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# Biofertilizers and the increment of tomato yield in different greenhouse systems

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**Abstract**

The increase in the world population will necessarily lead to an increased food demand. Conventional fertilizers have been used to increase crop yields, however, the intensive use of these types of products has revealed several negative impacts on the ecosystem, through the contamination of air, water, and soils.

Biofertilizers (cell-based preparations of specific microorganisms) have been identified as a promising tool for the transition to a more sustainable agricultural system, through the enhancement of food production, while contributing to soil health and ecosystem functionality.

In this work, two biofertilizers composed of two different bacterial consortia were used in order to study their effects in the production and productivity of tomato plants: biofertilizer 1, constituted by the consortium *Bacillus* + *Pseudomonas*, and biofertilizer 2, constituted by the consortium *Azospirillum* + *Pseudomonas.*

The application of these two biofertilizers were carried out in three greenhouse systems of commercial tomato production with different concentrations of total soil P and with different concentrations and compositions of soil organic matter.

In all the greenhouses, the application of both biofertilizers was effective in enhance tomato production and productivity. Even so, the different soil characteristics seem to influence the biofertilizers modes of action and consequently their effectiveness in the production and productivity of tomato.

The soil organic matter content and its C fractions were the main drivers of the biofertilizers influence on soil dynamic properties, mainly of its extracellular enzymatic activities. In its turn, the soil P content seemed to be determinant for the effectiveness of the biofertilizers in increasing plant P acquisition.

This work supports the Multiple Mechanism Theory that argues that the beneficial effects of the soil microorganisms for the plant development is not through a single but through a multiple potential mechanism that is specific to the present environmental conditions.

**Keywords:** Phosphorus solubilizing-bacteria; Soil phosphorus; Extracellular enzymes; Multiple Mechanism Theory;

**Resumo**

O aumento da população mundial e as alterações nos padrões de consumo têm vindo a aumentar a procura de alimentos. Os fertilizantes convencionais têm sido utilizados desde a Revolução Verde no sentido de aumentar a segurança alimentar. No entanto, a utilização intensiva deste tipo de produtos tem revelado vários impactos negativos no ecossistema, através da contaminação do ar, da água e dos solos.

Os fertilizantes convencionais à base de mineral de fósforo (P) são dos mais utilizados devido à importância e à limitação deste nutriente no crescimento e desenvolvimento das colheitas. A utilização deste tipo de fertilizantes tem permitido o aumento da produtividade. No entanto, a eficiência da sua utilização nos sistemas agrícolas é muito reduzida sendo que apenas uma parte do P aplicado ao solo pelo fertilizante é realmente utilizado pelas plantas devido à sua rápida conversão no solo em formas indisponíveis para as plantas. Para agravar esta situação os recursos mundiais de P com potencial para uso agrícola são limitados, o que faz com que o P disponível para fertilizante seja considerado um recurso não renovável. Por outro lado, a lixiviação e a erosão dos solos agrícolas enriquecidos em P provoca poluição e eutrofização das águas superficiais e freáticas. A presença de metais pesados nos fertilizantes minerais de P contribui também para a contaminação do solo e dos recursos hídricos. Esta questão é particularmente importante no contexto europeu, onde os solos agrícolas vão acumulando P ao longo do tempo.

Os biofertilizantes (preparações à base de células de microrganismos específicos) têm sido apontados como uma ferramenta promissora na transição para formas de agricultura mais sustentáveis, onde se reduzem os impactos ambientais da produção de alimento, contribuindo ao mesmo tempo para a saúde do solo e funcionalidade do ecossistema.

Neste trabalho utilizaram-se dois biofertilizantes compostos por dois consórcios bacterianos diferentes: biofertilizante 1, constituído por *Bacillus* + *Pseudomonas*, e biofertilizante 2, constituído por *Azospirillum* + *Pseudomonas*. Todas as estirpes de bactéria utilizadas nestes consórcios pertencem a géneros taxonómicos conhecidos pela sua capacidade de converter o P do solo de formas indisponíveis para formas disponíveis, disponibilizando-o assim para a planta. As bactérias utilizadas podem ser funcionalmente designadas como solubilizadoras de P.

A aplicação destes dois biofertilizantes foi feita em três sistemas de estufas de produção comercial de tomate com diferentes concentrações de P total do solo e com diferentes concentrações e composições da matéria orgânica. Os solos da estufa 1 e 3 eram caracterizadas por conter uma maior concentração de P total e de matéria orgânica que o solo da estufa 2. Para além disso o solo da estufa 1, ao contrário das restantes duas estufas, não tinha sido remobilizado há mais de três anos.

Uma vez que um dos problemas associados à utilização de biofertilizantes é a variabilidade da resposta das plantas, neste trabalho pretendeu-se estudar a forma como as condições reais de cultivo de tomate em estufa na zona Oeste de Portugal (a zona de maior produtividade do país) afetam a resposta do tomateiro aos biofertilizantes.

Desta forma aplicaram-se dois biofertilizantes em três sistemas de estufas diferentes para responder às seguintes questões: (1) Será que o efeito da aplicação dos biofertilizantes na produção de tomate é independente das condições das estufas? (2) Será que o mesmo biofertilizante vai mostrar modos de ação diferentes de acordo com as condições e características de cada estufa? (3) Será que os biofertilizantes melhoram a saúde do solo nas três estufas independentemente das condições iniciais? (4) Serão os biofertilizantes capazes de diminuir o P total acumulado nos solos em qualquer dos sistemas de estufas testados?

Para responder a estas questões foi criado nas três estufas diferentes um dispositivo experimental composto por três blocos iguais, onde cada bloco era composto por três parcelas que correspondem a cada um dos tratamentos: um controlo (onde não foi aplicado nenhum biofertilizante), biofertilizante 1 (onde foi aplicado o biofertilizante 1) e o biofertilizante 2 (onde foi aplicado o biofertilizante 2). Foram

definidos quatro tempos de amostragem (0, 30, 120 e 150 dias após a transplantação) onde se recolheu amostras de solo para se realizar análises no sentido de se obter uma resposta às questões mencionadas.

A resposta à primeira questão foi encontrada com base na produtividade comercial de cada tratamento por estufa. Para responder às outras perguntas foram medidas em cada tratamento por estufa, algumas atividades enzimáticas relacionadas com a atividade microbiana total do solo e com a disponibilização de nutrientes, a concentração de P total e de P disponível bem como a concentração do solo em matéria orgânica ao longo do desenvolvimento da cultura.

A utilização de qualquer dos biofertilizantes mostrou tendência para aumentar a produção de tomate nos três sistemas de estufas em todas as amostragens. O aumento da produção de tomate pela introdução dos biofertilizantes sem que isso represente um aumento na fertilização convencional pode ser interpretado como uma forma mais sustentável para aumentar a produção de alimento.

Ainda assim a eficácia dos biofertilizantes no aumento da produção de tomate diferiu consoante as características das estufas. Para o biofertilizante 1 a maior tendência para o aumento da produção foi observada em solos com concentrações mais altas de matéria orgânica composta por maiores concentrações das frações de carbono mais acessíveis à utilização pelos microrganismos do solo. Isto poderá ser devido ao facto de que uma das ações mais relevantes e eficazes do biofertilizante 1 parecer ser a estimulação da atividade de enzimas extracelulares, o que beneficiará de solos com maiores concentrações deste tipo de matéria orgânica. Por sua vez, o biofertilizante 2 parece ter sido mais eficaz em solos com menor conteúdo em P total o que poderá ter evidenciado o potencial do consórcio em promover a produção de fito-hormonas (ex: IAA) beneficiando a produtividade pela disponibilização de P e pela influência na fisiologia e morfologia da planta.

De forma resumida o teor de matéria orgânica do solo e as suas frações de carbono foram os principais fatores que influenciaram a ação biofertilizantes nas propriedades dinâmicas do solo, principalmente as atividades enzimáticas extracelulares. O menor teor de P total no solo pareceu ser determinante para a eficácia dos biofertilizantes no aumento da aquisição de P nas plantas.

Observou-se assim que existem certas características e condições do solo que vão permitir aos biofertilizantes ser mais eficazes no desenvolvimento da planta e aumento da produção. No entanto, devido ao facto de em todas as estufas ter havido uma tendência para um aumento da produção pela introdução dos biofertilizantes, aliado à observação de que os solos com biofertilizante apresentaram desempenhos diferentes nos parâmetros estudados nas diferentes estufas, poderá indicar que os biofertilizantes não têm apenas um modo de ação definido, mas, pelo contrário, um conjunto variado e diverso de mecanismos que beneficiam a planta e onde os diferentes fatores do solo vão modelar a intensidade de cada um destes mecanismos. Este trabalho suporta a teoria dos mecanismos múltiplos que defende que os microrganismos dos solos interagem com a planta através de diversos modos de ação não sendo possível definir para cada biofertilizante um único mecanismo de ação.

Para além do aumento da produtividade os biofertilizantes mostraram uma tendência para promover certos processos essenciais para o melhoramento da saúde do solo como por exemplo uma maior ciclagem de nutrientes. Ainda assim, a eficácia do melhoramento da saúde do solo pelos biofertilizantes esteve também dependente das condições presentes do solo em estudo.

A aplicação dos biofertilizantes não diminuiu o P total acumulado no solo nas três diferentes estufas entre o início e o fim do ciclo de vida da planta pelo que se concluiu que num sistema de fertirrega a constante introdução de P no solo faz com que a taxa de solubilização e fornecimento de P à planta seja menor do que a taxa de introdução deste nutriente no solo. Uma maior eficiência da solubilização e fornecimento de P à planta pela ação dos biofertilizantes poderia reduzir a quantidade de P acumulado nos solos agrícolas diminuindo os danos ambientais e económicos associados a este fenómeno, contribuindo ao mesmo tempo para um menor desperdício de P.

Mais estudos são necessários por forma a conceber biofertilizantes com níveis de eficácia mais altos e com a capacidade de produzirem efeitos benéficos em solos diversos e/ou heterogéneos. Segundo a

literatura, os biofertilizantes compostos por consórcios de microrganismos têm-se mostrado mais eficazes na promoção do desenvolvimento das plantas e na produtividade do que biofertilizantes compostos por apenas uma espécie de microrganismos. No contexto europeu será necessário perceber as diferentes características e fatores dos solos agrícolas, bem como das principais culturas produzidas, no sentido de se formarem consórcios de microrganismos que tenham capacidade de beneficiar as colheitas produzidas nos diferentes tipos de solos existentes na Europa. Desta forma os biofertilizantes serão uma peça fundamental na redução dos impactos ambientais associados à atividade agrícola.

**Palavras-Chaves:** Bactérias solubilizadoras de fósforo; Fósforo no solo; Enzimas extracelulares; Teoria dos mecanismos múltiplos

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**List of abbreviations**

| | |
|---|---|
| Al | Aluminium |
| ANOVA | Analysis of variance |
| ATP | Adenosine triphosphate |
| C | Carbon |
| Ca | Calcium |
| CAP | Common agricultural policy |
| Cd | Cadmium |
| $CO_2$ | Carbon dioxide |
| Corr. slope | Corrected Slope |
| DAT | Days after transplantation |
| Dw | Dry weight |
| D.M soil | Dry Matter soil |
| EPC | Equilibrium Solution Phosphorus Concentration |
| ESWS | Enzymatic substrate working solution |
| EU | European Union |
| Fe | Iron |
| Fw | Fresh weight |
| H | Hydrogen |
| $H_2O$ | Water |
| $H_2PO_4^-$ | Dihydrogen phosphate ion |
| $H_2SO_4$ | Sulfuric acid |
| HCl | Hydrochloric acid |
| $HCO_3^-$ | Hydroxidodioxidocarbonate |
| Hg | Mercury |
| $HPO_4^{2-}$ | Monohydrogen phosphate ion |
| IAA | Indole-3-acetic acid |
| INT | Iodotetrazolium chloride |
| INTF | Iodonitrotetrazolium formazan |
| LOI | Loss on ignition |
| M | Molar |
| Min. | Minute |
| Mo | Molybdenum |
| MES | 2-(N-Morpholino) ethanesulfonic acid |
| MU | Methylumbelliferone |
| N | Nitrogen |

| | |
|---|---|
| $N_2$ | Dinitrogen |
| O | Oxygen |
| O.M | Organic matter |
| P | Phosphorus |
| $P_2O_5$ | Phosphorus pentoxide |
| $P_i$ | Inorganic Phosphorus |
| $P_o$ | Organic Phosphorus |
| PAE | Phosphorus acquisition efficiency |
| Pb | Lead |
| PGPR | Plant growth-promoting rhizobacteria |
| PSB | Phosphate solubilizing bacteria |
| PVA | Polyvinyl alcohol |
| Ra | Radium |
| RL | Regression line |
| Rpm | Rotations per minute |
| s | second |
| SD | Standard deviation |
| U | Uranium |

## 1. Introduction

### *1.1. Phosphorus: Essential nutrient for life*

Phosphorus (P) is one of the most essential nutrients for the biological processes associated with life. This nutrient is involved in energy storage (in the form of ATP molecules), genetic information (9% of the nucleic acid mass is P), formation of the phospholipid membrane and in the photosynthesis reaction (in photosynthetic organisms), among others (Smit et al., 2009; Elser, 2012).

In agriculture, this nutrient, in its orthophosphate form ($H_2PO_4^-$ and $HPO_4^{2-}$), is essential for crop development. However, P acts as a limiting yield factor of productivity in many agricultural soils (Elser, 2012). About 30-40% of the crop yield of the world arable land is limited by P availability, thus, it is considered essential to supply P to the soil solution, in order to meet the nutritional needs of plants (Vance et al., 2003; Pierzynski et al., 2005). In agricultural soils, P inputs are mainly via P fertilizers. One of the major problems concerning P fertilization is that about 70 - 90% of the applied inorganic P fertilizer is made unavailable for plant uptake in the short term, representing a reduced fertilization efficiency (Walpola and Yoon, 2012; Ziadi et al., 2013).

In the rhizosphere, symbiotic and coordinated relationships between plants and soil microorganisms developed several types of mechanisms to convert the soil unavailable P to an available form. These symbiotic relationships have been studied by the soil scientists in order to improve the P availability status in the soil and contribute to a more sustainable agriculture.

### *1.2. Phosphorus conventional fertilization and soil health: The environmental price for food production*

Conventional agriculture uses large inputs of inorganic fertilizers to boost crop productivity. The increasingly world population, with forecasts pointing to 10 billion people in 2050, will consequently increase the world food demand which will require the increase of food production, making conventional agriculture resort to conventional fertilizers (Fróna et al., 2019). There are, however, limits to increasing crop yields through the use of conventional fertilizers (Gilland, 2002). Additionally, the use of this products can represent environmental and human health threats (Louekari et al., 2000).

The soil health definition has been changing over time, as more knowledge has been acquired about the soil and its processes, creating an increasingly holistic definition where soil health is related to the health of other ecosystem compartments. According to FAO, soil health can be defined as: "the continued capacity of the soil to function as a vital living system, within ecosystem and land-use boundaries, to sustain biological productivity, promote the quality of air and water environments, and maintain plant, animal, and human health" (Doran and Zeiss, 2000; FAO, 2008).

The need of P for the good development of the crops makes P fertilizers mandatory use in almost all conventional agricultural systems (Fageria and Oliveira, 2014). Most of the P fertilizers have high concentrations of heavy metals in its composition, originated from both the parent rock and the reagents and materials used in the fertilizer design, which may lead to heavy metals accumulation in the soils, negatively affecting the soil health, and the normal functioning of the soil microbiota, and all the constituents of the food chain. In its turn, runoff and leaching from agricultural soils can contaminate water resources, through the accumulation of these heavy metals in the water (Dissanayake and Chandrajith, 2009). From the heavy metals found in P fertilizers, cadmium (Cd), uranium (U), mercury (Hg), lead (Pb), molybdenum (Mo) and radium (Ra) are the most common (Guzmán, et al., 2006; Oyedele et al., 2006; Takeda et al., 2006; Dissanayake and Chandrajith, 2009; Cakmak et al., 2010). Some of these heavy metals are related with great environmental and/or human health negative impacts (Louekari et al., 2000; Patra and Sharma, 2000; Schnug and Lottermoser, 2013).

The soil dehydrogenase activity, a proxy for the soil microbial activity, has been reported as being lower in heavily P fertilized soils, which may be due to the potential toxic effects driven by the accumulation of heavy metals (Beauregard et al., 2010; Piotrowska-Dlufosz and Wilczewski, 2014). In addition, it was suggested that the P fertilization can change soil pH, organic C and N content, soil cation exchange capacity, and other soil characteristics and composition, which can negatively impact the native soil microbiota (Cakmak et al., 2010).

In freshwater and marine systems, P is often a primary limiting nutrient. The enrichment of P in these systems, through runoff and leaching from P fertilized agricultural soils, will lead to eutrophication events, i.e., a fast growth and proliferation of algae or other water plants, which in turn will contribute to the implementation of anoxia conditions, as well as modifications in the biodiversity structure (Bennet et al., 2001; Ngatia and Taylor, 2018). These events negatively affect water quality and biodiversity (Ngatia and Taylor, 2018).

In addition to the environmental impacts, these events also represent economic impacts. The degeneration of water resources results in investments for the treatment of the water damaged by eutrophication, it represents a loss of aquatic biodiversity with consequences for the fishing industry and may pose a threat to tourism and recreational activities (Ngatia and Taylor, 2018). Additionally, and concerning to the toxicity of agricultural soils, it may represent a decrease in the crops production (Shahid et al., 2015).

Since P fertilizers are necessary to maximize crop yields, effective agricultural management is necessary in order to combine crop maximization with reduced environmental and soil health impacts associated with P fertilization.

### *1.3. Phosphorus: A non-renewable resource*

The Green Revolution, led by advances in the agronomic field, allowed the increasing of crops yield and a decreasing in the world starvation, through the fertilizer use (Hazel, 2002).

Phosphorus is the 11$^{th}$ most abundant element in the earth's crust, representing 0.12% of its weight, but just a small percentage of world's total P is present in enough high concentrations to be mined (Schröder et al., 2010; Samreen and Kausar, 2019). Furthermore, P is one of the least biologically available major nutrients (Lavelle et al., 2005).

Phosphorus comes mainly from mined rock phosphate, a non-renewable resource formed 10-15 million years ago. About 90% of the world's rock phosphate is used in agriculture, more specifically in the fertilizer production field (Cordell et al, 2009; Prud'Homme, 2010). The continued phosphate extraction and consumption is leading to a decrease of the existing rock phosphate deposits (Samreen and Kausar, 2019). This is of great concern as no substitute for P in agriculture has yet been found and the use of P based fertilizers is of essential importance for food production (U.S. Geological Survey, 2019). Furthermore, the expected growing world population, reaching 10 billion of people by 2050, will result in an increase demand for food production and consequently an increase demand for fertilizers (Schröder et al., 2010; De Ridder et al., 2012; Calicioglu et al., 2019). The growing demand for P fertilizer, added to the fact that rock phosphate is a non-renewable resource, has led to speculations about the amount of the remaining rock phosphate reserves. Due to a diversity of factors, there is no clear idea about the longevity of this resource, however, some hypotheses point out that the price of rock phosphate will peak in 30-300 years, when the reserves will start to decrease, and the demand will outstrip supply (Van Kauwenbergh, 2010; Cordell and White, 2013). This is of great concern since the decrease in rock phosphate reserves availability will tend to increase its prices as well as the P fertilizers, which in its turn will increase the crops prices associated with P fertilizer use, jeopardizing the access to food for the most disadvantaged populations (Cordell and White, 2011). In addition, the fact that only a few countries have exploitable P reserves, such as Morocco, China, Algeria, Syria, South Africa, and

Russia, makes this problem more complex, since most countries in the world are dependent on P supply, which poses a threat to food production autonomy and sovereignty in these countries (Schröder et al., 2010). The consequences to food sovereignty represented by the P monopoly from these few countries can be illustrated when China, in 2008, increased rock phosphate export tariffs up to 135% in order to protect its domestic supplies. The demand for this resource led to a market response where the P price spike in 800%. Thus, geopolitical tensions can disrupt the supply of P to the countries that are dependent of the import of P (Schröder et al., 2010; De Ridder et al. 2012).

Concerning to European Union (EU), most of the imported P comes from Russia, North Africa, and Middle East. The political instability in Middle East and in North Africa, as well as the unstable relations between Russia and the EU, can disrupt the phosphorus supply to EU. The EU Common Agricultural Policy (CAP) has as one of its pillars to ensure security and autonomy on food production. The EU's dependence on other countries for P supply represents a threat to this goal (De Ridder et al., 2012).

There is an urgent need to find solutions that reduce the EU's external dependence on the supply of P by increasing European food autonomy and sovereignty. Most of the European soils contains more P accumulated than the recommended concentration (Barberis et al., 1995; MacDonald et al., 2011). The development of tools that would allow the access of the accumulated P in the soil making it available for crop uptake would be an efficient strategy for a higher food production autonomy. Greater knowledge about the behaviour of P in the soil, as well as the use of biofertilizers are presented as promising solution.

### *1.4. Phosphorus cycle*

Soil minerals are able to interact with P in the soil, and dependent of the soil pH, different soil minerals are more or less likely to bind to P, making it unavailable for plants (Penn and Camberato, 2019). Additionally, soil organic matter can also interact with P making it unavailable for plant uptake as well (Stewart and Tiessen, 1987). Despite all this diversity of soil P forms, the total soil P can be classified in two major different fractions: organic P ($P_o$) and inorganic P ($P_i$). $P_o$ is into a C-H bond and it is present in biogenic compounds, like organic matter and microbial biomass, while $P_i$ doesn't need a biogenic process to be formed. These different P fractions are related to different processes: $P_o$ is controlled by biological constraints and the $P_i$ is controlled by the soil chemical equilibrium (Kruse et al. 2015; Weihrauch and Opp, 2018). $P_o$ can even be adsorbed to the soil minerals. These two pools interact to replenish the available P in the soil solution (Weihrauch and Opp, 2018).

Plants are only capable of taking up $P_i$ as monovalent or divalent orthophosphates anions present in the soil solution, $H_2PO_4^-$ and $HPO_4^{2-}$, respectively, known as available P (Richardson, 2001; Owen et al., 2015). However, soil microorganisms can also take up some forms of low molecular weight $P_o$, like glycerophosphate, nucleotide phosphates, and sugar phosphates (Turner et al., 2005). To reach the available form, unavailable $P_i$ and $P_o$ suffers a solubilization and mineralization processes, respectively, to be converted in the orthophosphate form and consequently be available in the ecosystem. From the total soil P, just a tiny percentage (less than 1%) is in an available form for plant and microorganism's uptake, and it is estimated that from the total soil P, only 10% can interact with the plants and microorganisms (Frossard et al., 2000). This is of particular relevance since the continuous addition of P in agriculture soils leads to the accumulation of this nutrient in the soil without being available for plant or microorganisms.

As a consequence of the high reactivity of P ions, P solubility in the soil solution is a highly sensitive process since P ions can become quickly unavailable for plant and microorganism's uptake, through sorption processes. The adsorption (or just "sorption") process it is one of the most common reaction that leads to the unavailability of P for plant and microorganism's uptake. This process occurs when the soluble orthophosphate ions are removed from the soil solution by getting attached to the surface of the

inorganic and organic soil particles, without penetrating in its structure, making it unavailable for the plant and microorganisms (Sims et al., 2005; Kerr et al.,2011; Weihrauch and Opp, 2018).
The adsorption degree, i.e., the strength of the P ion adsorption to a particle, will influence its bioavailability, being more likely to enter in the soil solution the lower the strength of the P adsorption to a soil particle (Yang and Post, 2011; Costa et al., 2016a).
The desorption process is the opposite process where an adsorbed P ion gets detached from the soil matrix surface being able to be incorporated in the soil solution (Sims et al., 2005; Kerr et al., 2011; Weihrauch and Opp, 2018). In order to be clearer, the process of binding P to inorganic compounds will be called adsorption while its release from these compounds will be called desorption, on the other hand, the binding of P to organic compounds will be called immobilization while the release of these compounds will be called mineralization.

A scheme of the P cycle is shown in Figure 1.1, where it is represented the different soil processes concerning to the available and unavailable P forms.

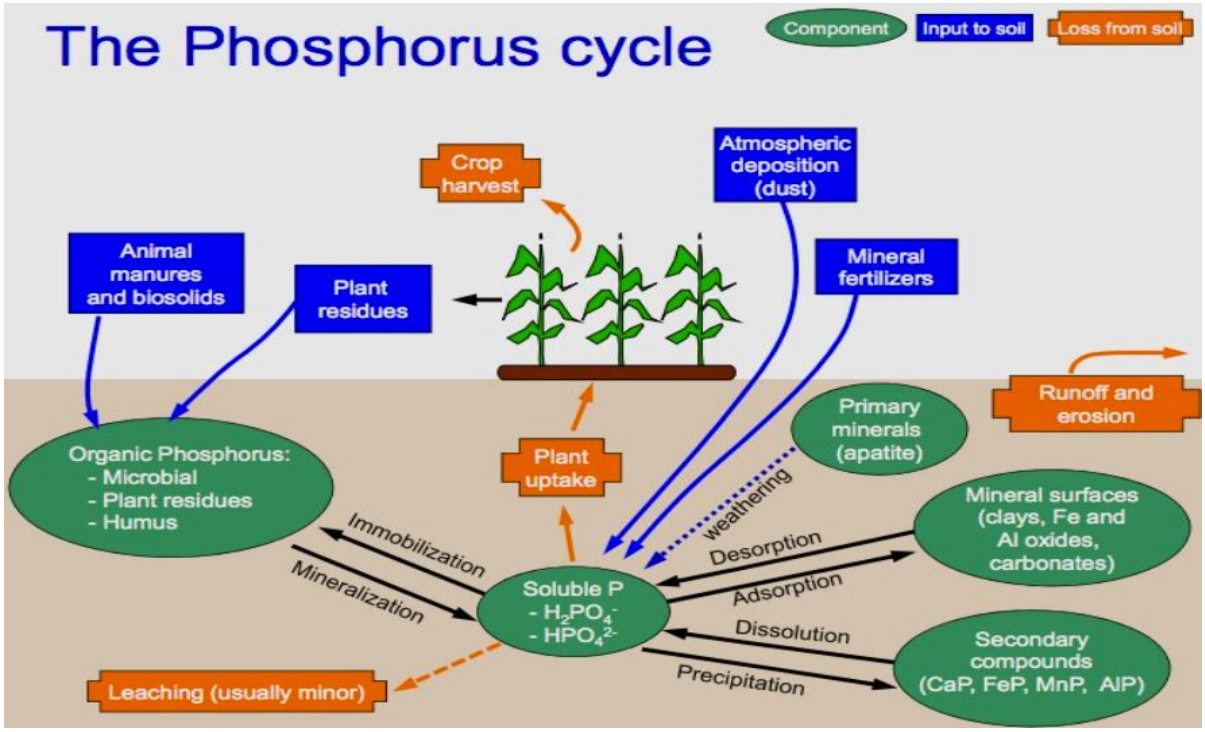



**Figure 1. 1:** Representation of the soil P cycle. Soil P can be made available and unavailable through diverse forms. P inputs to the soil is represented by mineral fertilizers application, atmospheric deposition of dust as well as plant residues and animal manures that have P in its composition. The inputted P will suffer diverse reactions in the soil by organic and inorganic compounds. The immobilization of orthophosphates ($H_2PO_4^-$ and $HPO_4^{2-}$) by organic compounds, and consequently prevention of P consumption by the plant, is represented here by microorganisms orthophosphate consumption and consequent integration of the consumed P in its structure and by the P present in the plant residues and hummus composition as well as its immobilization in the surface of these compounds. In order to get available (or soluble) P from this organic sources it is needed a mineralization process which is commonly driven by soil enzymes, particularly phosphatases. Concerning to the soil inorganic compounds the adsorption of orthophosphates in the surface of soil minerals (such as Fe and Al oxides, carbonates, and clay minerals) is one of the most relevant soil processes in making P soil unavailable to plant uptake. The desorption process is driven by organic acids, rhizosphere acidification and by the soil chemical equilibrium, and consists of the solubilization of the previous adsorbed orthophosphate, making it available for plant acquisition. The precipitation process is when available P gets precipitated with the soil minerals, turning into a solid form and consequently unavailable for plant acquisition. The dissolution of these precipitated minerals can make the previous unavailable P into an orthophosphate form and thus able to be in the soil solution and be acquired by the plants. Runoff and erosion as well as the leaching processes are responsible for the lost of P in the soil, since the soil P is transported to another site, where it can reach hydrological resources and contribute for eutrophication phenomenon. (Source: https://en.wikipedia.org/wiki/Phosphorus_cycle).

### *1.4.1. Organic P pool*

Soil organic P can represent 20 - 80% of the total soil P, depending on the soil organic matter content (Dalai, 1977).

Organic matter performs a key role in P supply since it is the major source of $P_o$ in the majority of soils, providing available P for ecosystem functioning (Cross, 1995). Regarding this P pool there are two essential processes: immobilization and mineralization. Immobilization occurs when P from soil solution gets bound to organic compounds and becomes unavailable for plants and microorganisms. Mineralization in turn is the release of the P linked to the organic matter to the soil solution by enzymatic hydrolysis, driven by phosphatases, making it available for plant and microbial uptake. The main $P_o$ sources present in the soil are phospholipids, inositol phosphate, nucleic acids, and sugar-based phosphates (Leytem et al., 2002). The inositol phosphates in general are the most predominant fraction of the $P_o$, however, due to its structure and strong bounds it is hydrolysis resistant, being needed the presence of phytase, a specific phosphatase, in order to degrade this molecule. The most common $P_o$ forms where orthophosphate is acquired are the $P_o$ monoester and diester forms, which are linked to organic matter by a phosphomonoester and a phosphodiester bond, respectively (Dodd and Sharpley, 2015; McLaren et al, 2016). The microbial P is an important pool of organic P since the amount of the microbially mediated turnover of organic P represents a substantial proportion of the annual plant P demands. From the soil microorganisms, bacteria and fungi are important contributors to this P pool. Additionally, microorganisms play a crucial role in the P availability by assimilating $P_i$, preventing it from being adsorbed, or participating in other fixation processes by the soil matrix, which can become available for plants when the microbial residues are decomposed (Tiessen et al., 1994; Khan and Joergensen, 2009; Turan et al.,2012).

Therefore, mineralization process is dependent on microbial activity (McGill and Cole, 1981; Oehl et al., 2004; Jones and Oburger, 2010; Kruse et al. 2015).

### *1.4.2. Inorganic P pool*

Soil $P_i$ is often adsorbed as phosphate anion to the mineral's surfaces such as Fe and Al oxides, Al silicates and Ca carbonates and clay minerals or be in a precipitated form (Raychaudhury, 1976; Sanyal and De Datta 1991).

The sorption and desorption processes are regulated by the equilibrium between the sorbed and desorbed P, known as "Equilibrium Solution Phosphorus Concentration" (EPC). At equilibrium, the rate between P adsorption and desorption is the same (Sims et al., 2005; Kerr et al.,2011; Weihrauch and Opp, 2018). This P equilibrium is often disturbed by plant and microorganism uptake (Ryan and Rashid, 2005).

The sorption capacity is dependent on many soil factors, pH being one of the most impactful. Soil pH determines the dominant electrical surfaces charges that are present in the soil. In more acidic soil surfaces electrical charges from the soil particles will be predominantly positive and in more alkaline conditions particles surfaces will be predominantly negative. Therefore, in alkaline conditions, phosphate anions will experience a repulsion effect, resulting in a decrease in the soil P sorption ability. The opposite effect happens in acidic conditions where the P sorption ability increases since the dominant positive charges in soil have an attractive effect for soil phosphate anions (Barber, 1995; Gérard, 2016; Weihrauch and Opp, 2018). However, depending on the pH different cations can form complexes with P: in alkaline pH, phosphate become unavailable through binding of $Ca^{2+}$, forming Ca-P precipitates and in acidic soils there is an increasing of Fe and Al solubility which allows orthophosphates to bind to $Fe^{3+}$ and $Al^{3+}$ forming Fe-P and Al-P precipitates, respectively. These precipitates are poorly soluble and not available for plant and microorganism uptake (Sanyal and De

Datta, 1991; Satyaprakash et al., 2017; Kalayu, 2019). Within the soil constituents clay minerals, Fe/Al oxides and humic substances are the ones with a major impact in the sorption processes since they are the most reactive fraction (Gérard, 2016).

### *1.4.3. Biological P acquisition: Soil microorganisms and the enhancement of P availability*

Given the great relevance of P for the normal development of the ecosystem, several strategies have evolved to obtain this often-unavailable nutrient by the plant and soil microorganisms. In conditions of P deficiency, the plant enhances the transcription of genes involved in certain P deficiency response mechanisms such as: morphological changes of the root, phosphatases and organic acids production, production of high affinity P transporters, among others (Liu, 1998; Dodd and Sharpley, 2015; Chen et al, 2018). In addition to these mechanisms, the plant takes advantage of soil microorganisms to overcome these nutritional limitations. Thus, we can observe the establishment of symbiotic relations between soil biota as a strategy to obtain P. Plant growth promoting rhizobacteria (PGPR) are bacteria present in the rhizosphere which are involved in symbiotic relationships with plant, enhancing plant growth and development (Parewa et al., 2014). Plant roots contribute directly to plant P nutrition through uptake of the available P. In addition, the root system also serves the propose of plant nutrition by producing and delivering chemoattractant to soil microorganisms that are able to create symbiotic relationships, making nutrients available for plant acquisition, in this case, the conversion of unavailable organic and inorganic P to available forms (Feng et al., 2018). The microbial inoculation in the soil have been described to play an influence in the relationship between the plant and P, promoting a greater plant P acquisition and use efficiency, benefiting its productivity (Pacheco et al., 2021).

One common type of PGPR used in the agriculture are the Phosphorus Solubilizing Bacteria (PSB) (Khan et al., 2007). *Bacillus* and *Pseudomonas* are the bacteria genera which contains the most P solubilizing bacteria species known so far (Rodríguez and Fraga, 1999).

Distinct PSB may act through distinct mechanisms, the most relevant and better understood are the described below.

#### *1.4.3.1. Rhizosphere acidification trough the excretion of organic acids*

One of the most common mechanisms used by plants and soil microorganisms to obtain available P is the release of organic acids, a type of negative charged carboxylates (Hocking, 2001).

Under P deficiency, plants increase the acidification of the rhizosphere through $H^+$-efflux and organic acids releasing, which promotes the local availability of P while increasing the growth of plant growth-promoting microorganisms (PGPM) (Imas et al., 1997; Gupta Sood, 2003; Ahmed et al., 2017). This microbial recruitment is of great importance since organic acids released by soil microorganisms are considered as the major mechanism of mineral phosphate solubilization. Soil microorganisms produce a wide range of organic acids in limited nutrients conditions, contributing to the nutrient's availability (Jones, 1998; Rodríguez and Fraga, 1999).

The main processes involved in the demobilization of inorganic P by organic acids are the ligand exchange reaction, the formation of metal-organic complexes, and the dissolution of low soluble minerals (Gypser and Freese, 2020). Concerning to the ligand exchange reaction, the negative charge of the organic acids promotes its adsorption to the positively charged soil particles, forming metal complexes in the same binding site where P is adsorbed, displacing the previous adsorbed phosphorus. The formation of metal-organic complexes is another effective strategy, since the organic anions compete with available P for the same adsorption sites of the soil metal cations, preventing future P adsorption at these sites. In its turn a ligand enhanced dissolution is a process where the adsorption and/or chelation by organic anions to Fe or Al oxides in a different sorption site than the one where P is

adsorbed, releases the previous occluded phosphorus due to the weakening of the mineral bonds, by changing the mineral's electric surface potential, putting it more negative and consequently affecting the binding strength of the adsorbed phosphate anions (Bowden et al., 1980; Akhtar et al., 2009; Oburger et al., 2010; Wang and Lambers, 2019).

The rhizosphere acidification in P stressed conditions is also a common strategy to available P acquisition and it is related with organic acid production. The rhizosphere acidification enhances the P solubility, increasing this nutrient uptake by the plants (Jones, 1998; Ryan et al., 2001; Sas et al., 2001; Lei et al., 2015). The extrusion of $H^+$ to the soil solution will enhance the protonation of the phosphates which will decrease its negative charge making it less vulnerable to be adsorbed by the soil cations (Jones and Oburger, 2010). Contrary to what was previously thought, it is not the released organic acids that affects the rhizosphere acidification. The releasing of organic anions is coordinated with the proton's extrusion to the rhizosphere, the latter being the process responsible for soil acidification (Dakora and Phillips, 2002; Jones and Oburger, 2010; Wang and Lambers, 2019). In order to maintain electrical neutrality, the balance between cations and anions will control the extrusion of $H^+$ or $HCO_3^-$, decreasing or increasing the soil pH, respectively (Dakora and Phillips, 2002; Hinsinger et al., 2003). In this case the releasing of protons to the medium is a response to balance the increase in negative charges by organic anions release. Thus, organic acids do not act as acids but instead as their conjugate base (Jones and Oburger, 2010).

In Figure 1.2 it is schematized the main mechanisms played by organic acids in the availability of soil P.

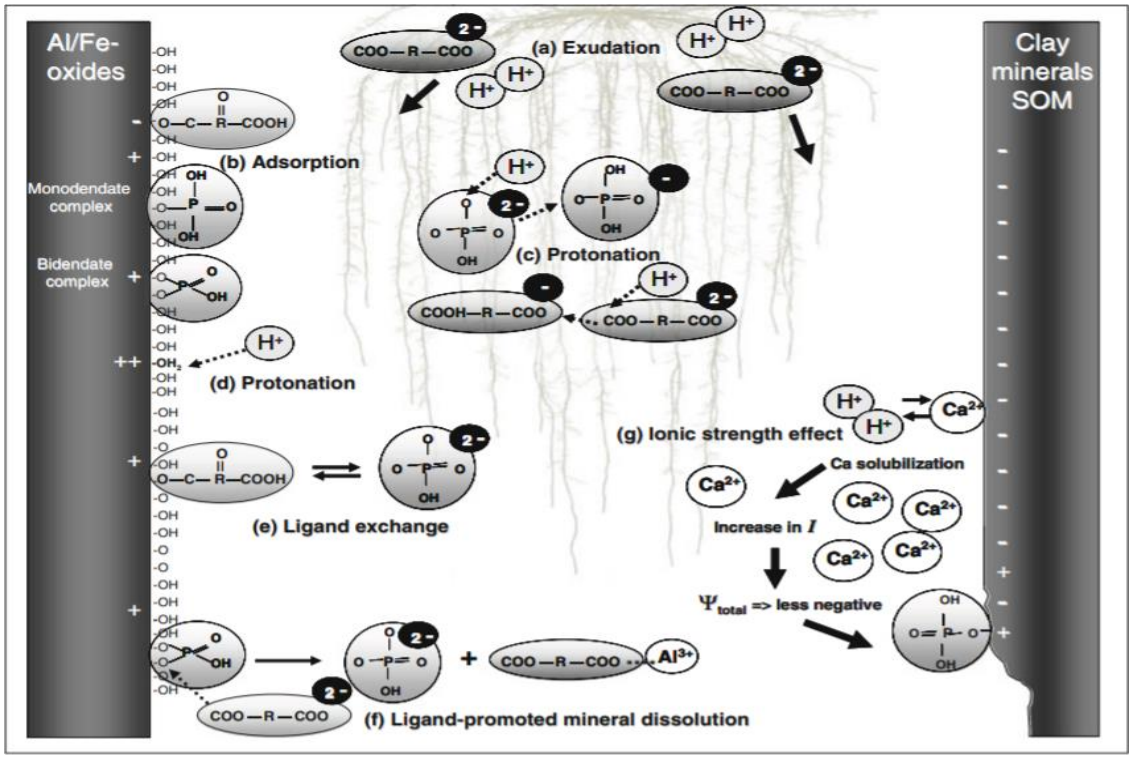



**Figure 1. 2:** Organic acids processes in turning P from an unavailable to an available form.; (a) the exudation of organic acids from plant or soil microorganisms origin.; (b) the adsorption of organic acids to the mineral surface will decrease the positive surface charge and in consequence the bond between the phosphate and mineral surface gets weaker.; (c) the protonation, i.e., the connection between protons and anions, like phosphates and organic acids, will decrease anions negative charge, which will, in consequence, decrease the phosphate and organic acids adsorption affinity to the positive surface of soil minerals.; (d) in this type of protonation the proton gets adsorbed to the mineral surface increasing the adsorption affinity of anions. This can increase the adsorbed phosphates but increases as well the organic anions adsorption.; (e) the ligand exchange reaction is when there is a substitution between the adsorbed phosphate and the organic acid, releasing the previous adsorbed phosphate.; (f) the ligand-promoted mineral dissolution is when there is a detachment of a complex between an organic acid-mineral releasing the phosphate.; (g) the ionic strength effect is more common in negative surfaces compounds as soil organic matter and clay minerals. The replacement of cations bounded to these compounds by protons increase the cations concentration in the soil

solution and the surface electrical potential turns less negative, which increases the phosphate retention in the soil matrix; (Source: Oburger et al., 2010).

#### *1.4.3.2. Phosphatase production and P mineralization*

The soil biota, fungi, bacteria, plants, among others, can solubilize P through the production of a wide range of phosphatases, releasing P ions to the soil (Margalef et al., 2017).

Comparing to the bulk soil, rhizosphere has a higher microbial and enzymatic activity. The greater activity by roots and soil microorganisms in rhizosphere is translated in a greater enzyme exudation. Thus, there is a greater amount and diversity of phosphatases in the rhizosphere than in the bulk soil (Tarafdar and Jungk, 1987; Fraser et al., 2017).

Phosphatases can catalyse its substrate in acidic or alkaline pH conditions. Acidic phosphatases show optimal catalytic activity in acidic pH ranges. For alkaline phosphatases, the alkaline pH values are associated with a better catalytic performance. Alkaline phosphatases are exclusively produced by the soil microbiota while the acid phosphatases are produced by both soil microbiota and the plant. Organic P from the soil organic matter is the main substrate for the phosphatases activity (Acuña et al., 2016).

Two different phosphatases play a crucial role for orthophosphate acquisition. When phosphate is into a phosphomonoester bond a phosphomonoesterase, is needed to release the phosphate group (Turner and Haygarth, 2005b). In its turn when phosphate is linked by a phosphodiester bond, it will require a phosphodiesterase and a phosphomonoesterase: first a phosphodiester molecule is hydrolysed by a phosphodiesterase resulting in a phosphomonoester which in turn is cleaved by a phosphomonoesterase freeing an orthophosphate ready for plant and microorganism's uptake (Turner and Haygarth, 2005b).

The available P concentration seems to regulate the phosphatase production: there is a higher phosphatase production when there is a significant decrease on the available P concentration and in its turn the phosphatase activity decreases when abundant available P is available (Rodríguez and Fraga, 1999; Baldwin et al., 2001; Wasaki et al., 2003).

Total P comprises all the P forms present in the soil, including those forms that are not compatible as a substrate for phosphatases like the recalcitrant occluded forms of P. Since just a small fraction of the organic P pool is able to release free orthophosphates through phosphatase reactions, the total P pool is a poor predictor of the phosphatase activity. Soils with high organic P pool were positively correlated with potential acid phosphatase activity and thus, being the organic P pool a natural substrate for phosphatase, it is an indicator of the system capacity to obtain P through phosphatase activity (Margalef et al., 2017).

#### *1.4.3.3. Phytohormones involved in P acquisition*

Plant hormones (phytohormones) or plant growth regulators are organic compounds that have an impact in the lifecycle and plant physiological processes. These organic compounds are related with plant development, cell division processes, root and stem elongation/inhibition, development of buds and branches and in chlorophyll production, among others (Wong et al., 2015).

There are five main groups of phytohormones: cytokinin, auxin, gibberellin, ethylene, and abscisic acid. Plants with high tolerant genotypes to P deficiency showed higher concentrations of auxin and ethylene under low P availability suggesting the existence of a signalling network of hormones in response to P deficiency (Nadira et al., 2016).

Despite the plant production of phytohormones, soil microbiota is also a great reservoir of these organic compounds which can be supplied to the plants and enhance its growth through plant-PGPR symbiotic interactions (Grappelli and Rossi, 1981; Chandra et al.,2015). PGPR can have a positive

impact in nutrient uptake by stimulating the increasing of the root length and root surface area through the releasing of phytohormones (Vessey, 2003).

Under low P availability the plant root system is highly branched compared to non-deprived P conditions. Thus, the low P concentration impacts the structure and morphology of the root system and induces a phenotype typical of P scarcity. The increasing of the root to shoot biomass ratio, i.e., increasing of the nutrient-absorbing surface and the topsoil exploration by the root systems, through the enhancement of the root hairs and increasing the lateral root system over the primary root system, is one common strategy to increase P consumption that is dependent of the phytohormones action. Since the macronutrients are more abundant in the topsoil, the increase of the lateral roots is an effective strategy for the enhancement of P uptake (López-Bucio,2002; Ticconi and Abel, 2004; Pérez-Torres et al., 2008; Jindo et al., 2016).

Indole-3-acetic acid (IAA) is one common auxin type which is released by PGPR that stimulates the increase of the root system. The supply of IAA to the plant in low P conditions leads to a reduced primary root while the density of the lateral roots gets higher due to the increasing of the lateral root number (Nacry et al., 2005).

Under low P availability the root system is more sensible to the presence of auxin in the medium regarding the length of the primary root and the density of the lateral roots. However, in sufficient P conditions the auxin sensitivity is lower, thus, the phytohormones production and supply to the plant doesn't trigger the stimulation of the responses by the root system related to the scarcity of P (López-Bucio, 2002; Nacry et al., 2005). Actually, it was reported that this higher auxin sensitivity in low P conditions by the plant was due to higher transcriptions of the genes encoding IAA, auxin receptors, and auxin response factors that activates the transcription of the auxin responsive genes involved in the plant growth and development (Pérez-Torres et al., 2008; Nadira et al., 2016). *Azospirillum* species are described to produce indole-3-acetic acid and other phytohormones at low P availability, being important PGPR to overcome the negative impacts in the plant due to the shortage of P (Fukami et al., 2018).

Despite auxin, ethylene also plays a role in the plant adaptation responses to P deficiency conditions. At low P availability there is the enhancement of the transcription of genes related to ethylene biosynthesis and the plant increase its ethylene sensitivity by changes in the expression of genes that are related to the ethylene signalling pathway. The formation of adventitious roots is a plant response to low P conditions and is dependent on the ethylene action (Borch et al., 1999; Zhang et al., 2003; Kim et al., 2008; Song and Liu, 2015). At low P concentration levels, ethylene can stimulate the lateral root initiation. However, it seems that the stimulatory effect of ethylene of the lateral root initiation happens only below a defined threshold level of ethylene concentration, since above this threshold the ethylene concentrations inhibit this adaptation response (Stepanova et al., 2005; Ivanchenko et al., 2008). At low P availability, ethylene can stimulate auxin biosynthesis, like IAA, which contributes to the growth of lateral roots and root hairs through the auxin response mechanism (Stepanova et al., 2005; Ivanchenko et al., 2008). Ethylene was also observed to be related and needed for the expression of the genes responsible for the transduction of high affinity phosphate transporters in the roots at low P conditions. Additionally, at low P conditions it was reported that the ethylene also plays a role in the regulation of acid phosphatase activity (Lei et al., 2011; Li et al., 2011).

Thus, understanding the mechanisms associated with available P acquisition through the interactions between soil microorganisms and plant, may reveal possible strategies for the transition to a more sustainable agriculture.

### *1.5. Biofertilizers: The key for sustainable agriculture*

Biofertilizers are microbial inoculant preparations formed by live or latent cells from specific microorganisms species that are applied to seed, plant surfaces, or soil, colonizing the rhizosphere, the surfaces or interior of the plant, with the goal to significantly increased growth and yield in the crop production through symbiotic relationships between the plant and the microbiome associated with the plant. This goal can be reached by diverse modes of action (Vessey, 2003; Hazarika and Ansari, 2007; Srivastava and Ngullie, 2009; Chandra et al., 2015).

The relation between plant growth promoting rhizobacteria (PGPR) and plant can occur at three levels: rhizosphere, endosphere or phyllosphere. At the rhizosphere level, PGPR colonize this area, in general attaching to the plant root surface. At the endophytic level, PGPR are actually within the apoplastic spaces of the host plants. This type of bacteria develops its symbiotic relations with the host on the interior of the plant cells but never rich the cytoplasm (Lugtenberg et al., 2001; Vessey, 2003; Chandra et al., 2015). At the phyllosphere level the introduced microorganisms colonize the foliar surface (Giri and Pati, 2004). The biofertilizers tested in this work were designed to acted at the rhizosphere level and therefore more attention will be paid to this biofertilizer-plant interaction.

For a successful plant colonization by the PGPR it is needed that the introduced PGPR can successfully compete with the indigenous microbiota for a stable colonization (De Weger et al., 1995).

Depending on the type of microorganisms present in the applied biofertilizer, different modes of action may be involved to achieve the expected results. The most common direct modes of actions driven by biofertilizers are the increase in the nutrient availability, as for example through P solubilization and/or mineralization; the production of growth-promoting substances, like phytohormones; production of vitamins; the enhancement of other beneficial symbioses with the host microbiome and finally the combination of different modes of action (Vessey, 2003; Hazarika and Ansari, 2007; Srivastava and Ngullie, 2009; Chandra et al. 2015).

Concerning to the enhancement of available P acquisition to the plant and rhizosphere microbiome, the biofertilizers should have P solubilizing microorganisms (PSM) in its composition. One of the main modes of action by biofertilizers composed by PSM is the solubilization and mineralization of unavailable soil P through organic acids and phosphatase release (Vessey, 2003). The effectiveness of the biofertilizers for P acquisition for the plants will depend on diverse factors. Dependent on the soil conditions, many different forms of phosphate can be in the soil, affecting the effectiveness of available P acquisition by the specific PSM species on the biofertilizers. Thus, the P acquisition will vary with the different phosphate nature, general soil characteristics and types of phosphorus-solubilizing microorganisms present in the biofertilizer (Saxena and Sharma, 2003).

The microorganism strains used in the biofertilizers tested in this work are from the *Bacillus*, *Pseudomonas* and *Azospirillum* genera, which are among the most used genera in the production of biofertilizers. The main characteristic of *Bacillus* and *Pseudomonas* is the increase of the availability of P for the plant, being one of the most effective PSM's, while *Azospirillum* is more related with the fixation of atmospheric $N_2$, as well as the production of phytohormones, like IAA, important for increased plant development and resilience under nutrition deficient conditions (Ramasamy et al., 2011; Bhattacharjee and Dey, 2014; Fukami et al., 2018; Aloo et al., 2019).

The biofertilizers composed of more than one species of microorganisms, i.e., mixed inoculations, proved to be more efficient in providing nutrients and thus, contributing more for the plant development than biofertilizers made up of only one microbial species. The higher diversity of modes of action and compounds produced in the mixed inoculation, due to the presence of more diverse microbial species, is the main explanation of its greater effects in the plant when compared with single species formulations (Thomloudi et al., 2019).

Apart from promoting plant growth, the biofertilizer also contributes to enhance soil health. This feature contrasts with conventional fertilization methods associated with soil degradation and low efficiency in the use of natural resources, as described in 1.2. section.

In general, the use of biofertilizers do not represent any environmental pollution risk, not threatening the environmental sustainability (Khan et al., 2007). Additionally, when the biofertilizer application is effective in contributing for the good plant development, can effectively reduce the conventional fertilizer application, thus reducing the negative effects associated with the use of this type of fertilizers (Dakora and Phillips, 2002; Yazdani et al., 2009). Finally, since the biofertilizers modes of action enhance the soil microbial activity and promotes beneficial symbiotic relationships and ecological interactions, and consequently the promotion of crucial soil processes, as nutrient cycling, it contributes for a more diverse and resilient soil environment, increasing the soil health as well as its long-term sustainability (Bhardwaj et al., 2014).

### *1.6. Soil quality bioindicators*

The physical, chemical, and biological soil properties can be used as indicators in order to access the soil quality (Adetunji et al., 2017). Soil quality indicators are of great importance in the sense that it is urgent to evaluate and monitor the impact of specific soil managements and farming practices on soil health (Bending et al., 2002; Alvear et al., 2005). Here, we will focus on the biological indicators or just "bioindicators" and its importance in assessing the impact of different agriculture managements in order to improve agriculture into a more sustainable activity.

A bioindicator is used to address the quality status of all or just a part of an ecosystem and it can be an organism, a part of an organism, a product of an organism, a collection of organisms or biological processes (Killham and Staddon, 2002). A good bioindicator should capture the complexity of the assessed ecosystem although it must be simply enough in order to be easily and frequently monitored. The challenge is to use a set of usable bioindicators that are representative of all the ecosystem components while respect the criteria described above (Dale and Beyeler, 2001; Dale et al., 2008).

One of the most common used bioindicators to access soil information are the soil enzymatic activities. Soil enzymes are intrinsically correlated with soil organic matter, soil chemical and physical properties and with microbial activity and biomass (Dick et al., 1997). Thus, the measurement of its activity is being classified as a suitable bioindicator for the soil status and quality, since it responds rapidly to soil changes, is easily measurable, is strongly related with the soil biodiversity and plays a role in a lot of important soil processes and reactions, such as nutrient cycling, transformation of the native organic matter, catalysation of most biological reactions, etc (Bending et al., 2002; Alvear et al., 2005; Utobo and Tewari, 2015). It is important to notice, however, that enzyme assays illustrate the potential enzyme activity of the soil tested and not the *in-situ* activity since this assays are developed under a strict set of conditions like pH, temperature and substrate concentration which will be different in the natural environment conditions (Dick et al., 1997).

While accessing the soil quality through the analysis of enzyme activity, it is necessary to consider that monitoring one single enzyme activity doesn't result in an accurate soil quality assessment. This is because one single enzyme that is responsible for catalysing only one particular reaction cannot be representative of the whole soil microbiological activity or the whole soil nutritional status due to the high diversity of microorganisms, substrates, and processes in the soil system. The soil fertility, soil quality and soil microbial processes are the result of a diverse set of different enzymatic reactions. A diverse set of enzymatic activities should be selected to be monitored in order to have a suitable representation of the diversity of the metabolic soil processes (Nannipieri et al., 2012).

In this work, enzymatic activities were the most used bioindicators in order to understand the effects of biofertilizers application in different greenhouse systems, specifically the soil dehydrogenase, β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase activities.

Dehydrogenase is an intracellular enzyme that plays a role in the microbial respiration chain which can be used as a bioindicator of the overall soil microbial activity (Piotrowska-Dlufosz and Wilczewski, 2014). This enzyme activity is associated with soil organic biological oxidation by transferring $H^+$ ions from organic to inorganic compounds. Dehydrogenase activity thus, reflect the metabolic capacity of the soil and it is proportional to the active microbial biomass in the soil. Therefore, the dehydrogenase activity has been pointed out as a good bioindicator of the microbial oxidative activities of the soil (Zhang et al., 2010).

The extracellular enzyme activities of β-xylosidase and β-glucosidase were selected since are considered as key enzymes in the processes of obtaining C. In its turn, *N*-acetylglucosaminidase and phosphatase were selected due to its key role in the processes leading to release of inorganic nitrogen and phosphorus, respectively. These enzymes catalyse the terminal reactions that produce assimilable products from the main C, N and P sources present in the soil (Sinsabaugh et al. 2008).

Cellulose is the most abundant polymer in the biosphere, and it is the major component of plants biomass. It is considered the main carbohydrate and carbon source for the soil microorganisms and herbivorous animals (Stone, 2001; Klemm et al., 2005; Štursová et al., 2012). The degradation process of cellulose is driven by an enzyme complex called cellulase. Cellulase is constituted by three different enzymes that breaks down cellulose β-1,4-glycosidic bonds: endo- β-1,4-D-glucanase, exo- β-1,4-D-glucanase and β-glucosidase, being the β-glucosidase the enzyme that catalyses the last reaction to obtain glucose. The synergistical work between this three enzymes can increase the rate of the cellulose hydrolysis significantly while each enzyme alone is not able to completely degrade the cellulose complex (Xi et al., 2013).

Hemicellulose, in general, is the second most abundant polymer in the plant biomass. Xylan is one of the most common types of hemicellulose (Nair et al., 2016; Bhardwaj et al., 2019). Xylan is a polymer made of xylose linked by β-1,4-glycosidic bonds. The degradation of the hemicellulose requires, as cellulose, an enzymatic complex called hemicellulase. The hemicellulase responsible for xylan degradation is called xylanase and is composed by endo-1,4- β-D-xylanase, α-glucuronidase, acetylxylanesterase, α-L-arabinofuranosidases, *p*-coumaric esterase, ferulic acid esterase and β-D-xylosidases. The degradation of xylan will result in xylose monosaccharides (Linton and Greenaway, 2004; Bhardwaj et al., 2019). β-xylosidase participates in the xylan degradation through xylooligosacharides hydrolyse, resulting in the xylose production (Margolles-Clark, 1996). Glucose is considered to be the preferred C source of soil microorganisms where xylose is only consumed as an alternative C source, when glucose is scarce in the soil (Sievert et al., 2017). Thus, it can be considered that glucose is a more labile and easily consumed C source whereas xylose can be considered to be a more recalcitrant C source, since its acquisition is more complex.

*N*-acetylglucosaminidase in its turn it is associated with obtaining N from chitin and peptydoglycan hydrolysis through the removal of the terminal *N*-acetylglucosamine. It is considered an important enzyme for N acquisition from organic N pools (Sinsabaugh et al., 2008; Su et al., 2016).

Finally, phosphatases are related with P acquisition by organic compounds (Tarafdar and Claassen, 1988).

The extracellular enzymes are part of the main mechanisms releasing nutrients in the soil solution through the degradation of more complex organic compounds. When there is a specific nutrient limitation, there is an increase in the enzymatic production in order to acquire the limiting nutrient (Allison and Vitousek, 2005).

The stoichiometry between the different enzymatic activities for nutrient availability can reflect the nutrient stoichiometric demand and nutrient constraints of the plant and rhizosphere microorganisms,

since these stoichiometries are related to resource availability and microbial and plant nutrient demand. The stoichiometry of the enzymatic activities expresses the relative abundance of enzymatic activity which reflects the nutritional needs of the soil system and the abundance of the available nutrients. There is the perspective that the microorganisms allocate its resources reserves in order to acquire the most limiting resources. Thus, in more N or P limiting environments it is expected that the microorganisms will potentially increase the N or P-acquiring enzymes, respectively. Through the stoichiometric enzymatic activities ratios, it is possible to understand where the plant and microorganisms are investing more concerning to the nutrients acquisition to meet its nutritional needs (Sinsabaugh et al., 2009; Peng and Wang, 2016; Zhang et al., 2019).

### *1.7. Objectives and strategies*

The growing world population will demand more sustainable food production management in order to avoid environmental and economic damage. Thus, this work will focus on testing two biofertilizers consortia in three different *Solanum lycopersicum* (tomato) producing greenhouses systems in order to understand if its application will be effective in increase the tomato production from the three studied greenhouses, without an increase in conventional fertilization. We will explore the possible modes of action of biofertilizers that benefit the development of plants as well as its impacts in the soil health.

This work was made in *Solanum lycopersicum* producing greenhouses due to the relevance of this species in the world food. This specie has an annual production around 122.9 million tonne, being the second most consumed vegetable in the world. Tomato consumption provides nutrients, antioxidants, vitamins, and other important components for human health (Dorais et al., 2008).

This work was done to answer the following questions:

(1) Is the effect of biofertilizer application on tomato production independent of the greenhouse conditions?

(2) Will the same biofertilizer show different modes of action according to the conditions of each greenhouse?

(3) Do biofertilizers improve soil health in the three greenhouses, regardless of the initial conditions?

(4) Will biofertilizers be able to decrease the total P accumulated in the soil in any of the tested greenhouse systems?

In order to answer the above questions, two biofertilizers were applied in three different greenhouses : greenhouse 1 (no-tillage regime, higher total P than greenhouse 2 but lower total P than greenhouse 3 and similar soil organic matter content than greenhouse 3); greenhouse 2 (tillage regime, lower total P and a lower soil organic matter content than greenhouse 1 and 3); and greenhouse 3 (tillage regime, higher total P than greenhouse 2 and greenhouse 1 and a similar soil organic matter content than greenhouse 1). In each greenhouse a complete randomized block with three replicate was done. Each block was divided into three equal plots: a control site (no biofertilizer application), biofertilizer 1 site (where it was applied the biofertilizer 1) and biofertilizer 2 site (where it was applied the biofertilizer 2). Four sampling times were defined where it was performed soil samplings: at 0 days after transplantation (DAT), 30 DAT, 120 DAT and 150 DAT.

The answer to the first question was found based on the commercial productivity of each treatment per greenhouse. To answer the other questions, it was measured some enzymatic activities related to the soil total microbial activity and to the turnover of nutrients, the concentration of total P and available P as well as the concentration of soil in organic matter throughout the development of the crop in each treatment of the three different greenhouses.

## 2. Materials and Methods

### *2.1. Greenhouses characterization and experimental design*

#### *2.1.1. Site description and greenhouse characterization*

The study site was located at Torres Vedras, west Portugal (39°07'03.8"N 9°21'54.7"W) and the sampling time occurred between the middle of January until the middle of July 2020. The study system consisted of three greenhouses for commercial production of tomato monocultures, namely greenhouse 1, 2 and 3. The soil texture of each greenhouse was determined by the laboratory "A2 – Análises Quimicas" using the manual field method which resulted in a sandy texture for all the three greenhouses.

All the greenhouses produced *Solanum lycopersicum*, however, each greenhouse was planted with a different variety: greenhouse 1 was planted with *Solanum lycopersicum var "*Coração de Boi", greenhouse 2 with *Solanum lycopersicum* var. "Chucha" and greenhouse 3 with *Solanum lycopersicum* var. "Salada". Each greenhouse was composed by three equal blocks that will be characterized in section 2.1.2.

Greenhouse 1 didn't suffer tillage for three years, contrary to greenhouse 2 and 3 that were characterized by tillage management before starting the planting season, according to the information provided by the farmer. In the tillage greenhouses, i.e., greenhouse 2 and 3, debris from old plants were incorporated into the soil before the planting season, contrarily to the no-tillage greenhouse, i.e., greenhouse 1, where no organic material has been incorporated since the last soil remobilization.

Before the beginning of the planting season and for each greenhouse, a composite soil sample was made by taking 3 random samples of the bulk soil in each block, followed by a mixing between all the different block soil samples in order to determine the percentage of organic matter, total P and available P of the soil from the three different greenhouses. The methods to determine those characteristics are described in 2.3. and 2.4. section from this chapter, respectively.

All the three greenhouses soils had a neutral pH value: 7.0; 6.9 and 7.0 for greenhouse 1, 2 and 3 soils, respectively, determined through potentiometry by "A2– Análises Quimicas" laboratory. The total soil nitrogen concentration was also similar in all greenhouses: 0.18%; 0.16% and 0.18% in greenhouse 1, 2 and 3 soils, respectively, and was determined through catarometry by the "A2 – Análises Quimicas" laboratory. The main characteristics of the three greenhouses are characterized in Table 2.1.

**Table 2. 1:** Total organic matter, total phosphorus, total nitrogen, pH and soil management from the different greenhouses

| **Green house** | **Tomato variety** | **Organic Matter (%)** | **Total P (**g/kg) | **Total N (%)** | **pH** | **Soil Management** |
|---|---|---|---|---|---|---|
| 1 | "Coração de boi" | 3.95 | 15.6 | 0.18 | 7.0 | No-Tillage |
| 2 | "Chucha" | 3.07 | 6.1 | 0.16 | 6.9 | Tillage |
| 3 | "Salada" | 3.87 | 18.5 | 0.18 | 7.0 | Tillage |

In this way, greenhouse 1 was characterized by a no-tillage management for 3 years, with an initial total P concentration of about 16 g P / kg of dry soil weight, with an initial soil organic matter content around 3.95%. The greenhouse 2, was characterized by being a system with intensive soil tillage, with the lowest initial total P concentration of the three studied greenhouse systems, around 6 g P / kg dry weight of soil, and with the lower soil organic matter content from the three greenhouses. Finally, greenhouse 3 was characterized by intensive soil tillage management, with the highest initial soil total P concentration of the studied greenhouses, around 19 g of P / kg dry weight of soil, and with a soil organic matter content similar to that of the greenhouse 1.

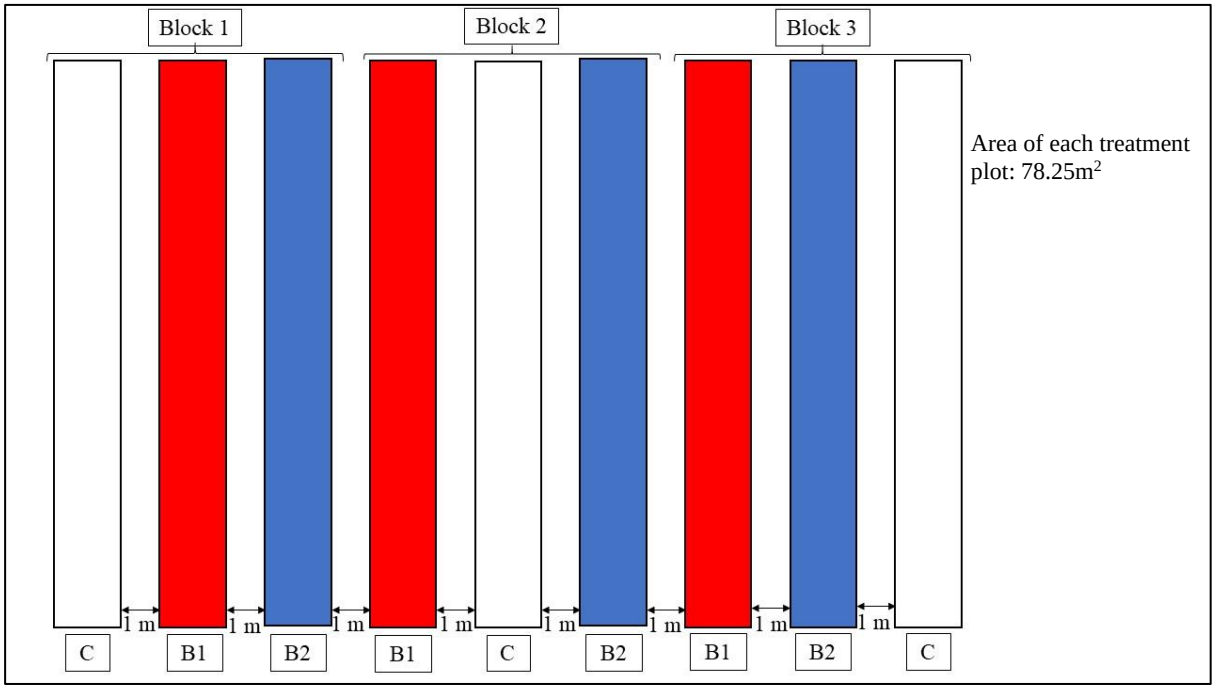
According to the farmer, greenhouse 2 would be a newer greenhouse with lower organic content due to a lower organic matter introduction into the soil. Visibly, this greenhouse had a less dark soil than the other greenhouses (no data shown), which may suggest a lower concentration of organic matter. Even so, the recent introduction of organic matter by the farmer may have increased the concentration of organic matter, even though it is suggested that this organic matter was not properly incorporated into the soil.

### *2.1.2. Study design, sampling times and biofertilizer application*

The design for all the greenhouses was a complete randomized block with 3 replicates. Each block was equally divided in three plots, one plot per treatment: control, where none biofertilizer was inoculated, biofertilizer 1, where the biofertilizer 1 was inoculated and biofertilizer 2, where biofertilizer 2 was inoculated. The experimental scheme of the different greenhouses is characterized in Figure 2.1.

Biofertilizer 1 was a biofertilizer composed by two strains from the *Bacillus* and *Pseudomonas* genera, and biofertilizer 2 was a biofertilizer composed by two strains from the *Azospirillum* and *Pseudomonas* genera. Both biofertilizers were developed by the start-up *Soilvitae*. The experimental design from greenhouse 1 was slightly different from the other greenhouses. While in greenhouse 1 each block was composed by three agricultural ridges, one per treatment, in the other two greenhouses each agricultural ridge was considered a block since it was divided in three equal plots, one per different treatment. In greenhouse 1 each agricultural ridge (and thus, each treatment plot) had an area of 78.25 $m^2$. In its turn, greenhouse 2 and 3 blocks had an area of 23.43 $m^2$, 7.81 $m^2$ per treatment plot. The blocks of all greenhouses were about 1 meter apart. Greenhouse 1 was a long-term assay from the *Soilvitae* start-up with the goal to study the effects of these two biofertilizers in the soil enzymatic activity as well as in the tomato plant production. In its turn, in greenhouse 2 and 3 this was the first time that an assay with the aim of studying the effect of these two biofertilizers in the tomato production was set up.

a)



**(b)**

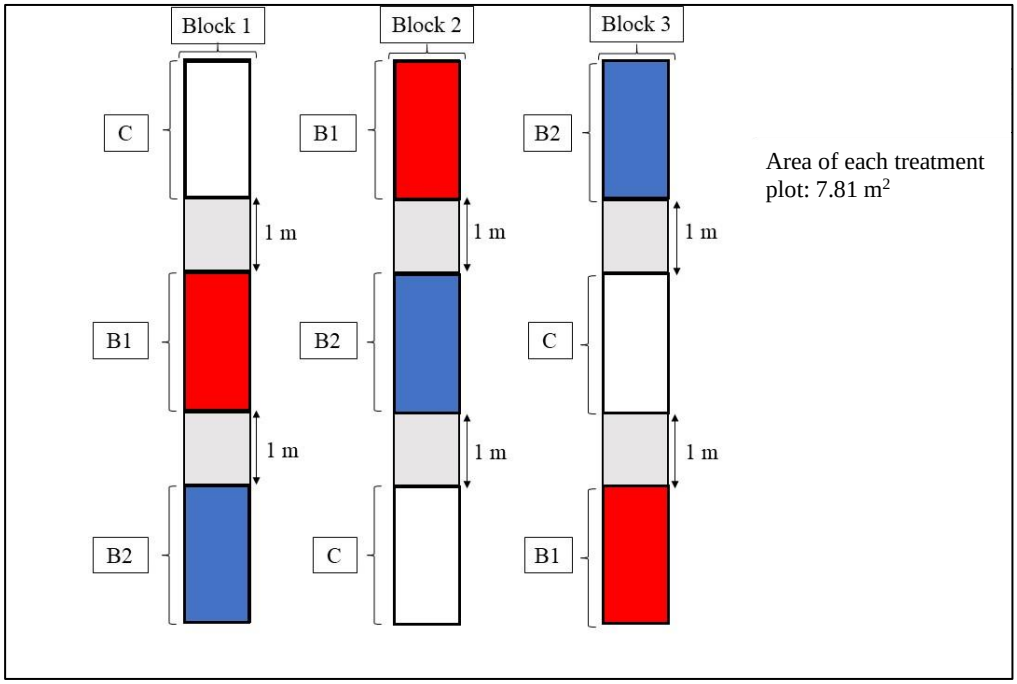



**Figure 2. 1**: (a) Greenhouse 1 experimental design: Each treatment plot of each block had an area of 78.25 $m^2$; (b) Greenhouse 2 and 3 experimental design: Each treatment plot of each block had an area of 7.81 $m^2$. In all the greenhouses, all the blocks were about 1 m apart; C-Control, B1- Biofertilizer 1, B2- Biofertilizer 2.

Four soil sampling times were designated for the three greenhouses: 0 days after transplantation (DAT), which corresponded to the time just before transplantation, i.e., bulk soil; 30 DAT, which was the time where the tomato plants started flowering; 120 DAT, due to the pandemic situation of covid-19 it was only possible to take samples at this time and not in the advance flowering as it was originally planned; and finally, at 150 DAT. Due to the different tomato plant genotypes, at 120 and 150 DAT sampling times, there was slightly differences in the phenological stages of the different greenhouses plants. In greenhouse 1 almost all the tomato production was harvested at 120 DAT where the plants were in a more advanced phenological stage than in the other greenhouses. Thus, for greenhouse 1 no harvest sample was made at 150 DAT, since, at this sampling time, the plants were entering in the end of production stage. However, in greenhouse 1 at 120 DAT it was possible to collect a representative sample of the total tomato yield produced by the plants. In greenhouse 2 at 120 DAT the plants were in the beginning of the fruit ripening stage, where it was possible to realize the first harvesting sample. Concerning to the 150 DAT sampling time, the greenhouse 2 plants were in the advanced fruit ripening stage, which allowed us to do the second harvesting sample. These two harvested samples represented almost all the tomatoes produced in greenhouse 2, thus, the sum of this two harvests were a good representation of the total tomato produced in this greenhouse. In greenhouse 3, and since this plant variety is grafted, i.e., for each root system four stems are formed, there was a delay in fruit formation. The delay in fruit production in grafted plants is commonly observed due to the fact that the grafting process causes stress on the plant, delaying the flower production and consequently the fruit production. In addition, the delay in the production is also explained since only one root system is in charge to allocate the required biomass for the formation of the four stems, which represents a delay in the plant development when comparing to non-grafted plants (Khah et al., 2006; Mourão et al., 2017). Thereby, at 120 DAT the plant was in the early stages of the fruit ripening, however it was possible to perform the first harvested sample. At 150 DAT in this greenhouse the plant was still in the mid-production stage, and thus it was not possible to have a representative sample of the total tomato produced by the plants. Nevertheless, it was possible to quantify the impact of the biofertilizers inoculation in the initial

fruit production. Again, due to the covid-19 pandemic, it was not possible to monitor this greenhouse until the end of production.

The soil samples were taken in the soil area in contact with the root in order to have a sample of the rhizosphere except at 0 DAT when there was still no rhizosphere present. For 0 DAT the bulk soil samples were taken from the place where the plants would be transplanted. In each treatment plot of each block, three sites were defined for the soil samples. Thus, for each treatment plot in each block, soil sampling was made up of all the three soil samples of the three defined locations. The collected soil samples were saved in plastic bags and were split in two parts: one half was stored at 4ºC for enzymatic analyses where the other half was store at room temperature for organic matter, total and available P, and texture analysis. Three biofertilizer applications (5L/ha per application) were applied in each greenhouse: the first application was at 0 DAT in the hole where the plant would be placed, the second application was at 15 DAT and the third application was at 30 DAT. The different tasks in each sampling time for the three greenhouses are outlined in Figure 2.2, however there are slightly differences in each greenhouse concerning to the Figure 2.2. In greenhouse 1 at 120 DAT the plants were in the middle of the ripening fruit stage and at 150 DAT there was no harvest sampling since the plants were at the end of the production stage. In greenhouse 2 at 150 DAT the plants were at the advanced fruit ripening stage.

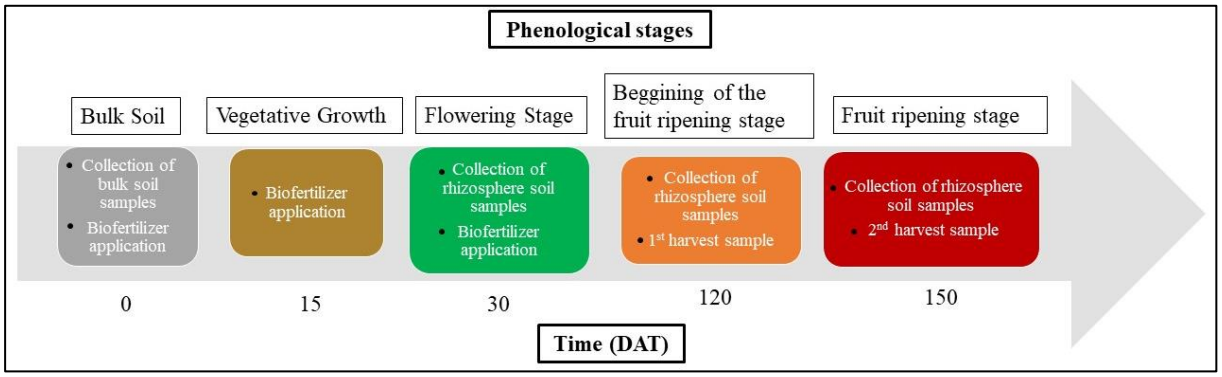



**Figure 2. 2:** Representation of the different tasks developed in the different sampling times and plant phenological stages in the three greenhouses. Slightly differences were found in greenhouse 1 were at 150 DAT the plants were already in the end of the production and thus no harvest sample was made. At 150 DAT the plants in greenhouse 2 were near to reach its final production and the greenhouse 3 plants were still in the middle of its production.

### *2.1.3. Farming practices*

All the greenhouses were characterized by the production of tomato monoculture for several consecutive years and by conventional agricultural fertilization. Conventional fertilizers were added to the soil by a drip-fertigation system with the concentrations indicated in Table 2.2.

**Table 2. 2:** Added nutrients (mg) per L of water in all three studied greenhouses.

| **Nutrients** | **Concentration (mg/L of water)** |
|---|---|
| N | 152 |
| $P_2O_5$ | 20 |
| $K_2O$ | 348 |
| MgO | 20 |
| CaO | 273 |
| $SO_3$ | 317 |

Before 30 DAT and between 120 and 150 DAT it was applied organic matter from Ferbio brand in every greenhouse however the farmer did not record the quantity supplied to the soil, yet all greenhouses received similar quantities of this product.

*2.2. Extracellular enzymatic potential activity*

In order to evaluate the extracellular enzymatic potential activities, the sampled soils were analysed by a fluorometric assay based on Marx et al. (2001). Four soil enzymes were tested by the microplate fluorometric assay using 4-methylumbelliferone (MU) as the fluorescent compound. The selected soil enzymes were β-xylosidase, β-glucosidase, *N*-acetylglucosaminidase and phosphatase.

*2.2.1. Enzymatic substrates*

All the used substrates were from Sigma-Aldrich namely: 4-Methylumbelliferyl-β-D-xylopyranoside (Xyl), 4-Methylumbelliferyl *N*-acetyl-β-D-glucosaminide (Nag), 4-Methylumbelliferyl β-D-glucopyranoside (Gls) and 4-Methylumbelliferyl phosphate (Pho) in order to follow β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase potential activities, respectively.

The enzymatic substrates were prepared based on the article Marx et al. (2001). Thus, enzymatic substrate stock solution was made to a concentration of 10 mM where 1:10 of the volume was pre-dissolved in methoxyethanol and 9:10 of the volume was dissolved in deionized and sterilized $H_2O$, except for Pho which was all dissolved in deionized and sterilized $H_2O$. Due to their sensitivity to light, the stocking solutions were kept protected from light at 4ºC and used within a maximum period of 48 hours. The enzymatic substrate working solutions at a concentration of 2mM were obtained through the dilution of the enzymatic substrate stock solutions with 0.1 M pH 7.8 2-[*N*-Morpholino] ethanesulfonic acid (MES buffer).

*2.2.2. Standard methylumbelliferone solution*

Methylumbelliferone (MU) standard stock solutions were needed to draw fluorescence calibration curves: 0.0176 g of MU was diluted in 10 mL of methanol to obtained 10 mL of standard MU stock solution at a concentration of 10 mM. To obtain the standard MU working solution, 0.1 ml from standard MU stock solution was diluted in 9.9 mL of 0.1 M MES buffer at pH 7.8, in order to obtain 10 ml at 100 µM of standard MU working solution.

*2.2.3. Soil sample suspension*

Soil sample suspensions were prepared for all the different soil samples taken from 0 DAT to 150 DAT. Soil sample suspensions were prepared by mixing 5 g (fresh weight) of each of the different collected soil samples with 50 mL of sterile and deionized $H_2O$ in a sterile plastic container. The soil sample suspension was shaken vigorously by hand for about 30 s., followed by a sonication bath for 120 s with an intensity of 90 kHz. The goal of the sonication process is to free the extracellular enzymes, which are attached to soil colloids and other types of enzyme-soil particles complex, to the soil sample suspension (Qin et al., 2013).

*2.2.4. Microplate setup*

The 96 well-microplates setups were based on the original paper Marx et al. 2001. The four enzymatic potential activities were tested for each of the soil samples. For each of the studied enzyme activity, three well-replicates were made for the detection of the fluorescence of the soil sample suspension being tested. Thus, for each well-replicate, it was added 50 µL of soil sample suspension, 50 µL of 0.1 M MES buffer and 100 µL of the enzymatic substrate working solution.

Each soil sample had a quenched standard which was made by adding 50 µL of each soil sample suspension to 6 microplate wells, then it was added 150, 130, 120, 110, 100 and 70 µL of 0.1M MES pH 7.8 buffer for the 1st, 2nd,3rd, 4th, 5th and 6th well, respectively. Finally, it was added 0, 20, 30, 40,50 and 80 µl of 100µM standard MU working solution for the 1st,2nd,3rd,4th,5th, and 6th well, respectively. Thus, regarding to the quenched standard, for the 1st,2nd,3rd,4th,5th, and 6th well the concentration of MU was 0, 2000, 3000, 4000, 5000 and 8000 pmol, respectively. The quenched standard will perform a calibration curve in order to link a fluorescence measurement to a MU concentration. Additionally, it will act as a quenched correction, which is of great important since the different soil turbidity and soil phenolics from the different soil sample suspensions can perform a quenched effect that will have an impact in the fluorescence intensity of MU (Marx. et al., 2001).

A control for the enzymatic substrate working solution was also done. For this, three replicates of each enzymatic substrate working solution was made. For each well it was added 50 µL sterilized and deionized water, 50 µL of 0.1 M pH 7.8 MES buffer and 100 µL of an enzymatic substrate working solution.

Finally, per assay was also made a MU standard control which consisted in the addition of 50 µL deionized and sterilized water in 6 wells, followed by the addition of 150, 130, 120, 110, 100, and 70 µL of 0.1M pH 7.8 MES buffer on the 1st,2nd,3rd,4th, 5th, and 6th well, respectively, and the addition of 0, 20, 30, 40,50 and 80 µL of 100µM standard MU working solution for the 1st,2nd,3rd,4th,5th, and 6th well, respectively. This standard MU control was needed to compare if different standard MU working solutions were similar. The microplate set up is schematized in Figure 2.3.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 50 µl of soil sample suspension + 50 µl of MES + 100 µl Xyl working solution | 50 µl of soil sample suspension + 50 µl of MES + 100 µl Xyl working solution | 50 µl of soil sample suspension + 50 µl of MES + 100 µl Xyl working solution | 50 µl of soil sample suspension + 50 µl of MES + 100 µl Nag working solution | 50 µl of soil sample suspension + 50 µl of MES + 100 µl Nag working solution | 50 µl of soil sample suspension+ 50 µl of MES + 100 µl Nag working solution | 50 µl of soil sample suspension + 50 µl of MES + 100 µl Gls working solution | 50 µl of soil sample suspension + 50 µl of MES + 100 µl Gls working solution | 50 µl of soil sample suspension + 50 µl of MES + 100 µl Gls working solution | 50 µl of soil sample suspension + 50 µl of MES + 100 µl Pho working solution | 50 µl of soil sample suspension+ 50 µl of MES + 100 µl Pho working solution | 50 µl of soil sample suspension + 50 µl of MES + 100 µl Pho working solution |
| B | 150 µl MES/0 standard MU working solution/ 50 µl soil sample suspension | 130 µl MES/20 standard MU working solution /50 µl soil sample suspension | 120 µl MES/30 standard MU working solution /50 µl soil sample suspension | 110 µl MES/40 standard MU working solution /50 µl soil sample suspension | 100 µl MES/50 standard MU working solution /50 µl soil sample suspension | 70 µl MES/80 standard MU working solution /50 µl soil sample suspension | 150 µl MES/0 standard MU working solution /50 µl $H_2O$ | 130 µl MES/20 standard MU working solution /50 µl $H_2O$ | 120 µl MES/30 standard MU working solution /50 µl $H_2O$ | 110 µl MES/40 standard MU working solution /50 µl $H_2O$ | 100 µl MES/50 standard MU working solution /50 $H_2O$ | 70 µl MES/80 standard MU working solution /50 µl $H_2O$ |
| C | 50 µl $H_2O$/50 µl MES/100 µl Xyl working solution | 50 µl $H_2O$/50 µl MES/ 100 µl Xyl working solution | 50 µl $H_2O$/50 µl MES/10 0 µl Xyl working solution | 50 µl $H_2O$/50 µl MES/10 0 µl Nag working solution | 50 µl $H_2O$/50 µl MES/10 0 µl Nag working solution | 50 µl $H_2O$/50 µl MES/100 µl Nag working solution | 50 µl $H_2O$/50 µl MES/10 0 µl Gls working solution | 50 µl $H_2O$/50 µl MES/10 0 µl Gls working solution | 50 µl $H_2O$/50 µl MES/10 0 µl Gls working solution | 50 µl $H_2O$/50 µl MES/10 0 µl Pho working solution | 50 µl $H_2O$/50 µl MES/100 µl Pho working solution | 50 µl $H_2O$/50 µl MES/10 0 µl Pho working solution |
| D | | | | | | | | | | | | |
| E | | | | | | | | | | | | |
| F | | | | | | | | | | | | |
| G | | | | | | | | | | | | |
| H | | | | | | | | | | | | |

**Figure 2. 3:** Schematic representation of 96-well microplate set-up for one soil sample being tested for the enzymatic activity detection assay. Xyl – β-Xylosidase substrate; Nag – *N*-acetylglucosaminidase substrate; Gls – β-glucosidase substrate; Pho-Phosphatase substrate.; The yellow wells represent the soil sample suspension being tested for the enzymatic activities.; The green wells represent the quenched MU standard; the blue wells represent the standard MU control.; The red wells represent the control of the enzymatic substrate working solution.

### *2.2.5. Fluorescence measurements and enzymatic potential activity detection*

Before fluorescence measure of the 96-well microplate, the microplate was shaken by 10 min. at room temperature, in a orbital shaker at 800 rpm, to homogenize the solutions contained in the wells. After that, the 96-well microplate was placed on a microplate fluorimeter and the fluorescence was measured by using the software "Gen5 version 3.0". The microplate was subjected to a reading cycle composed of seven fluorescence measurements at room temperature with a 5-minute interval between each fluorescence measurements (0, 5, 10, 15, 20, 25 and 30 min. after the beginning of the reading cycle). After the end of the reading cycle the software gave us the fluorescence measurements at the different reading time intervals which is going to be used in the extracellular enzymatic potential activity determination.

### *2.2.6. Determination of extracellular enzymatic potential activity*

The enzymatic activities were determined by g of dry soil or organic matter unit. The first was used when the different greenhouses soils were compared and the second was used when the different treatments soils from the same greenhouses were compared. The enzymatic activity by g of dry soil represents the enzymatic activity that is taking place in a g of the soil under study. The enzymatic activity by organic matter unit represents the enzymatic activity directed to the hydrolysis of organic matter, and therefore, the efficiency of these enzymatic activities in catabolizing their reactions.

In order to get the fluorescence values driven by the hydrolysis of one of the used substrate-MU of a given soil sample suspension, three replicates in the microplate were made (Figure 2.3- yellow wells). For each of these three-well replicates (n=1,2,3), it was made a linear regression between the obtained fluorescence values as a function of the fluorescence measurement time (t), i.e., at 0, 5, 10, 15, 20, 25, and 30 min. after the beginning of the microplate reading cycle. Once the linear regression was done, the slope of this regression line (RL) was calculated for each of the three well-replicates, which represents the rate of the fluorescence increase over time (equation 2.1).

$$2.1 \qquad slope_{replicate\ n}\left[RL\left(soil\ sample\ suspension\ fluorescence(t)\right)\right], \qquad n = \{1,2,3\}$$

In order to remove any contamination of the substrate and/or buffer from the previous calculated slope value, it was necessary to generate a corrected slope (corr. slope). In this way, and for each well-replicate, it was computed the slope from the RL of the linear regression between the fluorescence of the control of the same enzymatic substrate working solution (ESWS)in study (Figure 2.3- red wells) as a function of the fluorescence measurement times (t) (equation 2.2). Then, the average of the slope from the equation 2.2 was made between the three well replicates (equation 2.3). After that, it was subtracted from the slope value of each replicate from equation 2.1, the value obtained in equation 2.3, generating the corrected slope (equation 2.4).

$$2.2 \qquad slope_{replicate\ n}[RL(fluorescence\ of\ the\ control\ of\ ESWS(t))],\ n=\{1,2,3\}$$

$$2.3 \qquad average\left(slope_{replicate\ 1,2,3}\left[RL\left(fluorescence\ of\ the\ control\ of\ ESWS\ (t)\right)\right]\right)$$

$$2.4 \qquad corr.\ slope_{replicate\ n} = slope_{replicate\ n}[RL(soil\ sample\ suspension\ fluorescence\ (t))] - average\left(slope_{replicate\ 1,2,3}[RL(control\ of\ ESWS(t))]\right), \quad n=\{1,2,3\}$$

For the quenched MU standard (quenched MU std) calculation, it was generated the slope of the RL between the fluorescence of the different MU concentrations in the quenched MU standard wells (Figure 2.3 – green wells) and the different MU concentrations (0, 2000, 3000, 4000, 5000, 8000 pmol MU) This slope was calculated for each of the time intervals of the fluorescence measurements (equation 2.5). After, it was calculated the average of the quenched MU standard slopes between the different time intervals (equation 2.6).

$$2.5 \qquad slope_{t}[RL(fluorescence\ of\ each\ MU\ concentration(MU\ concentration))],\ MU\ concentration=\{0,\ 2000,\ 3000,\ 4000,\ 5000,\ 8000\ pmol\ MU\}$$

$$2.6 \qquad average\left(slope_{t}\left[RL(fluorescence\ of\ each\ concentration\ of\ the\ quenched\ MU\ std\ (MU\ concentration))\right]\right),\ MU\ concentration=\{0,2000,\ 3000,\ 4000,\ 5000,\ 8000\ pmol\ MU\}$$

To determine the enzymatic activity of each replicate from the soil sample, the corrected slope of each well-replicate (equation 2.4) was divided by the multiplication between the average of the quenched MU standard slope (equation 2.6), the percentage of the dry matter (D.M. (%))/percentage of soil organic matter (O.M(%)) of the soil sample and the soil amount presented in the 50 µL soil sample suspension pipetted in the well of the microplate ( i.e., 0.005 g) (equation 2.7/equation 2.8).

2.7 $$Enzymatic\ activity_{\text{replicate n}}\ by\ g\ of\ dry\ soil = \frac{corr.slope_{replicate\ n}}{average\left(slope_t\left[RL\left(fluorescence of each MU concentration(MU concentration)\right)\right]\right) \times \text{D.}M(\%) \times 0.005},\quad \text{n}=\{1,2,3\}$$

2.8 $$Enzymatic\ activity_{\text{replicate n}}\ by\ organic\ matter\ unit = \frac{corr.slope_{replicate\ n}}{average\left(slope_t\left[RL\left(fluorescence of each MU concentration(MU concentration)\right)\right]\right) \times O.M(\%) \times 0.005},\quad \text{n}=\{1,2,3\}$$

After the three replicates of the enzymatic activities from the soil sample were calculated (equation 2.7/ equation 2.8), it was made the average between the three-replicate enzymatic activity and the resulting value was considered as the potential enzymatic activity of the studied enzyme for the soil sample suspension in study (equation 2.9/ equation 2.10).

2.9 $$Soil\ sample\ enzymatic\ activity\ by\ g\ of\ dry\ soil = average(Enzymatic\ activity_{replicate\ 1,2,3}\ by\ g\ of\ dry\ soil)$$

2.10 $$Soil\ sample\ enzymatic\ activity\ by\ organic\ matter\ unit = average(Enzymatic\ activity_{replicate\ 1,2,3}\ by organic\ matter\ unit)$$

*2.3. Loss of ignition (LOI) and soil organic matter determination*

The LOI method consists in organic matter oxidation triggered by high temperatures resulting in the production of carbon dioxide ($CO_2$) and ashes. Basically, the difference between the pre-heated soil weight and the after-heated soil weight gives us the weight loss triggered by the high temperatures that corresponds to the weight of the organic matter present in the pre-heated sample (Dean,1974).

The soil organic matter percentages were determined for all the soils samples at the four sampling times. Soil samples were oven-dried for 72 h at 70 ºC and the crucibles used were oven-dried for 24 h at 60 ºC. The weight of the dry crucibles was measured and 5g of each of the 70ºC dry soils' samples were placed in a different dry crucible. The weight of the dry crucibles with the 5g of the 70ºC dry soils were registered. After that, the dry crucibles with the 5g of the 70ºC dry soils' samples were placed at the muffle which was programmed to reach a temperature of 550 ºC. The muffle took 1 hour to reach the programmed temperature, where from that point 6 hours were counted. After that time, it was allowed to cool down to 100 ºC in order to record the new weight of the crucibles plus the dry soil after being subjected to the 550ºC heat. The soil organic matter percentage was determined by first subtract the weight of crucible + dry soil after the 550 ºC heating from the weight of the crucible at 60 ºC which gave us the dry soil weight after 550 ºC heating ($DW_{550ºC}$). Next from it was applied the following formula (2.11) to get the soil organic matter percentage of the soil samples:

2.11 $$Soil\ organic\ matter\ (\%) = \frac{5g - DW550^{o}C}{5g} \times 100$$

*2.4. Malachite green colorimetric method for available and total phosphorus determination*

The phosphorus determination method was based on D'Angelo et al. (2001). All the collected sample soils have undergone this method. The basis of this method consists of the malachite green complexation with phosphomolybdate complex followed by the absorbance reading at 630 nm. Through a calibration curve was possible to get the available and total P values. The reagent 1 and 2 were prepared based on Van Veldhoven and Mannaerts, 1987 and Ohno and Zibilske, 1991: reagent 1 was 14.2 mmol ammonium molybdate tetrahydrate in 3.1 M $H_2SO_4$; and reagent 2 was prepared by dissolving 3.5 g/L polyvinyl alcohol (PVA) prepared with deionized water at 80ºC and after cooling to room temperature it was added 0.35 g/L malachite green carbinol hydrochloride. Therefore, the addition of reagent 1 to soil suspension will make a complex between the phosphates present in the soil suspension and the molybdate ions. After that, the addition of reagent 2 will enable the complexation of triarylmethane dye malachite green to the phosphomolybdate complex, allowing for the absorbance measurement to quantify the phosphate concentration present in the soil suspension (D'Angelo, 2011). The PVA function on reagent 2 it is of great importance since it stabilizes the colour of phosphomolybdate and the triarylmethane dye malachite green complex and prevented the agglutination and precipitation of the dye complex (Van Veldhoven and Mannaerts, 1987). This method was performed using a 96-well plate based on D'Angelo et al. (2001) which allowed for the absorbance measurement of different soil samples at the same time. As described in D'Angelo et al. (2001), 200 µl of soil sample suspension was added to the 96-well polystyrene microplate followed by the addition of 40 µl of reagent 1. After this, the 96-well microplate was placed in an orbital 96-well microplate shaker for 10 min. at 700 rpm at room temperature. After the 10 min. shaking, it was added 40 µl of reagent 2 followed by a 20 min. shaking on the orbital microplate shaker at 1000 rpm at room temperature. The absorbance was read in a microplate spectrophotometer at 630 nm.

It was generated a calibration curve to get the phosphate concentration from the absorbance values. For the calibration curve it was used a known concentration of 0.1 mg/L potassium phosphate solution. The potassium phosphate solution was used for the calibration curve since potassium was found to not interfere with the colour development and consequently, on the soil sample absorbance, in a wide range of potassium concentrations (D'Angelo et al., 2001). The absorbance of different concentrations of potassium phosphate were measured and it was created a calibration curve for phosphate concentration with a R=0.98. The following equations were generated:

2.12 $$Absorbance\ (630\ nm) = 0.3717 \times Phosphorus\ concentration\ \left(\frac{mg}{l}\right) + 0.216$$

2.13 $$Phosphorus\ concentration\ \left(\frac{mg}{l}\right) = \frac{Absorbance(630\ nm)}{0.3717} - 0.216$$

For the orthophosphate concentration detection, a soil suspension for each soil sample was made by mixing 5 g of dry soil (72 h at 70 ºC in the stove) with 50 ml of sterile deionized water. The use of water as phosphorus extractant it was used since it will extract the phosphorus in the soil solution fraction, i.e., the dissolved or readily soluble forms of P (Van der Paauw, 1971). The soil suspension was then sonicated by 2 min at 90 kHz. Dilutions of the soil suspensions were made so that the absorbance values could be within the range of values of the generated calibration curve. For each soil suspension three replicates were made, and the average of the three phosphate concentration values were used as the final phosphate concentration for each soil sample.

The total phosphorus was estimated through the addition of 1 mL of 1M HCl to the resultant calcinated product of the LOI process (section 2.3). Due to the calcination process the organic P present in the soil sample passed into inorganic form. The addition of 1M HCl extracted the total amount of P present in the soil samples (Wuenscher et al., 2015). After that 3 ml of sterile and deionized water was added and several dilutions were made to get the absorbance values in the range of the calibration curve values. The absorbance reading process and phosphorus concentration measurements were equal to the ones made in the available phosphorus measurement: 200 µL of the solution mixed with reagent 1, followed by the orbital mixing at 700 rpm by 10 min., after that was added the reagent 2 followed by 20 min. of orbital mixing at 1000 rpm and the measurements of absorbance at 630 nm.

*2.5. Determination of dehydrogenase activity*

The protocol used in this assay was based on ISO 23753-2:2005 (International Organization for Standardization, 2005). For this method just the soil samples from 0 and 30 DAT have undergone this method. Due to the covid-19 pandemic situation, it was not possible to carry out this analysis on the remaining soil samples from the other sampling times since this method is characterized by being very time consuming. Nevertheless, is important to refer that at 30 DAT, in vegetative growth/beginning of the flowering stage, is one of the plant phenological stages where the rhizosphere microbiome develops a crucial role in plant nutrition due to the fact that the plant is actively taking nutrients from the soil (Ramanathan and Krishnamoorthy, 1973; Jones, et al., 2011).

For this assay it was used iodotetrazolium chloride (INT) as the reagent to be reduced by dehydrogenase, forming iodonitrotetrazolium formazan (INTF). The INTF concentration can be determined by absorbance measurements. The INT reduction to INTF has been used to quantify the dehydrogenase activity (Benefield et al.,1977; Trevors et al., 1982; Griffiths, 1989).

INT is very sensitive to light, being reduced by it, so it was necessary to have a protocol control that indicated how much of the INTF formed was influenced by this abiotic factor. Because of that sensitivity all the processes of this assay were made in a low light intensity environment. Despite that, a blank for the absorbance reading was also needed to be prepared. For each of the protocol control and blank, it was prepared a composite samples per block, i.e., 2 g of each plot sample from the same block were mixed and it was weighted 2 g of that mixture for a large test tube. For all soil samples two replicates were done for this procedure. After this, the control tubes were autoclaved three times in 20-min. cycles at 121ºC.

For the soil samples tubes, 2 g of each soil sample were weighed to large test tubes. This process was made in replicate. It was added 2 mL of 0.5% (w/v) iodotetrazolium chloride (INT) to the sample test tubes and for the control test tubes. For the blank tubes it was added 2ml of 0.1M pH 8 tris-HCl buffer. The preparation of 100 ml of 0.5% (w/v) INT consisted in weighed 0.5g of INT substrate to an eppendorf. It was added 2ml of Diethylene Glycol and the mixture was vortexed. The resulting solution was mixed with 98 ml of 0.1 M pH 8 tris-HCl buffer in a glass storage bottle and it was again placed in ultrasonic bath until complete dissolution was obtained.

All the test tubes were incubated for 24 h in a horizontal vortex at 120 rpm, in the absence of light. After 24h, all the test tubes were strongly agitated in a vortex. It was added 10 ml of acetone for all the test tubes and the mixture was homogenised in a vortex. All the tubes were incubated 1h in a horizontal vortex at 28 ºC at 120 rpm in the absence of light. After that and within a maximum time of 1 h, all the test tubes were filtered through filter paper and the absorbance of the filtrate was read at 485 nm.

In order to get the dehydrogenase activity from the absorbance it was needed a calibration curve. To generate the calibration curve, it was weighed 25 mg of iodonitrotetrazolium formazan (INTF) being dissolved in 50 mL of acetone. After that, 0, 100, 200, 400, 600 and 800 µL of the INTF stock solution were pipetted into different test tubes. It was added 2 mL of buffer 0.1 M pH 8 tris-HCl and acetone was

added up to 10 mL. Finally, it was read the absorbance from the different test tubes at 485 nm. The calibration curve was made from that absorbance values of the known INTF concentration solutions and an equation was generated with a R=0.998 (equation 2.14; equation 2.15):

2.14 $$Absorbance(485\ nm) = 0.0486 \times INTF\ \left(\frac{\mu l}{ml}\right) - 0.01$$

2.15 $$INTF\ \left(\frac{\mu l}{ml}\right) = \frac{Absorbance(485\ nm)}{0.0486} + 0.01$$

The dehydrogenase activity was then calculated by the following equation:

2.16 $$Dehydrogenase\ activity = (Sample\ INTF\ concentration - Control\ INTF\ concentration) \times \frac{Solution\ volume + Extraction\ volume}{soil\ sample\ d.w \times incubation\ hours}$$

*2.6. Crop harvest*

Due to the covid-19 pandemic situation the harvest weighing was impaired because it was not possible to weight all the harvest. For greenhouse 1 it was only possible to carry out the first harvesting sampling of the tomato produced, on the 12$^{th}$ of May 2020, around 120 DAT, although, it represented almost the total tomato production from this greenhouse. For greenhouse 2 and 3 two production samples were taken on 12$^{th}$ of May 2020 and 4$^{th}$ of July of 2020 (around 120 and 150 DAT). For the greenhouse 2 a good representation of the total production was harvested in this two harvest samples, and in greenhouse 3 only the first and second tomato cluster were harvested. In greenhouse 3 it was only possible to count the initial production of the crop. The weight of the tomato produced in each of the treatments was recorded. It was only harvested and weighed the commercial fruits, i.e., the fruits that, according to the farmer, were ready to be harvested.

*2.7. Statistical analyses*

The two-way analysis of variance (two-way-ANOVA) was applied in order to compare the effects of the different treatments in the same greenhouse for the different analysed parameters: tomato production; extracellular enzymatic potential activities, dehydrogenase activities, soil available and total P, and soil organic matter content. The two factors consisted of the treatments and the blocks. This last factor was to ensure that the different blocks were functioning as replicas.

When comparing the different analysed parameters between the different greenhouses only the control soil samples from the three different greenhouses were compared. In order to perceive differences in the studied parameters at the greenhouse level, a one-way-analysis of variance (one-way ANOVA) was applied with the greenhouse being the only factor.

For the ANOVA's analyses, extracellular enzymatic potential activities and dehydrogenase activity values were log transformed, in order to improve normality that was checked by the shapiro-wilk test. The following ratios were calculated, ln(β-xylosidase + β-glucosidase activities)/ln(*N*-acetylglucosaminidase activity), ln(β-xylosidase + β-glucosidase activities)/ln(phosphatase activity) and ln(*N*-acetylglucosaminidase activity)/ln(phosphatase activity), to measure and compare the relative nutrients demand in the different treatments.

The soil extracellular enzymatic potential activities were interpreted by g of dry soil when this parameter was compared between the different greenhouses and by organic matter unit when this parameter was compared between the different treatments from the same greenhouse. When significant

effects were detected from the one and two-way ANOVA's ($p \leq 0.05$) it was applied a Tukey HSD post hoc test in order to reveal which treatments/greenhouse differed significantly from each other.
All the statistical analyses were made in "R studio (version 4.0.2)" software and the graphics were generated in "Excel (version 2011)".

## 3. Results

### *3.1. Effect of the biofertilizers treatments in the tomato production*

For the first and only harvested sampling in greenhouse 1, around 120 DAT, there weren't any significant effect between biofertilizers and control soils. However, the biofertilizer 2 soil presented the highest tomato production (10572 kg/ha), followed by biofertilizer 1 soil (10556 kg/ha) and control soil (9321 kg/ha). Thus, the biofertilizer 2 and 1 soils produced about 13.4% and 13.3% more tomato than control soil, respectively, at this harvested sample. This can suggest a trend for a higher tomato production in the biofertilizers soils than in the control soil, in greenhouse 1. In Figure 3.1 it is represented the tomato produced by each treatment soil in greenhouse 1 at the first harvested sample.

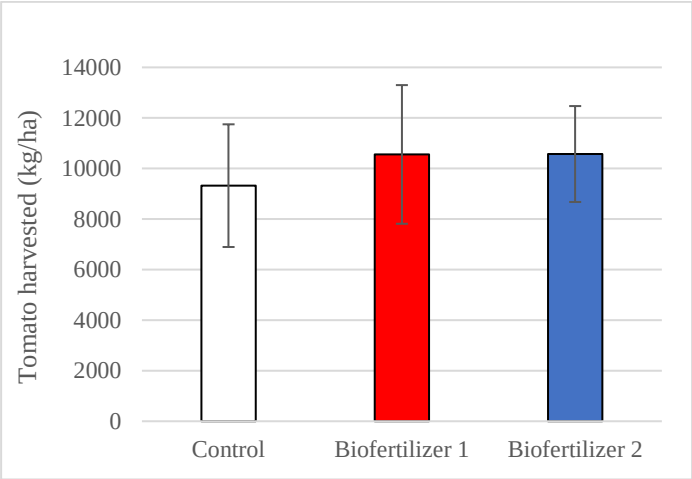



**Figure 3. 1:** Tomato production (kg/ha) in greenhouse 1 at the first and only harvested sample. No significant differences were found between the different treatments soils, however, the biofertilizers soils presented a higher tomato production than control soil.; Bars represent the mean of 3 replicates ± SD.

In greenhouse 2, in the first harvested sample, there were not significantly differences between biofertilizers and control soils, concerning to the amount of the tomato harvested. Even so, biofertilizer 1 soil presented the largest amount of produced tomato (19204 kg/ha), followed by biofertilizer 2 soil (17975 kg/ha) and control soil (16602 kg/ha). Thus, biofertilizer 1 and biofertilizer 2 soils presented a first tomato harvest that were 16% and 8% higher than the one from control soil, respectively. This can suggest a trend for a higher initial tomato productivity driven by the biofertilizers application than in control soil. In Figure 3.2a it is represented the tomato produced by each treatment soil in greenhouse 2 in the first harvested sample.

Regarding to the second harvest sample in greenhouse 2, the biofertilizer 2 soil presented a tomato production that was significantly higher than in control soil ($p<0.05$). The biofertilizer 2 soil presented the highest tomato production (46037 kg/ha) from all the treatments, followed by biofertilizer 1 soil (41351 kg/ha) and control soil (39465 kg/ha). Thus, concerning to the tomato production in the second harvested sample, the biofertilizer 2 and biofertilizer 1 soils were 17% and 5% higher than the tomato production from control soil, respectively. Thus, this shows that the biofertilizer 2 soil in greenhouse 2 enhanced significantly the late tomato produced over the control. Additionally, this also can show a trend for biofertilizer 1 soil to enhance the late tomato produced over the control. In Figure 3.2b it is represented the tomato produced by each treatment soil in greenhouse 2 at the second harvested sample.

In greenhouse 2, summing the two harvested samples, in order to have a proxy of the total tomato production, the biofertilizer 2 soil presented the highest total tomato production (64012 kg/ha), being significantly higher than control soil ($p=0.05$), followed by biofertilizer 1 soil (60555 kg/ha), with

control soil presenting the lowest total tomato produced from all the treatments (56067kg/ha). The total tomato produced in greenhouse 2 by biofertilizer 2 and 1 soils were 14% and 8% higher than the one from control soil, respectively. In Figure 3.2c it is represented a proxy of the total tomato produced by each treatment soil in greenhouse 2.

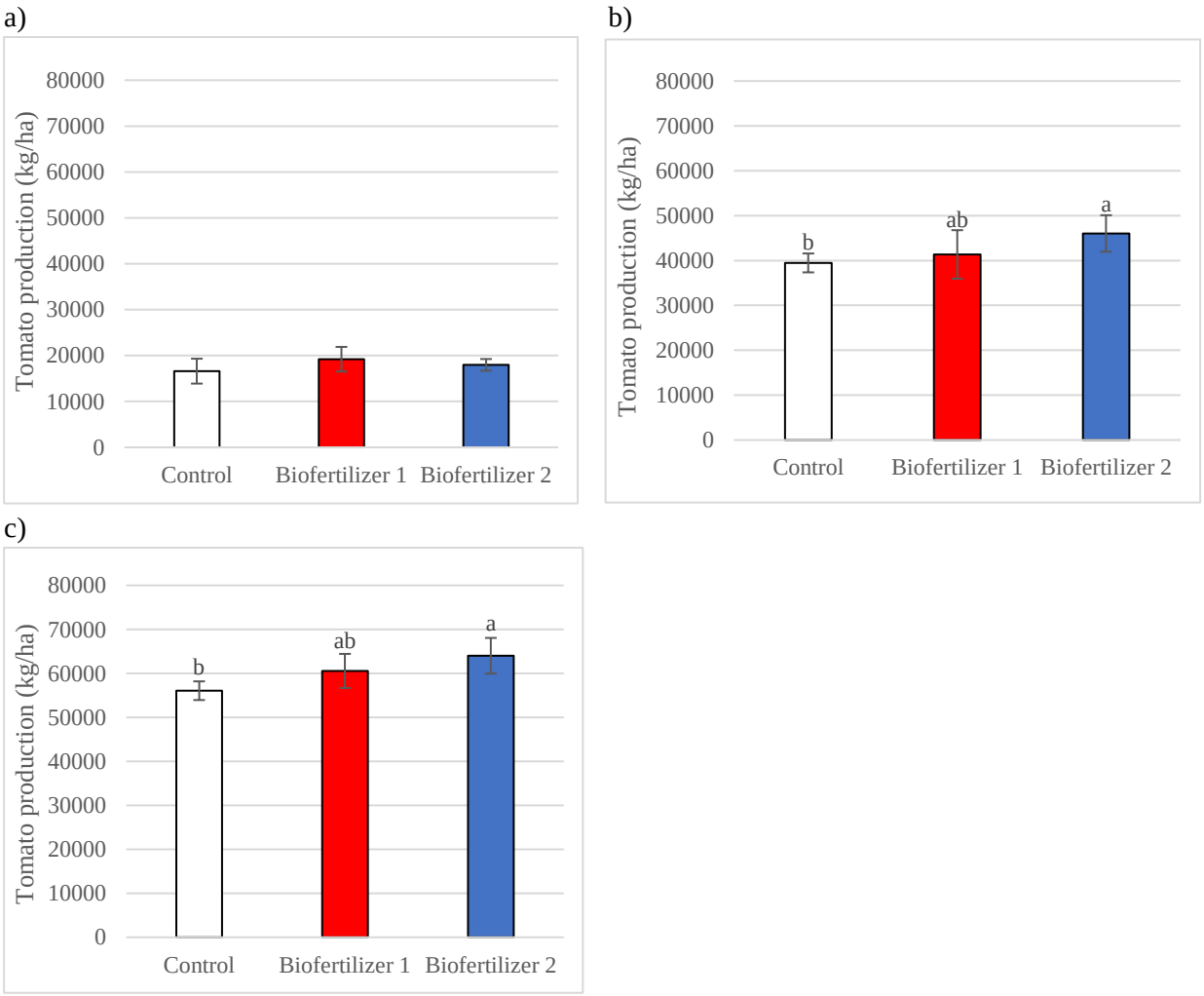



**Figure 3. 2:** (a) Tomato production (kg/ha) in greenhouse 2 by the different treatments soils at the first harvest sample. No significant differences were found between the different treatments soils. Nevertheless biofertilizer 1 and biofertilizer 2 soils presented a tomato production that were higher than the tomato produced in control soil.; (b) Tomato production (kg/ha) in greenhouse 2 at the second harvest sample by the different treatments soils. Biofertilizer 2 soil presented a tomato production significantly higher than control soil ($p<0.05$). Biofertilizer 2 and biofertilizer 1 soils presented a tomato production that were higher than the tomato produced in control soil.; (c) Total tomato production (kg/ha) in greenhouse 2 by the different treatments soils. Biofertilizer 2 soil presented a total tomato production significantly higher than control soil ($p=0.05$). Biofertilizer 2 and biofertilizer 1 soils presented a total tomato production that were higher than the tomato produced in control soil.; Bars represent the mean of 3 replicates ± SD. Bars sharing the same letter, in each sampling time, do not differ significantly at $p\leq0.05$, according to Tukey's HSD test.

At the first harvest sample in greenhouse 3 there was no significant differences between the treatment's soils. However, biofertilizer 2 soil presented the highest tomato production (13024 kg/ha), followed by biofertilizer 1 soil (12815 kg/ha) and control soil (12125 kg/ha). The tomato produced in the first harvest sample by biofertilizer 2 and 1 soils were 7% and 6% higher than in control soil. Thus, in greenhouse 3, it seemed that the biofertilizers soils showed a tendency to anticipate tomato production over control. In Figure 3.3a it is represented total tomato produced by each treatment soil in greenhouse 3 at the first harvested sample.

At the second harvest sample, in greenhouse 3, there were no significant difference between the different treatment's soils. However, biofertilizer 1 soil showed the highest tomato production (10215 kg/ha), followed by biofertilizer 2 soil (8442 kg/ha) and control soils (7490 kg/ha), which makes the tomato production at the second harvest sample by the biofertilizer 1 and biofertilizer 2 soils 36% and 13% higher than the one from control soil, respectively. This showed that, despite not significantly, the biofertilizers introduction to the soil present a trend to produce a higher tomato amount over the control at the middle of the ripening stage in greenhouse 3. In Figure 3.3b it is represented the tomato produced by each treatment soil in greenhouse 3 in the second harvested sample.

The sum of the two harvest samples from greenhouse 3, showed no significant differences between the treatments. However, biofertilizer 1 soil presented the highest tomato production value (23030 kg/ha) followed by biofertilizer 2 soil (21466 kg/ha) and control soil (19616 kg/ha). Thus, the biofertilizer 1 and biofertilizer 2 soils presented 17% and 9% higher tomato produced in the sum of the two harvested samples than control soil, respectively. In Figure 3.3c it is represented the sum of the tomato produced in the two harvested samples by the different treatment's soils in greenhouse 3 (Figure 3.3c).

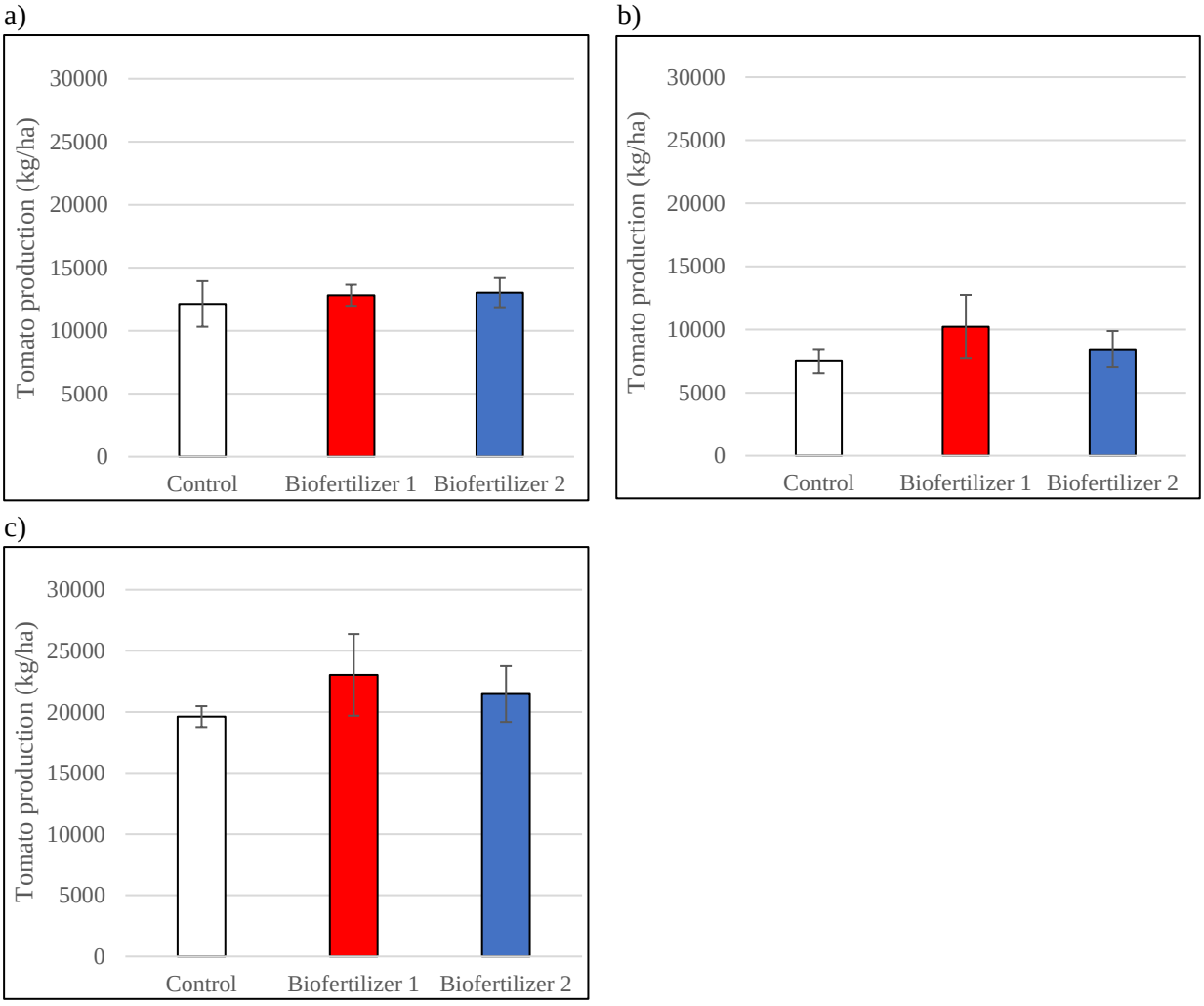



**Figure 3. 3:** (a) Tomato production (kg/ha) in greenhouse 3 by the different treatments at the first harvest sample. No significant differences were found between the treatments soils. Nevertheless biofertilizer 2 and biofertilizer 1 soils presented a tomato production that were higher than the tomato produced in control soil.; (b) Tomato production (kg/ha) in greenhouse 3 by the different treatments at the second harvest sample. No significant differences were found between the different soils. Nevertheless, biofertilizer 1 and biofertilizer 2 soils presented a tomato production that were higher than the tomato produced in control soil.; (c) The sum of the two harvest samples of the tomato production (kg/ha) in greenhouse 3 by the different treatments soils. No significant differences were found between the different treatments soils. Nevertheless, biofertilizer 1 and biofertilizer 2 soils presented a higher tomato production correspondent to the sum of the two harvest samples that were higher than the one from the control soil.; Bars represent the mean of 3 replicates ± SD.

### *3.2. Dehydrogenase activity in no-till and tilled systems*

Dehydrogenase activity was measured in the three studied greenhouses at 0 and 30 DAT. The objective was to evaluate the dehydrogenase activity on the bulk soil (0 DAT) of the different greenhouses systems, and, at the time of intensive vegetative growth and the beginning of the flowering stage (30 DAT), to understand the impact of the different greenhouses soils characteristics in soil dehydrogenase activity.

Comparing the activities of the different greenhouses, at 0 DAT, significant differences were just found between greenhouses 1 and 3 soils ($p<0.05$), where soil dehydrogenase activity of greenhouse 3 was 38% higher than that of greenhouse 1. There was a trend for greenhouse 3 to present the highest and greenhouse 1 the lowest dehydrogenase activity (Figure 3.4a).

At 30 DAT the dehydrogenase activities from greenhouses 2 and 3 soils were significantly higher than that from greenhouse 1 soil ($p<0.05$ and $p<0.01$, respectively). Greenhouses 2 and 3 soils presented higher dehydrogenase activity by 100% and 174% than the one from greenhouse 1 soil, respectively (Figure 3.4b).

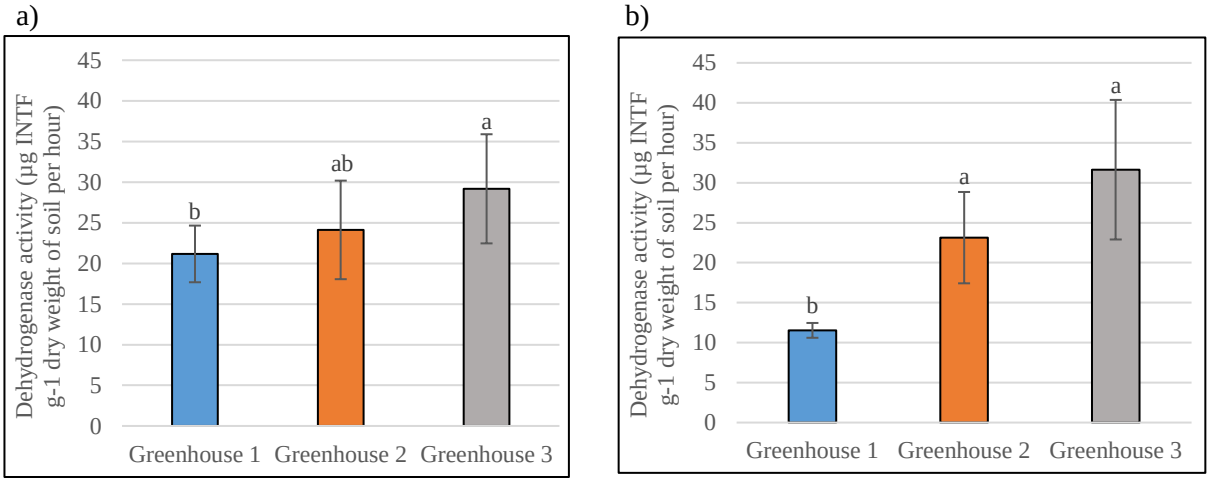
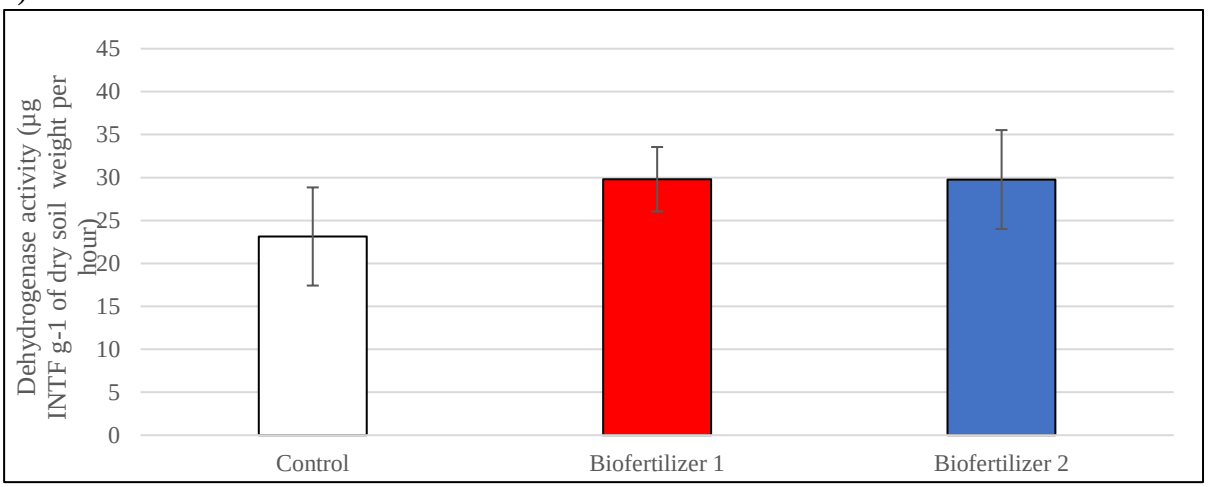


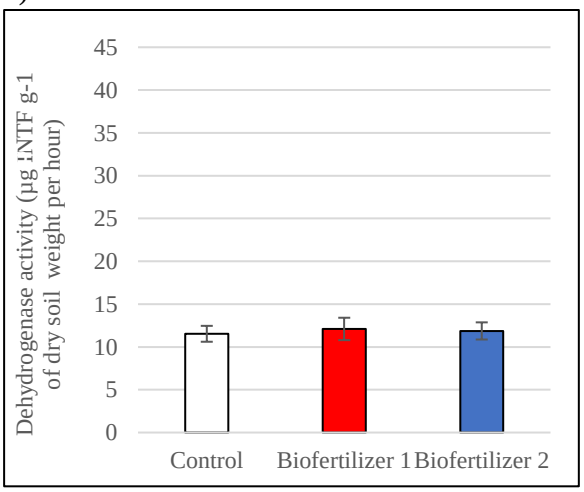

**Figure 3. 4:** (a) Dehydrogenase activity at 0 DAT in the different greenhouses soil systems. Greenhouse 3 soil was significantly higher than greenhouse 1 soil ($p<0.05$).; (b) Dehydrogenase activity at 30 DAT in the different greenhouses soil systems. Greenhouse 2 and 3 soils were significantly higher than greenhouse 1 soil ($p<0.05$ and $p<0.01$, respectively).; Bars represent the mean of 3 control replicates ± SD. Bars sharing the same letter do not differ significantly at $p\leq0.05$, according to Tukey's HSD test.

Concerning to the effects of the biofertilizers introduction in the dehydrogenase activity at 30 DAT, no significant differences between the dehydrogenase activities from the control and biofertilizers soils were found in any of the greenhouses soil systems. Nevertheless, in greenhouse 2, the introduction of biofertilizer 1 and 2 increased the soil dehydrogenase activity over the control soil by 29% (Figure 3.5a). On the opposite, in greenhouse 1 and 3 the introduction of both biofertilizers did not changed the soil dehydrogenase activity value over the control soil (Figure 3.5b,c).

a)




b)




c)

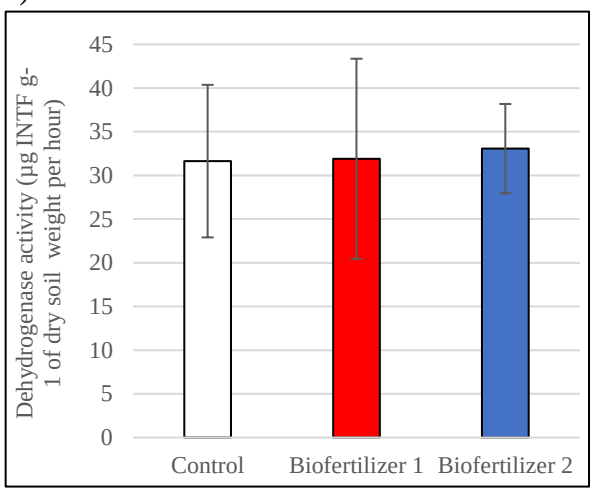



**Figure 3. 5**: (a) Dehydrogenase activity at 30 DAT in the greenhouse 2 by the different treatments soils. No significant differences were found between the different treatments soils. However, biofertilizer 1 and 2 soils presented a dehydrogenase activity that were higher than the one from control soils.; (b) Dehydrogenase activity at 30 DAT in the greenhouse 1 by the different treatments soils. No significant differences were found between the different treatments soils.; (c) Dehydrogenase activity at 30 DAT in the greenhouse 3 by the different treatments soils. No significant differences were found between the different treatments soils.; Bars represented the mean of 3 replicates ± SD.

### *3.3. Impact of biofertilizers in soil organic matter content in the different greenhouses systems*

Soil organic matter content was tested at the four sampling times (0, 30, 120 and 150 DAT) in the three different greenhouses.

Comparing the different greenhouse's bulk soil organic matter content (0 DAT) it was possible to conclude that there were no significant differences between all the three greenhouses systems. However, the Tukey's HSD test, when compared the greenhouse 2 soil organic matter content with greenhouse 1 and 3 soil organic matter contents, calculated a $p = 0.072$ and $p=0.098$, respectively. This may suggest that greenhouse 2 was closer to present a significantly lower organic matter content than in the other greenhouses. Although not significantly different, the organic matter content of greenhouse 2 was 22% and 21% lower than the ones from greenhouse 1 and 3, respectively (Figure 3.6a). The greenhouse 2 may have presented this non-significant value due to the fact that the farmer incorporated organic matter into the soil, yet we believe that it was not properly incorporated into the soil.

At 30 DAT the organic matter content of greenhouse 2 soil was significantly lower than the one from greenhouse 3 ($p<0.05$) by 23%. No significant differences were found between the soil organic matter content from greenhouse 1 and the ones from greenhouse 2 and 3. Even so, greenhouse 2 soil organic

matter content was 15% lower than the one from greenhouse 1. From the three greenhouses soils, greenhouse 3 and 2 presented the highest and lowest soil organic matter content at 30 DAT, respectively (Figure 3.6b).

At 120 DAT there were no significant differences between the greenhouses systems, concerning to the soil organic matter content. Nevertheless, from the three greenhouses systems, greenhouse 1 soil presented the highest organic matter content and greenhouse 2 soil presented the lowest organic matter content. Greenhouse 2 soil presented 16% and 13% lower organic matter content than the organic matter contents from greenhouse 1 and 3 soils, respectively (Figure 3.6c).

Finally, at 150 DAT, no significant differences were found between the greenhouses. Nevertheless, greenhouse 2 and 3 soils presented the lowest and highest organic matter content from the three studied greenhouses soils, respectively. The organic matter content in greenhouse 2 soil was 3% and 20% lower than the organic matter content of greenhouse 1 and 3 soils, respectively (Figure 3.6d).

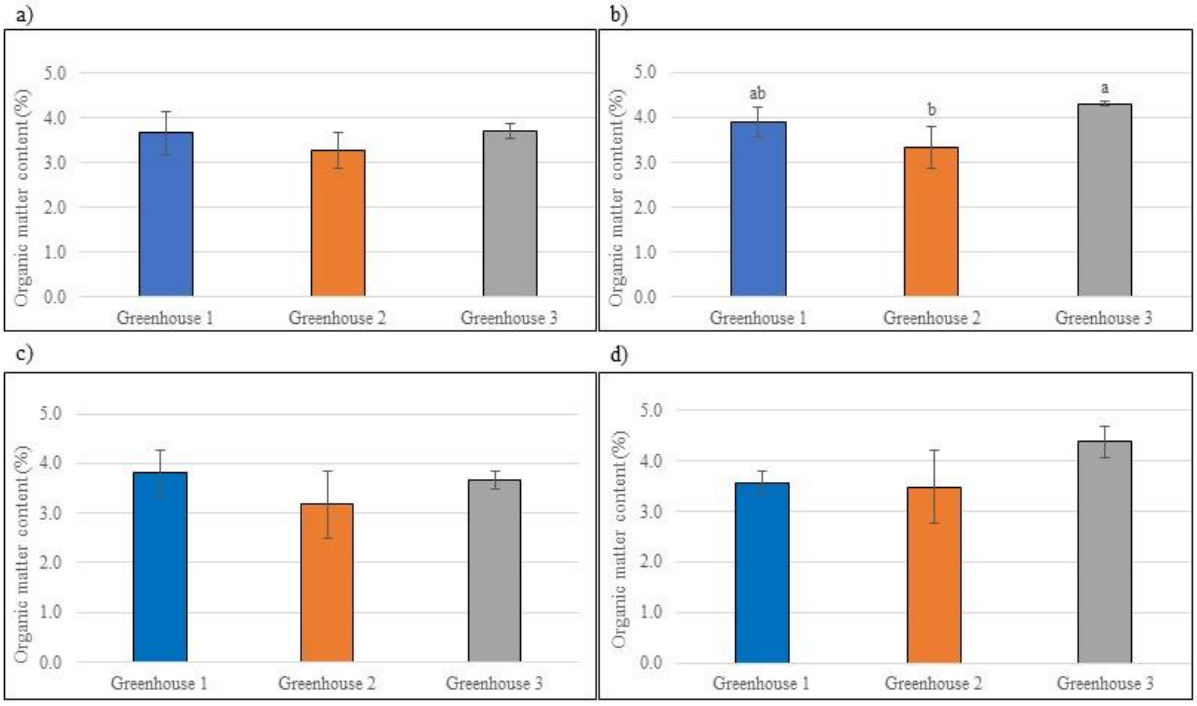


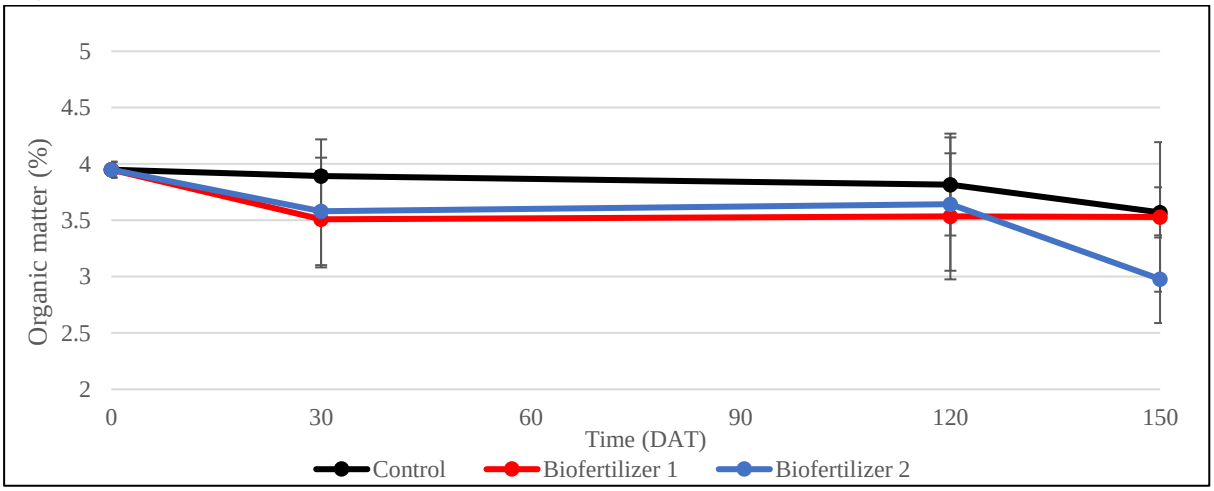

**Figure 3. 6:** (a) Organic matter content (%) in the different greenhouses at 0 DAT. No significant differences were found between the greenhouses soils.; (b) Organic matter content (%) in all the different greenhouses at 30 DAT. Organic matter content of greenhouse 2 soil was significantly lower than the one from greenhouses 3 soil ($p<0.05$).; (c) Organic matter content (%) in all the different greenhouses soils at 120 DAT. No significant differences were found between the organic matter content from the three greenhouses soils.; (d) Organic matter content (%) in the different greenhouses soils at 150 DAT. No significant differences were found between the organic matter content from the three greenhouses soils.; Bars represented the mean of the 3 control replicates ± SD. Bars sharing the same letter do not differ significantly at $p \leq 0.05$, according to Tukey's HSD test.

The introduction of the biofertilizers didn't changed significantly the organic matter content over the control soil in any of the studied greenhouses in any of the sampling times. However, it was still possible to suggest some trends driven by the biofertilizers application, dependent on the greenhouse (Figure 3.7).

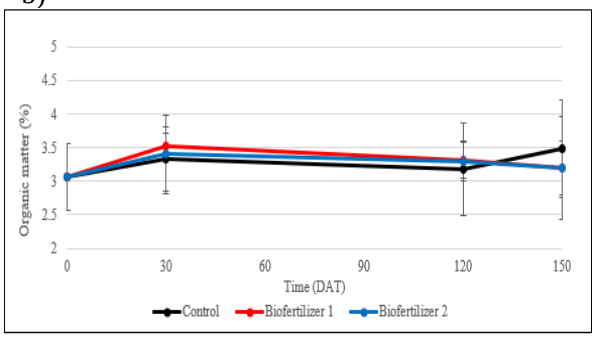
In greenhouse 1, despite not significantly differences were found between the biofertilizers and control soils, the observation of the different organic matters content in the different sampling times from the different treatments soils, made it possible to suggest that biofertilizer 2 application played a tendency towards greater degradation of organic matter compared to control soil. Additionally, it seems that biofertilizer 1 soil presented a trend for a higher initial organic matter degradation than in control soil (Figure 3.7a).

Concerning to the other greenhouses the biofertilizers application didn't presented any evident trend in the organic matter content over time compared to control soil (Figure 3.7b,c).

a)




b)




c)

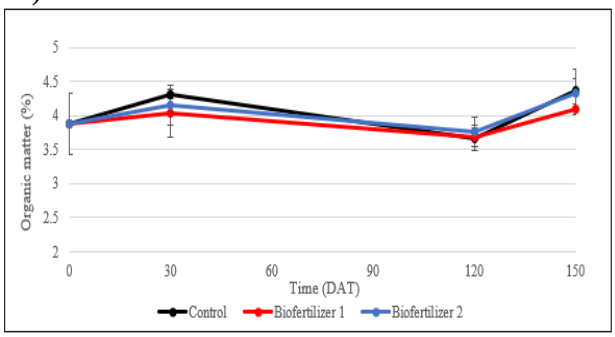



**Figure 3. 7:** (a) Organic matter content (%) in greenhouse 1 from the different treatments soils at the different sampling times. No significant differences in soil organic matter content between the different treatments soils were found. Nevertheless, it was possible to detect a higher initial soil organic matter degradation trend driven by the biofertilizers application and a more efficient degradation trend by biofertilizer 2 soil over time, when comparing to the control soil organic matter trend.; (b) Organic matter content (%) in greenhouse 2 from the different treatments soils at the different sampling times. No significant differences in soil organic matter content between the different treatments soils were found.; (c) Organic matter content (%) in greenhouse 3 from the different treatments soils at the different sampling times. No significant differences in soil organic matter content between the different treatments soils were found.; Each point represents the mean of 3 replicates ±SD.

## *3.4. Impact of the biofertilizers in available and total phosphorus in the different greenhouses systems*

### *3.4.1. Available phosphorus*

For this parameter, no comparison between the different greenhouses were done.

The available P concentration (mg available P/g of dry soil) of each treatment soil per greenhouse was assessed in the four sampling times (0, 30, 120 and 150 DAT) to understand if the biofertilizers application had an impact in soil available P trend.

Concerning to greenhouse 1, no significant differences between the available P content in the control and biofertilizers soils were found in all of the sampling times due to the high variability presented by the replicates of the different treatments soils. Biofertilizer 1 and control soils presented similar available P concentration in all the sampling times. However, despite not significantly different, from 0 to 120 DAT, the biofertilizer 2 soil seemed to present a trend for a higher available P concentration than the one from control soil. However, at 150 DAT, biofertilizer 2 soil seemed to show a trend to decrease its available P concentration, contrary to the control soil that increased its available P concentration over

time. The trend of the available P concentration along the time in the different treatments soils in greenhouse 1 is represented in Figure 3.8a.

In greenhouse 2 at 30 DAT, it seemed that in both biofertilizers soils, despite not significantly different, there was a trend for a higher available P accumulation than in control soil. At 120 DAT, the trend was the opposite, where control soil presented the highest available P concentration and biofertilizer 1 soil the lowest, with biofertilizer 1 soil presenting significantly lower available P concentration than the one from control soil ($p$<0.05) by 36%. Despite not significantly different, at 120 DAT, biofertilizer 2 soil also showed a trend for a lower available P concentration than the one from control soil. At 150 DAT both biofertilizer 1 and control soils presented similar available P concentrations values. However, biofertilizer 2 soil presented a significantly lower available P concentration than the available P concentration from control and biofertilizer 1 soils ($p$<0.001) by 84% and 85%, respectively. The trend of the available P concentration along the time in the different treatments soils in greenhouse 2 is represented in Figure 3.8b.

In greenhouse 3, no significant differences were found between the available P concentrations in the treatments soils in any of the sampling times. Biofertilizer 2 and control soils presented similar available P concentrations in all the sampling times. From 0 to 30 DAT, biofertilizer 1 soil presented a trend for a higher initial available P concentration than the one from control soil. However, in the following sampling times the available P concentration in the control and biofertilizer 1 soils were similar. The trend of the available P concentration along the time in the different treatments soils in greenhouse 3 is represented in Figure 3.8c.

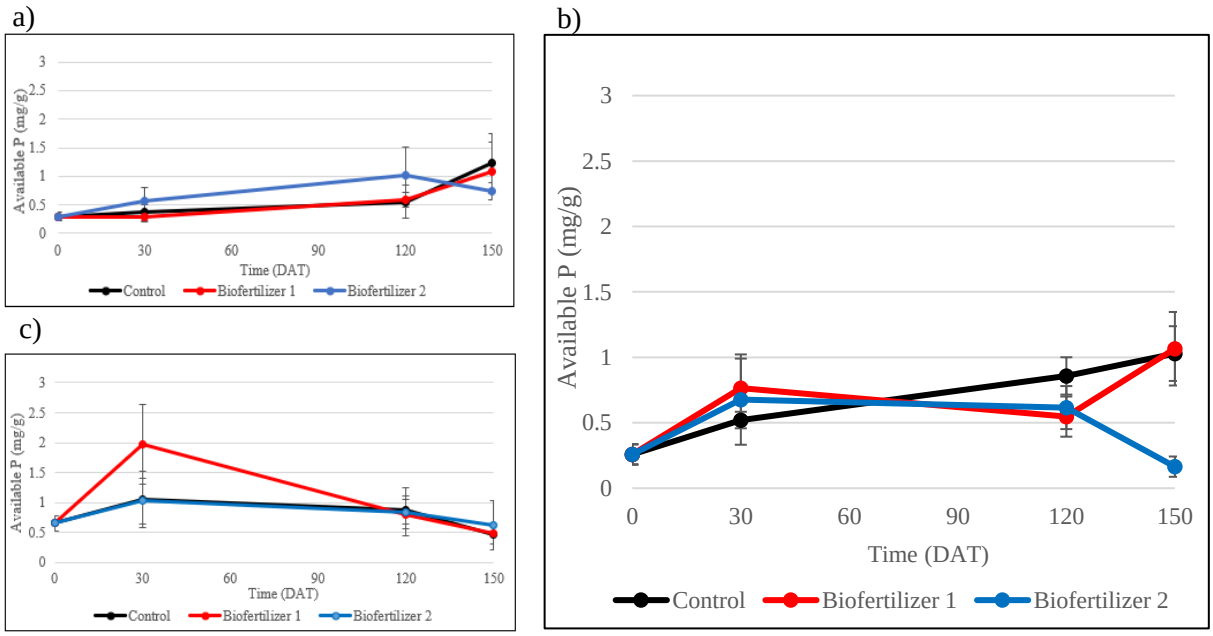



**Figure 3. 8:** (a) Available P concentration (mg/g) in greenhouse 1 soil influenced by the different treatments over the different sampling times. No significant differences were found between the available P concentration from the different treatments soils in all the sampling times.; (b) Available P concentration (mg/g) in greenhouse 2 soil influenced by the different treatments over the different sampling times. Two significant differences were detected: at 120 DAT where biofertilizer 1 soil presented a significantly lower available P concentration than control soil ($p$<0.05) and at 150 DAT where biofertilizer 2 soil presented a significantly lower available P concentration than control and biofertilizer 1 soils ($p$<0.001).; (c) Available P concentration (mg/g) in greenhouse 3 soil influenced by the different treatments over the different sampling times. No significant differences were found between the available P concentration from the different treatments soils.; Each point represents the mean of 3 replicates ±SD.

### *3.4.2. Total phosphorus*

Total phosphorus was measured at 0 and 150 DAT to access the concentration of the soil total P concentration in each greenhouse soil system. Additionally, we wanted to understand if the biofertilizers

application could effectively solubilize the soil P and provide it to the plant in order to reduce its total concentration in the soil of a drip fertigation agricultural system.

Concerning to the soil total P concentration (mg total P/g of dry soil) between the different greenhouse systems at 0 DAT, greenhouse 2 soil presented a total P concentration significantly lower than the one from greenhouse 1 soil ($p$<0.01) and greenhouse 3 soil ($p$<0.01) by 61% and 67%, respectively. At this sampling time, greenhouse 3 presented the highest total P concentration with a concentration of 18.5 mg total P/g of dry soil, followed by greenhouse 1 with a concentration of 15.6 mg total P/g of dry soil and greenhouse 2 presented the lowest total P concentration with a concentration of 6,1 mg total P/g of dry soil (Figure 3.9). At 150 DAT greenhouse 2 soil had significantly lower total P content than the one from greenhouse 1 and 3 soils ($p$<0.05). In this sampling time, greenhouse 3 presented the highest soil total P concentration, with a total P concentration of 20.6 mg total P/ g of dry soil, followed by greenhouse 1 soil with a concentration of 19 mg total P/ g of dry soil and greenhouse 2 soil with a concentration of 9 mg of total P/ g of dry soil. In this way, at 150 DAT, greenhouse 2 soil presented a soil total P concentration that was 52% and 55% lower than the soil total P concentration from greenhouse 1 and greenhouse 3 soils, respectively (Figure 3.9).

From 0 to 150 DAT all the greenhouses increased its soil total P concentration.

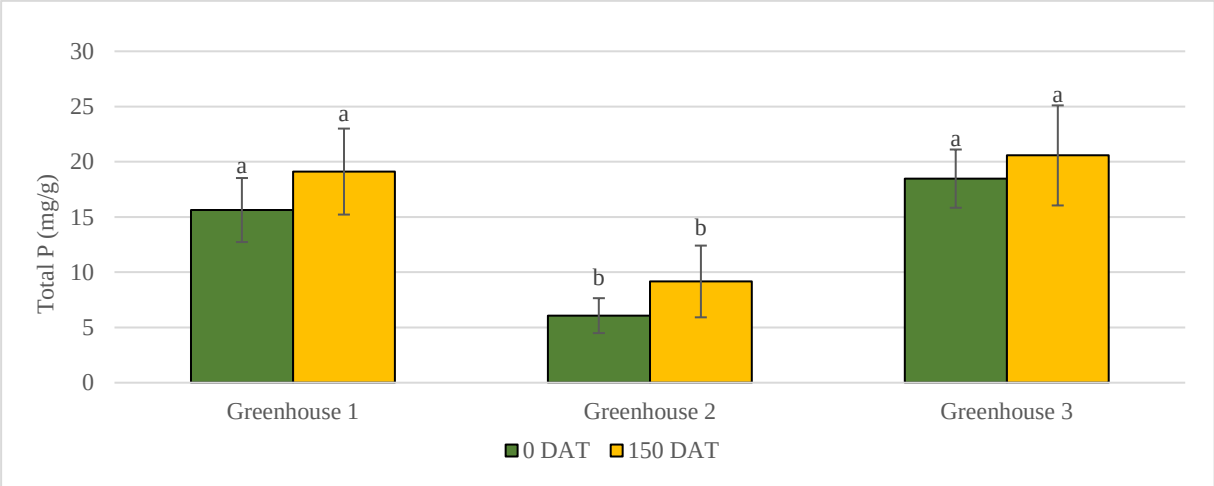



**Figure 3. 9:** Total P content (mg/g) in the different greenhouses at 0 DAT and 150 DAT. The green bars represent the total soil P concentration at 0 DAT and the yellow bars represent the total soil P concentration at 150 DAT, in the different greenhouses soils. At 0 DAT, greenhouse 2 soil presented a significantly lower total P concentration than the total P concentration from greenhouse 1 and greenhouse 3 soils (p<0.01). At 150 DAT, greenhouse 2 soil presented a total P concentration that was significantly lower than the total P concentration from greenhouse 1 and 3 soils ($p$<0.05). From 0 to 150 DAT, all the greenhouses soils increased its total P concentration.; Bars represent the mean of 3 control replicates ± SD. Bars sharing the same letter in the same sampling time, do not differ significantly at $p \leq 0.05$, according to Tukey's HSD test.

Concerning to the effect of the biofertilizers application in the soil total P concentration at 150 DAT there wasn't any significant differences in total P concentration between the control and biofertilizers soils, in all the greenhouses.

In greenhouse 1, while control and biofertilizer 2 soils presented an increase in its total P contents from 0 to 150 DAT, the biofertilizer 1 soil presented a trend for a decrease of its total P concentration between the two sampling times (Figure 3.10a). In greenhouse 2, the control and biofertilizers soils presented a similar total P concentration at 150 DAT (Figure 10b). In greenhouse 3, the control and biofertilizers soils presented a similar total P concentration at 150 DAT (Figure 3.10c).

Except biofertilizer 1 in greenhouse 1, all the controls and biofertilizers soils from all the different greenhouses showed an increase in its total P concentration from 0 DAT to 150 DAT.

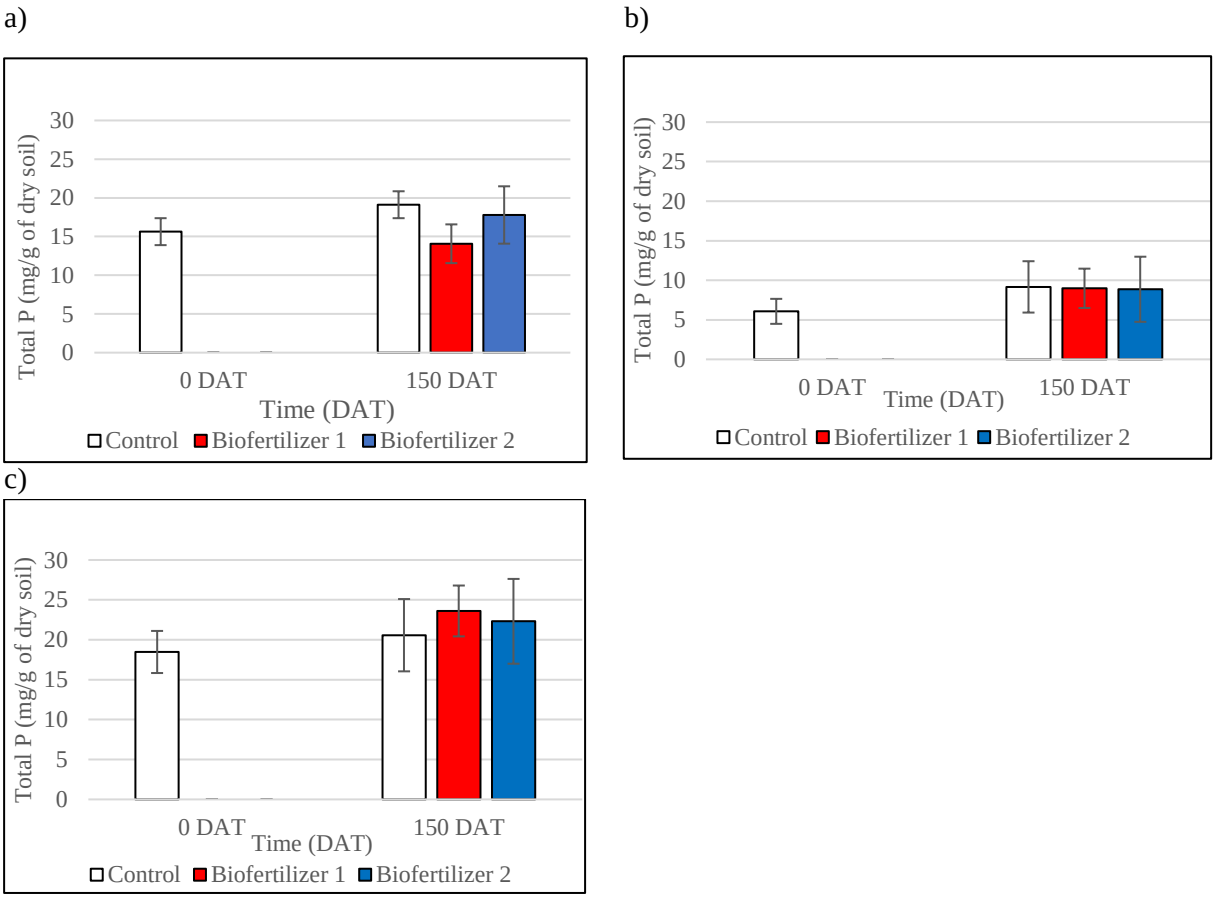



**Figure 3. 10:** (a) Total P concentration (mg/g) in greenhouse 1 soil at 0 DAT (bulk soil) and at 150 DAT by the different treatments soils. No significant differences were found between the total P concentration of control and biofertilizers soils.; (b) Total P concentration (mg/g) in greenhouse 2 soil at 0 DAT (bulk soil) and at 150 DAT by the different treatments soils. No significant differences were found between the total P concentration of control and biofertilizers soils.; (c) Total P concentration in greenhouse 3 (mg/g) soil at 0 DAT (bulk soil) and at 150 DAT by the different treatments soils. No significant differences were found between the total P concentration of control and biofertilizers soils.; Bars represent the mean of 3 replicates ± SD.

## *3.5. Biofertilizers and extracellular enzymatic potential activities*

### *3.5.1. Overall soil extracellular enzymatic potential activity in the different greenhouses systems*

It was compared the potential enzymatic activities by g of dry soil between the different greenhouses in order to access the impact of the different greenhouse characteristics in the soil potential enzymatic activities.

In order to have an idea of the overall soil extracellular enzymatic potential activity per greenhouse, all the four studied extracellular enzyme potential activities by g of dry soil from the control soils of each greenhouse, i.e., β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase activities, were summed. The overall extracellular enzymatic potential activities from the three greenhouses in the four sampling times are represented in Figure 3.11.

At 0 DAT, i.e., in the bulk soil, all the greenhouses soils presented a significantly different overall soil extracellular enzymatic potential activity between them ($p_{Greenhouse2 - Greenhouse1,3}$<0.00001; $p_{Greenhouse1-3}$<0.0002). The greenhouse 2 soil presented the highest overall soil extracellular enzymatic potential activity and greenhouse 1 soil presented the lowest. At this sampling time the overall soil extracellular enzymatic potential activity of greenhouse 2 soil was about 570% and 280% higher than the ones from greenhouse 1 and 3 soils, respectively. In its turn, greenhouse 3 soil presented an overall extracellular enzymatic potential activity that was about 76% higher than the one from greenhouse 1 soil.

At 30 DAT, greenhouse 2 and 3 soils presented a significantly higher overall extracellular enzymatic potential activity than the one from greenhouse 1 soil ($p$<0.05). At this sampling time, greenhouse 2 and 3 soils presented an overall extracellular enzymatic potential activity that were 80% and 106% higher than the one from greenhouse 1 soil, respectively.

At 120 DAT, greenhouse 2 and 3 soils overall extracellular enzymatic potential activities were significantly higher than the one from greenhouse 1 soil ($p$<0.05 and $p$<0.01, respectively). The overall extracellular enzymatic potential activities from greenhouse 2 and 3 soils were 73% and 147% higher than the one from greenhouse 1 soil.

Finally, at 150 DAT, concerning to the overall extracellular enzymatic activities no significant differences were found between the different greenhouses. Nevertheless, greenhouse 2 and 3 soils presented an overall extracellular enzymatic potential activity that were 20% and 40% higher than the one from greenhouse 1 soil.

This trend led us to conclude that in bulk soil, i.e., at 0 DAT, greenhouse 2 and 1 soils presented the highest and lowest overall soil extracellular enzymatic potential activity from the studied greenhouses soils, respectively. However, with the presence of the rhizosphere, i.e., at 30, 120 and 150 DAT, the trend changed, with greenhouse 3 soil starting to present the highest overall extracellular enzymatic potential activity and greenhouse 1 soil the lowest, from the three studied greenhouses soils.

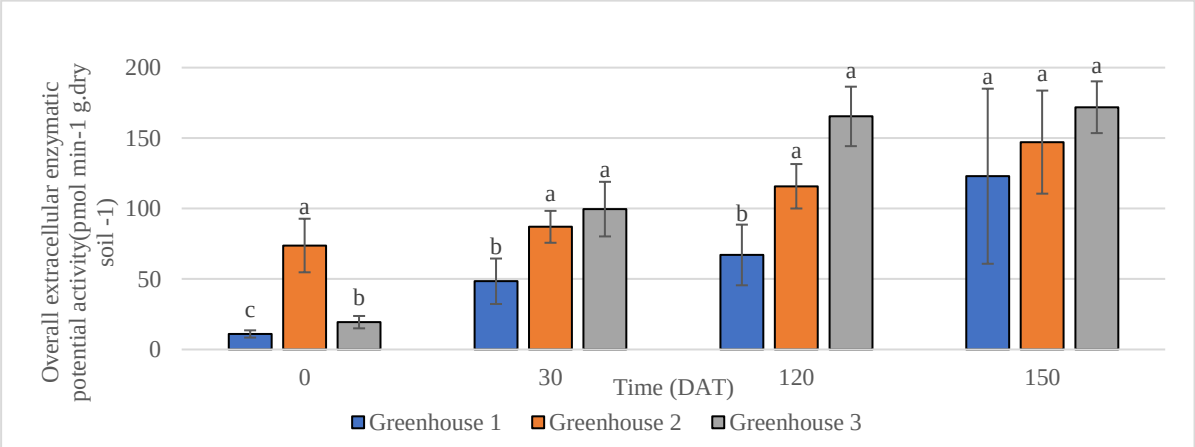



**Figure 3. 11:** Overall extracellular enzymatic potential activity from the different greenhouses control soils at the four sampling times (0, 30, 120 and 150 DAT). Greenhouse 1 soil showed a significantly lower overall extracellular enzymatic potential activities than greenhouse 2 and 3 soils at 0, 30 and 120 DAT ($p$<0.05). Bars represent the mean of the 3 control replicates ± SD.; Bars sharing the same letter, in each sampling time, do not differ significantly at $p$≤0.05, according to Tukey's HSD test.

### *3.5.2. β-glucosidase potential activity in the different greenhouses systems*

When analysing the four studied enzymatic activities, i.e., β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase activities, there was an explicit tendency for the greenhouse 1 soil to present the lowest values of β-glucosidase potential activity from the three studied greenhouses soils in all the sampling times (Figure 3.12). For all the four sampling times, the β-glucosidase potential activity from greenhouse 1 soil was significantly lower than the one from greenhouse 2 and 3 soils ($p$<0.05). Greenhouse 1 soil presented a significantly lower β-glucosidase potential activity than greenhouse 2 by 95%, 73%, 58% and 65% at 0 DAT, 30 DAT, 120 DAT and 150 DAT, respectively. Concerning to the differences between the β-glucosidase potential activity of greenhouse 1 and 3 soils, the β-glucosidase potential activity from greenhouse 1 soil was 76%, 74%, 74% and 61% lower than the one in greenhouse 3 soil at 0 DAT, 30 DAT, 120 DAT and 150 DAT, respectively.

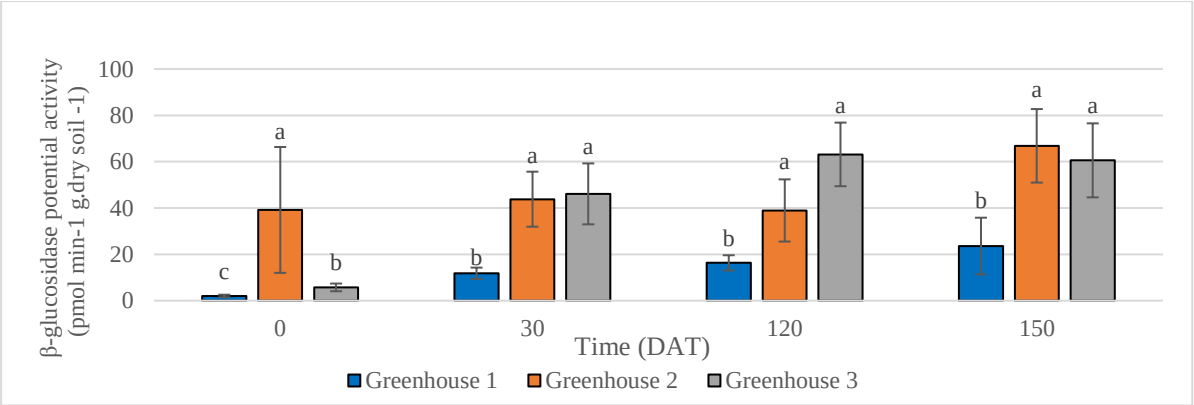



**Figure 3. 12:** β-glucosidase potential activity in the three different greenhouses soils at the four sampling times (0, 30, 120 and 150 DAT). Greenhouse 1 soil presented a β-glucosidase potential activity that was significantly lower than the one from greenhouse 2 and 3 soils in all the sampling times ($p$<0.05).; Bars represent the mean of 3 control replicates ± SD. Bars sharing the same letter, in each sampling time, do not differ significantly at $p$≤0.05, according to Tukey's HSD test.

### *3.5.3. Ratios between the β-glucosidase and β-xylosidase potential activities in the different greenhouses systems*

Due to the fact that in all the sampling times the β-glucosidase potential activity was significantly lower in greenhouse 1 soils than the one from the other greenhouses soils, we decided to calculate the relationship between the two studied extracellular enzymatic potential activities responsible for C availability through a ratio, i.e., β-glucosidase activity:β-xylosidase activity. This would allow us to access the information about the relationships of the different C fractions from the different greenhouses soils. It is important to notice that β-glucosidase is related to the decomposition of cellulose, i.e., a more labile C fraction, whereas β-xylosidase is more related to the decomposition of hemicellulose, i.e., a more recalcitrant fraction of C than cellulose (DeMartini et al., 2013; Costa et al., 2016b).

In all the sampling times, the ratio value between the β-glucosidase activity and β-xylosidase activity was significantly lower in greenhouse 1 soil than in greenhouse 3 soil ($p$<0.05). When comparing this ratio value in greenhouse 1 and 2 soils, the greenhouse 1 soil presented a significantly lower ratio value in all the sampling times ($p$<0.05), except at 120 DAT where no significant differences were found between these two greenhouses soils. However, at 120 DAT, greenhouse 1 soil still presented a lower ratio value than greenhouse 2 soil, which gives robustness to the hypothesis that there was a tendency for greenhouse 1 soil to present a lower value of this ratio than greenhouse 2 soil over time. In Figure 3.13 it is represented the β-glucosidase activity:β-xylosidase activity from the different greenhouses soils.

At 0 DAT, greenhouse 1 soil presented a value of the β-glucosidase activity/β-xylosidase activity that was 68% and 60% lower than the ones from greenhouse 2 and 3 soils, respectively. At 30 DAT, greenhouse 1 soil presented a value of β-glucosidase activity/β-xylosidase activity that was 64% and 65% lower than the ones from greenhouse 2 and 3 soils, respectively. At 120 DAT, greenhouse 1 soil presented a value of β-glucosidase activity/β-xylosidase activity value that was 39% and 53% lower than the ones from greenhouse 2 and 3 soils, respectively. At 150 DAT, greenhouse 1 soil presented a value of β-glucosidase/β-xylosidase that was 73% and 59% lower than the one presented in greenhouse 2 and 3 soils, respectively.

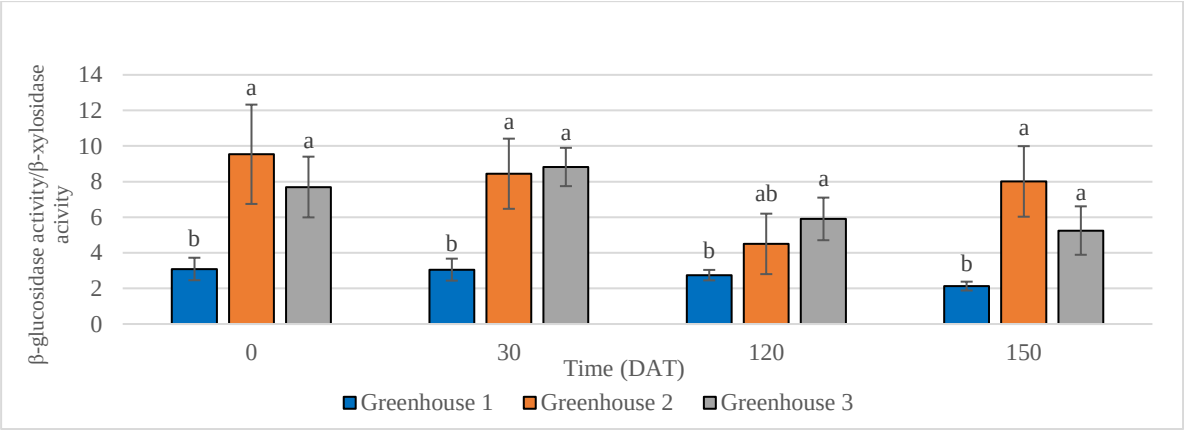


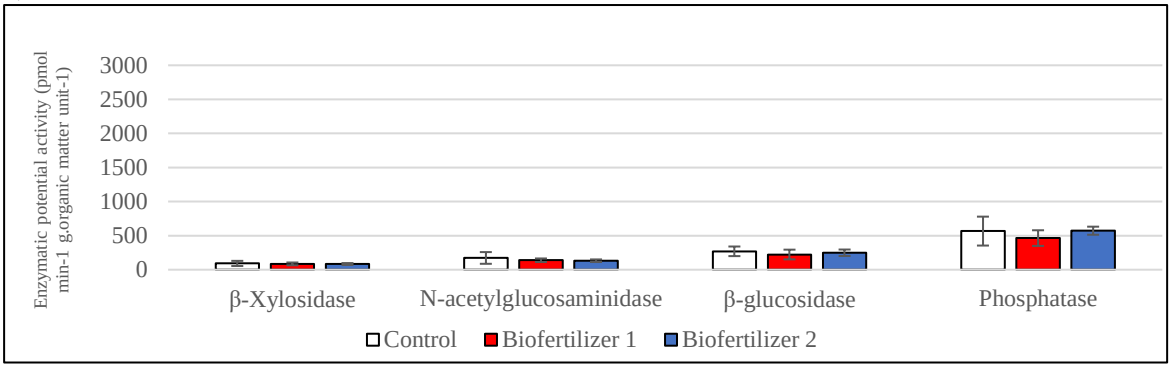

**Figure 3. 13**: Ratio between β-glucosidase activity/β-xylosidase activity in the different greenhouses control soils in all the sampling times. For all the sampling times, greenhouse 1 presented significantly lower β-glucosidase activity/β-xylosidase activity ratios than the other greenhouses ($p$<0.05), except at 120 DAT where no significant differences were found between greenhouse 2 soil.; Each bar represents the mean of 3 control replicates ± SD. Bars sharing the same letter, in each sampling time, do not differ significantly at $p$≤0.05, according to Tukey's HSD test.

### *3.5.4. Effect of the biofertilizers in soil extracellular enzymatic potential activity*

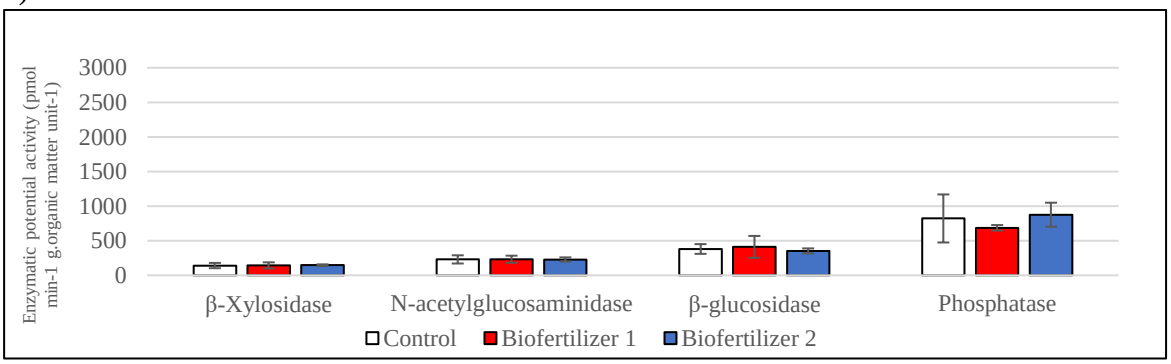
The direct effect of biofertilizers application in the studied soil extracellular enzymatic potential activities was not the same for the different greenhouses soils, indicating that different soil characteristics and agricultural practices will influence the biofertilizers action in this parameter. The sampling time, i.e., the tomato plant lifecycle phase, was also another factor that influenced the biofertilizers effect on the soil extracellular enzymatic potential activities. The studied extracellular enzymatic potential activities were interpreted by organic matter unit.

Both biofertilizers application in greenhouse 1 soil (no-tillage management and higher organic matter content than greenhouse 2) showed no significant differences on the soil extracellular enzymatic potential activities when compared with control soil, in any of the sampling times. Thus, the introduction of the biofertilizers in greenhouse 1 seemed to didn't play any role in the studied soil extracellular enzymatic activities. The four studied extracellular enzymatic activities in the soils of the different treatments in greenhouse 1 at 30, 120 and 150 DAT are represented in Figure 3.14.

a)



b)




c)

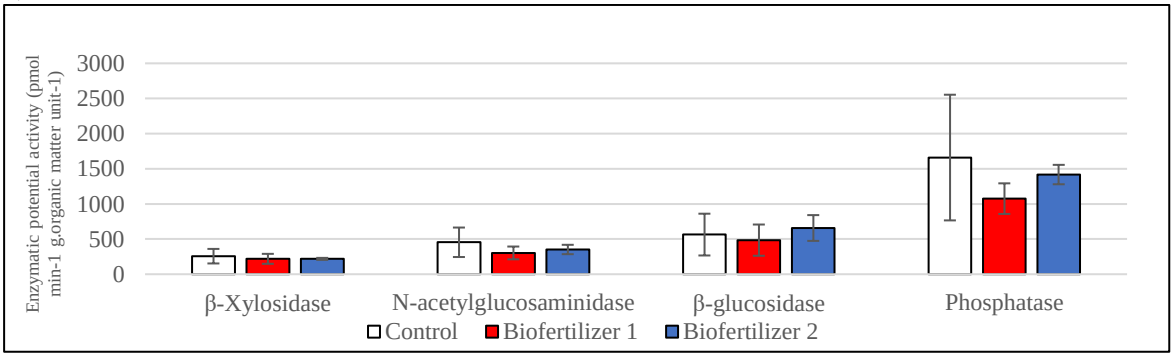



**Figure 3. 14:** (a) Extracellular enzymatic potential activities by organic matter unit of the enzymes β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase in greenhouse 1 at 30 DAT in the different treatments soil. No significant differences were found in any of the extracellular enzymatic potential activities between the different treatments soils.; (b) Extracellular enzymatic potential activities by organic matter unit of the enzymes β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase in greenhouse 1 at 120 DAT in the different treatments soils. No significant differences were found in any of the extracellular enzymatic potential activities between the different treatments soils.; (c) Extracellular enzymatic potential activities by organic matter unit of the enzymes β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase in greenhouse 1 at 150 DAT in the different treatments soils. No significant differences were found in any of the extracellular enzymatic potential activities between the different treatments soils.; Bars represent the mean of 3 replicates ± SD.

In greenhouse 2 (tillage management and lower soil organic matter content from all the three studied greenhouses), at 30 DAT (beginning of flowering stage), the β-xylosidase potential activity was significantly higher in biofertilizer 1 soil than in control soil ($p<0.05$) by 42%. However, not statistically significant, the other extracellular enzymatic potential activities were higher in the biofertilizer 1 soil than in control soil, showing a positive trend for biofertilizer 1 in stimulating the other soil enzymatic activities. Control and biofertilizer 2 soils presented similar extracellular enzymatic potential activities for all the studied enzymes activities. The extracellular enzymatic potential activities in greenhouse 2 from the different treatments soils at 30 DAT are represented in Figure 3.15a.

At 120 DAT (beginning of the fruit ripening stage), in greenhouse 2, the β-xylosidase, *N*-acetylglucosaminidase and phosphatase activities where significantly lower in biofertilizer 2 soil than control soil ($p\leq0.05$) by 24%, 25% and 32%, respectively. At this sampling time, no significant differences were found between control and biofertilizer 1 soils in any of the studied potential enzymatic activities. The extracellular enzymatic potential activities in greenhouse 2 from the different treatments soils at 120 DAT are represented in Figure 3.15b.

At 150 DAT (advanced fruit ripening stage), in greenhouse 2, no significant differences between the biofertilizers and control soils were found for all the studied extracellular enzymatic potential activities. The extracellular enzymatic potential activities in greenhouse 2 from the different treatments soils at 150 DAT are represented in Figure 3.15c.

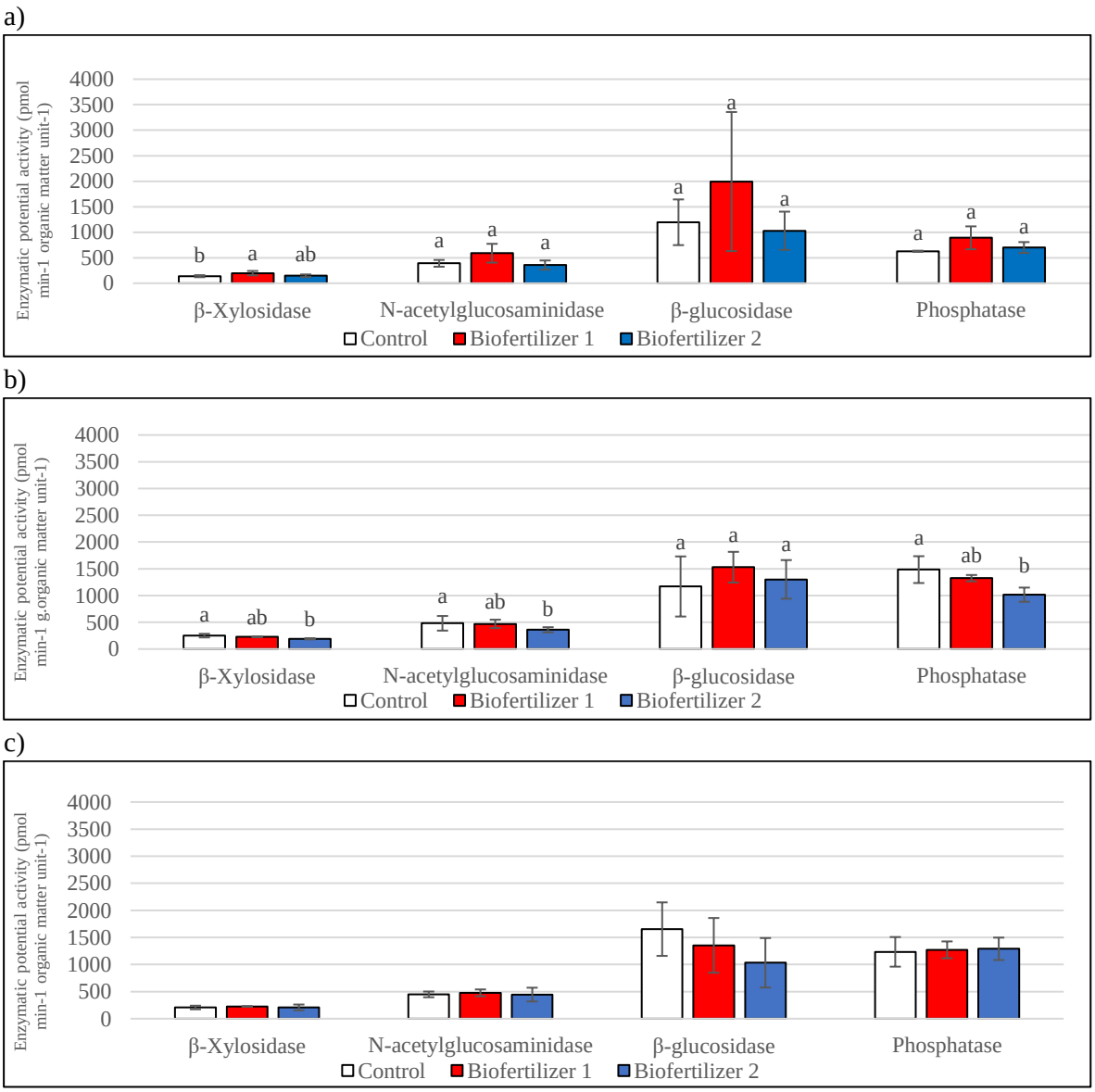



**Figure 3. 15:** (a) Extracellular enzymatic potential activities by organic matter unit of the enzymes β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase in greenhouse 2 at 30 DAT by the different treatments soils. Biofertilizer 1 soil presented a significantly higher β-xylosidase potential activity than the one from control soil ($p<0.05$).; (b) Extracellular enzymatic potential activities by organic matter unit of the enzymes β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase in greenhouse 2 at 120 DAT in the different treatments soils. Biofertilizer 2 soil presented a significantly lower β-xylosidase, *N*-acetylglucosaminidase and phosphatase potential activities than in control soil ($p\leq0.05$).; (c) Extracellular enzymatic potential activities by organic matter unit of the enzymes β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase in greenhouse 2 at 150 DAT in the different treatments soils. No significant differences were found in any of the extracellular enzymatic potential activities between the different treatments soils.; Bars represent the mean of 3 replicates ± SD. Bars sharing the same letter do not differ significantly at $p\leq0.05$, according to Tukey's HSD test.

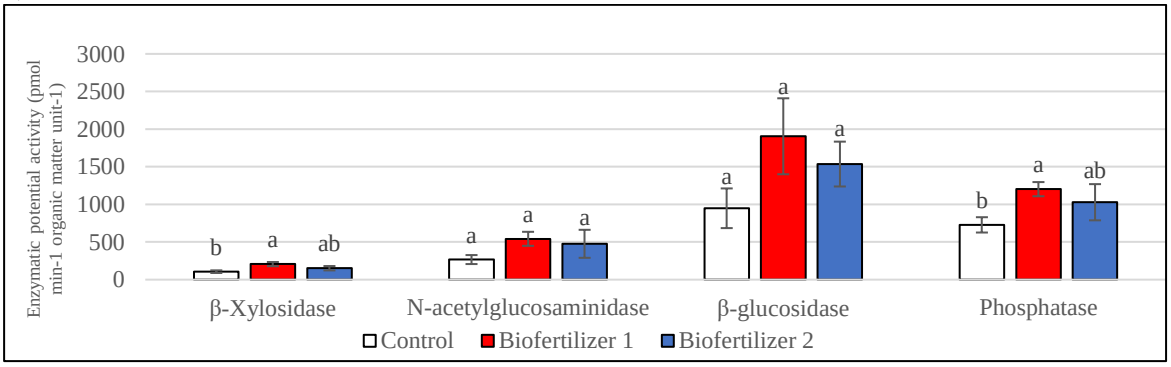
In greenhouse 3 (tillage management and higher soil organic matter content than greenhouse 2), at 30 DAT (beginning of the flowering stage), β-xylosidase and phosphatase potential activities were significantly higher in biofertilizer 1 soil than the ones from control soil ($p<0.05$) by 95% and 65%, respectively. However, not statistically significant, *N*-acetylglucosaminidase and β-glucosidase potential activities were close to be significantly higher in biofertilizer 1 soil than that from control soil ($p=0.099$ and $p=0.077$, respectively). At 30 DAT, for all the studied extracellular enzymatic potential activities, biofertilizer 1 soil presented the highest potential activities values from all the treatments soils. The biofertilizer 2 and control soils didn't present any significant differences between them concerning

to all the studied extracellular enzymatic potential activities. However, biofertilizer 2 soil presented higher activities values than control soil for all the studied extracellular enzymes potential activities. This trend can suggest that the biofertilizers soils, and on a larger scale, the biofertilizer 1 soil, were effective in enhancing all the studied potential enzymatic activities over the control at 30 DAT in greenhouse 3. The extracellular enzymatic potential activities in greenhouse 3 from the different treatments soil at 30 DAT are represented in Figure 3.16a.

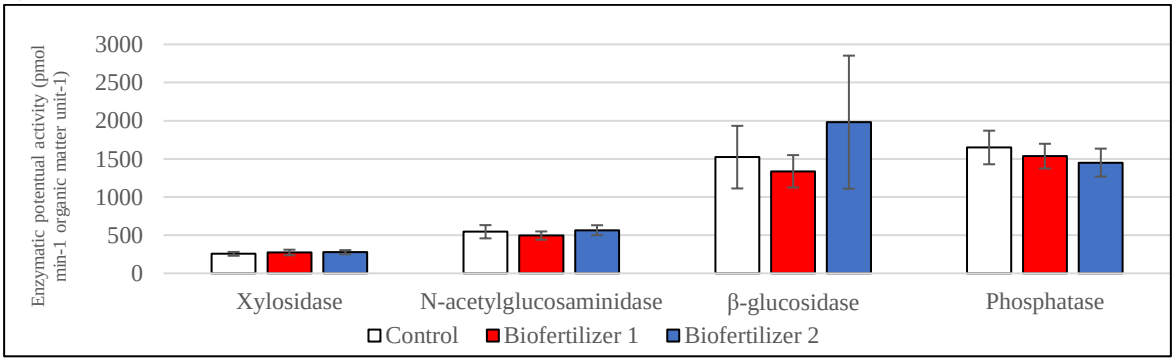
At 120 DAT (beginning of fruit ripening) there were no statistically significant differences in all the studied extracellular enzyme activities between control and biofertilizers soils. The extracellular enzymatic potential activities in greenhouse 3 from the different treatments soil at 120 DAT are represented in Figure 3.16b.

At 150 DAT (middle fruit ripening stage), β-xylosidase and phosphatase activities were significantly higher in biofertilizer 1 soil than that from control soil ($p<0.05$ and $p<0.01$, respectively) by 44% and 56%, respectively. Concerning to biofertilizer 2 soil there was no significant differences in all the studied extracellular enzymatic potential activities between control soil. The extracellular enzymatic potential activities in greenhouse 3 from the different treatments soil at 150 DAT are represented in Figure 3.16c.

a)




b)




c)

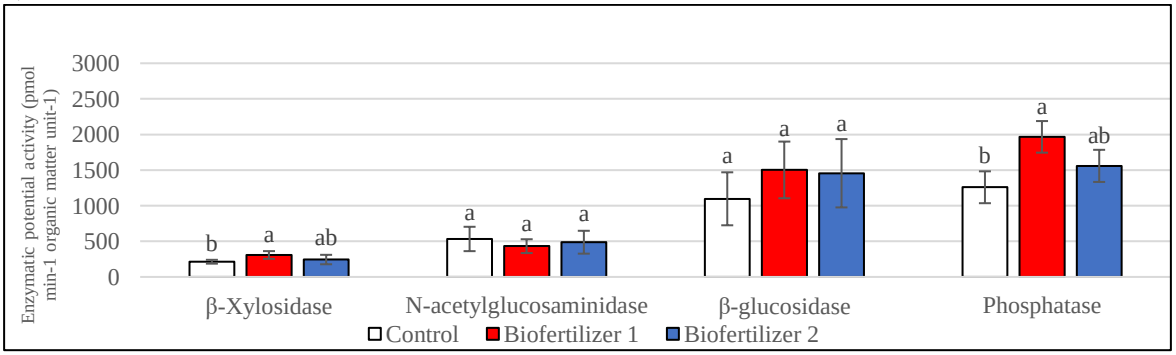


**Figure 3. 16:** (a) Extracellular enzymatic potential activities by organic matter unit of the enzymes β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase in greenhouse 3 at 30 DAT in the different treatments soils. Biofertilizer 1 soil presented significantly higher β-xylosidase and phosphatase potential activities than that from control soil ($p<0.05$).; (b) Extracellular enzymatic potential activities by organic matter unit of the enzymes β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase in greenhouse 3 at 120 DAT in the different treatments soils. No significant differences were found between the different treatments soils.; (c) Extracellular enzymatic potential activities by organic matter unit of the enzymes β-xylosidase, *N*-acetylglucosaminidase, β-glucosidase and phosphatase in greenhouse 3 at 150 DAT in the different treatments soils. Biofertilizer 1 soil presented a significantly higher β-xylosidase and phosphatase potential activities ($p<0.05$ and $p<0.01$, respectively) than that from control soil.; Bars represent the mean of 3 replicates ± SD. Bars sharing the same letter do not differ significantly at $p\leq0.05$, according to Tukey's HSD test.

### *3.5.5. Effect of biofertilizer on the enzymatic activity ratios*

The ratios between the extracellular enzymatic potential activities related with nutrient availability can illustrate the nutrients demands by the plants and soil microorganisms (Sinsabaugh et al., 2008).

Enzyme activities related with C availability ($C_{enz}$) was represented by the sum of β-xylosidase and β-glucosidase activities values. The enzyme activity related with N availability ($N_{enz}$) was represented by *N*-acetylglucosaminidase activities values. Finally, the enzymatic activity related with P availability ($P_{enz}$) was represented by phosphatase activities values. All the enzymatic activities were treated by organic matter unit.

In order to access the ratios between the studied enzymatic nutrient availability related activities, it were generated $\ln(C_{enz}):\ln(N_{enz})$, $\ln(C_{enz}):\ln(P_{enz})$ and $\ln(N_{enz}):\ln(P_{enz})$ ratios. The $\ln(C_{enz}):\ln(N_{enz})$ and $\ln(C_{enz}):\ln(P_{enz})$ ratios represents the enzymatic resources directed towards the acquisition of organic N and organic P relative to the enzymatic activity for C acquisition by soil microorganisms and plant, respectively, and the $\ln(N_{enz}):\ln(P_{enz})$ ratio represents the enzymatic resources directed towards the organic P acquisition relative to the organic N acquisition activity by soil microorganisms and plants (Sinsabaugh et al., 2008). The generated ratios between the selected soil enzymes activity were influenced differently by several factors: the biofertilizers treatments, the different greenhouses, and the different tomato lifecycle phases (represented by the sampling time).

In greenhouse 1, at 30 and 120 DAT, no significant differences were found between the different treatments. The enzymatic activities ratios values from the different treatments soils in greenhouse 1 at 30 DAT and 120 DAT are represented in Table 3.1 and Table 3.2, respectively.

**Table 3. 1:** Enzymatic activities ratios at 30 DAT in greenhouse 1. Each ratio value represents a mean of 3 replicates.

| **Treatments** | **$\ln(C_{enz}):\ln(N_{enz})$** | **$\ln(C_{enz}):\ln(P_{enz})$** | **$\ln(N_{enz}):\ln(P_{enz})$** |
|---|---|---|---|
| Control | 1.158 | 0.931 | 0.805 |
| Biofertilizer 1 | 1.158 | 0.931 | 0.804 |
| Biofertilizer 2 | 1.190 | 0.913 | 0.768 |

**Table 3. 2:** Enzymatic activities ratios at 120 DAT in greenhouse 1. Each ratio value represents a mean of 3 replicates.

| **Treatments** | **$\ln(C_{enz}):\ln(N_{enz})$** | **$\ln(C_{enz}):\ln(P_{enz})$** | **$\ln(N_{enz}):\ln(P_{enz})$** |
|---|---|---|---|
| Control | 1.154 | 0.942 | 0.816 |
| Biofertilizer 1 | 1.156 | 0.961 | 0.832 |
| Biofertilizer 2 | 1.142 | 0.917 | 0.803 |

At 150 DAT, the $\ln(C_{enz}):\ln(N_{enz})$ ratio values were significantly higher in biofertilizer 1 ($p<0.01$) and biofertilizer 2 ($p<0.01$) soils than the one from control soil by 4% and 5%, respectively. Concerning to the $\ln(C_{enz}):\ln(P_{enz})$ and $\ln(N_{enz}):\ln(P_{enz})$ ratio no significant differences were found between the treatments. The enzymatic activities ratios values from the different treatments soils in greenhouse 1 at 150 DAT are represented in Table 3.3.

**Table 3. 3:** Enzymatic activities ratios at 150 DAT in greenhouse 1. For each column, the values (mean of 3 replicates) sharing the same letter do not differ significantly at $p \leq 0.05$, according to Tuckey's HSD test.

| **Treatments** | **ln($C_{enz}$):ln($N_{enz}$)** | **ln($C_{enz}$):ln($P_{enz}$)** | **ln($N_{enz}$):ln($P_{enz}$)** |
|---|---|---|---|
| Control | $1.10^{b}$ | $0.906^{a}$ | $0.826^{a}$ |
| Biofertilizer 1 | $1.143^{a}$ | $0.933^{a}$ | $0.816^{a}$ |
| Biofertilizer 2 | $1.155^{a}$ | $0.932^{a}$ | $0.807^{a}$ |

In greenhouse 2 at 30 DAT no significant differences were found in the three studied ratios between the different treatments soils. The enzymatic activities ratios values from the different treatments soils in greenhouse 2 at 30 DAT are represented in Table 3.4.

**Table 3. 4:** Enzymatic activities ratios at 30 DAT in greenhouse 2. Each ratio value represents a mean of 3 replicates.

| **Treatments** | **ln($C_{enz}$):ln($N_{enz}$)** | **ln($C_{enz}$):ln($P_{enz}$)** | **ln($N_{enz}$):ln($P_{enz}$)** |
|---|---|---|---|
| Control | 1.200 | 1.111 | 0.925 |
| Biofertilizer 1 | 1.190 | 1.116 | 0.936 |
| Biofertilizer 2 | 1.200 | 1.075 | 0.895 |

At 120 DAT, in greenhouse 2, there was significant differences in the studied ratios driven by the biofertilizers application. Concerning to ln($C_{enz}$):ln($N_{enz}$) ratio, biofertilizer 2 soil presented a significantly higher ratio value than control soil ($p<0.05$) by 6%. The ratio between ln($C_{enz}$):ln($P_{enz}$) was significantly higher in biofertilizer 1 and biofertilizer 2 soils comparing to that from the control soil ($p<0.05$), by 5% and 7%, respectively. For the ln($N_{enz}$):ln($P_{enz}$) ratio, no significant differences were found between the treatments soils. The enzymatic activities ratios values from the different treatments soils in greenhouse 2 at 120 DAT are represented in Table 3.5.

**Table 3. 5:** Enzymatic activities ratios at 120 DAT in greenhouse 2. For each column, values (mean of 3 replicates) sharing the same letter do not differ significantly at $p \leq 0.05$, according to Tuckey's HSD test.

| **Treatments** | **ln($C_{enz}$):ln($N_{enz}$)** | **ln($C_{enz}$):ln($P_{enz}$)** | **ln($N_{enz}$):ln($P_{enz}$)** |
|---|---|---|---|
| Control | $1.169^{b}$ | $0.985^{b}$ | $0.843^{a}$ |
| Biofertilizer 1 | $1.215^{ab}$ | $1.038^{a}$ | $0.855^{a}$ |
| Biofertilizer 2 | $1.24^{a}$ | $1.054^{a}$ | $0.850^{a}$ |

At 150 DAT, in greenhouse 2, concerning to ln($C_{enz}$):ln($N_{enz}$) ratio, there were no significant differences between the treatments soils. The ln($C_{enz}$):ln($P_{enz}$) ratio value in biofertilizer 2 soil was significantly lower than the one from control soil ($p<0.05$) by 7%. For the ln($N_{enz}$):ln($P_{enz}$) there was no significant difference between the different treatments soils. The enzymatic activities ratios values from the different treatments soils in greenhouse 2 at 150 DAT are represented in Table 3.6.

**Table 3. 6:** Enzymatic activities ratios at 150 DAT in greenhouse 2. For each column, values (mean of 3 replicates) sharing the same letter do not differ significantly at $p \leq 0.05$, according to Tuckey's HSD test.

| **Treatments** | **ln($C_{enz}$):ln($N_{enz}$)** | **ln($C_{enz}$):ln($P_{enz}$)** | **ln($N_{enz}$):ln($P_{enz}$)** |
|---|---|---|---|
| Control | $1.229^{a}$ | $1.058^{a}$ | $0.860^{a}$ |
| Biofertilizer 1 | $1.188^{a}$ | $1.026^{ab}$ | $0.863^{a}$ |
| Biofertilizer 2 | $1.164^{a}$ | $0.987^{b}$ | $0.848^{a}$ |

In greenhouse 3 there were no significant differences between the different treatments soils, in all the studied ratios and in all the sampling times. The enzymatic activities ratios values from the different treatments soils in greenhouse 3 at 30 DAT, 120 DAT and 150 DAT are represented in Table 3.7, Table 3.8, and Table 3.9, respectively.

**Table 3. 7:** Enzymatic activities ratios at 30 DAT in greenhouse 3. Each ratio value represents a mean of 3 replicates.

| **Treatments** | **ln($C_{enz}$):ln($N_{enz}$)** | **ln($C_{enz}$):ln($P_{enz}$)** | **ln($N_{enz}$):ln($P_{enz}$)** |
|---|---|---|---|
| Control | 1.247 | 1.054 | 0.846 |
| Biofertilizer 1 | 1.215 | 1.077 | 0.886 |
| Biofertilizer 2 | 1.216 | 1.073 | 0.883 |

**Table 3. 8:** Enzymatic activities ratios at 120 DAT in greenhouse 3. Each ratio value represents a mean of 3 replicates.

| **Treatments** | **ln($C_{enz}$):ln($N_{enz}$)** | **ln($C_{enz}$):ln($P_{enz}$)** | **ln($N_{enz}$):ln($P_{enz}$)** |
|---|---|---|---|
| Control | 1.186 | 1.008 | 0.850 |
| Biofertilizer 1 | 1.190 | 1.006 | 0.846 |
| Biofertilizer 2 | 1.211 | 1.055 | 0.871 |

**Table 3. 9:** Enzymatic activities ratios at 150 DAT in greenhouse 3. Each ratio value represents a mean of 3 replicates.

| **Treatments** | **ln($C_{enz}$):ln($N_{enz}$)** | **ln($C_{enz}$):ln($P_{enz}$)** | **ln($N_{enz}$):ln($P_{enz}$)** |
|---|---|---|---|
| Control | 1.147 | 1.000 | 0.874 |
| Biofertilizer 1 | 1.218 | 0.984 | 0.808 |
| Biofertilizer 2 | 1.201 | 1.031 | 0.859 |

## 4. Discussion

Biofertilizers use in the agriculture have been proving its efficiency in improving crops productivity while decreasing the environmental impact of the farming activities (Pacheco et al., 2021). The results obtained in this work support this view and provide pathways for future research towards the design and production of better and more efficient biofertilizers. The use of biofertilizers (biofertilizer 1= *Bacillus* + *Pseudomonas* consortium; biofertilizer 2= *Azospirillum* + *Pseudomonas* consortium) tended to increase the total commercial tomato yield, as well as that of each harvest in all the studied greenhouses (Figure 3.1; Figure 3.2 and Figure 3.3). This trend suggests that not only the total production was enhanced, but also an anticipation of the fruit production was driven by the biofertilizers inoculation which may represent higher economic benefits for the farmer, since an earlier tomato production can represent higher market prices (Mourão et al., 2017).

The consistency of this trend across greenhouses with distinct characteristics and harvests occasions, supports the hypothesis that the inoculation of soils with biofertilizers have the potential to increase crop productivity and production in a range of fertility conditions due to their beneficial effects on the soil-plant interactions.

The total final crop yield is assumed as an integrated proxy for all the preharvest technological, environmental, and biological factors that influenced fruit production along all the plant lifecycle stages (Liliane and Charles, 2020). In this work, from all the factors affecting the final crop yield, we focused on the soil fertility factor, which is considered as one of the most relevant, especially in long-term farming soils (Wang, 2014). Soil fertility is the ability of the soil to provide the required optimum amounts of available nutrients in order to reach the optimum crop growth. Additionally, the soil fertility is related to a greater diversity of the soil microbial community and improved soil structure (Mader, 2002; Wang, 2014).

The different soil characteristics of each greenhouse affected the biofertilizers soil performance and their effect on the crop, highlighting possible distinct paths of the biofertilizers action. In general, the soil organic matter content and its C fractions were the main drivers of the biofertilizer influence on soil dynamic properties, mainly of its extracellular enzymatic activities. The soil P content and the plant P demand seemed to be determinant for the effectiveness of the biofertilizers in increasing plant P acquisition.

In resume, our data suggests that:

(1) biofertilizer 1 application was more effective in increasing tomato productivity in soils with higher organic matter contents with higher labile C fractions concentrations, i.e., greenhouse 3, suggesting that biofertilizer 1 increased nutrient availability through the promotion of nutrient cycling driven by soil extracellular enzymatic activities (Sinsabaugh et al., 2008; Bowles et al., 2014; Zhou and Staver, 2019).

(2) biofertilizer 2 was more effective in increasing tomato production in soils with lower total P and organic matter contents, i.e., greenhouse 2, highlighting its potential to increase P availability to the plant under conditions where P is more limiting.

The trend for a higher tomato yield by biofertilizer inoculation, without increasing conventional fertilization, can be a promising strategy to generate more food without representing an increase in conventional fertilization practices.

*4.1. The different greenhouse characteristics and their influence in the enzymatic activity*

The enzymatic activities of the control soils from the different greenhouses were compared in order to understand the relation between the greenhouses characteristics and soil enzymatic activities.

Our first hypothesis was that the greater the amount of soil organic matter, the greater the soil enzymatic activities. However, greenhouses with similar levels of organic matter (i.e., greenhouse 1 and 3) displayed distinct levels of enzymatic activities (Figures 3.4; Figure 3.11). From this observation we understand that the relationship between soil enzymatic activities and organic matter levels is more complex than expected.

Taking in consideration that greenhouse 1 had similar organic matter content than in greenhouse 3 and was characterized by no tillage management, higher soil dehydrogenase and extracellular enzymatic activities would be expected for greenhouse 1 than for greenhouses 2 or 3. This hypothesis is based on the rational that soil fungal community is the main contributor to soil extracellular enzymatic activities and the most affected by tillage (Balota et al., 2003; Mirás-Avalos et al., 2011; Lu et al., 2018). However, greenhouse 1 soil presented the lowest dehydrogenase and extracellular enzymatic activities (Figure 3.4; Figure 3.11). This observation led to our second hypothesis that not only the amount of soil organic matter, but also the quality of organic matter can influence soil enzymatic activities. The observed trend in greenhouse 1 soil for a relatively higher β-xylosidase over β-glucosidase activity in comparison to the soils of the other greenhouses supported that not just the quantity but also the quality of organic matter is an important factor that impacts the enzymatic activity (Figure 3.12; Figure 3.13). β-xylosidase is part of the xylanase enzymatic complex, acting mainly on hemicellulose and producing xylose as the main product, while β-glucosidase is part of the cellulase enzymatic complex, acting mainly on cellulose and having glucose as the main product (López-Mondéjar et al., 2016; Houfani et al., 2019). In the soil xylose is used as a main carbon source by the majority of microorganisms only when glucose is scarcer (Sievert et al., 2017).

In this way the lower ratio between β-glucosidase and β-xylosidase activities observed in greenhouse 1 (Figure 3.13) may be an indirect evidence of a higher recalcitrant C fraction relatively to the other more labile C fractions of the soil organic matter in the greenhouse 1, in comparison to the soil organic matter content of the other greenhouses. The no tillage practices in greenhouse 1 and the no introduction of old plant debris, contrarily to the other two greenhouses, may have led to a higher cellulose consume over the years by the soil microbiota, contributing to a relatively higher hemicellulose to cellulose ratio in the soil of greenhouse 1 in comparison to that of greenhouses 2 and 3.

This relative increased availability of xylose over glucose in greenhouse 1 could also explain the down regulation of the soil microbial activity, since glucose is the most efficient carbon source for the majority of the soil microbial community (Yang et al., 2012; Juan et al., 2015; Li et al., 2018). Concerning to the extracellular enzymatic potential activity, many studies demonstrate that their correlation with organic matter is dependent on the recalcitrance degree of the organic matter. Usually, the more labile soil organic matter fractions are those presenting a higher correlation with hydrolytic extracellular enzymatic activities (Phiri et al., 2001; Štursová and Baldrian, 2011; Verma et al., 2017; Zhao et al., 2016; Zhang et al., 2020). These results corroborate the idea that not just the amount, but also the quality of organic matter affects the microbial development and its interaction with the soil plant system.

Additionally, we suggest that the ratio between β-glucosidase and β-xylosidase activities should be explore as a potential indicator of the recalcitrant level of the soil organic matter.

The higher soil dehydrogenase activity and extracellular enzymatic activities observed in greenhouse 3 relatively to the other greenhouses (Figure 3.4 and Figure 3.11) support the above hypotheses and are supported by the literature where higher organic matter soils, especially the labile C fraction, have been

noticed to host higher microbial biomass and extracellular enzymatic activities (DeMartini et al., 2013; Costa et al., 2016b; Phiri et al., 2001; Demoling et al., 2007; Murphy et al., 2011; Šturs ová and Baldrian, 2011; Verma et al., 2017; Zhao et al., 2016; Zhang et al., 2020).

*4.2. Soil dehydrogenase activity in the different greenhouses: the influence of biofertilizers*

Plants and microorganisms are co-evolving over millions of years. Plant microbial relationships are extremely important for the plants to overcome stress and these interactions are modulated by the abiotic and biotic environment in reciprocal ways (Badri et al., 2009; Goh et al., 2013).

From the three studied greenhouses, only in greenhouse 2 both biofertilizers application showed a trend to increase the soil dehydrogenase activity (Figure 3.5). In greenhouse 2, both biofertilizer application increased the soil dehydrogenase activity by around 29% over the control (Figure 3.5a), whereas no differences between control and biofertilizers soils were found in the other greenhouses, regarding to this parameter (Figure 3.5 b,c).

Considering that from the three greenhouses, the greenhouse 2 was the one with the lowest soil content of total P (Figure 3.9) and presented a tendency towards a lower soil organic matter content (Figure 3.6), it is possible to suggest that the application of biofertilizers had higher impact on soil microbial activity under higher nutritional demand conditions.

The nutritional limitation is considering as one of the main limiting factors for microbial growth. It is possible to advocate that under the lower P and C conditions, observed in greenhouse 2, the action of biofertilizers in making nutrient available has a higher impact in microbial activity than under higher fertility conditions, as observed in greenhouses 1 and 3 (Demoling et al., 2007). Additionally, in lower P nutritional soil conditions, the soil microorganisms and plants may adequate their interactions through root exudates compositions and physiological activity to optimize nutrient bioavailability (Bais et al., 2006; Carvalhais et al., 2013; Yuan et al., 2015).

Although all the strains composing biofertilizers 1 and 2 have high potential for P solubilization and mineralization, their effective action in the soil is modulated by a plethora of factors that can justify distinct performances in response to specific conditions. This will be translated in distinct efficiencies of the biofertilizers interaction with the soil and plants according to the biotic and abiotic conditions. In greenhouse 1 and 3, although the application of biofertilizers did not influence soil microbial activity in relation to the control, the composition of the rhizosphere microbial community may have changed so that these biofertilizers were able to have an impact on other studied (and not studied) parameters.

All these observations support the biofertilizer Multiple Mechanism Theory which states that PGPR's do not act through a single but through a multiple potential mechanism that get in action according to specific conditions of environment, plant genotype and soil microbial community among others (Cassán and Diaz-Zorita., 2016; Cassán et al., 2020). All those factors can shape the intensity and effectiveness of each one of the mechanisms developed by the inoculated PGPR's. Thus, in different conditions, it is expected a different combination of modes of actions by the PGPR's and consequently a different effect on the crops (Bashan and Levanony, 1990; Bashan and De-Bashan, 2010; Cassán et al., 2020).

*4.3. Influence of biofertilizers on plant available P acquisition in the different greenhouses*

Low P uptake by the plant is linked with lower yields (Smit et al., 2009; Dixon et al., 2020). The two tested biofertilizers influenced the trend of P acquisition by the tomato plants differently in each greenhouse, suggesting that total soil P and soil organic matter contents influence the performance of biofertilizers, regarding this parameter. Both biofertilizer 1 (*Bacillus* + *Pseudomonas*) and biofertilizer 2 (*Azospirillum* + *Pseudomonas*) microorganisms are known for their ability to increase the plant P

acquisition efficiency (PAE) through both direct and/or indirect mechanisms (Ramaekers et al., 2010; Pacheco et al., 2021). From the three studied greenhouses, it seemed that both biofertilizers had more impact in the available P plant uptake trend in greenhouse 2.

Greenhouse 2 presented the lower total soil P and soil organic matter contents (Figure 3.6; Figure 3.9), and thus the highest P demand by the plant and rhizosphere microbial community from the three studied greenhouses.

In greenhouse 2, at the time of high vegetative growth and beginning of the flowering stage, 30 DAT, both biofertilizers showed a trend to increase the soil available P concentration over the control (Figure 3.8b). Based on the dehydrogenase activity at 30 DAT, both biofertilizers showed a trend for stimulating microbial activity (Figure 3.5a), suggesting that in lower P conditions, the biofertilizers application may have contributed to a higher soil available P concentration via stimulation of the PGPR community.

The decrease in soil available P concentration observed between 30 to 120 DAT in both biofertilizers soils and between 120 DAT and 150 DAT by biofertilizer 2 soil, in relation to the control soil, reveals that in biofertilizers soils, the available P plant consumption rate was higher than the P that was being released by the soil particles into the soil solution (Figure 3.8b). The time interval between 30 to 120 DAT corresponds to the crop bloom period and the beginning of the fruit formation, a time of high consumption of P by the plant since fruits are relevant sinks of plant P (Tagliavini et al., 2005; Juárez-Maldonado et al., 2017).

The mechanisms of biofertilizers stimulation of P consumption by the plant may involve the microbial production of phytohormones. Both biofertilizers integrated microorganisms are known for their ability to produce phytohormones, namely IAA, especially *Azospirillum sp.* from biofertilizer 2 (Ramaekers et al., 2010; Cassán et al., 2014; Egamberdieva et al., 2017). The production of phytohormones by microorganisms as well as the plant phytohormone sensitivity may be triggered by P deficiency (López-Bucio, 2002; Nacry et al., 2005). The enhancement of root hairs and changes in the root architecture (as a higher root-to-shoot ratio) are efficient strategies for the enhancement of the P acquisition by the plant in lower P conditions that are triggered by the phytohormones action, especially the IAA (López-Bucio, 2002; Ticconi and Abel, 2004; Nacry et al., 2005; Pérez-Torres et al., 2008; Jindo et al., 2016).

Comparing the influence of the two biofertilizers on the trend of soil available P, it was possible to observe that biofertilizer 2 was more effective in promoting the consumption of P up to a more advanced stage of the plant life cycle than biofertilizer 1 (Figure 3.8b). The greater promotion of P consumption by biofertilizer 2 compared to biofertilizer 1 may explain the differences in the tomato production of the two biofertilizers (Figure 3.2).

In greenhouses 1 and 3, with higher total soil P and organic matter contents, the influence of biofertilizers in the trend of soil available P was not so clear (Figure 3.8a,c). This can be attributed to a more stochastic and less deterministic relation between plant, soil, and microbial community (Bryla and Koide, 1998). In this way the soil total P content and the P demand may had shaped the effectiveness of the distinct modes of actions of each biofertilizer in supplying P to the plant.

The different impacts on the available P trends in the different greenhouses by the two biofertilizers highlight, once again, the multi-mechanistic action of biofertilizers: in systems with lower soil total P and thus, higher P needs, the biofertilizers were effective in providing the needed P to the plant via a phytohormonal pathway, and in systems with higher soil total P concentration and thus, lower P needs, the impact of the biofertilizers in the available P trend was not so clear since the phytohormonal pathway was no longer as effective in providing P to the plant as in lower total soil P content conditions.

*4.4. Impact of the biofertilizers on the extracellular enzymatic activities in the different greenhouses*

The effects of the biofertilizers in the soil extracellular enzyme activities were greenhouse-specific, showing that several soil conditions determine the mode of action and the results obtained with each one.

Biofertilizers may enhance extracellular enzyme activities due to: (1) direct production of enzymes by the inoculated microorganisms; (2) indirect mechanisms, by the microbial inoculant stimulation of enzyme production or activity made by the autochthonous microbial soil community; or (3) a combination of both (Castro-Sowinski et al., 2007).

The enhancement of the soil extracellular enzyme activities by the biofertilizers application tended to be higher in greenhouse 3 in comparison to the other greenhouses. In this greenhouse, from the two biofertilizers, biofertilizer 1 was able to stimulate more soil enzymatic activities than biofertilizer 2.

At 30 and 150 DAT, biofertilizer 1 application was able to significantly increase soil phosphatase and β-xylosidase activities over those of the control soils. Additionally, at 30 DAT, the application of this biofertilizer in greenhouse 3, showed an increase in *N*-acetylglucosaminidase and β-glucosidase activities that were close to be significantly higher than the enzymatic activities from the control soil.

During the initial phases of vegetative plant development, a higher phosphatase activity, with increased P availability to the crop, may had contributed for the plant development. A lower P plant uptake in the vegetative growth is related with P allocation for the root, decreasing the allocation of this nutrient in other sinks, such as fruits, and consequently decreasing the fruit production. The proper root establishment associated with proper P may increase P exports to fruits and therefore increasing the crop productivity (Hansen and Lynch, 1998; Ma et al., 2020).

At 150 DAT (fruit ripening stage), the enhancement driven by biofertilizer 1 of the soil phosphatase and β-xylosidase activities may have contributed for the supply of the required amounts of nutrients to the plant and rhizosphere microorganisms contributing as well to the soil fertility by enhancing the nutrient cycling.

The enhancement of the soil extracellular enzymatic activities by biofertilizer 1 contributed for the improvement of crucial soil processes, such nutrient cycling, benefiting the plant development and productivity (Sinsabaugh et al., 2008; Bowles et al., 2014; Zhou and Staver, 2019).

Biofertilizer 2 was not as effective as biofertilizer 1 in increasing the soil extracellular enzymatic activities in greenhouse 3, however, biofertilizer 2 microorganisms may have beneficiated from the higher labile organic matter content since, at 30 DAT, it presented a trend for higher enzymatic activities than control soil for all the studied enzymatic activities. This suggests that the two biofertilizers established different relationships with the plant, interacting and benefiting the plant and the rhizosphere microbial community through different ways.

Nevertheless, in greenhouse 3, it was the biofertilizer 1 soil that presented the highest tomato production. In this way it is possible to suggest that in higher organic matter content with higher labile C fractions and higher soil total P contents, the enhancement of the extracellular enzymatic activities is an effective mode of action to increase the tomato production. Even so, the fact that biofertilizer 2 induced a trend for a higher tomato production in relation to control, illustrates its ability to increase the tomato productivity through other mechanisms than the enhancement of the soil enzymatic activities.

No significant differences were found in the extracellular enzymatic nutrient acquisition activities ratios in any of the sampling times in greenhouse 3. The higher organic matter content and the suggested higher labile C fraction in this greenhouse may had led that in every treatment soil the nutrient requirement may had been supressed more easily, even in the absence of the biofertilizers.

In greenhouse 1, the suggested higher recalcitrant C fraction of the soil organic matter may have prevented the enhancement of the enzymatic activities by the biofertilizers as originally hypothesized.

The enzymatic activity is found to be more co-related with the more labile C fraction instead of the total organic matter content (Zhao et al., 2016; Verma et al., 2017; Zhang et al., 2020). In this way the biofertilizers mode of actions based on the increase of the enzymatic activity, especially by biofertilizer 1, proved to be ineffective in higher C recalcitrant conditions.

Concerning to the soil extracellular enzymatic ratios the only significant differences between the control and biofertilizers soils were observed at 150 DAT (Table 3.3). At this sampling time, in the end of tomato production stage, the significantly higher $\ln(C_{enz}):\ln(N_{enz})$ ratio in the soils treated with biofertilizers soils in relation to the control, may characterize a stage of high carbon abundance provided by the crop residues (including the roots) and a lower nutrient demand since the plant is not active any longer in soil nutrient acquisition at this final stage of its lifecycle.

In greenhouse 2, the promotion of the extracellular enzymatic activity by the biofertilizer 1 application was not as efficient as in greenhouse 3. It was only observed a trend for a higher enhancement of the soil enzymatic activities during the initial plant development (30 DAT). Despite the high plant nutrient demand observed at this stage, the absolute amount of nutrients consumed by the plant is lower than in more advanced phenological stages (Juárez-Maldonado et al., 2017).

Although in this greenhouse soil, the soil organic matter content had similar cellulose:hemicellulose ratio than greenhouse 3 soil, the trend for the lower organic matter content may had result in a lower organic substrate content for the soil extracellular enzymes express its activity. Thus, it is suggested that the lower organic substrate content may had limited the biofertilizer 1 mode of action in enhancing the soil extracellular enzymatic activity.

Concerning to biofertilizer 2, at 120 DAT, this biofertilizer soil presented a significantly lower β-xylosidase, *N*-acetylglucosaminidase and phosphatase activities than from control soil (Figure 3.15b). However, in every harvest the biofertilizer 2 presented a higher tomato production (Figure 3.2), which can illustrate that the beneficial effects of this biofertilizer in the plant development was not in fact driven by enzymatic activity stimulation and also that a higher soil enzymatic activity in soils with relatively lower organic matter is not correlated with a higher yield.

A possible explanation for the lower enzymatic activities presented by the soil where biofertilizer 2 was applied when compared to the control soil at 120 DAT is that since the end products of the enzyme activity represses the same enzymatic activity by a negative feedback, the biofertilizer 2 mode of action may had acquire those end products through other mechanisms than the stimulation of enzymatic activity (Olander and Vitousek, 2000; Xiao et al., 2004; Rasmussen et al., 2006; Maillard et al., 2018). Interestingly, at 120 DAT it was observed that both biofertilizers soils, especially the biofertilizer 2 soil, presented higher $\ln(C_{enz}):\ln(N_{enz})$ and $\ln(C_{enz}):\ln(P_{enz})$ ratios (Table 3.5). The higher ratio values in the biofertilizers soils may suggests that the biofertilizers application contributed for a lower N and P demand by the plant and rhizosphere microorganisms when compared to the control soil. This lower demand suggests that the required nutrients by the plant and soil rhizosphere microorganisms were available in more optimum amounts in biofertilizers soils than in the control soil. This result gives robustness to the idea that in lower organic matter content the enhancement of the soil enzymatic activity shouldn't be the main strategy of the biofertilizers in benefiting the plant and that other mechanisms are being implemented by the biofertilizers which allowed the biofertilizers soils to present a lower demand for N and P than control soil, despite a lower enzymatic activity by biofertilizer 2 soil.

As already suggested, the beneficial mode of actions driven by the biofertilizers in greenhouse 2 were probably by the enhancement of P solubilization and PAE as discussed above (section 4.3). The lower organic matter and total P may had shaped the symbiotic relationship between the applied biofertilizers microorganisms and plant differently than the one observed in greenhouse 3.

Interestingly at 150 DAT the trend changed with the control soil presenting higher $\ln(C_{enz}):\ln(N_{enz})$ and $\ln(C_{enz}):\ln(P_{enz})$ ratios than the biofertilizers soils (Table 3.6). It is suggested that at 150 DAT, in the

end of the production cycle, the soil microorganisms and the plant from the biofertilizers soils faced a greater shortage of available nutrients due to the greater consume of soil nutrients by the plant to form the higher tomato production than control soil, presenting at this sampling time a higher demand for nutrients. We hypothesized that the biofertilizers application in greenhouse 2 contributed effectively to provide nutrients to the plants in the lifecycle stages where these nutrients were more needed.

The different amounts of C fractions in the greenhouses soil systems influenced the impact of the biofertilizers in the soil extracellular enzymatic activities, especially in biofertilizer 1 which is suggested to be one of the main and more effective mode of action of this biofertilizer.

Thus, in more labile C soil conditions, it is suggested that the enhancement of the soil extracellular activity is an important mode of action of biofertilizer 1 in enhancing the nutrient cycling and availability, contributing to soil fertility which may repercuss in the higher tomato productivity. On the opposite it seemed that in soil systems with a lower labile C to recalcitrant C ratio didn't allowed the biofertilizers to increase the soil extracellular enzymatic activities.

This again support the Multiple Mechanisms Hypothesis, which assumes that no single mechanism is involved in the promotion of plant growth by microorganisms (Cassán and Diaz-Zorita, 2016; Cassán et al., 2020). The soil microorganisms diversity and activities perhaps adapt to the characteristics of the soil in such a way that they are able, from the present conditions, to have the most possible effective effect on the symbiotic relationship between plant and soil microorganisms.

### *4.5. Impact of biofertilizers in soil organic matter content*

The use of biofertilizers in the crop management may enhance the decomposition of organic matter at least during the early stages of plant development where the root exudates may not be in enough amounts to support the establishment of the rhizosphere microbiome due to carbon limitations (Six and Jastrow, 2002).

In greenhouse 1, it was possible to detect a trend for a higher initial organic matter decomposition rate promoted by both biofertilizers and a higher and more efficient organic matter degradation in the soil where it was applied the biofertilizer 2 (Figure 3.7a). None of the analysis performed in this work explain this trend. It is possible that these trends were mediated by non-enzymatic hydrolysis processes, such as oxidation, which is effective in degrade the more recalcitrant fractions of the soil organic matter (Fritsche and Hofrichter, 2005; Tian and Shi., 2014).

Concerning to greenhouses 2 and 3, the biofertilizers application showed no obvious tendency to change organic matter degradation compared with the control.

### *4.6. Impact of biofertilizers in soil total phosphorus in the different greenhouses*

One of the goals of using P solubilizing microorganisms in agriculture is to make available to the plant the P immobilized and accumulated in the soil, reducing its concentration in the soil. The accumulation of P in the agricultural soils is a problem waiting for urgent solutions.

The continuous addition of P through drip fertigation led to P accumulation in all the greenhouse soils from 0 DAT to 150 DAT (Figure 3.9). Only a small fraction of the added P fertilizer (from 5% to 25%) is taken up by the crops, despite that in long term the P recovery by the plant might be higher (Schröder et al., 2011; Noor et al., 2017). Nevertheless, the low effectiveness of P fertilizers at the short term and the need for continuous P fertilizers application leads to unavailable P accumulation in the soil, representing a relevant environmental and sustainable issue (Smit et al., 2009; Van Vuuren et al., 2010). Therefore, a rational and sustainable use of P is mandatory.

The conversion of accumulated P from an unavailable to an available form, and its consequent plant and microbial use, associated with the stimulation of the soil biological functionalities, by the application of biofertilizers, has been referred to as an innovative and sustainable strategy to improve P crop use efficiency. Consequently, this could mitigate the problem of the depletion of the P reserves, since this would decrease the current P waste and a higher P consume efficiency (Saeid, 2018).

Our results support the potential of using biofertilizers to improve P acquisition efficiency in higher nutritional demand systems (Figure 3.8b). However, with regard to soil total P, all the greenhouses increased its total P concentrations from 0 to 150 DAT. This may be explained by the continuous addition of P by the drip fertigation system in a higher rate than the P plant consume rate, which led to the accumulation of P in the soil. Despite the high P introduction, other variables, such as pH, texture, or organic matter content, have been pointed out as being involved in soil-crop system response to biofertilizers (Khare and Arora, 2015).

So, in order to achieve a reliable performance of the P solubilizing biofertilizers in terms of more universal solutions, further studies should be done in order to create consortia that are able to perform well across distinct combinations of the efficiency determinant variables, especially in higher soil total P conditions, to reduce the soil total P concentration. This can be a promising strategy for securing P for future generations.

## 5. Conclusion

The expected increase in the world population will require greater food production in order to ensure the nutritional needs of the world population.

This work illustrated that the application of biofertilizers based on microbial consortia are promising tools to increase tomato monoculture production under greenhouse conditions. The increase in tomato production without an increase in the conventional fertilization use can be viewed as an open door to a more sustainable food production system. The less reliance on conventional fertilizers to form greater crop production is in itself a way to contribute to soil health and resources sustainability.

Although many biofertilizers are available in the market, their actual use is still far from their full potential use. One of the main reasons is the lack of consistency in the biofertilizer performance across crops, soil, and climatic conditions. Most of the farmers and technicians look for a mode of action that is characteristic of the biofertilizer and that can be recognized in all circumstances. This work supports a change of paradigm by showing that one biofertilizer does not have a defined "mode of action" but a plethora of potential actions. In this way, the actual "mode of action" will result from the integration of many variables by the distinct actors involved in the soil-plant interactions.

Data suggested that biofertilizer 1 may provide more benefits to the plant in greenhouses soils with higher levels of organic matter composed of higher concentrations of labile C fractions, while biofertilizer 2 may provide more benefits to the plant in greenhouses soils with low organic matter and low total P content and therefore a high P plant demand.

Soil organic matter content and its labile fraction were key drivers of the biofertilizers influence on soil dynamic properties, mainly the extracellular enzymatic activities. In its turn, the soil P conditions determined the influence of biofertilizers in the supply of P to the plant.

However, the versatility of the biofertilizer's responses towards the enhancement of the tomato production was in accordance with the biofertilizer's Multiple Mechanisms Hypothesis which argues that the promotion of plant growth and development by microorganisms is not limited to a single mode of action but instead, operates through a combination of mechanisms (Cassán and Diaz-Zorita, 2016; Cassán et al., 2020).

Apart from improving crop productivity and nutrient use efficiency, biofertilizers may also contribute to improve soil health through the promotion of microbial mediated soil processes, such as the nutrient cycling.

Further studies are needed to develop a biofertilizer that can be as efficient as possible in the most diverse soil conditions so these biological products can be presented as an efficient alternative to conventional fertilizers based on non-renewable products.

It is suggested that the scientific community of EU should continue to invest in the knowledge of European soils ecology, as well as of the most produced crops, in order to develop biofertilizers that are compatible with the diversity of soils and crops in the EU. In this way, biofertilizers will be important allies in the transition to a more sustainable agricultural system.

## 6. References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