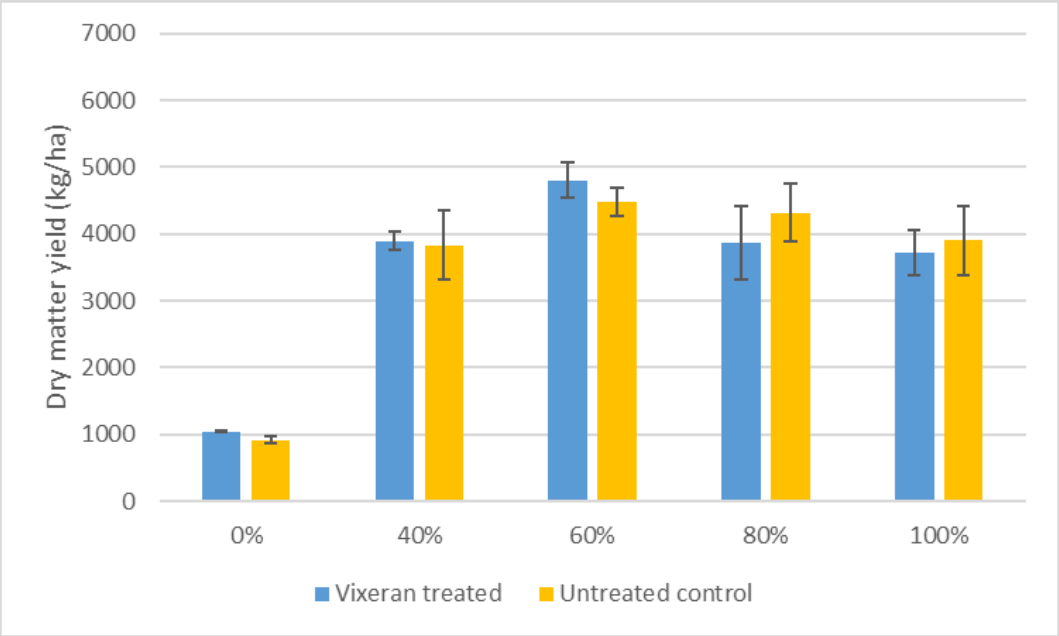

Figure 8 Dry matter yield (kg/ha) of wheat for Vixeran treated and untreated control for different N-levels in the low temperature chamber. The different N-levles are 100%=154 kg N/ha, 80%= 123 kg N/ha, 60%= 92 kg N/ha, 40%= 62 kg N/ha, 0%= 0 kg N/ha. Bars shows the average yield and error bars indicate standard deviation.

4.2.2 N-concentration in wheat

The N-concentration in Vixeran treated and the untreated control did not differ significantly in the low temperature treatment (p=0,0756). The untreated control wheat showed in average 0,14% percentage points higher N-concentration than the Vixeran treated wheat (Figure 9).

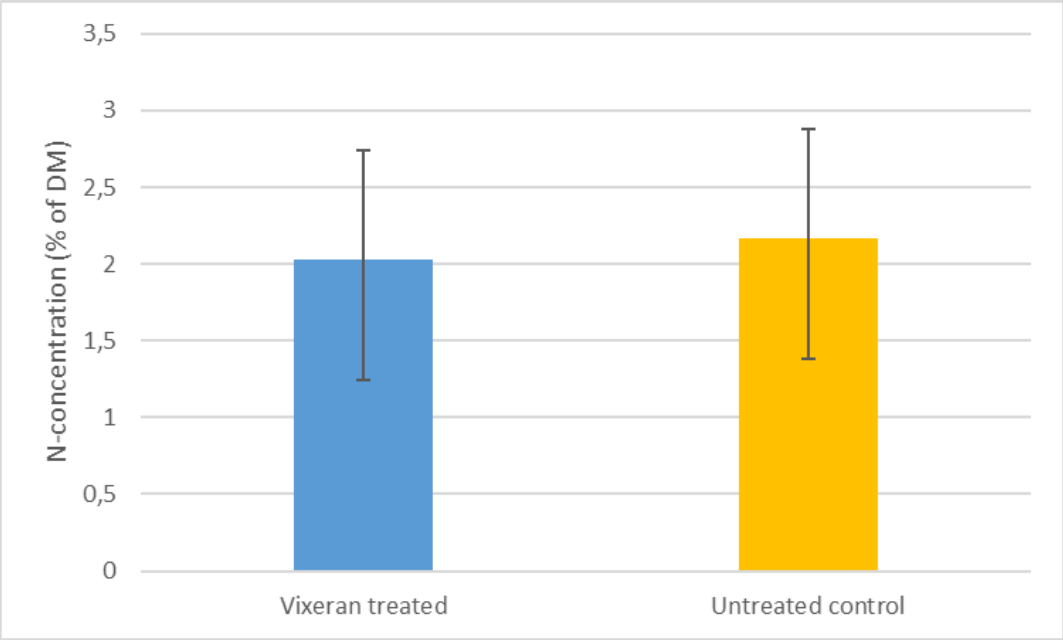

Figure 9 N- concentration (% of DM) of wheat for Vixeran treated and untreated control in the high temperature chamber. Bars show an average N-concentration for all included N-levels (n=40) and error bars indicate standard deviation.

Since there was no significant interaction effect between N-level and Vixeran treatment (p=0,8360), no separate analysis for the different N-levels has been conducted. However, the untreated control wheat had a slightly higher N-concentration than the Vixeran treated wheat in all N-levels (Figure 10).

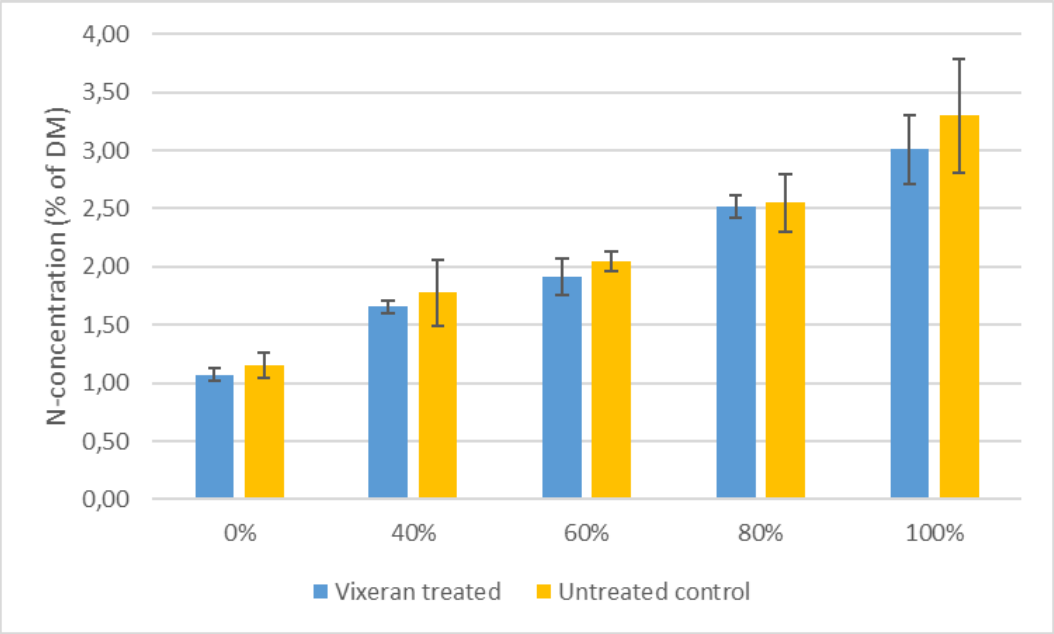

Figure 10 N- concentration (% of DM) of wheat for Vixeran treated and untreated control for different N-levels in the low temperature chamber. The different N-levles are 100%=154 kg N/ha, 80%= 123 kg N/ha, 60%= 92 kg N/ha, 40%= 62 kg N/ha, 0%= 0 kg N/ha. Bars show the average N-concentration and error bars indicate standard deviation.

4.2.3 N-accumulation in wheat

In the low temperature treatment, the results showed significant differences in N-accumulation between Vixeran treated plants and the untreated control (p=0,0121). The untreated control wheat had on average 5,90 kg higher N-uptake per hectare then the Vixeran treated wheat (Figure 11).

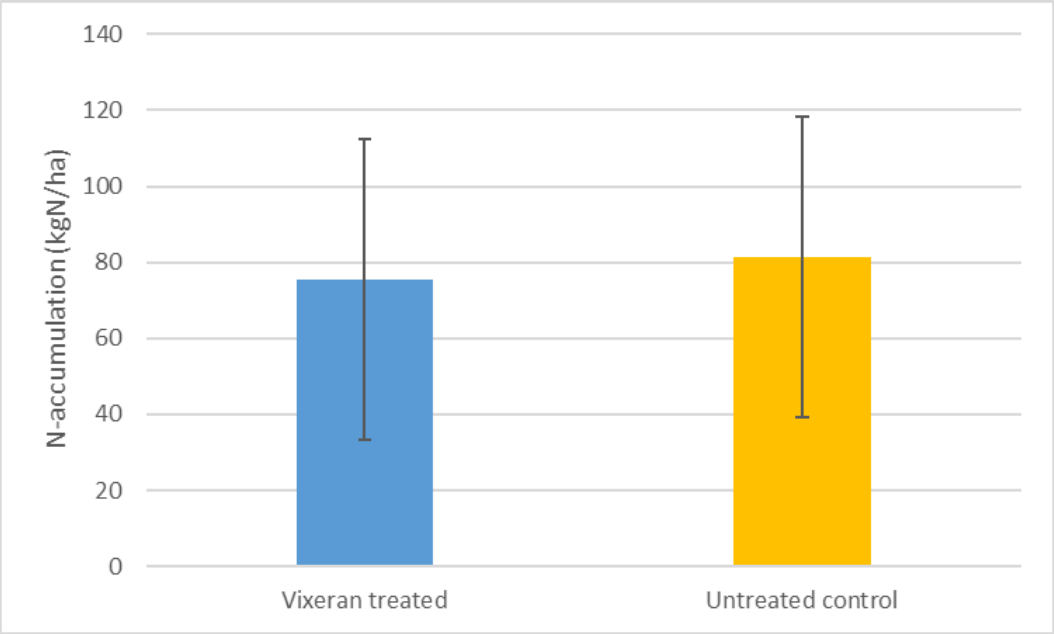

Figure 11 N- accumulation (kgN/ha) of wheat for Vixeran treated and untreated control in the low temperature chamber. Bars show an average N-uptake for all included N-levels (n=40) and error bars indicate standard deviation.

Since there was no significant interaction effect between N-level and Vixeran treatment (p=0,0820), no separate analysis for the different N-levels has been conducted. However, the Vixeran treated wheat had a slightly lower N- uptake than the control treated wheat except for the N-levels 60% and 0% (Figure 12).

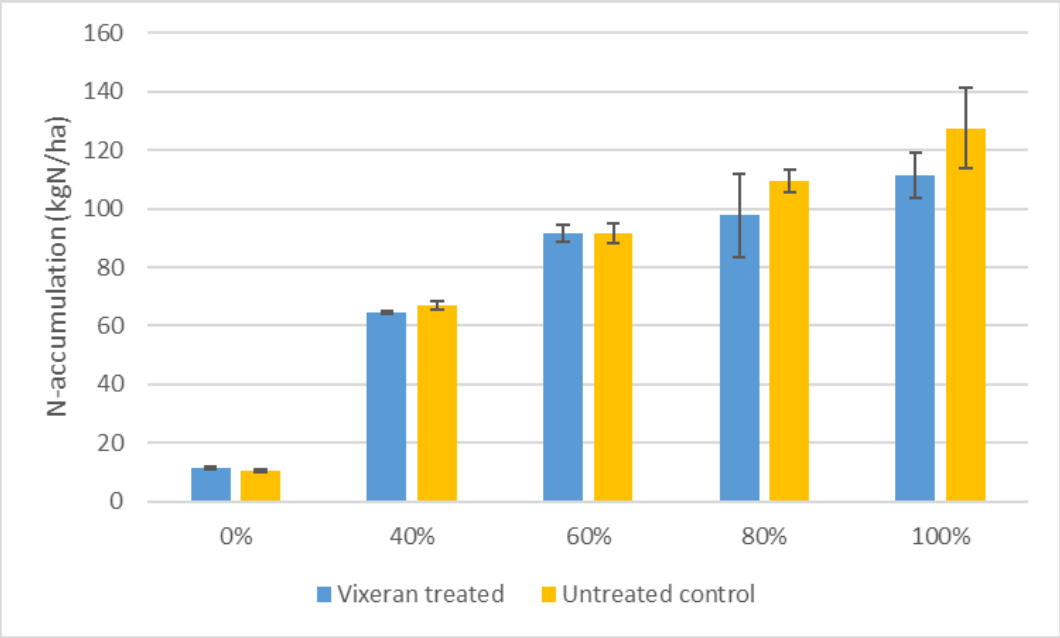

Figure 12 N- accumulation (kgN/ha) of wheat for Vixeran treated and untreated control for different N-levels in the low temperature chamber. The different N-levles are 100%=154 kg N/ha, 80%= 123 kg N/ha, 60%= 92 kg N/ha, 40%= 62 kg N/ha, 0%= 0 kg N/ha. Bars show the average N-accumulation and error bars indicate standard deviation.

5. Discussion

Nitrogen-fixing biostimulants in wheat are new types of products, and there is a need for more agronomic knowledge how these products perform under different conditions. The purpose with this study was to increase the knowledge about how Vixeran (*Azotobacter salinestris CECT 9690)* work under different conditions. Vixeran generally had a negligible effect on the dry matter yield, N-concentration, and N accumulation in the wheat crop studied. The Vixeran treatment worked different in the different temperatures where it was a positive effect of the Vixeran treatment in the high temperature and a negative effect of the Vixeran treatment in the low temperature. There was no interaction effect between N-level and Vixeran treatment in either temperatures.

5.1 Influence of temperature on the effect of Vixeran in wheat

Azotobacter salinestris have an optimal growth at around 35 ˚C, when temperatures become higher or lower than 35 ˚C the growth starts to decrease (Chennappa et al. 2017). In the present study, the low-temperature treatment had an average temperature of 13 ˚C, and the high-temperature treatment had an average temperature of 17 ˚C. Both of these temperatures was below the optimal temperature for growth of the bacteria. This indicates that both temperature treatments limited bacteria growth and colonoization and that this was most pronounced in the low temperature (Figure 11 & 12). For wheat grown in the higher temperature, the nitrogen fixation from *Azotobacter salinestris CECT 9690* probably already had begun before harvest in DC 41-43, and these 4 kg N/ha higher N-accumulation in Vixeran treated wheat can potentially be the result of this (Figure 5 & 6). In the low temperature, *Azotobacter salinestirs CECT 9690* were probably still colonizing the wheat at harvest at DC 41-43, no nitrogen fixation from the bacteria had yet occurred and the colonization process was enegy demanding at the time of harvest. This could explain why there was a higher N uptake in the untreated control in the low temperature (Figure 11). The result would

probably have been different if the wheat had been grown to maturity, such as in the study Kızılkaya (2008).

Kızılkaya (2008) conducted field trials where they tested different strains of *Azotobacter,* both non-indigenous and indigenous, and their effect on yield and N-concentration in spring wheat. The indigenous strains were found in the soil used for the trial, and the non-indigenous were not. The trial was conducted in Turkey, with average temperatures in April, May, June, and July of 9.3, 17.5, 18.5, and 21.3 °C, respectively. The soil was a clay loam with a pH value of 7,1. They saw that the indigenous strains of Azotobacter performed better concerning grain and straw yield as well as N-concentration than the non-indigenous strains of *Azotobacter*. The author mean that indigenous strains are adapted to the environment and are more compatible in these locations than non-indigenous strains. This shows that biostimulants containing microorganisms must be tested in different locations worldwide to determine their effectiveness. Even if a biostimulant works well in one location it does not automatically mean it will work the same way at other locations. This can be an explaination to the small or negative effect of the *Azotobacter salinestris CECT 9690* on N-accumulation in the current study. It is possible that the bacteria may not be adapted and competitive in the soil and to the environmental conditions used in the pot trial.

The positive effect of inoculation with *Azotobacter* on N-concentration and straw yield in the study by Kızılkaya (2008) could not be seen in the present study. In the high temperature treatment there was significant 620 kg/ha higher dry matter yield in Vixeran treated wheat compared to the untreated control (Figure 1). Considering the N-concentration, there are statistically significant 0,11 percetage points higher N- concentration in the untreated control wheat compared to the Vixeran treated wheat (Figure 3). In the low temperature treatment, there was not statistical significance, but 22 kg/ha higher dry matter yield in the untreated control compared to the Vixeran treated wheat (Figure 7). Considering the N-concentration, there is also not any statistical significance but the untreated control had 0,14 percentage points higher N-concentration than the Vixeran treated wheat (Figure 9). Both dry matter yield and N-concentration are factors in the formula to calculate the N-accumulation. The relationship between dry matter yield and N-concentration clearly affected the N-accumulation.

5.2 Influence of N-levels on the effect of Vixeran in wheat

Yanni and El-Fattah (1999) conducted field trials in Egypt where they inoculated rice seeds with *Azotobacter* and studied the effect on N in straw yield at three different N-levels. The different N- levels were 36 , 72 and 108 kg N/ha. The study showed that there was an effect of *Azotobacter* that ranged from 5 to 6 kg N/ha. It seems that the biggest effect of *Azotobacter* could be seen in the two highest N-levels. Due to the very small differences in effect of the *Azotobacter* treatment between the N-levels, around 1 kgN/ha difference, it was difficult to determine any interactrion effect between bacteria treatment and N-levels. The present study showed similar results where there were no interaction effect beween bacteria trearment and N-levels in any of the temperatures (Table 8 & 9).

Rai and Gaur (1988) conducted pot trials where they inoculated wheat seeds with *Azotobacter*. They had four different N- levels 0 kgN/ha, 40 kgN/ha, 80 kgN/ha, and 120 kgN/ha. In N-rates of 0 and 80 kg N/ha, there was a positive effect on treatment with *Azotobacter* which corresponded to 3-5 kg N/ha. However, in the N-level 120 kg N/ha, the control treatment accumulated around 4 kg N/ha more than the *Azotobacter* treated wheat (Rai & Gaur 1988). These results indicate that the bacteria fix more nitrogen in lower N- levels. In the very high N-levels, where the N-concentration is over optimal, it seems to instead be a disadvantage for the nitrogen fixation from the bacteria, since the control treatment generated a higher N-uptake. In the present study a smiliar effect can be seen in the low temperature treatment. The growth rate was relatively low in the low temperature, which led to a low N demand. This could have implied that the applied N-levels was too high which could be the reason why a negative effect of Vixeran was seen in the low temperature. Low growth and too high N-levels could explain why there was an average 6 kg N/ha higher N- accumulation in the untreated control compared to the Vixeran treated wheat in the low temperature (Figure 11). In the high temperature treatment, the growth rate was higher and therefore the N-levels were better adapted, which led to a 4 kgN/ha positive effect of Vixeran treatment (Figure 5). The interaction effect showed by Rai and Gaur (1988) that *Azotobacter* should fix more nitrogen in lower N-levels can not be seen in the present study. There were no interaction effect of N-level and bacteria treatment on N-accumulation in any of the temperature treatments (Figure 6 & 12).

5.3 Other factors that could have influenced the result

The bacteria thrive best in neutral to alkaline soil, and to grow in pH values ranging from 6-9 but has its optimal growth at pH ranging from 7,2-7,5 (Page & Shivprasad

1991). The soil from Krusenberg had a pretty low pH but was limed with the intention to raise the pH from 5,5 to 6,5. The risk here could be that the liming did not have enough time to work in the soil during the pot trial and did not increase the pH value, which could have led to that the amount of Vixeran that ended up in the soil did not have any effect on the nitrogen fixation. According to Rodriguez[2], Vixeran has a triple mode of action in the soil, root, and leaf. However, in this kind of soil condition, it set very high demands that the nitrogen fixation through the leaves actually works, as mentioned.

Kızılkaya (2008) and Mrkovac & Milic (2001) thinks that it is crucial to test different cultivars together with different strains of the bacteria to see which ones that worked best together. It is vital to have a compatible partner to have a high success rate with nitrogen-fixing biostimulants such as *Azotobacter*. One potential reason why the effect of Vixeran did not show any evident effect can be that it was a bad match between *Azotobacter salinstris CECT 9690* and the spring wheat variety Diskett.

5.4 Improvement potential

A few things would have been done differently if the experiment would have been repeated. First, the wheat should have been grown for a longer time to a later stage of development. This gives the bacteria more time to colonize the wheat and fix nitrogen. It would also been intresting to have a optimal temperature for the bacteria to see the potential of the N-fixation. Another part that would have been done differently is the pH value of the soil. It should have been ensured that the pH value was optimal before the experiment started. Finally, it would be interesting to see if different varieties react different to the Vixeran treatment. If the experiment had been redone, more varieties would have been included in the experiment.

[2] Esteban Rodriguez, Product Manager Microbiologist & Probiotics specialist, Ceres Biotics, mail 2023-01-11

6. Conclusion

The tested biostimulant Vixeran (*Azotobacter salinestris CECT 9690*) seems to have little or no effect on the N-accumulation in spring wheat. Due to different results in the different temperatures with a certain effect on N-accumulation in the high temperature and no effect in the low temperature it can not be determined that the differences in N-accumulation depend on the effect of Vixeran. The interaction effect between N-levels and Vixeran treatment were not significant which means that the different N-levels didn't play any role in the effect of Vixeran. More research is needed under field conditions to understand how the bacteria works and what leads to success and what does not. It is essential to conduct research on how *Azotobacter salinestris CECT 9690* works under different environmental conditions such as different soil types, pH and temperatures. Last, it is also important to research how the bacteria works with different wheat cultivars. If all four parameters, soil type, pH, temperature and cultivar, are optimal for the bacteria there will probably be a better effect. This study shows that more research is needed in the field to guarantee N-fixation from *Azotobacter salinestris CECT 9690.*

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Acknowledgements

I want to thank my supervisor Karin Hamner with all the help and support through my thesis. I would also like to thank Oskar Björling from Syngenta who gave me the opportunity to write about Vixeran and provided my trails with the product. Finally I want to thank Esteban Rodriguez from Ceres Biotics who has been very helpful and answerd all my questions about the bacteria.