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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída

Giuliana Gigliotti, UALG

Title

Effect of microalgal biomass on the strawberry (*Fragaria x ananassa*) yield and quality

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Resumo

O aumento da população mundial, que pode chegar a 10 bilhões de seres humanos em 2050, exige um aumento na demanda de produção de alimentos. No entanto, esse aumento acarreta algumas dificuldades que necessitam de soluções, tal como, a otimização da produção de fertilizantes e estimulantes químicos na agricultura, para incrementar a produção de alimentos. O uso excessivo, continuo e insustentável dos agroquímicos sintéticos pode levar à poluição antropogénica por nutrientes e à degradação ambiental. A aplicação intensiva de fertilizantes químicos na agricultura resulta numa quantidade excessiva de nutrientes nos oceanos, uma vez que, esses fertilizantes são lixiviados dos solos e transportados por escoamento (desencadeado pelas chuvas, irrigação em excesso ou derretimento da neve). Como consequência, há um excesso de reprodução de algas microscópicas que se depositam no mar e posteriormente são decompostas por bactérias - eutrofização. Estas utilizam todo o oxigênio disponível e, assim, provocam a morte de outros seres vivos marinhos por hipoxia. As áreas onde ocorre o depósito dessas microalgas são zonas mortas e podem servir de alerta para a urgência da aplicação de medidas contrárias, uma vez que, essas zonas são inabitáveis até se recomporem, o que pode levar muitos anos (biorremediação). A procura por soluções mais eficazes para a produção agrícola tem aumentado com o passar dos anos, considerando a necessidade crescente de melhorar a saúde humana e o meio ambiente.

O biofertilizante e o bioestimulante de microalgas representam uma alternativa promissora para alcançar uma agricultura mais sustentável e moderna, uma vez que contêm uma ampla gama de macro e micronutrientes, aminoácidos, vitaminas, minerais e fito hormônios promotores de crescimento de plantas. Os bioestimulantes promovem os processos naturais do vegetal, como absorção de nutrientes e tolerância a stresses abióticos, enquanto biofertilizantes são produto líquido gerado a partir da decomposição de matéria orgânica que, quando aplicados ao solo, melhoram as qualidades físicas, química e biológicas. Os recursos marinhos podem ser usados como biofertilizantes e bioestimulantes por suas propriedades ecologicamente corretas. O estudo das microalgas tem suscitado interesse na agricultura moderna como uma alternativa promissora para área de sustentabilidade agrícola, devido ao seu potencial para melhor germinação, crescimento de plantas, produtividade e eficiência no uso de nutrientes. Além disso, é uma alternativa mais ecológica e económica quando comparado ao uso de fertilizantes químicos.

A espécie *Chlorella vulgaris* é uma microalga verde (*Chlorophyta*), eucariótica, unicelular, encontrada em ambientes marinhos, ambientes de água doce e que contribui para a sustentabilidade do mundo, transformando CO_2 em O_2. O uso da *C. vulgaris* melhora as propriedades do solo aumentando o rendimento agrícola. Assim, esta microalga é a mais cultivada no mundo, sendo usada em diversas aplicações. *Fragaria x ananassa* Duch. (morango) são populares entre os consumidores em quase todas as partes do mundo, e a versatilidade de cultivo e adaptabilidade torna a sua produção global constante. O principal objetivo desse estudo é determinar o efeito de *Chlorella vulgaris* (gerada comercialmente) na produção de morangos. A finalidade deste trabalho é avaliar quais das quatro diferentes concentrações de extrato seco de *Chlorella vulgaris*, em suspensão em água destilada (utilizando um controle positivo - Algaman B e um controle negativo - água destilada), obtém o melhor rendimento e melhor qualidade de frutos e, por fim, determinar as condições ideais de pulverização de microalgas nos morangos.

O estudo foi desenvolvido no Horto da Universidade do Algarve, numa estufa coberta por plástico, sem aquecimento e com cobertura de tetos ajustáveis. A metodologia passou pelo cultivo dos morangos em sacos com substratos comerciais e fertilizante encapsulado, com liberação inicial rápida de nutrientes. Foram utilizadas estruturas metálicas para elevar os sacos de cultivo do chão, e canais plásticos, colocados embaixo dos sacos, para coleta da água de drenagem, com declividade de 2%. O projeto foi realizado em dois ensaios consecutivos, com o mesmo delineamento e procedimentos: cada ensaio foi constituído de 6 tratamentos (C+, C-, T1, T2, T3 e T4) em quatro blocos selecionados ao acaso (I, II, III e IV). Um total de 24 sacos de cultivo foram usados para cada ensaio e o período de cultivo, desde o plantio até a primeira colheita, pode levar cerca de três meses, de acordo com as condições meteorológicas. Os ensaios de cultivo foram realizados por um período de quatro meses, durante a primavera e o verão. Durante o período de cultivo de *F. x ananassa* realizaram-se medições para monitorizar o desenvolvimento das plantas e frutos, tais como, variações diárias de temperatura do ar, umidade, condutividade elétrica, pH da água de drenagem e controle de irrigação. A determinação mais específica ocorreu quando as folhas começaram a se desenvolver. Assim, o SPAD (*Soil Plant Analysis Development*) foi utilizado para medir o teor nutricional das plantas, uma vez por semana, em três novas folhas emergentes por tratamento. O crescimento das plantas foi monitorizado uma vez por semana através da contagem do número de folhas por planta, número de cachos de flores por planta e número de flores e frutos por planta. Os frutos foram também testados organolepticamente por um painel de provadores da Universidade do Algarve, numa sala de provas. O teste consistiu em um questionário padrão referente aos

aspetos e sabor da fruta. Durante o período de colheita os frutos foram recolhidos duas vezes por semana. Quando se atingiu um nível de maturidade pré-estabelecido, estes foram contados, pesados e medidos os graus de °Brix. No fim dos ensaios, 30-50 g de frutos e folhas foram secos e moídos e posteriormente enviados a um laboratório para analisar o teor de nutrientes.

A técnica de aplicação foliar é uma ferramenta vantajosa no enriquecimento de nutrientes para as plantas; a absorção de nutrientes pela superfície da folha é significativamente mais rápida do que através das raízes. Neste estudo, o tratamento de aplicação foliar T3, da suspensão de *C. vulgaris* na concentração de 3,6 g L^{-1}, melhorou significativamente o desenvolvimento das plantas de folhas, cachos, flores, frutos, bem como o número e o peso dos frutos, em comparação com o controle positivo e negativo. As evidentes mudanças positivas no crescimento vegetativo, rendimento e no desenvolvimento de *F. x ananassa* podem ser resultado do efeito de proteína rica, com alta quantidade de micro- e macronutrientes, contendo todos os aminoácidos essenciais de uma forma equilibrada, sendo também rica em vitaminas e minerais, ferro, potássio, fósforo e cálcio. Além disso, as fitohormonas, auxinas e citocinas, apresentados em *C. vulgaris* têm sido relatadas como tendo um papel crucial na divisão celular, alongamento celular e desenvolvimento de raízes e rebentos.

Palavras-chaves: *Fragaria x ananassa*; sustentabilidade; bioestimulantes; microalga; biofertilizante; *Chlorella vulgaris*

Abstract

The rapid growth of the human population requires an equivalent increase in food consumption. However, modern society demands healthy and nutrient-rich foods through more sustainable agricultural alternatives aimed at improving human health, consequently a better quality of life, and preservation of a sustainable environment. As fertilizer runoff seriously affects marine life, the use of biofertilizers and biostimulants might be a future strategy to decrease this harmful effect as, in general, they do not contain any chemicals harmful to the soil. They are also a source of essential nutrients for plants, and they are able to increase soil fertility, which is favoring their adoption over chemical fertilizers all around the world. Biofertilizers are products that contain live microorganisms capable of increasing the availability of nutrients to host plants, while biostimulants provide plant growth-promoting molecules at lower doses but may require additional chemicals to sustain growth. In the presented study, the objective was to investigate the effect of applying a suspension of *Chlorella vulgaris* in distilled water, in comparison with other commercial biostimulants, to the cultivation of *Fragaria x ananassa*, for different growth and quality parameters, such as plant growth and development of fruits. The experimental design included a positive control (Algaman B), a negative control (distilled water) and four different concentrations of *C. vulgaris* applied by foliar spray. Strawberry cultivation was done in commercial substrate plastic bags. The results showed that the treatment of foliar application of a *C. vulgaris* suspension at a concentration of 3.6 g L^{-1} significantly improved the development of the plants of leaves, bunches, flowers, fruits, as well as the number of fruits and the weight of the fruit, compared to the controls. These results, thus, suggest that microalgae might be effective sources of plant biostimulants.

Keywords: biofertilizers; biostimulants; microalgae; sustainability; *Chlorella vulgaris*

Table of Contents

Indices of Figures

Indices of Tables

Chapter 1

1.1. Human population growth

1.1.1. *The search for a more sustainable agriculture alternative*

The fast expansion of human numbers from 2.5 billion in 1950, 6.1 billion in 2000 to over 7 billion today (Southgate, 2009) has resulted in a rising demand for food production. Since the dawn of agriculture, more than 10,000 years ago, every major advance has allowed the global human population to rise. The rising dependency on chemical fertilizers and pesticides to satisfy the high demand for food by growing populations has encouraged the industries to produce life-threatening chemicals as a form of plant protection products (PPP) or fertilizers (Mahanty et al., 2017). These chemicals are not merely hazardous for human consumption but can also severely affect the biological balance of the environment. Ensuring healthy diets for an expected global population of 10 billion by 2050, according to the World Bank (2010), while improving the life quality at the same time, will require a more sustainable farming system. Nevertheless, a more sustainable method for agricultural purposes needs urgently to be explored due to its benefits for the environment, public health, and animal welfare. Considering the dangerous effects of chemical fertilizers, biofertilizers and biostimulants are a safe alternative to eliminate or at least minimize chemical input that might cause ecological disturbances. Environmentally friendly fertilizers and stimulants are becoming increasingly popular in many countries and for many crops, because they can act as a potent alternative that not only is able to feed a larger number of consumers but also save the agriculture from the severity of various environmental stresses. The European biostimulants industry council (EBIC) reported that in 2012 over 6.2 million hectares were treated with biostimulants in Europe (European Biostimulants Industry Council 2013). Biofertilizers are well known, promising, cost-effective, eco-friendly, renewable source of plant nutrients for supplementing chemical fertilizers (Kannaiyan, 2002) and for improving the overall health status of plants. They possess great potential to significantly enhance the productivity of agricultural land, using an integrated approach to determine the most favorable plant-microorganism interaction, one of the most crucial factors that might result in the augmentation in productivity (Mahanty et al., 2017).

1.2. Fertilizers

Fertilizers are any material of natural or synthetic origin (other than limiting materials) that are applied to soil or to plant tissues to supply one or more essential nutrient to plant growth (Mercy et al., 2014)). Recent European legislation defines a "fertilizing product' as a "substance, mixture, microorganism or any other material, applied or intended to be applied to plants or their rhizosphere or to mushrooms or their mycosphere, or intended to constitute the rhizosphere or mycosphere, either on its own or mixed with another material, for the purpose of providing the plants or mushrooms with nutrients or improving their nutrition efficiency" (Regulation (EU) 2019/1009). This legislation considers six Product Function Categories (PFCs), including the Biostimulants (6th Category, Annnex 1), which can be either microbial or non-microbial products.

Many sources of fertilizer exist, both natural and industrially produced. Although responsible for a tremendous increase in crop yields during the 20th century, the indiscriminate use of chemical fertilizers to meet the growing demand for food supply has unquestionably led to contamination and brutally damaged habitats and human-friendly insects (Mahanty et al., 2017), apart from the accumulation of heavy metals in the soil. These chemicals found in fertilizers can upset the soil's symbiotic relationship of microbes. The long-term overuse of chemical fertilizers has resulted in many problems, such as serious imbalance of nitrogen, phosphate and potassium ratio, soil hardening, salinization, and nutrient reduction (Guo et al., 2020). Their excessive use can also cause harm to the surrounding environments, since the hazardous chemical cannot be taken up by the plants, they start accumulating in ground water, making their way into the larger bodies of water, and causing eutrophication (Savci, 2012).

1.3. The impact of chemical fertilizers in the oceans

The overuse of chemical fertilizers and stimulants leads to accumulation of hazardous chemical in the soils and surrounding environments, prominent to negative organic reactions. Excessive amounts of nutrients are reaching the ocean, from intensive agricultural application of chemical fertilizers, which are washed away by soil erosion, runoff triggered by rainfall, over-irrigation, and snowmelt. This high availability of nutrients such as nitrogen and phosphorus induce a beyond normal level of growth in the phytoplankton population in aquatic environments. This abnormal growth is often called "algae blooms". As the algal mass sinks, it is decomposed by the bacteria, using all the oxygen available, thus leading to a large-scale mortality of marine creatures, and causing a phenomenon called "dead zones". Those dead zones may decrease with time, but an affected ecosystem can take many years to recover and return to its healthy state. There are many other reasons for the formation of dead zones. Those can be also caused by upwelling currents that bring nutrients from the bottom to the upper layers of the oceans causing that visible collapse of marine life. Moreover, increase in temperature due to climate change can cause ocean stratification, which exacerbates the problem (Diaz & Rosenberg, 2008).

According to United Nation (2004), about 75% of the world's fish and crustacean stocks are already being consumed faster than desirable, but UNEP says that the dead zones, which now number a total of 150 worldwide, will prove to be an even greater threat. Worldwide, around 120 million tons of nitrogen are used as fertilizer each year – more than 90 million tons produced naturally. Of this total, only 20 million are retained in food, while the rest is carried over to the sea by rivers and other water streams. The United Nation Environment Programme (UNEP) published a report mentioning that "Many of the dead zones are less than one square kilometer in area, while others are as large as 70,000 square kilometers, as is the case in the Baltic and Black Seas and some parts of the Adriatic. One of the best known is that of the Gulf of Mexico, showed in Figure 1, affected by nutrients carried over there by the Mississippi River. Other zones have also been recorded near South America, Japan, China, Australia, and New Zealand. Some of them only appear occasionally, while others appear on an annual basis."

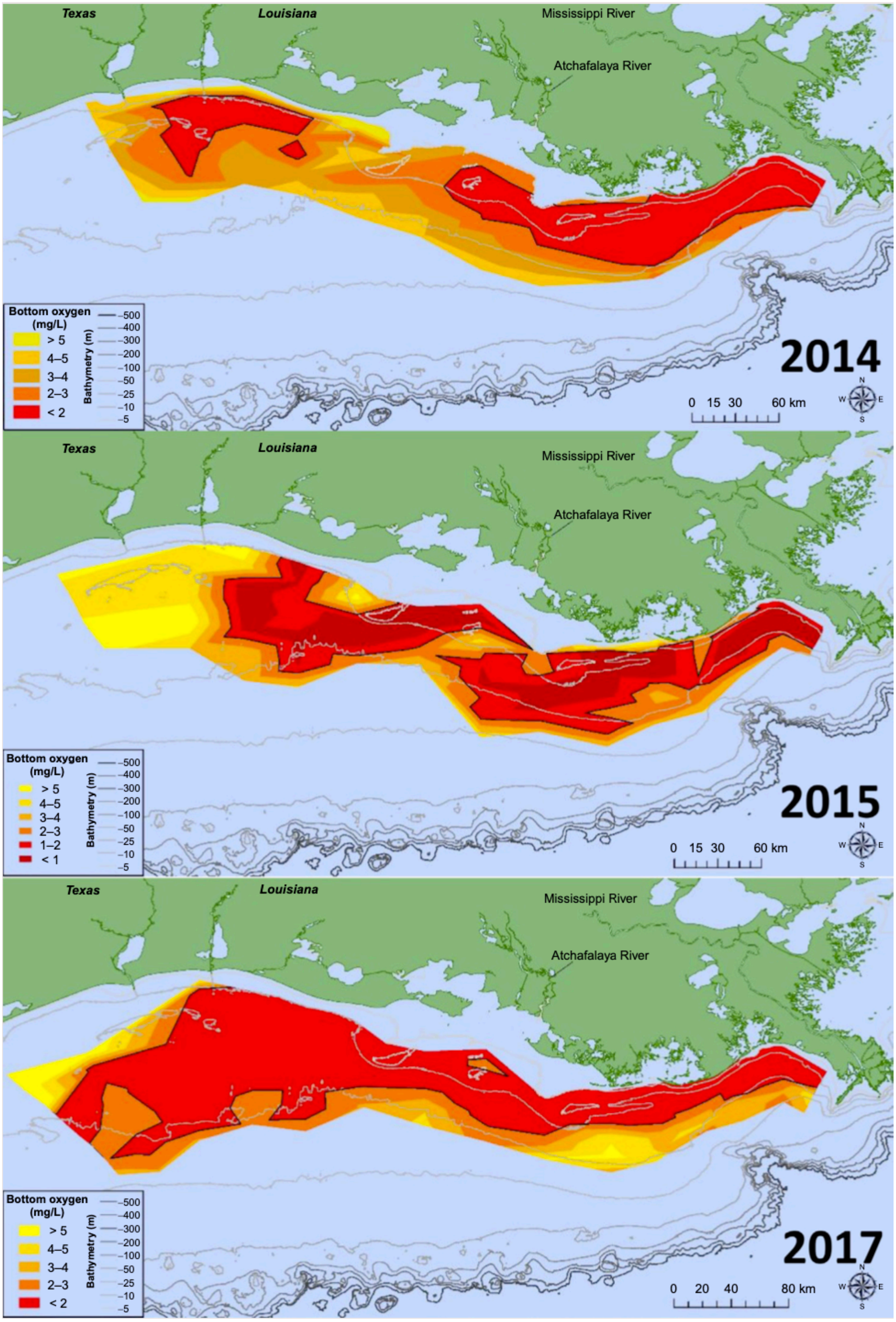


Figure 1: The Northern Gulf of Mexico "dead zone" in three time periods in 2014, 2015 and 2017 (Altieri & Diaz, 2019)

1.4. The alternative to minimize dead zones

1.4.1. Biofertilizers

Biofertilizers are products containing living microorganisms or natural substances which when applied to seeds, plants, or soils, colonizes the rhizospheres or the interior of the plants, promoting plant growth by increasing the supply of nutrients to the host plant (Vessey, 2003). They are considered green, healthy, and pollution-free, as well as improve soil chemical and biological properties, and restore soil fertility (Ronga et al., 2019. As previously mentioned, according to Regulation (EU) 2019/1009, these fertilizers are now included in the 6th PFC. It was reported by Bhardwaj et al. (2014) that the use of biofertilizers elevated the crop yield by 10-40% by increasing contents of protein, essential amino acids, vitamins, and nitrogen fixation. The commercialization of biofertilizers started in India in the year of 1934 with the production and marketing of about less than a ton per year; however, this same country has been purported as the largest biofertilizer user in the world (Kannaiyan, 2002). The recent developments in the biofertilizer technology such as immobilization of cyanobacteria for maximizing ammonia production in rice soil ecosystems and endophytic nitrogen fixation in wheat have also been included, which are considered as potential technologies for the future (Kannaiyan, 2002).

1.4.2. Biostimulants

The term “biostimulant” was first proposed by Zhang and Schmidt (1997) to indicate “materials that, in minute quantities, promote plant growth”. According to the new Regulation (EU) 2019/1009, “A plant biostimulant shall be an EU fertilizing product the function of which is to stimulate plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: i) nutrient use efficiency, ii) tolerance to abiotic stress, iii) quality traits, or iv) availability of confined nutrients in the soil or rhizosphere”. Biostimulants includes diverse substances and microorganisms, or any material of natural or synthetic origin (other than limiting materials) that are applied to soil or to plant tissues to supply one or more plant nutrients essential to the growth of plants.. They are products able to regulate and enhance the crop's physiological processes, when applied in low doses to seeds, crops, or soil (Zhang & Schmidt, 1997). They act on the plant's physiology through different pathways and simulate natural nutritional processes in the plant or around the roots to enhance nutrient uptake, nutrient efficiency, increase tolerance to abiotic stress, and the shelf life of harvested products by increasing the fertility of the soil (Rouphael & Colla, 2018). Some biostimulants have been found to stimulate the plants' natural defense systems, increasing their resistance to pest and diseases.

1.4.3. Microalgae-based biofertilizers and biostimulants

Microbial biostimulants and biofertilizers are attracting interest in modern agriculture as a promising alternative to increase agricultural sustainability, due to their potential to improve germination, great competence in improving the physical and chemical properties of the soil, seedling and plant growth, yield, nutrient use efficiency, as well as tolerance to a wide range of abiotic stresses (Du Jardin, 2015). Moreover, a more environmentally friendly, cost-effective alternative to the use of synthetic chemical fertilizers and stimulants is urgently needed. According to Dineshkumar et al. (2020), microalgal biofertilizers can replace chemical fertilizers, as they are renewable and cost-effective, which can help farmers to produce healthy organic foods and create a healthy chemical-free organic mankind through microalgal biofertilizers. Microalgae may be applied in agriculture in different ways such as: soil amendment, seed priming, foliar spray, or fertigation (Chiaiese et al., 2018). Microalgae can not only fix CO_2 and N_2 by assimilation to increase soil fertility but also secrete amino acids and phytohormones, such as auxins and cytokinins, which are able to stimulate growth crop and form a symbiotic system to improve soil structure.

This field of study remains largely unexploited, with a promising future, even though these aquatic microorganisms could play a major role in the development of a more sustainable agriculture due to their contents in plant-stimulating compounds. Besides the implementation for agriculture, microalgae have also been used for other environmentally friendly applications, such as those indicated in Figure 2.

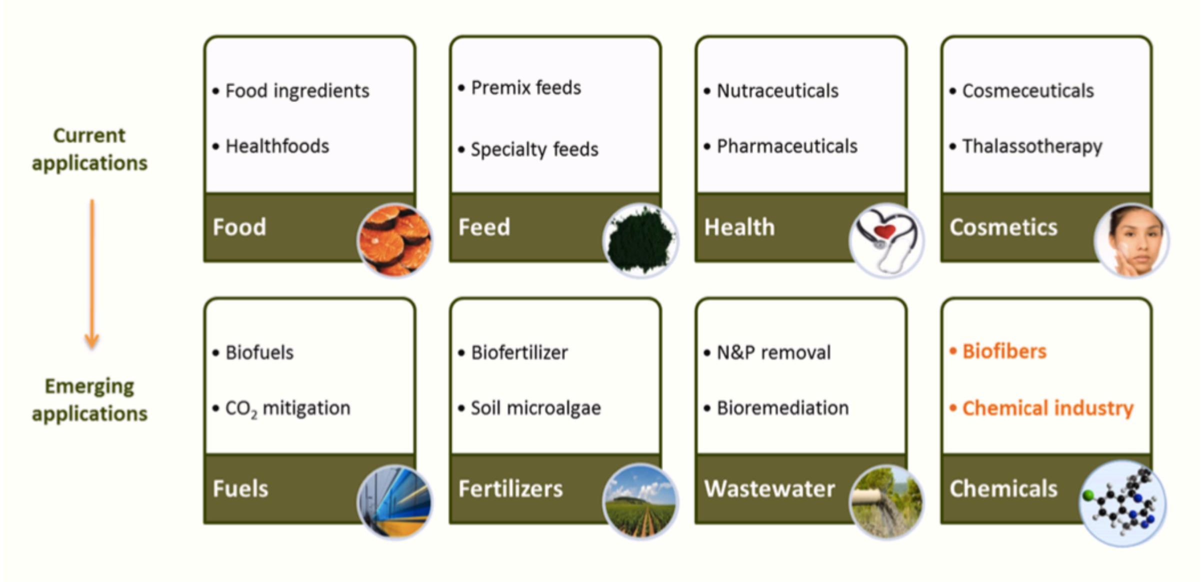


Figure 2: Current and emerging applications of microalgae (Fernández et al., 2020)

1.4.4. *Chlorella vulgaris*

Microalgae are microscopic eukaryotic unicellular photosynthetic organisms that are available in both marine and freshwater environments, and contribute to the planet's sustainability, mainly by transforming CO_2 into O_2. Microalgae have the potential to prevent nutrient losses through a gradual release of N, P and K, as an organic fertilizer, which meets the requirements for plant growth (Mulbry et al., 2007). They have an enormous biodiversity from which about 40,000 have already been described or analyzed (Hu et al., 2008). One of the most remarkable microalgae is the green eukaryotic chlorophyte *Chlorella vulgaris.* It is widely distributed in freshwater, marine, and terrestrial environments, and has high photosynthetic ability and the capability for rapid growth under autotrophic, mixotrophic and heterotrophic conditions (Tomaselli, 2004). This species is one of the most cultivated microalgae in the world, used extensively in a diverse array of applications, such as human nutrition in the form of dried biomass processed as powder, or commercialized in capsules or tablets (Görs et al., 2010). It is rich in protein, with high amount of macro- and micronutrients, containing all the essential amino acids, displaying also high chlorophyll contents and a lipid profile with up to 60% of polyunsaturated fatty acids (Safi et al., 2014). *C. vulgaris* is known for its high protein content, even higher than most animal-based proteins per gram (Guo et al., 2020). *Chlorella* and "Spirulina" (*Arthrospira platensis*) microalgae have been reported to constitute up to 70%

protein with respect to its own mass, they also contain well-balanced essential amino acids content required for human consumption (Koyande et al., 2019). They are also rich in vitamins and minerals such as vitamin B_{12} (synthesized by associated planktonic cyanobacteria), iron, potassium, phosphorus, and calcium. This green microalga can be easily cultured with inexpensive nutrient regimes and has a faster growth rate under autotrophic, mixotrophic and heterotrophic conditions, as compared to terrestrial energy crops, and high biomass productivity (Ru et al., 2020). *Chlorella* spp. is among one of the few that are allowed for human consumption as whole-cell biomass in Europe according to the European Union food regulations (Caporgno & Mathys, 2018).

1.4.5. Microalgae as fertilizing feedstocks

Microalgae-based biofertilizers is a topic that has been gaining a lot of interest due to its many benefits. Many studies are being performed to better understand the effect of the utilization of microalgae, such as: (I) Rice cultivation with marine microalgae (*Chlorella vulgaris* and *Spirulina platensis*) as soil drench increased rice yields up to 7 – 20.9% respectively (Dineshkumar et al., 2018); (II) Onion cultivation showed improved growth parameters, yield, composition, anti-nutritional composition, and minerals upon the presence of microalgae-based biofertilizer mixed with cow manure (Dineshkumar et al., 2020); (III) Maize cultivation revealed plant growth during the early stages, up to 51.1%, upon 60 days after planting (Dineshkumar et al., 2019); (IV) Microalgae-based fertilizers can be used as an organic slow-release product to improve the quality of tomato produced, through an increase in sugar and carotenoid contents of the fruit (Coppens et al., 2016); (V) An increase of up to 186%, on lettuce cultivation, in several characteristics like growth, and biomass and pigment content in seedlings (Lee & González Nariño, 2011); (VI) *Chlorella Vulgaris* suspension can enhance the germination of tomato and cucumber seeds, and improve its the root and shoot length (Bumandalai & Tserennadmid, 2019); (VII) Water extract *Chlorella vulgaris* as foliar fertilizer led to more than 140% yield increase and more than 40% grain weight increase in wheat plants (Shaaban, 2001).

1.5. Strawberry

1.5.1. *Fragaria x ananassa*

Strawberry is one of the most economically important berry fruits consumed for its pleasant flavor, and its great nutritional and medicinal properties. The most cultivated strawberry plants (*Fragaria* ✕ *ananassa* (Weston) Duchesne *ex* Rozier), as can be seen in Figure 3, are hybrids of two American species, *Fragaria chiloensis* L.P. Mill. and *Fragaria virginiana* Duch. (ITIS, 2021; Jamal Uddin et al., 2012). Strawberry is a non-climacteric fruit with a very short postharvest life (Hernández-Muñoz et al., 2006), rich in vitamin C and appreciated for their unique flavor and nutritional qualities. It may be consumed fresh, frozen, or processed by a variety of industries.

Figure 3: *Fragaria x ananassa* collected at the horto University of Algarve

Aside from its sweet taste and organoleptic properties, it has also been observed to aid in the treatment of chronic diseases such as cancer and heart disease (Šamec, et al., 2016; Zhang et al., 2008). There are over 40 species of strawberry around the world, some native to Europe. They are one of the most important temperate berry fruits which can also be cultivated in sub-tropical regions and grow up to 3000 meters above mean sea level in humid and dry regions (Kumar et al., 2022). The largest strawberry producers in the world are China, the United States of America, Spain, and Japan (FAOStat). Strawberry production exceeded 7.7 million tons in 2013, following by an increase by 142% over the previous 20 years (FAOStat), with commercial production in 76 countries, covering all continents except Antarctica (Simpson, 2018). Strawberries are cultivated all over the world due to their adaptability, being grown under different cultivation settings, from cool temperatures to sub-tropical conditions, either on soil or soilless culture, on open air or under protected cultivation. Plant breeding continuously develops new cultivars with improved adaptation to consumers taste, cultivation conditions and disease resistance.

1.5.2. Biofertilizers on strawberry cultivation

Many studies have been conducted on the use of biofertilizers on strawberry cultivations, such as: (1) Pešaković et al. (2013) reported that plant growth promoting rhizobacteria provided the most favorable conditions for the development of soil microorganisms over the chemical fertilizer; (2) Kumar et al. (2019) showed a 25% substitution of mineral fertilizer with either *Azotobacter* or *Azospirillum* biofertilizers increased the number of fruits/plant along with improving juice content; and (3) Hassan (2015) indicated that rhizobacterial isolates of diazotrophic nature application in organic farming can be used to increase the fertilizer use efficiency. However, the use of microalgae as a biofertilizer / biostimulant is highly innovative. Currently, there are no published studies on microalgae as biostimulants for the cultivation of strawberry. Moreover, many products in the market are based on macro- rather than microalgae. An example of this is AgriAlgae®, a brand under which AlgaEnergy (Spain) develops, produces, and commercializes an innovative range of agricultural biostimulants. The results were more vigorous plants, better fruit coloration, better crop development and fruit quality, and yield increase by around 19% (AgriAlgae, 2016).

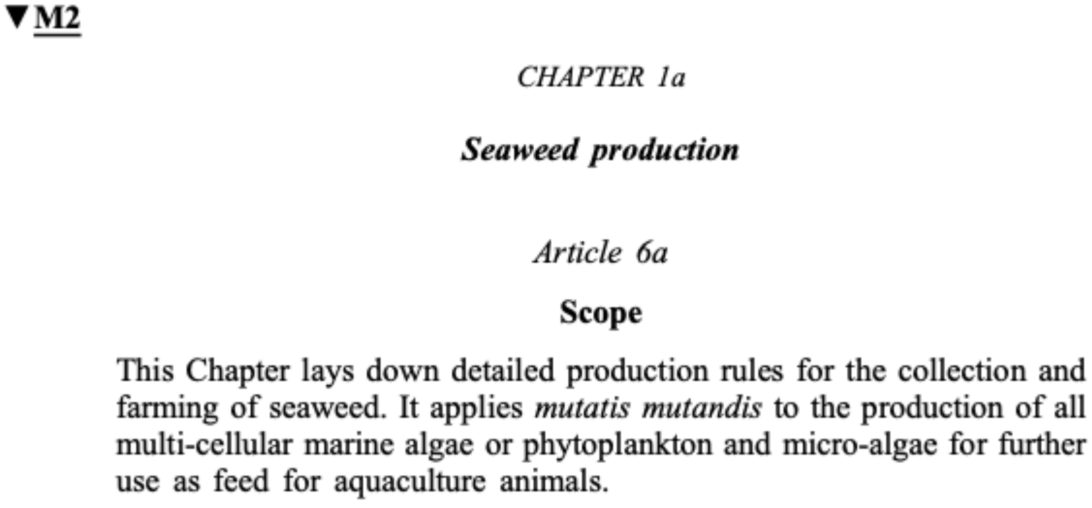
1.6. Portuguese legislation

Another interesting point to raise is the current Portuguese law regarding the use of microalgae to produce biofertilizers and/or biostimulants. Although the legislative situation is not yet entirely clear in Portugal, a future Allmicroalgae product might fall into the COMMISSION REGULATION (EC) Nº 884/2018 of 5 September 2008 regarding the use of biofertilizers and biostimulants by the organic production. This regulation establishes rules for the implementation of Regulation (EC) Nº 834 / 2007 of the Council (annex), which in ANNEX I: Fertilizers and soil amendments referred to in Article 3 (1), states:

A - Authorized under Regulation (EEC) No 2092/91 and taken over by Article 16 (3) (c) of Regulation (EC) No 834/200.

Seaweed and seaweed products (including microalgae; Figure 2). Since obtained directly by:

i) Physical processes, including dehydration, freezing, and crushing
ii) Extraction by means of water or aqueous acidic and / or alkaline solutions
iii) Fermentation



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CHAPTER 1a

Seaweed production

Article 6a

Scope

This Chapter lays down detailed production rules for the collection and farming of seaweed. It applies *mutatis mutandis* to the production of all multi-cellular marine algae or phytoplankton and micro-algae for further use as feed for aquaculture animals.

Figure 4: Annex I to COMMISSION REGULATION (EC) No 848-2018 chapter 1a, article 6a, scope

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Chapter 2

2.1. Introduction

As the world population is expected to reach 10 billion people by 2050, requiring an estimated 70% increase in food production, and keeping up with this increase in food demands is a challenge in need of solutions (Smil, 2001). Feeding a growing population comes at a cost. Pesticide runoff, water-intensive crops, and petrochemical-based fertilizers have taken a toll on the health of people and our planet (Tilman & Clark, 2014). The continuous and unsustainable overuse of synthetic agrochemicals can lead to anthropogenic nutrient pollution and environmental degradation. According to BBC Brazil, Worldwide, about 120 million tonnes of nitrogen are used each year as fertilizer - more than 90 million tonnes produced naturally. Of this total, only 20 million are retained in food, while the majority is lost in rivers and the sea. This leads to an excess of reproduction of microscopic algae that sink in the sea and are decomposed by bacteria, which will use all the available oxygen and, thus, promoting the death of other marine living beings due to hypoxia. Areas are known as “dead zones” or are just one of many warning signs that counteractive measures are urgently needed (Diaz & Rosenberg, 2008). Dead zones can affect bodies of water from small canals and harbors to large basins of inland sea such as the Baltic Sea where the dead zone cover over 60,000 km^2, with permanent features (Karlson et al., 2002). They vary tremendously in size, severity and duration year to year based on freshwater inputs and external conditions as in the Gulf of Mexico (Altieri & Diaz, 2019).

Healthier and sustainable solutions have been a greater search for more sustainable agricultural alternatives to improve human health, the environment, and the world. Microalgae biofertilizer and biostimulant represent a promising alternative to achieve a more sustainable, modern agriculture, since they contain a wide array of plant growth-promoting macro- and micronutrients, amino acids, vitamins, minerals, and phytohormones (Shereni, 2019). All these elements and molecules can further increase crop yields by stimulating root and shoot growth, prevent disease and stimulate the overall plant productivity. They usually do not contain chemicals that are damaging to the soil, being also a source of essential nutrients for plants and increase soil fertility. Therefore, they are being adopted over chemical fertilizers across the globe. Biofertilizers are products that contain living microorganisms that can increase the nutrient availability to host plants (Win et al., 2018), whereas biostimulant provide plant-growth promoting molecules at lower doses but may require additional chemicals to sustain growth (González-Pérez et al., 2022). Additionally, microalgae can be applied as a non-polluting approach for wastewater treatment of agricultural drain waters, as they uptake N and P. Another important feature of these microorganisms is their detoxification capacity because there is evidence that microalgae can withstand the effects of PPP (plant protection products) and other chemicals due to their ability to sequester these molecules (Nicolopoulou-Stamati et al., 2016).

The marine-based resources can be used as biofertilizers and biostimulants for its eco-friendly properties. Algal biofertilizers will become the best alternative source of nitrogenous chemical fertilizers as they are fuel independent, cost effective, and easily available (Dineshkumar et al., 2020). Algae have the remarkable potential to survive in the presence of highly concentrated organic and inorganic chemical in varying waste streams, which are toxic to living organisms, making it an important feature, and enabling more sustainable and efficient production in agriculture (Win et al., 2018). Many studies published from 2015 to 2017 used *Chlorella* sp. as the model system. *Chlorella vulgaris* is rich in amino acids, which can act as chelating agent and phytosiderophores that facilitate the penetration and absorption of micronutrients though various parts of the plants (Shaaban, 2001a). Several research findings suggest that *C. vulgaris* improves agricultural yield (nutrient content), by improving soil properties such as water holding capacity (the amount of water that given soil can hold for a crop use) and aeration (Faheed & Fattah, 2008; Shaaban, 2001b), and at the same time reduces the environmental impact compared to chemical fertilizer (Ru et al., 2020).

Fragaria x ananassa Duch. (strawberry) are popular with consumers in nearly all parts of the world and the steady increase in global production, due to their versatility crop in terms of its adaptability. They can grow from cold temperatures to sub-tropical environments, and in the variety of production systems to adapt to the local conditions (Simpson, 2018). One way for cultivation of *Fragaria x ananassa* is grow bags. There are many benefits in cultivating nourishments in bags, such as: (1) versatility of use and easy crop monitoring; (2) allows high yields; (3) prevents over-watering by easy percolation through the substrate; and (4) the white color (inside black) of the bags will increase radiation received by the plants.

Chlorella vulgaris is produced using industrial tubular photobioreactors; it is then harvested using pumps and centrifuges. During this process, a remnant part of the biomass cannot be collected with the requirements needed for food production. To valorize this remainder biomass, this study aims to verify and compare, with other commercial biostimulants, if *C. vulgaris* biomass can be used as biofertilizer/biostimulant for the cultivation of *Fragaria x ananassa*. The new purpose of this biomass will be its transformation into a novel plant growth-promoting product for agriculture and reduce the use of chemical fertilizers and stimulants, creating a more sustainable agricultural culture and assist on the reduction of the dead zones in the oceans.

2.2. Material and Methods

The study was conducted at the Horto of the University of Algarve – Gambelas Campus. It took place inside of an unheated plastic covered and greenhouse, with adjustable roof vents. The project consisted of the evaluation of development, yield, and quality of *Fragaria x ananassa* (*F. x ananassa*) 'PLARED (0822)' (Planasa innovation, Spain) in white commercial substrate plastic bags with foliar application of green microalgae *Chlorella vulgaris* every 14 days, as shown in Figure 5.

The cultivation bags were filled with 30L of commercial substrate (SIRO Morango Hobby®, Portugal) that consisted of a mix of forestry residues compost (namely pine bark), white peat, and composted horse manure, amended with 1 kg.m^{-3} of Exactyon®, an encapsulated fertilizer with an initial fast nutrient release, and a controlled release fertilizer and 2 kg.m^{-3} of controlled-release fertilizer from Osmocote®, of 5 to 6 months of release. The cultivation bags were 1 m long and filled with 30 L of substrate, and they can hold up to 20 plants per bag on soilless systems. However, to reduce the likelihood of occurrences of diseases related to poor aeration and to facilitate flowering and pollination, only 10 plants per bag were used.

The Trials were determined to last 4 months each trial one, and the springtime was the best opportunity as it is the most favorable time to grow strawberries, as is often the case, which in this case corresponded to a low precipitation period. Conversely, summers are not so advantageous due to high temperatures, but in the greenhouse is controllable in some extent, although that factor could be migrated by means of growing strawberries in a greenhouse.

Figure 5: *F. x ananassa* plants just after plantation in the cultivation plastic bags filled with an organic substrate. The white arrows point to the direction of the drainage water, the red box shows one of the dripper with microtubes, the green boxes indicate where the drainage water is collected and the black circles on the bottom bags shows the pre-plantation holes that were not necessary to be used for this crop.

2.2.1 Plantation design

Metallic frameworks were used to support the cultivation bags, at a 1 meter from the ground, and the plastic channels, placed underneath the bags, to collect the drainage water, with a slope of 2%, as seen in Figure 6. Ten *F. x ananassa* plants were planted per bag, in the pre-marked holes on each cultivation bag. Final plant density was 10 plants m^{-2}.

Figure 6: Cultivation bags on framework at a decline angle for the capture of water drainage, the arrow indicates the distance between the floor and the framework, which shows a reduction of the distance toward the end of the channel, indicating a decline slope.

The treatment consisted of 4 different concentrations of dry extract *Chlorella vulgaris* (*Cv*) suspension on distilled water suspension; a positive control (Algaman B®, Hubel verde, Portugal) and a negative control (distilled water), as indicated in Table 1.

Algaman B is a biostimulant formulated from *Ecklonia maxima* seaweed extract and boron that includes a range of natural hormone-like substances, and its pulverization dosage is 2g.L^{-1}. *Chlorella vulgaris* is a green eukaryotic unicellular microalga. The concentrations of *Chlorella* *vulgaris* were calculated based on Algaman B's phytohormones content (auxins and cytokinins), which is 11.031 mg.L^{-1} (9192.5 ng.g^{-1}), however as the dosage is 2g.L^{-1}, the phytohormone quantity is multiplied by 2 to have 22.062 mg.L^{-1} (18385 ng.g^{-1}).

Table 1: The 6 treatments for the trials with the dry extract microalgae suspensions concentrations, Algaman B and distilled water

Treatments*		Concentration (g.L^{-1})
C+	Algaman B	2
C-	Distilled water	-
T_1	*Cv*	0.04
T_2	*Cv*	0.4
T_3	*Cv*	3.6
T_4	*Cv*	36

*C+ = positive control   C- = negative control   T = treatment

According to Gouveia et al. (2022), the average *C. vulgaris* phytohormone content is 5075.27 ng.g^{-1}, therefore, to achieve the same quantity of Algaman B, 3.622 g.L^{-1} of the microalgae would have to be used. This average concentration was reduced and multiplied by a factor of 10, to obtain the rest of the other tested concentrations. this work included two consecutive crops with the same design and procedures: each trial consisted of 6 treatments (C+, C-, T1, T2, T3 and T4) in four completely randomize blocks (I, II, III and IV). A total of 24 cultivation bags were used for each trial (Figures 7).

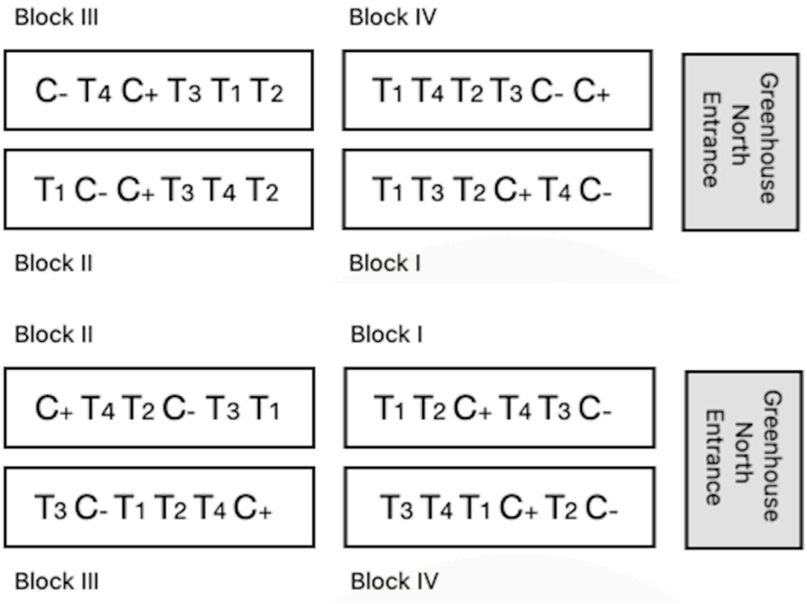

Figure 7: Sketch of the random distribution of the cultivation bags for Trial 1 (upper panel) and Trial 2 (lower panel).

The drainage water from consecutive blocks was collected together (Blocks I & II, and Blocks III & IV). The experiments were carried out in a double-blind way, meaning that the person in charge of foliar spaying did not know the treatments applied to the plants, to generate a more accurate result. The sprayer bottles were covered with black tape to hide the color of suspension inside. The plant irrigation system was automatic, controlled by a timer, trough drippers (2L.h^{-1}) with microtube and spikes, inserted in the cultivation bags, as seen in Figure 4. The irrigation frequency was based on the drainage water volume, EC, pH, and weather. Irrigation frequency ranged from 2 to 4 hours, for 2 to 3 minutes per irrigation.

2.2.2 Measurements and analysis

The cultivation period from plantation to the first harvest can take roughly three months, according to weather conditions. The cultivation trials were held for a period of four month, during spring and summer time:

1st Trial: from March 24th to July 29th (128 days)

2nd Trial: from May 12th to September 19th (130 days)

During the F. *x ananassa* cultivation period, different technical operations were performed to ensure healthy plants and fruits. Measurements to monitor plant development included daily measurements of air temperature, humidity, electrical conductivity and pH of the drainage water and irrigation control. Those measurements were done to evaluate if all blocks were getting the same amount of sun, water, and nutrients. The pH and electric conductivity were measured with a portable meter (HANNA HI 9813-5). Substrate temperature was also measured twice a week, with a TP3001 digital contact thermometer. The plants were foliar sprayed every 14 days with each treatments, while ensuring that plants were spayed evenly, and cross-contamination of the surrounding plants was avoided.

More specific determination took place once the leaves started developing. The Soil Plant Analysis Development (SPAD) meter was used for determinations once a week on 3 new emerging leaves per treatment (each bag) per block, with a portable equipment (Minolta SPAD-502, Japan), which indicates the nutritional state of the plants. The SPAD meter measures, accurate and non-destructively, leaf chlorophyll concentrations. Plant growth was monitored once a week by counting the number of leaves per plant, number flowers trusses per plant, and number of flowers and fruits per plant. The fruits were also organoleptic tested by a taster panel at the University of Algarve, in a tasting room. The teste consisted of a standard questionnaire regarding the fruit aspects and flavor.

During the harvesting period, the fruits were collected twice a week, when a preestablished maturity level was reached: number 3 as observed in Figure 8. The fruit maturity stage was assessed by visually evaluation and color comparison, always done by the same person. Fruits were counted, weighted and the degrees of Brix (sugar content) were determined, with a HI 96801 digital refractometer. A small amount of fruit squeezed juice was place in the refractometer, to measure the refractive index of the juice to determine the mineral/sugar ratio (°Bx), as shown in Figure 9.

Figure 8: Strawberries at different maturity stages, from green (1) to the fully matured (3) self

Figure 9: ºBx measurement with juice in the refractometer

At the end of the Trials, 30-50 g of dry and milled fruits and leaves were sent to a laboratory for nutrient content analysis. The dehydration processes required the material to be placed in an oven at 103° C for a minimum period of 48 hours. The samples, dried and ground in a mill (basic microfine grinder drive MF 10, IKA Werke, Germany), through a 1mm mesh sieve and sent to the laboratory (A2, Análises Químicas Lda, Guimarães, Portugal) due to the large number of samples. The leaves and fruits were analyzed for humidity and mineral content determination, namely: nitrogen (Ntotal), phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), sulfur (S), iron (Fe), manganese (Mn), boron (B), copper (Cu), zinc (Zn), sodium (Na), and aluminum (Al).

2.2.3 *The organoleptic test*

The organoleptic test was only conducted for the 1st trial. The analyses were of appearance, smell, taste, texture, sweetness, and acidity of the fruit for each treatment. A total of 38 responses were acquired. However, only 26 for T1 due to the low productivity. Figure 10 shows the display of the fruits for the tasters to analyze their appearance, while Figure 11 shows the plate in which each taster received, with two slices of strawberries, from each treatment.

Figure 10: Organoleptic test set up for Trial 1 at the University of Algarve

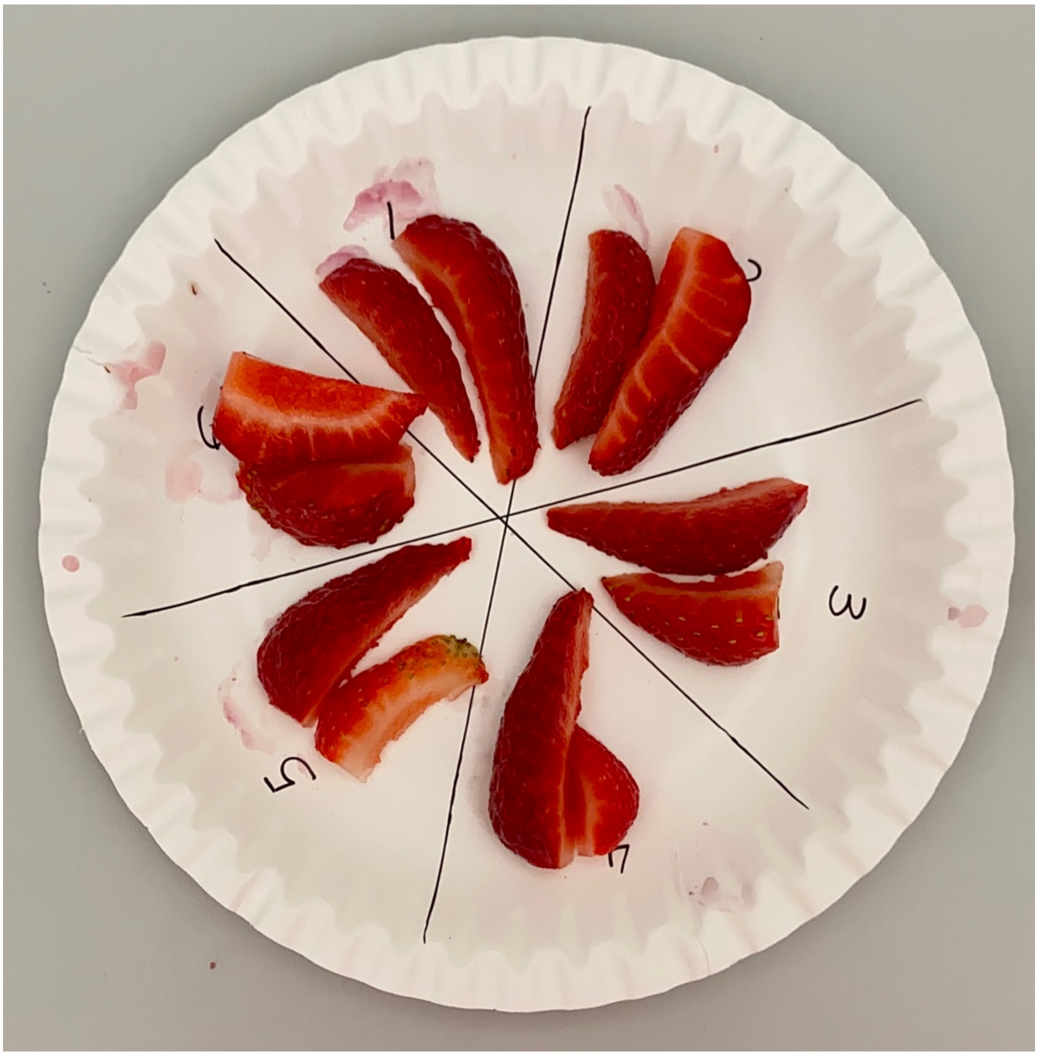

Figure 11: Plate with the strawberries that the volunteer testers received during the organoleptic test for Trial 1.

The questions of appearance, odor, flavor, and taste of the fruit, had 5 answers, including: excellent, very good, good, bad, and very bad. The sweetness questionary had 4 possible answers: little sweet, sweet enough, optimal sweetness and too sweet. For acidity there were only 3 possible responses: little, adequate, or too much acidity. The final question was regarding the possibility of future consumption of this fruit with 3 answers: no, maybe, or yes.

2.2.4 Statistical analysis

The data are presented as a mean ± standard deviation. The variables were analyzed using IBM SPSS statistic, version: 28.0.1.0 (142). One-way ANOVA was performed to determine whether the results were significantly different among the different treatment and the control. Once a statistically difference was obtained with the One-way ANOVA, then Tukey HSD was performed to ensure which specific groups' means are different, by comparing all possible pairs of means. Statistical significance was acceptable to a level of $p < 0.05$.

2.3. Results

2.3.1. *Environmental conditions of the experiment*

2.3.3.1 Measurements of pH and electric conductivity (EC)

The daily measurements consisted of pH and EC of the drainage water. As previously shown in Figure 7, it is observed that blocks I and II shared the same drainage channel, while blocks III and IV shared the other one. Trial 1 had 93 measurements of pH and EC while Trial 2 had 81. The daily pH measurements are presented in Figure 12 and 13, for Trials 1 and 2, respectively. For the 1st Trial, the highest pH record for Blocks I & II was 8.9, and the lowest was 5.7, while for Blocks III & IV were 8.6 and 5.2. The high variation of pH for the first few weeks are due to a high saturation on the growth bags, and early plantation of the *F. x ananassa*. The bags should have been prewashed until EC was below 2 $dS.m^{-1}$, and after the reduction to the desired EC value, the plants could then be planted. The variation is observed for both pH and EC results.

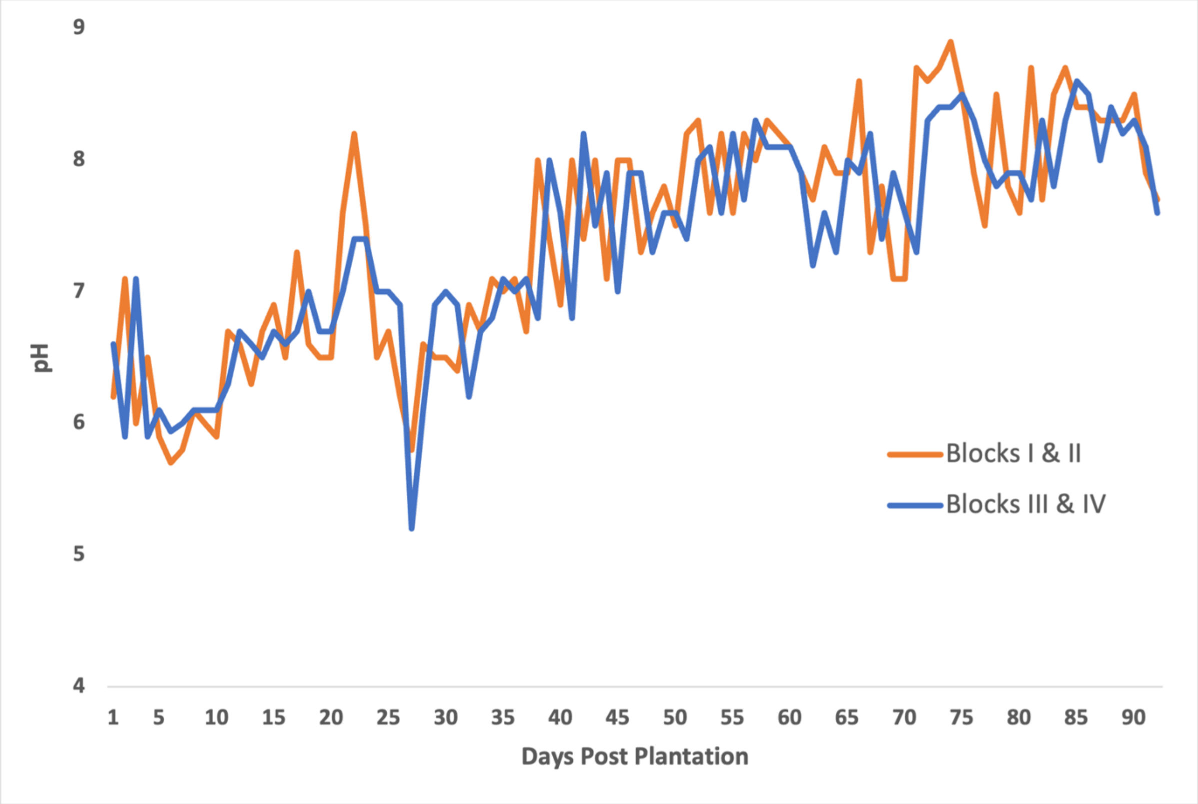

Figure 12: Daily pH measurements of the drainage water for Trial 1, for a period of 93 days.

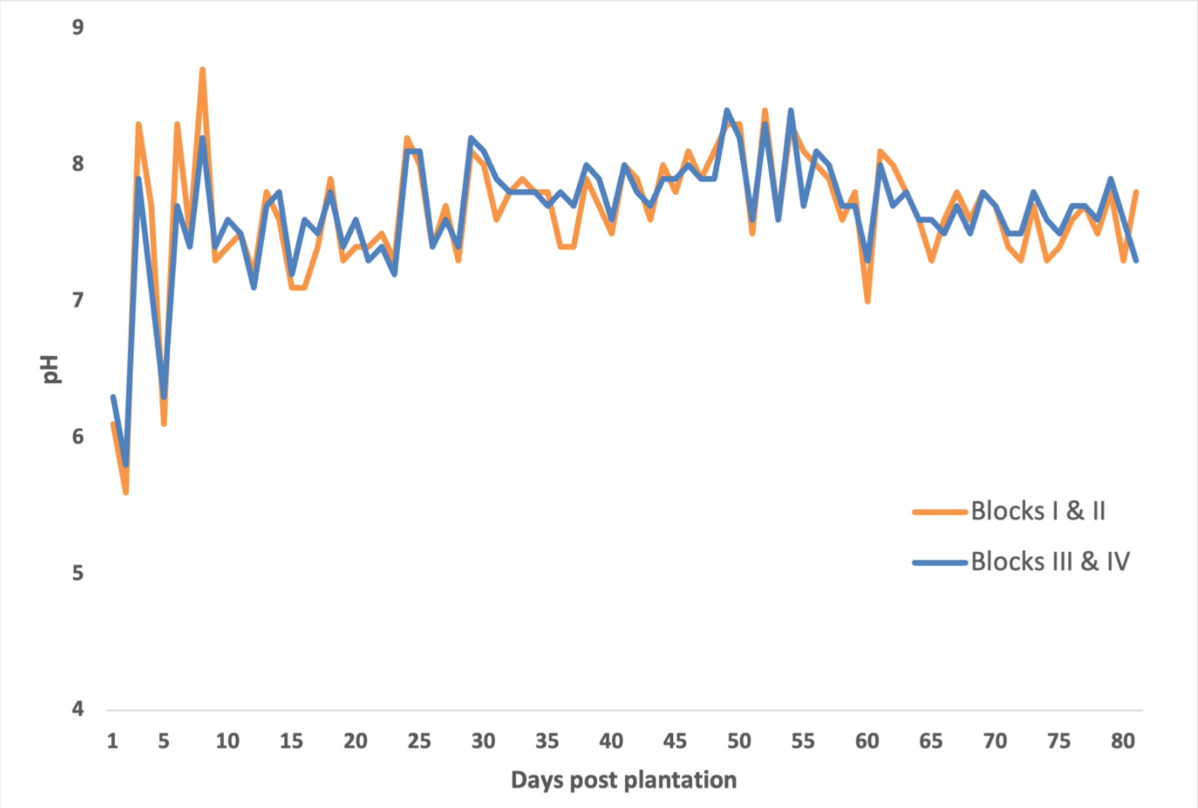

Figure 13: Daily pH measurements of the drainage water for Trial 2, for a period of 81 days.

The daily EC measurements are presented in Figure 14 and 15. The variation in EC, for the 1st Trial, during the project range from 6.23 dS.m^{-1} for Block I & II and 6.25 dS.m^{-1} for Block III & IV to 0.93 dS.m^{-1} and 0.81 dS.m^{-1}, respectively. The higher values were recorded within 6 days post plantation. Moreover, for the 2nd Trial, EC values ranged 3.23 dS.m^{-1} for Block I & II and 3.3 dS.m^{-1} for Block III & IV to 0.9 dS.m^{-1} and 0.82 dS.m^{-1}, respectively. Both high EC values were recorded 4 days post plantation, while the bags were still too saturated.

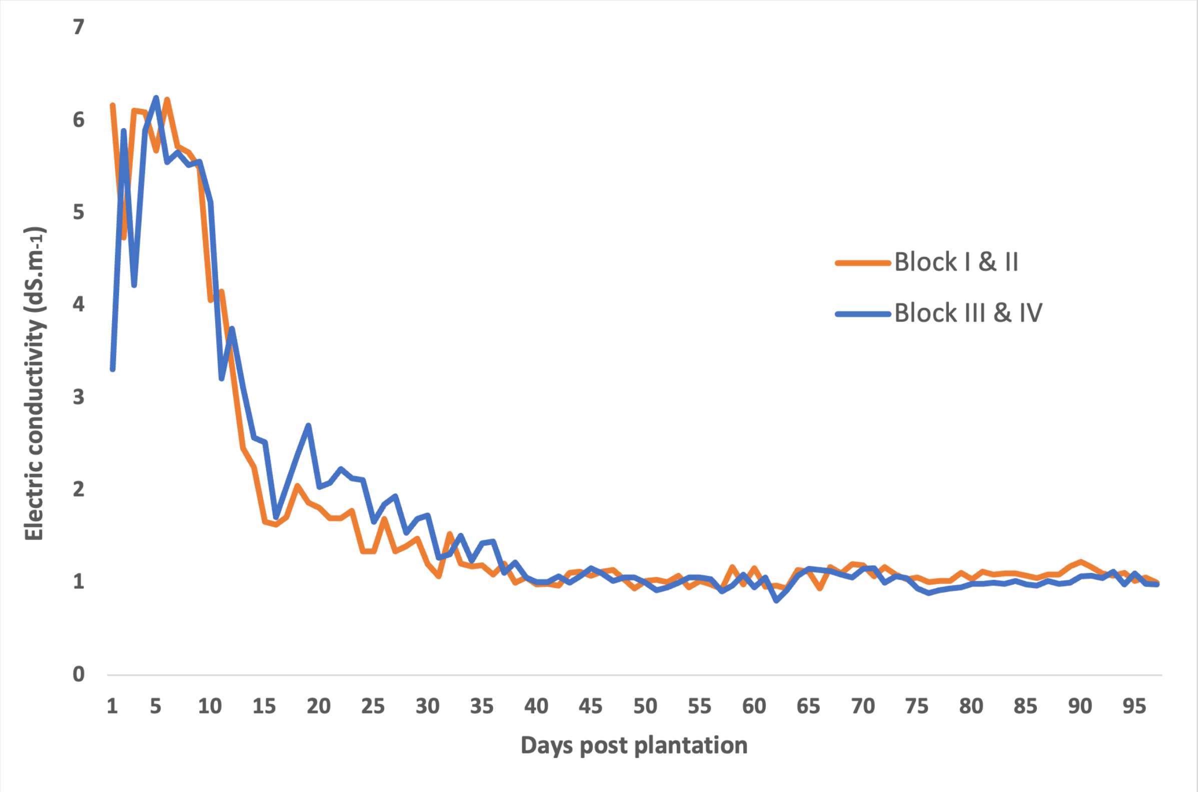

Figure 14: Daily EC measurements of the drainage water for Trial 1, for a period of 93 days.

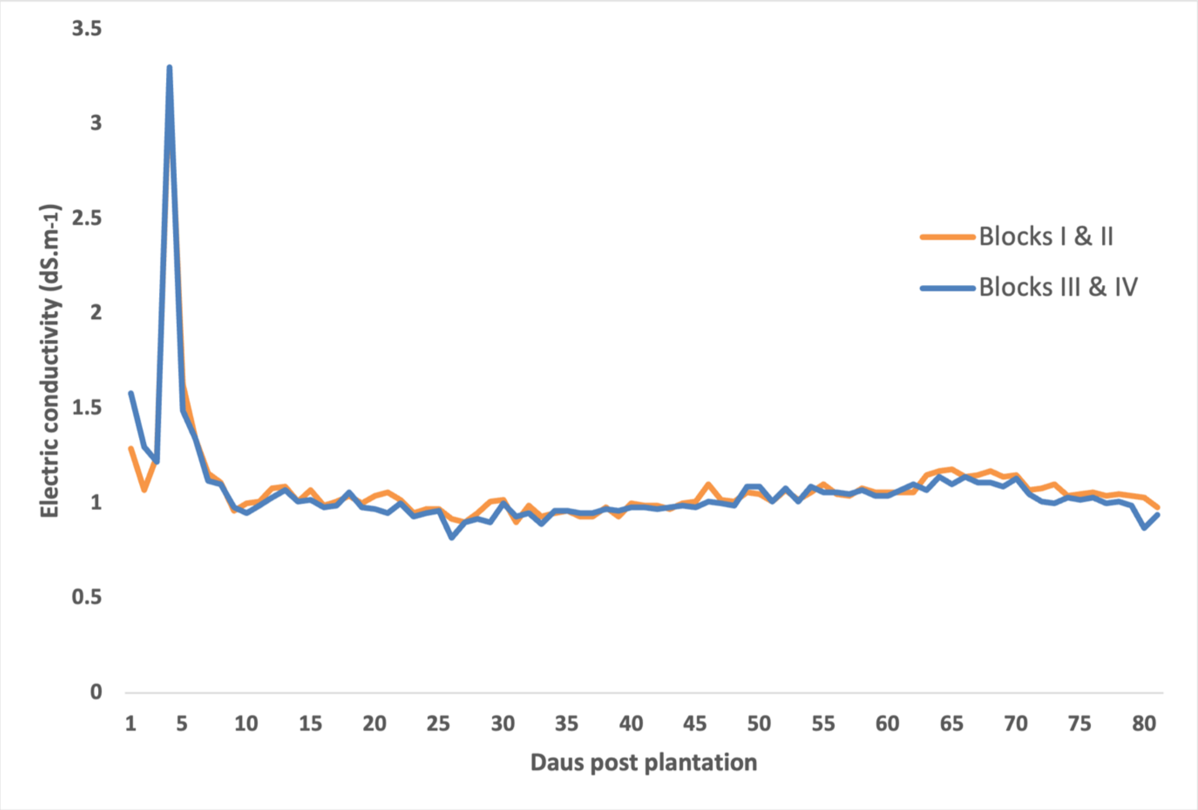

Figure 15: Daily EC measurements of the drainage water for Trial 2, for a period of 81 days.

Within the same trial, the values were consistent, without a significant variation between the blocks (p>0.05). The ANOVA test was performed for each trial separately, and no statistically significant differences were observed among the EC (p = 0.920) and pH (p = 0.629) values of Trial 1, and the EC (p = 0.685) and pH (p = 0.847) values of Trial 2. The mean values ± standard deviations are presented in Table 2.

Table 2: The mean values of pH and electric conductivity of the drainage (Trial 1 n=91; Trial 2 n=81)

Variables	Blocks	Trial 1	Trial 2
pH	**I & II**	7.4 ± 0.9	7.6 ± 0.5
	III & IV	7.4 ± 0.8	7.7 ± 0.4
Electric conductivity (dS.m^{-1})	**I & II**	1.72 ± 1.4	1.08 ± 0.3
	III & IV	1.74 ± 1.4	1.06 ± 0.3

2.3.3.2 Temperatures and humidity of air

The values of humidity and temperature of the greenhouse can be observed in Figures 16 and 17. The humidity graphs shows a pattern of higher humidity for May and June, with a decrease for months July and August, and a significant increase for September. The average humidity for Faro was 69%.

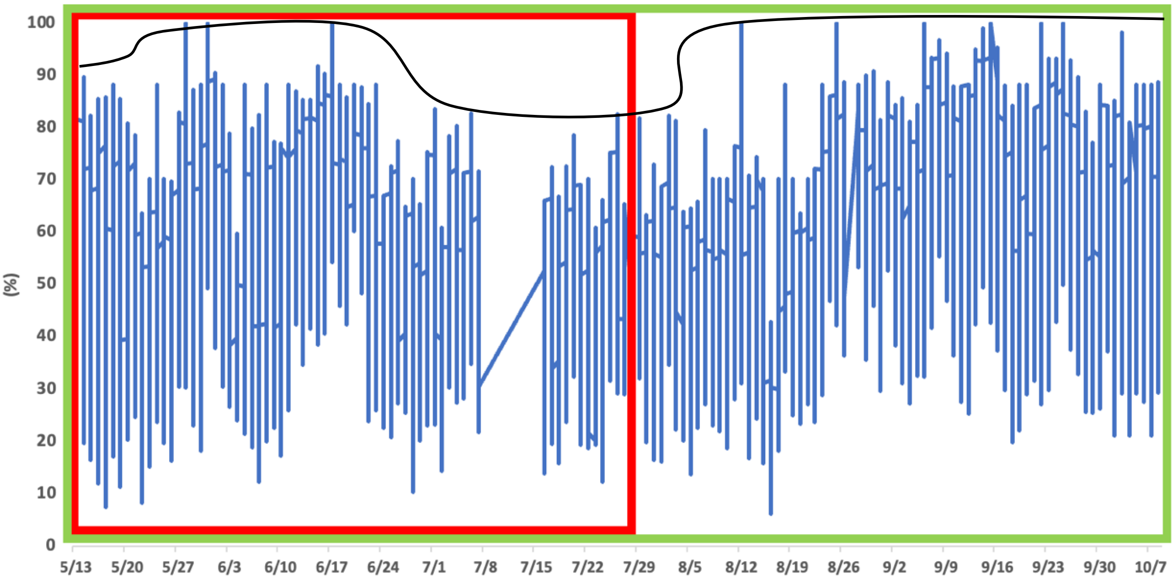

Figure 16: Humidity inside of the greenhouse. The red box presented values for Trial 1, while the green box present values for Trial 2 and the black line shows the pattern of the graph (*n* = 19838).

The highest temperature recorded was at 47.48°C on the 16th of August of 2021. Conversely, the lowest temperature was registered on the 24th of May of 2021, with 13.22°C. The humidity and temperature showed an opposite pattern, i.e., lower temperatures were accompanied by higher humidity.

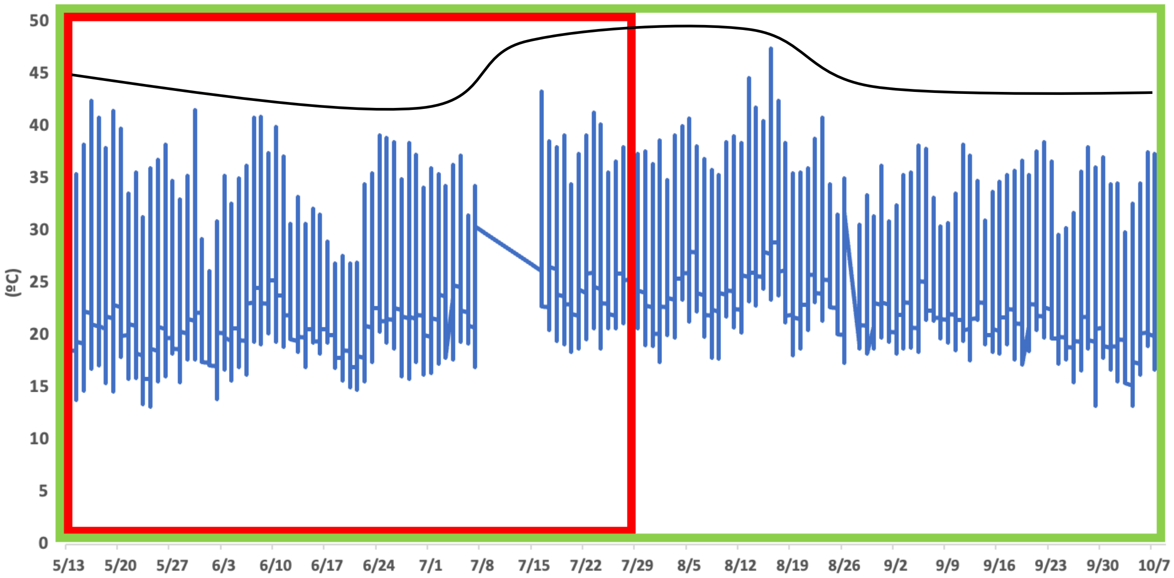

Figure 17: Temperature changes inside of the greenhouse. The red box presented values for Trial 1, while the green box present values to Trial 2 and the black line shows the pattern of the graph (*n* = 19838).

2.3.3.3 Measurements of substrate temperature

The substrate temperatures were measured weekly. The bags were manually randomly selected for the measurements, and values are presented in Figures 18 and 19. The temperatures were measured at different times of the day, to ensure that none of the bags were overheating, which could have caused thermal stress in the plants. All blocks had the same patterns in temperature range. The difference in temperatures is done due to different times of the day measurements, some days the measurements were done in the morning and other days in the afternoon. The temperatures ranged, for the 1st Trial, from 35.3°C (measured in Block II) to 17.9°C (Block IV), whereas for Trial 2 the highest temperature registered was 37.5 °C (Block I), whereas the lowest temperature observed was to 22.3°C (Block III).

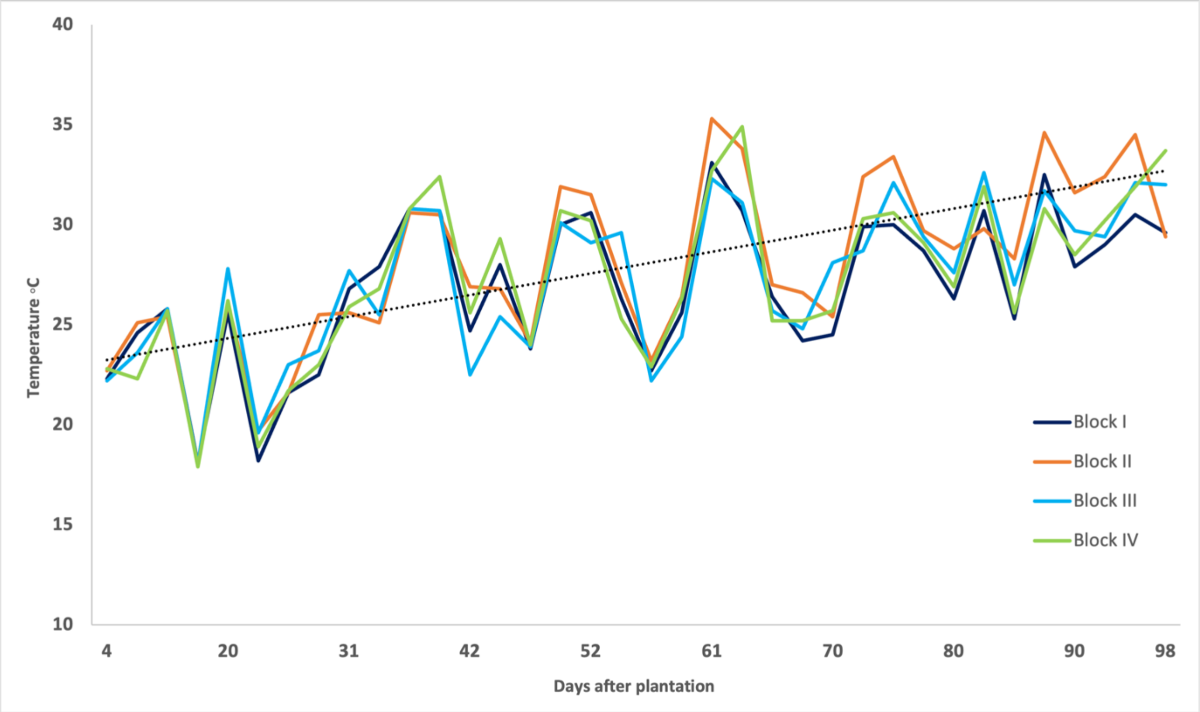

Figure 18: Temperate profile upon plantation for Trial 1.

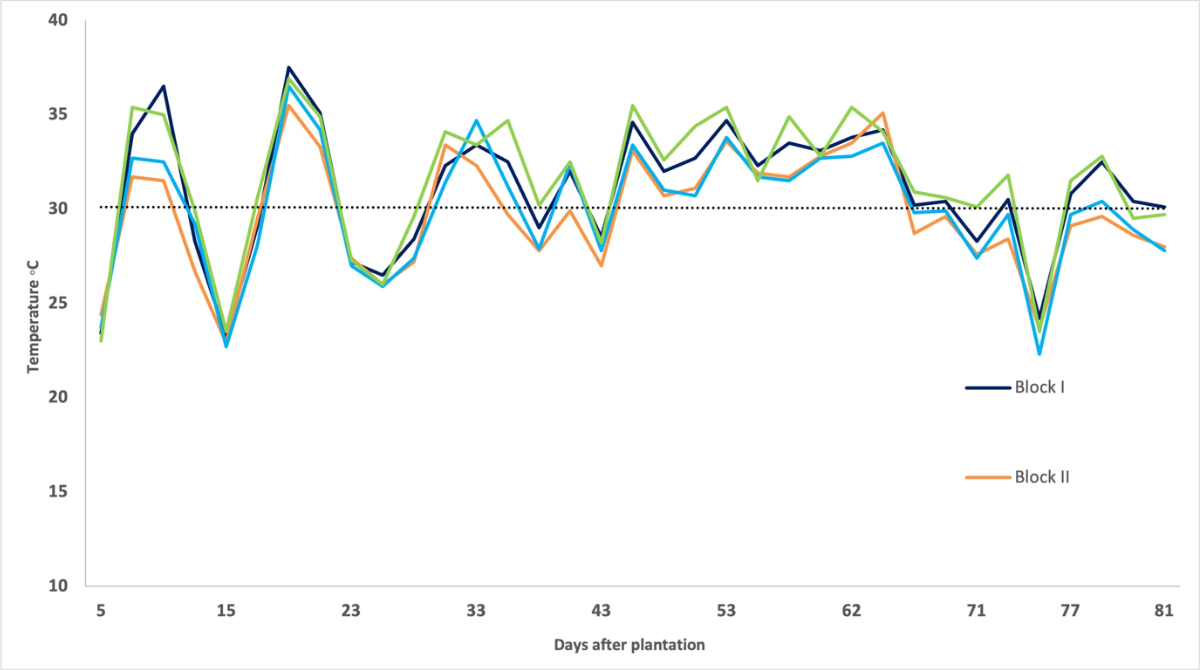

Figure 19: Temperate profile upon plantation for Trial 2.

The most important factor is that all the temperatures within the same day are statistically alike, and no significant differences were observed for the 1st trial ($p = 0.619$), nor for the 2nd trial ($p = 0.163$). The mean values ± standard deviations for temperature are seen in Table 3. The temperatures for Trial 2 were higher, as it was carried out during the summer seasons with temperatures ranging from 22°C to 28°C, as compared to those of springtime, which ranged from 15°C to 21°C.

Table 3: Substrate mean temperature of randomly selected bags for each block (*n* = 36)

Variables	**Blocks**	**Trial 1**	**Trial 2**
Temperature (°C)	I	26.6 ± 3.8	31.0 ± 3.5
	II	28.0 ± 4.2	29.8 ± 3.1
	III	27.2 ± 3.8	30.1 ± 3.3
	IV	27.7 ± 4.1	31.7 ± 3.4

2.3.2. *Growth and development of the plants*

2.3.2.1 The SPAD analysis

The SPAD measures the nutritional state of the plant, and those are measured on newly leaves, when existing. The SPAD results ranged from 57.9 to 17.7 for Trial 1, and from 35.6 to 19.8 for Trial 2. The highest values in both trials correspond to T1(*Cv* 0.04 g.L^{-1}) leaves, while the lowest are from T3 (*Cv* 3.6 g.L^{-1}) and positive control (C+) leaves. The SPAD results are observed in Figure 20 and 21. For the 1st Trial, all treatments follow the same pattern.

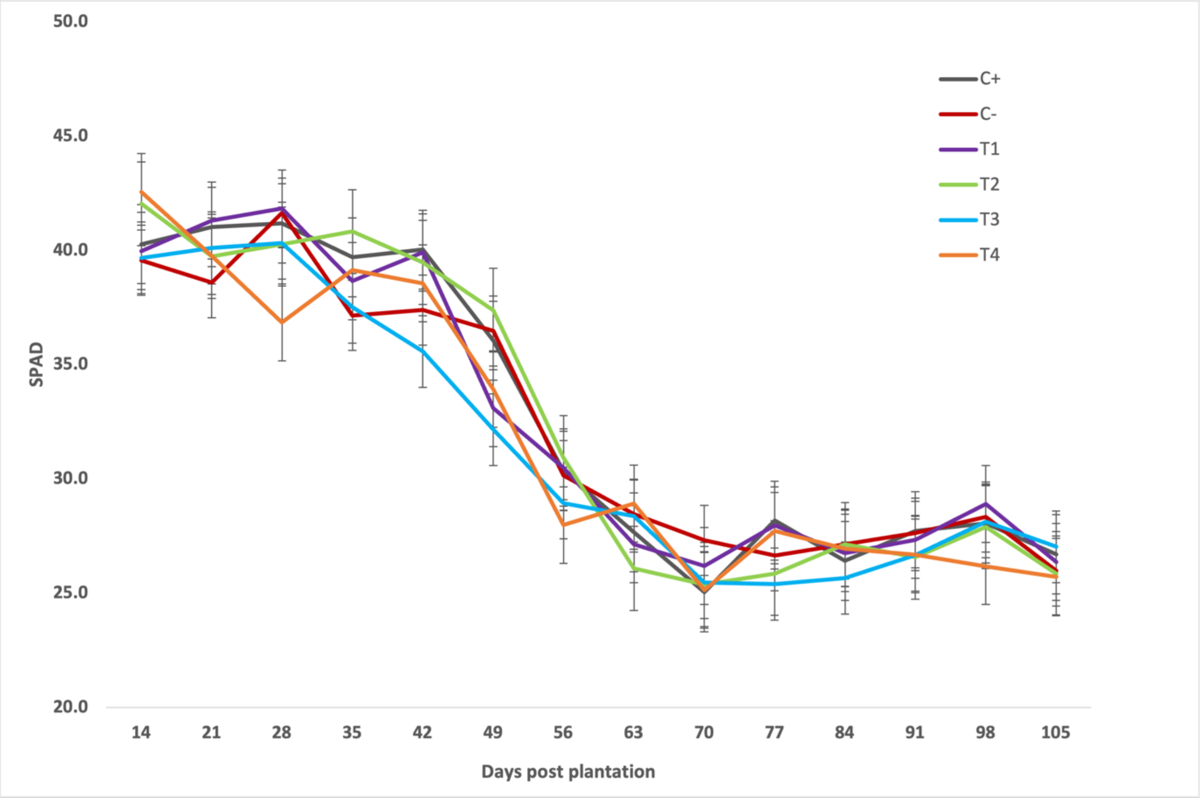

Figure 20: The mean SPAD of newly emerging leaves for Trial 1 (*n* = 189). C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

For the 2nd Trial, T2 (*Cv* 0.4 g.L^{-1}) SPAD showed the widest range of values, with 5.6 difference between the highest and the lowest value. Aside from negative control, with a different peak value at day 28 days post plantation, all other treatments showed similar patterns. During the 1st Trial a decrease in the SPAD values was observed, while the same was not observed for the 2nd Trial, with values dropping at 35 days post plantation and increasing again after that.

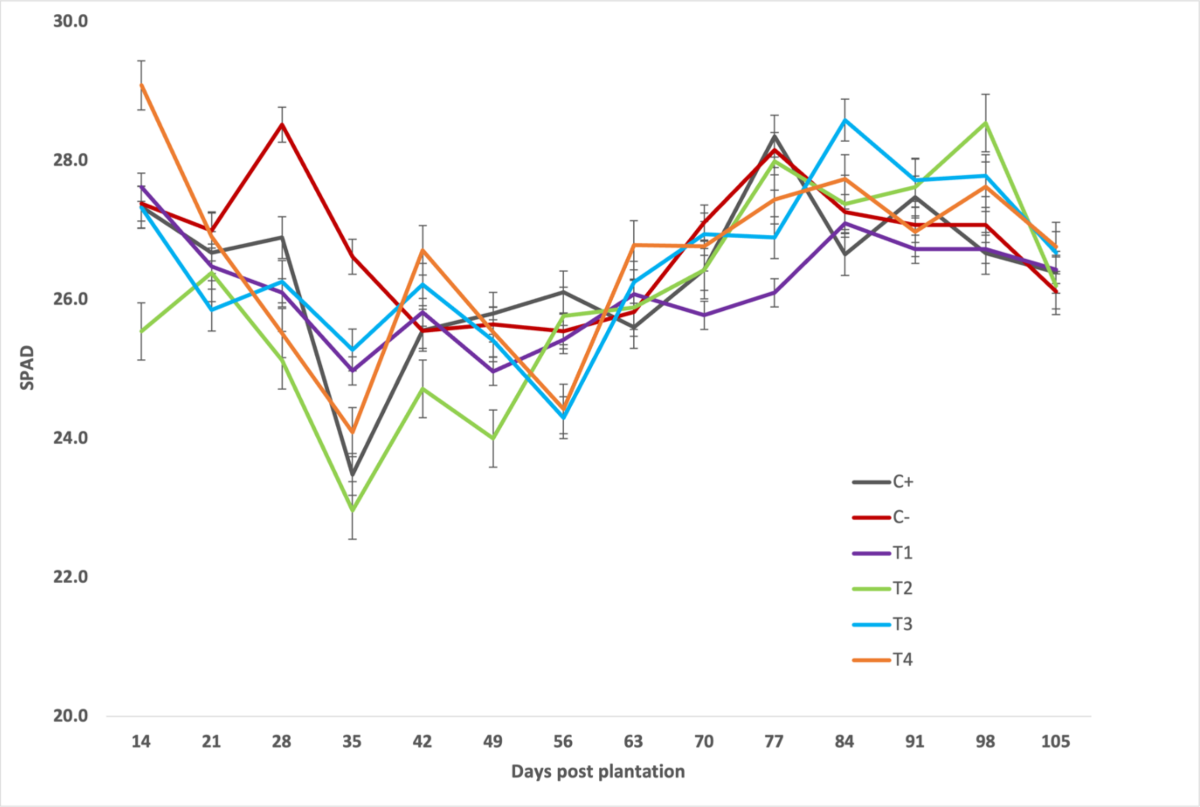

Figure 21: The mean SPAD of newly emerging leaves for Trial 2 (*n* = 163). C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

A total of 189 measurements were taken for Trial 1, and 163 for Trial 2. The mean values of SPAD measurements are presented in Table 4.

Table 4: The mean SPAD values of newly emerging leaves for both Trials.

Treatment	**SPAD**	
	Trial 1	**Trial 2**
C+	32.1 ± 7.7	26.4 ± 2.8
C-	31.6 ± 6.9	26.8 ± 2.6
T_1	32.0 ± 7.0	26.2 ± 2.5
T_2	31.9 ± 7.5	26.0 ± 3.0
T_3	31.0 ± 6.8	26.5 ± 2.8
T_4	31.3 ± 7.0	26.6 ± 2.9

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

No statistically significant differences among the SPAD values of plants under different treatments were observed for Trial 1. However, for Trial 2, significant differences were observed on the 28th day and 35th day post plantation. On the 28th day, the significant differences were observed between negative control (C-) SPAD, with the highest value (28.5), and T2 (*Cv* 0.4 g.L^{-1}) and T4 (*Cv* 36 g.L^{-1}) SPAD, which are statistically similar, with the lowest values of 25.1 and the second lowest of 25.5, respectively (Table 5). On the 35th day, the significant differences were between negative control SPAD, with again the highest value of 26.6, and T2 (*Cv* 0.4 g.L^{-1}) SPAD with the lowest (23.0).

Table 5: Tukey analyses of SPAD measurements for Trial 2, on 28 and 35 days post plantation. The values are presented as the mean SPAD.

Treatments	28 days	35 days
C+	26.9^{ab}	23.5^{ab}
C-	28.5^{a}	26.6^{a}
T$_1$	26.1^{ab}	25.0^{ab}
T$_2$	25.1^{b}	23.0^{b}
T$_3$	26.3^{ab}	25.3^{ab}
T$_4$	25.5^{b}	24.1^{ab}

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}). For each column, the values followed by the same letter, do not present statistical differences for $p<0.05$, according to Tukey test.

The growing parameters were measured once a week, by counting the number of leaves per plant, number of flowers trusses per plant, number of flowers and fruits per plant. The measurements were done on days 37, 44, 51, 58, 65, 72, 79, 86, 93, 100 and 107 after plantation for both trials. The visually count was done on 2 randomly selected bags per treatment per block, adding to a total of 8 counts per days.

2.3.2.2 Number of leaves per plant

The average values of leaves per plant for Trial 1 were acquired and the results are presented in Figure 22 A growth pattern is observed for all treatments, in special T3 (*Cv* 3.6 g.L^{-1}) and T4 (*Cv* 36 g.L^{-1}), reaching up to 32 leaves per plant on day 100. From day 37 to 107, T3 plants grew on average 16 leaves per plant, while T4 plants only grew 11 leaves. The lowest growth rate was observed for T1 (*Cv* 0.04 g.L^{-1}) plants which only grew 7 leaves over those 70 days. T3 also presented the highest growth rate after the 51st day after plantation compared to the remaining treatments.

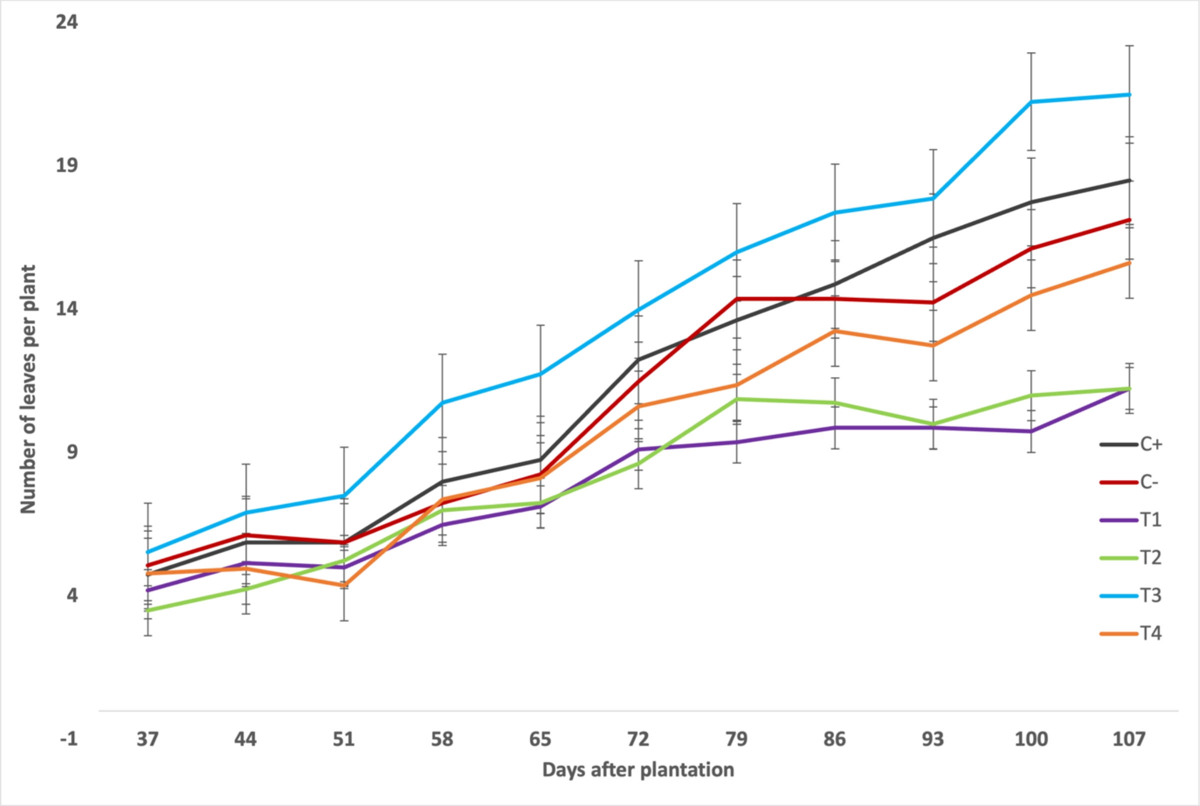

Figure 22: The mean number of leaves according to the number of days after plantation for Trial 1. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

For the 2nd Trial, T2 (*Cv* 0.4 g.L^{-1}) showed the highest growth development over the days post plantation, with values always higher than the remaining treatments, as seen in Figure 23. However, from days 37 to 107, the same average growth value, of 11 leaves per plant, was seen for both T2 and T3 (*Cv* 3.6 g.L^{-1}). Negative control presented the lowest development over time, with a growth of 7 leaves per plant. Whereas the remining treatments had the same values. Negative control plants had the highest number of leaves per plant, with 28 leaves at once.

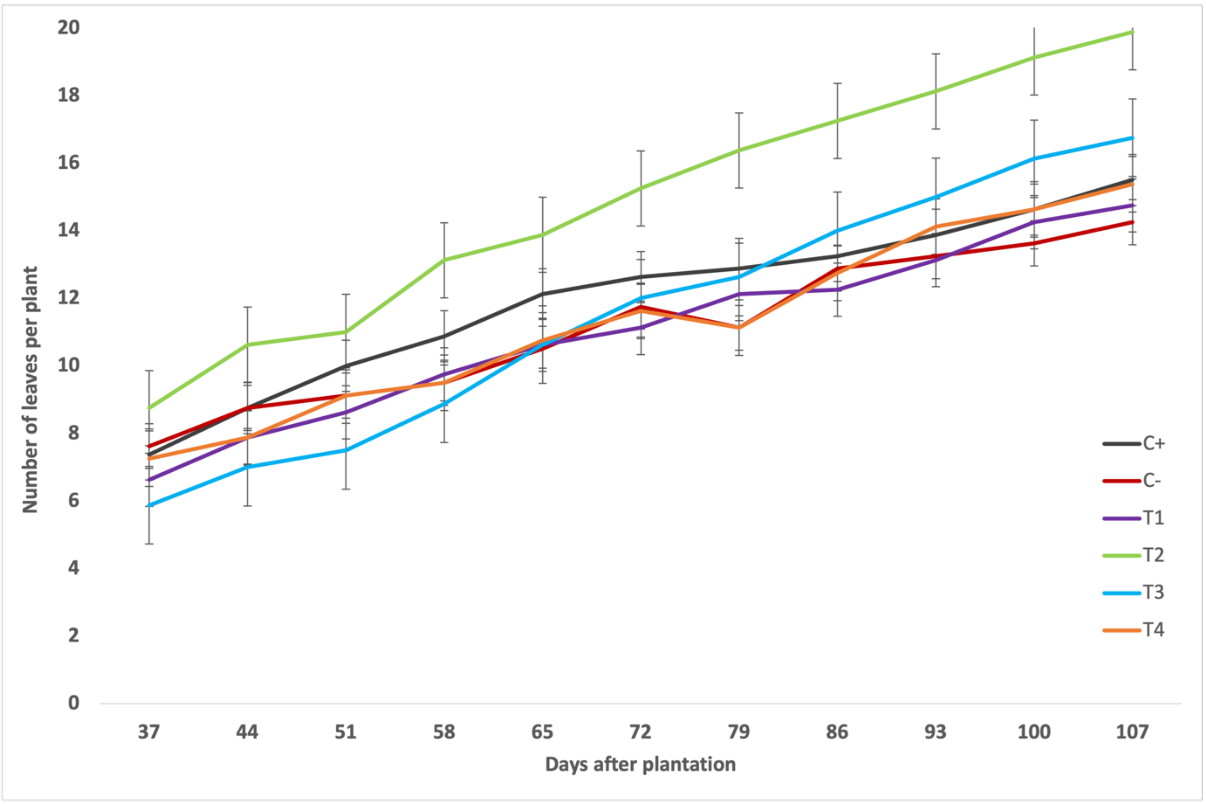

Figure 23: The mean number of leaves according to the number of days after plantation for Trial 2. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

When statistically comparing the trials, separately, no statistically significant differences were observed for Trial 2. However, the same cannot be said for the 1st Trial, as statistically significant differences were observed between T1 (*Cv* 0.04 g.L^{-1}) and T3 (*Cv* 3.6 g.L^{-1}) plants, which presented the lowest (9.8) and highest (21.3) values, respectively (Table 6).

Table 6: Number of leaves per plant count for Trial 1, on day 100th post plantation.

Treatment	Day 100
C+	17.8^{ab}
C-	16.1^{ab}
T$_1$	9.8^{b}
T$_2$	11.0^{ab}
T$_3$	21.3^{a}
T$_4$	14.5^{ab}

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}). For each column, the values followed by the same letter, do not present statistical differences for $p<0.05$, according to Tukey test.

The total number of leaves developed resulted with 1204 leaves for T3 (*Cv* 3.6 g.L^{-1}), as the highest value, while T1 (*Cv* 0.04 g.L^{-1}) produced the lowest with 698 leaves, for Trial 1. For Trial 2, T2 (*Cv* 0.4 g.L^{-1}) produced the highest with 1307, whereas T1 produced the lowest with 969. The leaf development is observed in Appendix 1.

2.3.2.3 Number of trusses per plant

The average number of trusses per plant, for Trial 1, can be seen in Figure 24. From day 58 to 93, there is an increase in the trusses per plant development except for negative control who encountered a slight drop on day 72. Positive control showed the greatest growth, with 4 trusses per plant along the days. The total number of trusses was obtained, and it showed that negative control produced the most trusses with 266, while T1 (*Cv* 0.04 g.L^{-1}) had the lowest trusses total number with 129. Positive control had the highest number of trusses per plant at once, with 17 trusses.

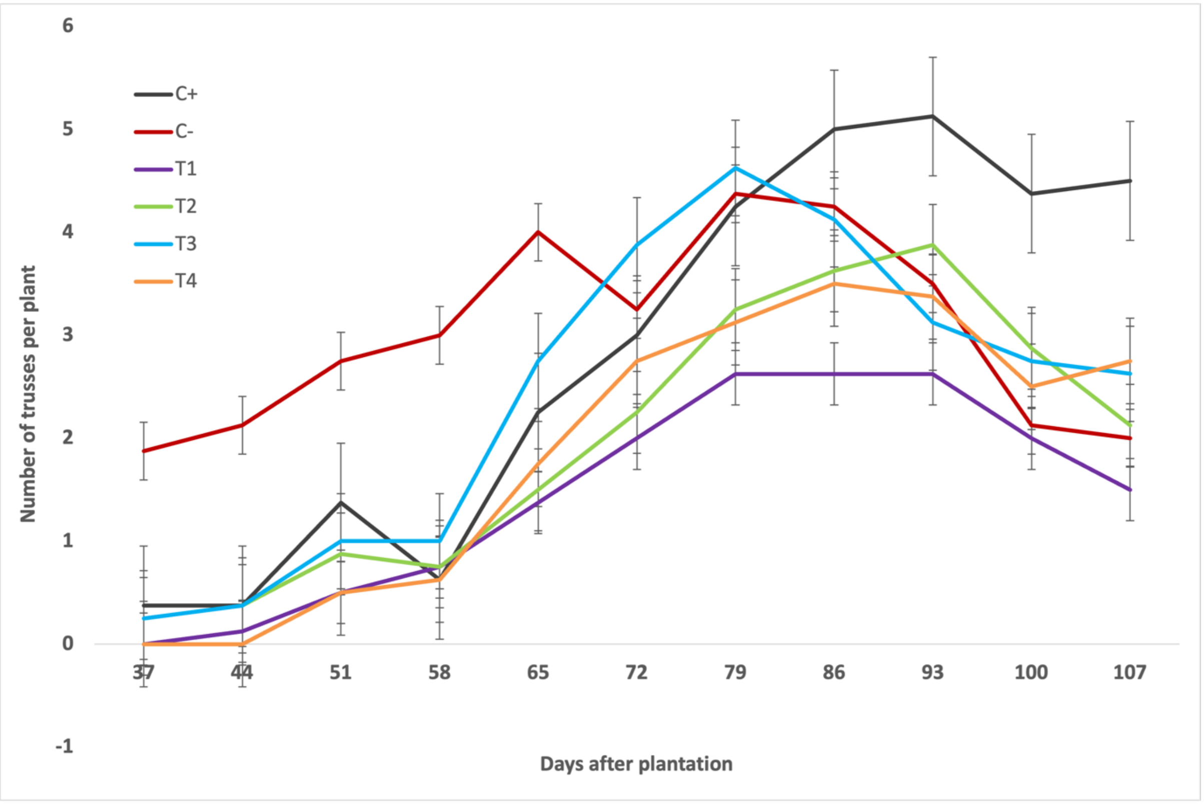

Figure 24: The average number of trusses per plant according to the days after plantation for Trial 1. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

For the 2nd Trial, from the beginning of the measurement to the 86th day, the plants had a linear growth, with T3 (*Cv* 03.6 g.L^{-1}) and positive control reaching the highest value of 8 trusses per plant, as seen in Figure 25. T3 had sample plants reaching 8 trusses per plant, as positive control only had 1. After the 86th day, all treatments dropped, and only T2 (*Cv* 0.4 g.L^{-1}) and T3 (*Cv* 3.6 g.L^{-1}) had a slight growth of 1 truss per plant. The total number of trusses produced reached 224 for T3 and 143 for T4, being the highest and lowest production, respectively.

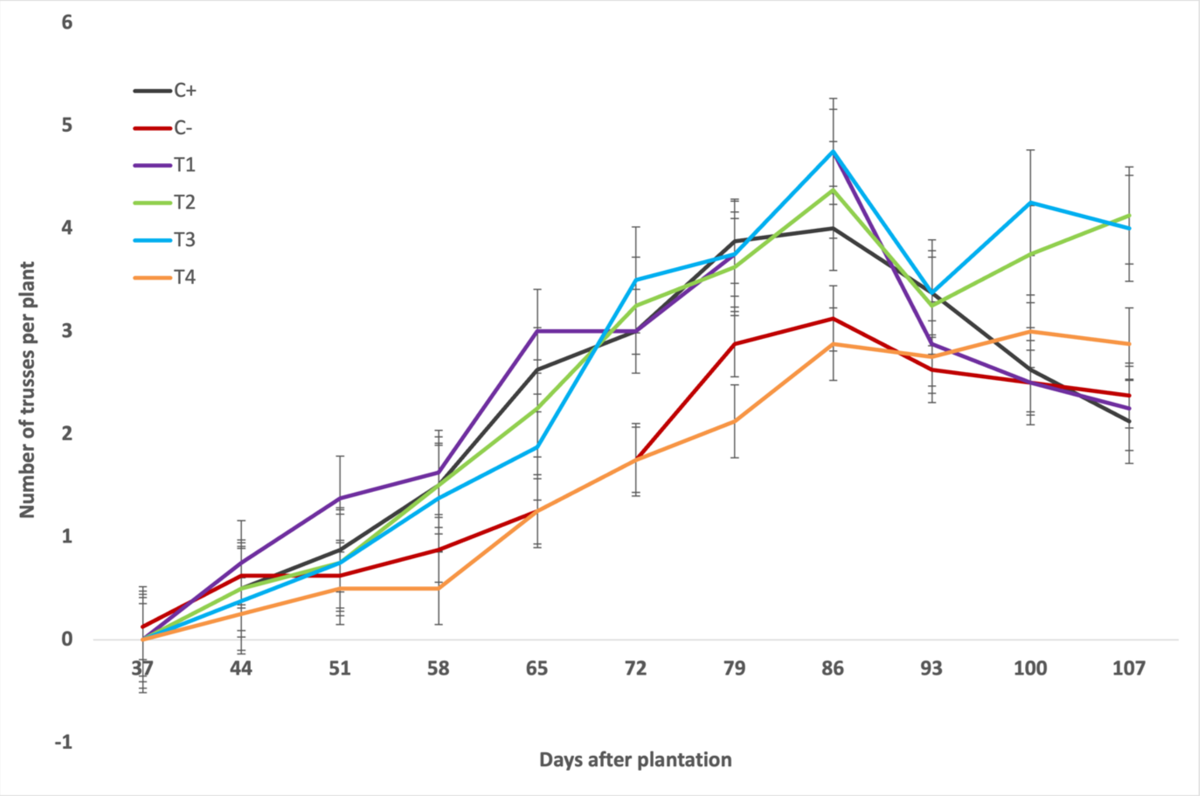

Figure 25: The average number of trusses per plant according to the days after plantation for Trial 2. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

The peak of trusses per plant for all treatments occurred on the 86th days after plantation. No statistically significand differences were observed with p-values above the significant level.

2.3.2.4 Number of flowers per plant

The highest number of flower per plant (22 flowers), for Trial 1, was achieved by positive control and T2 on the 72nd and 86th day, respectively (Figure 26). A decrease in flowers development is observed after day 86, for most Trials. A total of 145 flowers was produced for T3, being the highest number, while T1 only produced 74 flowers.

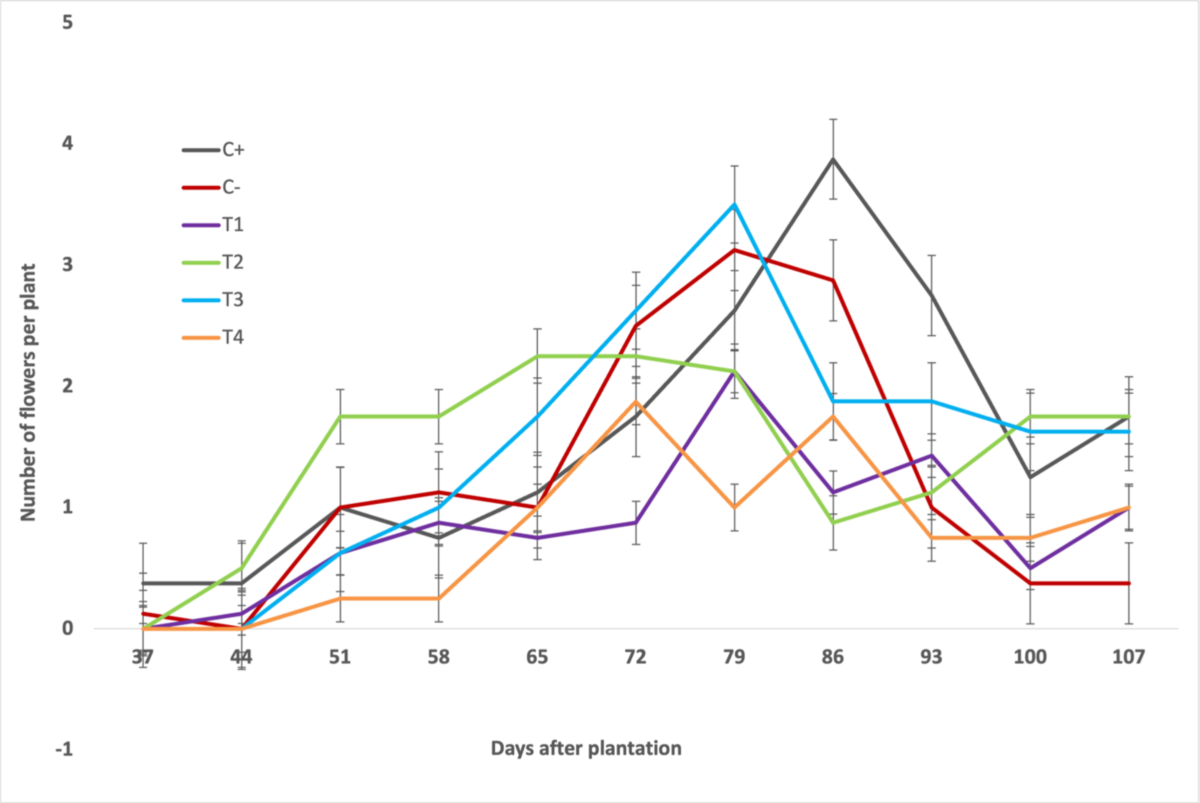

Figure 26: The average number of flowers according to the days after plantation Trial 1. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

For Trial 2, the highest number of flowers per plant was observed for positive control plant with 9 flowers. There are three peaks of production of flower, on the 72nd, 79th and 86th days post plantation (Figure 27), with the highest average value of 4 flowers per plant for T3 (*Cv* 3.6 g.L^{-1}) plants on the 79th day. Aside from T2 (*Cv* 0.4 g.L^{-1}), all other treatments follow the same pattern of a growth to a peak and then a decline. A total of 132 flowers was produced for T3, being the highest number, while T4 only produced 69 flowers.

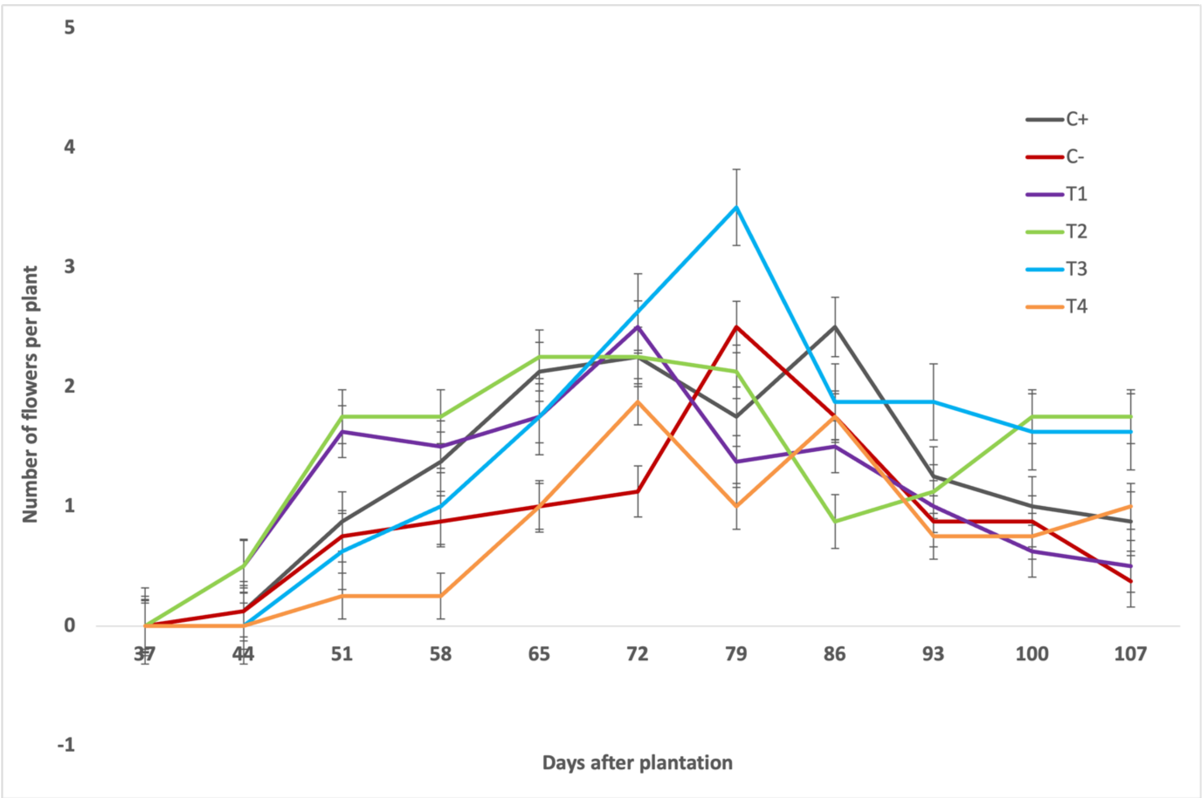

Figure 27: The average number of flowers according to the days after plantation Trial 2. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

No statistically significand differences were observed for Trial 1, regarding the number of flowers per plant. However, for the 2nd Trial, T3 (*Cv* 03.6 g.L^{-1}) and T4 (*Cv* 36 g.L^{-1}) plants displayed the highest (3.5) and the lowest (1.0) number of flowers per plant, respectively, on day 79 post plantation (Table 7). The first flowers to emerge for Trial 1 were from positive and negative control, and T3 (*Cv* 36 g.L^{-1}) plants, whereas for Trial 2 the first flowers were from positive and negative control, T1 (*Cv* 0.04 g.L^{-1}), and T2 (*Cv* 0.4 g.L^{-1}).

Table 7: Number of flowers per plant count for Trial 2, on day 79th post plantation.

Treatment	Day 79
C+	1.75^{ab}
C-	2.5^{ab}
T_1	1.38^{ab}
T_2	2.13^{ab}
T_3	3.5^{a}
T_4	1.0^{b}

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}). For each column, the values followed by the same letter, do not present statistical differences for $p<0.05$, according to Tukey test.

2.3.2.5 Number of fruits per plant

The plants with the most fruits at once were registered to be those of the positive control with 28 fruits. The high fruit productivity is observed between the 68th and 100th days (Figure 28). The period of fruit production was longer for C+, with an increase in fruits number until the 100th day. Positive control also produced the highest number of fruits, 390, and the lowest was produced by T1 with 158 fruits.

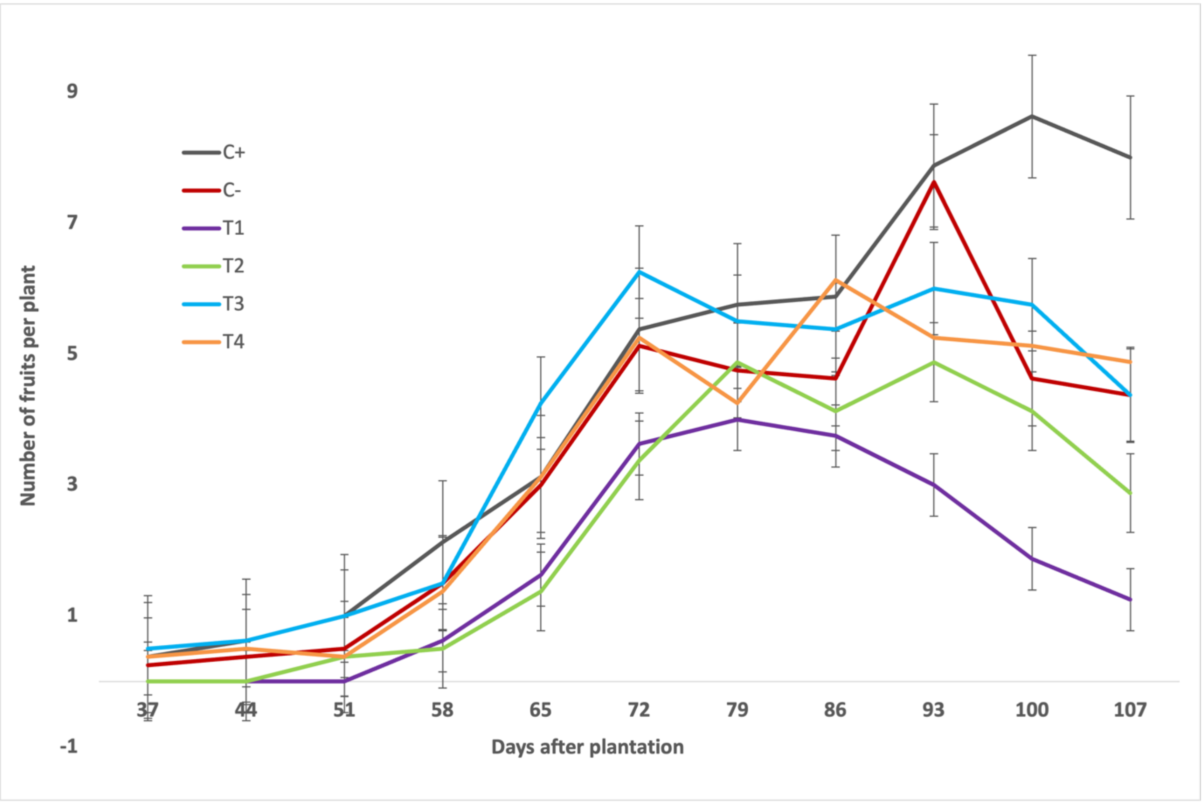

Figure 28: Number of fruits according to the days after plantation for Trial 1. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

For Trial 2, the highest number of fruits per plant were observed for T3 (*Cv* 3.6 g.L^{-1}) plants, 23 fruits, on the 86th day post plantation. The greatest average value of fruits per plant was also observed on day 86 post plantation, 11 fruits, for both positive control and T3. An exponential growth is seen from the 37th day until the 86th day, the highest peak in fruit production (Figure 29). T3 produced the greatest amount of total fruit with 412, whereas T4 had the least fruits with 240.

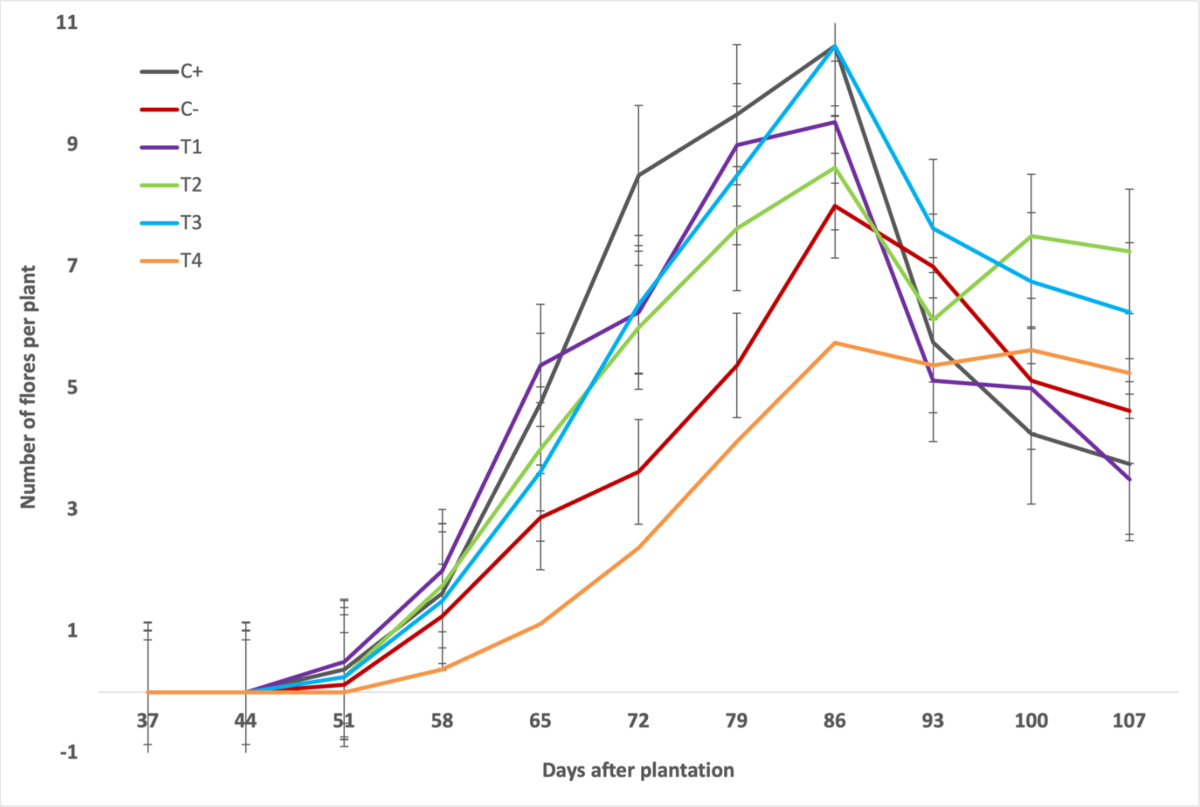

Figure 29: Number of fruits according to the days after plantation for Trial 2. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

The first fruits to emerge for Trial 1 belonged to positive and negative control, T3 (*Cv* 3.6 g.L^{-1}) and T4 (*Cv* 36 g.L^{-1}) plants, whereas for Trial 2, fruits from plants under all treatments, except T4 (*Cv* 36 g.L^{-1}), emerged together. The first fruits to be collected were on May 14th and July 2nd, for the 1st and 2nd Trial respectively. They were both collected exactly 51 days post plantation. No statistically differences were observed among all treatments.

2.3.3. *Productivity and fruit quality*

2.3.3.1 Fruit quantity

A total of 6192 strawberries were collected along the experiment, in which 2835 were collected during the 1st Trial and 3357 during the 2nd Trial , as can be seen in Figure 30. Trial 2 had a higher productivity with more 522 strawberries than those of Trial 1.

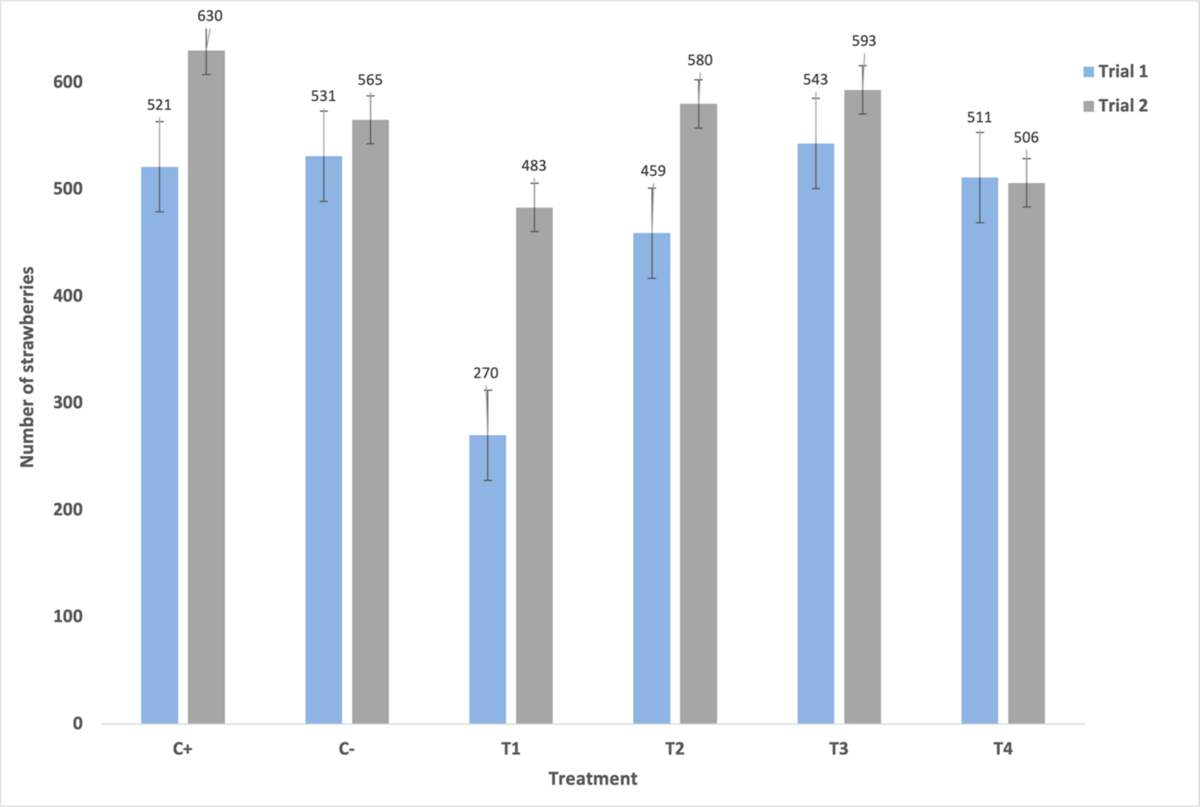

Figure 30: Total number of strawberries per treatment for Trials 1 and 2. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

For the 1st Trial, the T3 (*Cv* 3.6 g.L^{-1}) plants produced the most strawberries, 543, while T1 (*Cv* 0.04 g.L^{-1}) plants had the least with only 270. It is observed a much smaller productivity for T1, with an average of only 136 strawberries, in which the results presented a statistically significant difference among all other treatments. T3 resulted in a productivity 2 times higher than that of T1 but was similar to that of the controls (Table 8).

Table 8: The mean of the productivity of fruits per treatment for Trial 1.

Treatment	Mean number of fruits
C+	261^{a}
C-	266^{a}
T1	136^{c}
T2	230^{b}
T3	272^{a}
T4	256^{ab}

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}). For each column, the values followed by the same letter, do not present statistical differences for $p<0.05$, according to Tukey test.

For the 2nd trial, T1 (*Cv* 0.04 g.L^{-1}) plants also led to the lowest productivity among all treatments, with only 483 strawberries collected. However, the fruit number was statistically similar to that of T4 (*Cv* 36 g.L^{-1}) plants, which produced 506 fruits. Positive control had the highest productivity among both trials, with 630 fruits collected, but was statistically similar to those of T2 (*Cv* 0.4 g.L^{-1}) and T3 (*Cv* 3.6 g.L^{-1}), as seen in Table 9.

Table 9: The mean of the total amount of fruits per treatment for Trial 2.

Treatment	Mean number of fruits
C+	316^{a}
C-	283^{b}
T1	242^{c}
T2	291^{ab}
T3	297^{ab}
T4	254^{c}

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}). For each column, the values followed by the same letter, do not present statistical differences for $p<0.05$, according to Tukey test.

The total number of fruits for both trials was added, and the percentage is displayed in Figure 31, with the positive control (C+) and T3 (*Cv* 3.6 g.L^{-1}) plants showing the highest percentage of fruits collected during the experiment, with 1151 and 1136 fruits, respectively. Conversely, T1 (*Cv* 0.04 g.L^{-1}) resulted in the lowest amount of collected fruits (753).

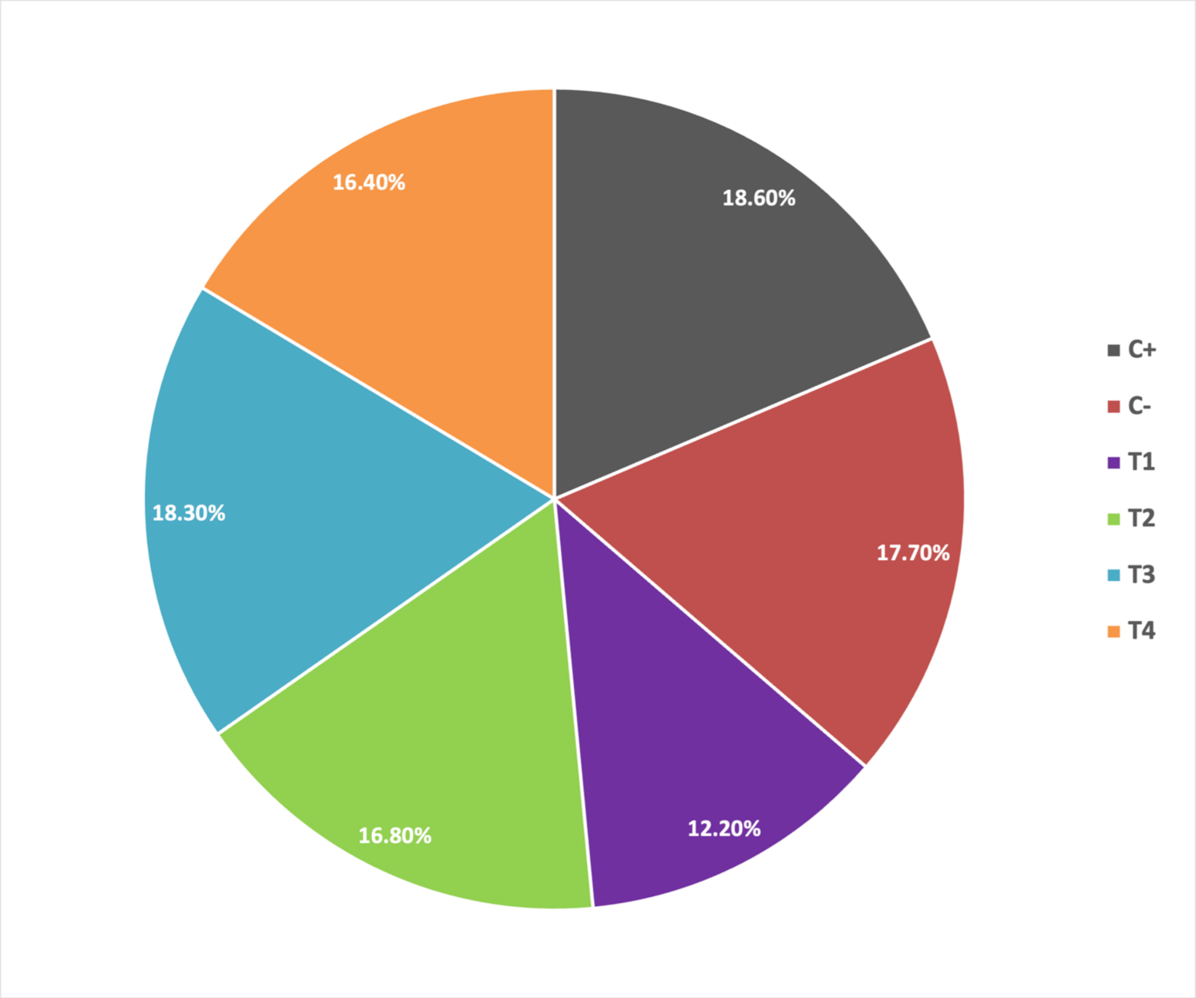

Figure 31: Percentage of strawberries collected per treatment for both trials together. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

2.3.3.2 Fruit weight

The *F x ananassa* fruits were collected and individually weighed twice a week and the mean values can be observed in Figure 32. The largest fruit was recorded to weigh 43.8 g (Fig. 33) from Trial 1 under the T4 (*Cv* 36 g.L^{-1}) treatment, which also resulted in the highest mean weigh among all other treatments and trials. The smallest fruit collected weighed only 0.52 g. The 1st Trial had a lower productivity but heavier fruits; conversely, in the 2nd Trial the opposite was observed. The highest total weight of collected *F x ananassa* fruits in the 1st Trial (5095.9 g) corresponded to those coming from negative control plants; the lightest fruit total weight (1980.1 g) was measured for T1 (*Cv* 0.04 g.L^{-1}) plants. For the 2nd Trial, maximum (4206.1 g) and minimum (2868.4 g) total weights were obtained for positive control and T1 fruits, respectively.

Concerning average fruit weighs, T1 (*Cv* 0.04 g.L^{-1}) plants displayed the smallest value (8.4 g) compared to those under all other treatments for Trial 1. However, for Trial 2, the highest fruit weigh was observed in positive and negative control plants, which were significantly different from those coming from T2 (*Cv* 0.4 g.L^{-1}) and T1 (*Cv* 0.04 g.L^{-1}) plants. Fruits with the lowest mean weight were recorded to belong to T2 plants, weighing 6.1 g on average. However, the largest difference in weight was observed between trials, most probably due to the season in which the fruits were grown and collected, in that the 2nd Trial was held on hot summer days, which are known to be less favorable for this soft-fruit crop as compared to those grown during springtime (Trial 1).

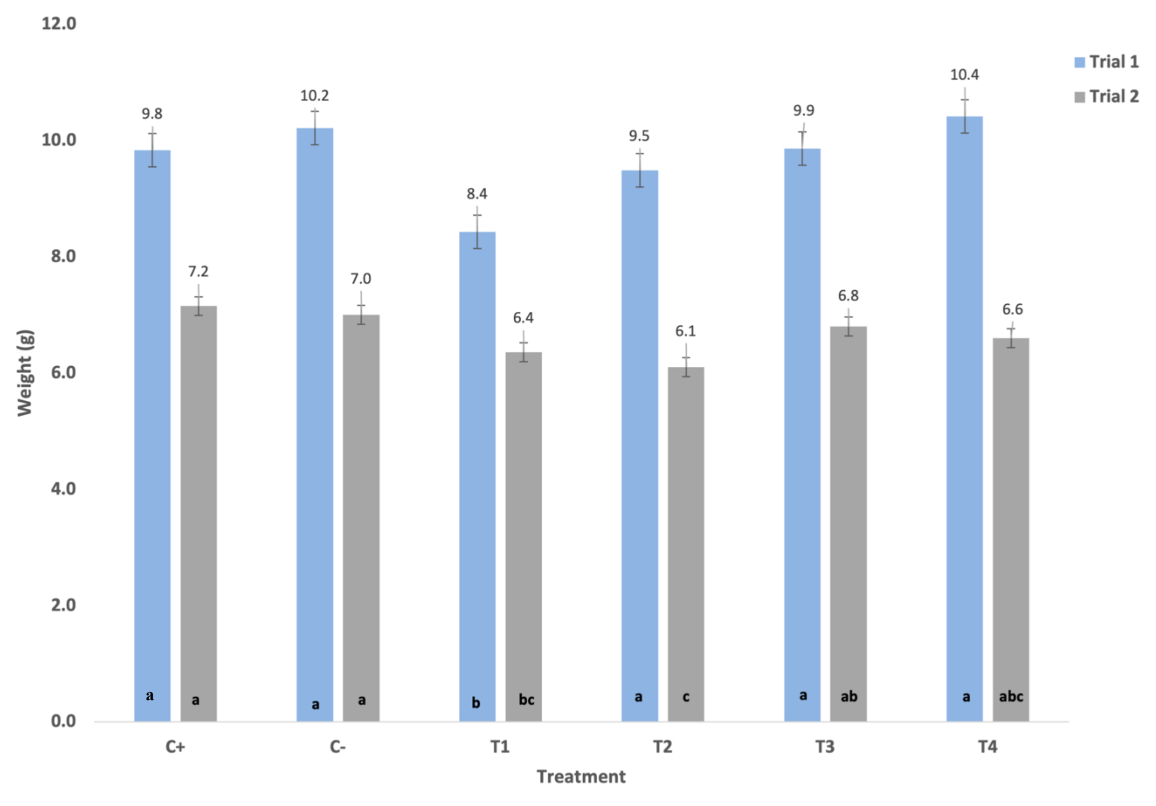

Figure 32: The mean weight of strawberries, displayed on the top of the bars, for both Trials. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

Figure 33: The largest strawberry collected in this project (43.8g) belonging to T4 (*C. vulgaris* 36 g.L^{-1}).

2.3.3.3 Measurements of ºBrix

ºBrix values were measured on 20 randomly selected fruits (manually selected) for each block. A total of 80 measurements per treatment for each trial. On the 2nd Trial, negative control fruits had a total of 74 measurements, while T1 had 77. For the 1st Trial, the values ranged from 20 to 4.6, being the highest from T3 (*Cv* 3.6 g.L^{-1}) and the lowest from T1 (*Cv* 0.04 g.L^{-1}) fruits, while for the 2nd Trial they ranged from 18.5 to 2.6, from negative control and T2 (*Cv* 0.4 g.L^{-}

^{1}) fruits, respectively (Figure 34). No statistically significant differences were found on Brixº values within each trials.

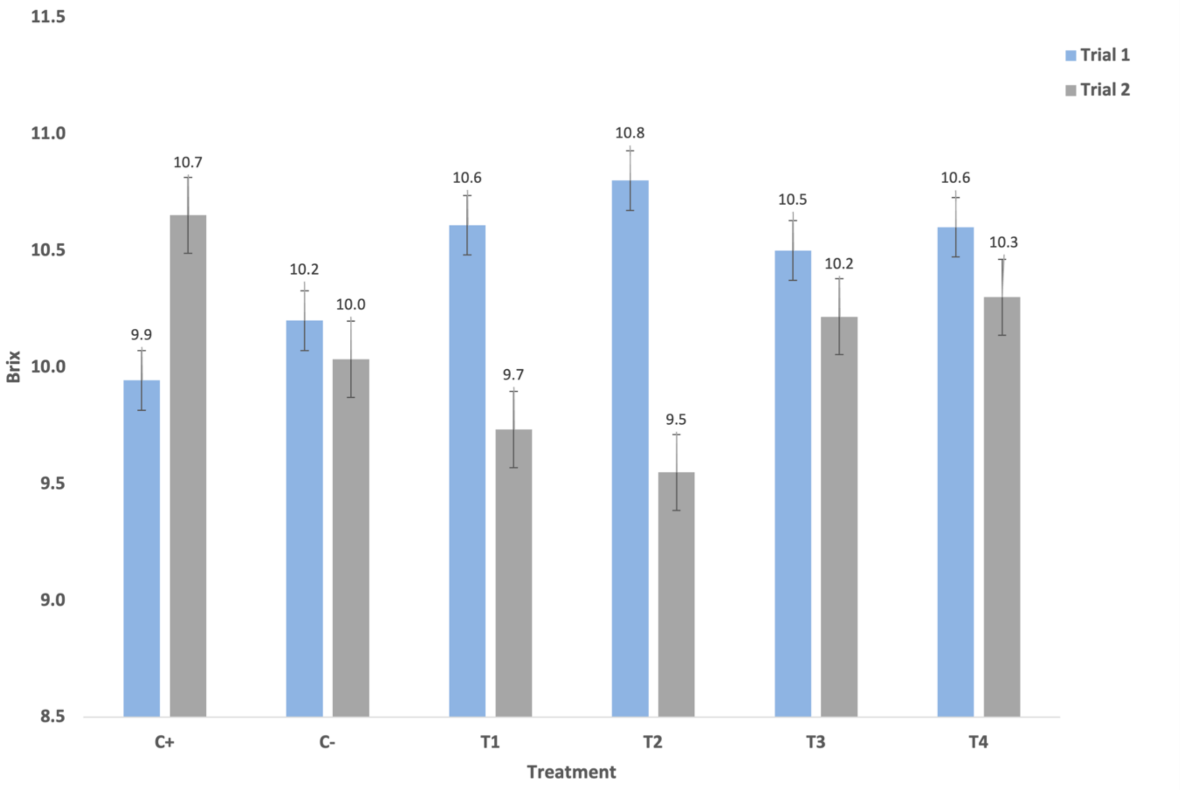

Figure 34: The mean Brixº of strawberries, displayed on the top of the bars, for both Trials. C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

2.3.3.4 Nutrient content of leaves and fruits

The leaves were analyzed for macronutrients (P, K, Ca, Ma, and S) and micronutrients (Fe, Mg, B, Zn and Al). For this project, elements Cu, Na and N$_{total}$ were under the detection limit due to low quantities of this specific element. The results of the analysis for both trials can be seen in Table 10, as well as the reference maximum and minimum range values.

For Trial 1, the only significant difference is for element Fe. Negative control leaves (44.78 mg.kg^{-1}) present statistically different Fe contents as compared to T4 (104.63 mg.kg^{-1}) leaves. When the treatments are compared to the reference values, Ca, Mg, Mn, B and Al contents are within the minimum and maximum reference value for all treatments. Conversely, P and S showed significant lower values compared to reference leaves values, for all treatments ($p<0.001$). Negative control, T1 (*Cv* 0.04 g.L^{-1}), T2 (*Cv* 0.4 g.L^{-1}) and T4 (*Cv* 36 g.L^{-1}) leaves had K contents below the reference minimum value. However, only T4 leaves showed K contents that were significantly different from the reference values.

For Trial 2, elements K, Ca, Mg and Mn are statistically similar among treatments of leaves and are all within the minimum and maximum of the reference values range. T4 (*Cv* 36 g.L^{-1}) leaves were statistically different from all other treatments on P, S and Fe contents, even though only S contents were within the reference range. For P, only T4 (*Cv* 36 g.L^{-1}) leaves were within the reference range, although it was statistically different from all other treatments. T4 leaves had statistically higher S contents than those from other treatments; yet, when compared to the reference values, a significant difference was observed with values below the minimum. T4 leaves (423.50 mg.kg^{-1}) had a Fe content about 4 times higher than the maximum reference value of 250 mg.kg^{-1}, making it statistically quite different ($p < 0.001$). Boron (B) contents of positive control leaves (78.05 mg.kg^{-1}) were significantly higher than those of the T3 (*Cv* 3.6 g.L^{-1}) leaves and were above the reference maximum of 70 mg.Kg^{-1}. Zn values for T4 (20.95 mg.kg^{-1}) leaves were the only ones within the reference range (20 – 50 mg.kg^{-1}), making them statistically higher than those of leaves under almost all treatments, except those of the negative control (14.43 mg.kg^{-1}). All the Al values were within the range of reference, but due to the wide range of values obtained, Al contents of T4 (154.68 mg.kg^{-1}) and positive control (54.33 mg.kg^{-1}) leaves were significant different. Interestingly, T4 plants presented the highest values for P, S and Fe.

Table 10: Macro- and micronutrients content of 500g of dried leaves for Trials 1 and 2, as well as the reference minimum and maximum values for comparison.

Treatment	%					mg.kg-1				
	P	**K**	**Ca**	**Mg**	**S**	**Fe**	**Mn**	**B**	**Zn**	**Al**
						Trial 1				
C+	0.140	1.593	1.533	0.380	0.111	57.35^{ab}	109.73	50.20	22.45	57.68
C-	0.148	1.453	1.583	0.410	0.139	44.78^{b}	101.25	54.35	18.58	53.63
T1	0.110	1.400	1.495	0.378	0.109	56.40^{ab}	91.43	47.48	13.45	55.38
T2	0.119	1.413	1.553	0.384	0.129	60.15^{ab}	94.90	51.05	12.94	44.33
T3	0.150	1.540	1.498	0.381	0.132	71.28^{ab}	104.60	56.23	22.28	52.08
T4	0.139	1.245	1.430	0.370	0.129	104.63^{a}	107.00	47.98	16.46	53.15
						Trial 2				
C+	0.172^{b}	1.943	1.490	0.376	0.111^{b}	50.73^{b}	100.83	78.05^{a}	9.86^{b}	54.33^{b}
C-	0.160^{b}	1.918	1.503	0.374	0.111^{b}	74.78^{b}	95.23	58.38^{bc}	14.43^{ab}	75.98^{ab}
T1	0.144^{b}	1.628	1.628	0.391	0.126^{b}	90.40^{b}	122.75	68.33^{ab}	9.09^{b}	81.15^{ab}
T2	0.153^{b}	1.783	1.495	0.370	0.122^{b}	89.55^{b}	108.38	62.23^{bc}	6.47^{b}	69.63^{ab}
T3	0.158^{b}	1.880	1.415	0.364	0.109^{b}	102.40^{b}	95.45	55.75^{c}	10.26^{b}	69.63^{ab}
T4	0.252^{a}	1.780	1.420	0.381	0.162^{a}	423.50^{a}	97.13	59.20^{bc}	20.95^{a}	154.68^{a}
						Reference				
Min	0.25	1.5	0.7	0.3	0.4	60	50	30	20	1
Max	0.4	2.5	1.7	1.5	0.6	250	200	70	50	300

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}). For each column, the values followed by the same letter do not present statistical differences for $p<0.05$, according to Tukey test.

The fruits were evaluated for macronutrients (Ntotal, P, K, Ca, and Mg) and micronutrients (Fe, Mn, Cu, Zn and Na). For this project, elements S, B, Mo and AL were disregarded due to the lack of data for comparison. No statistically significant differences were observed among treatments for either trial (Table 11). However, some statistically differences were observed when the results from the fruit analyses was compared to the reference values

For Trial 1, Ntotal ($p < 0.05$) results for all treatments were significant below the reference value (1024 mg.kg^{-1}).). For potassium, the results were significantly higher than reference value (890 mg.Kg^{-1}), T2 (*Cv* 0.4 g.L^{-1}) fruit two times higher (2088.5 mg.Kg^{-1}). For both Na and Cu, all plants in trial showed statistically lower contents than the reference values (*p* <0.001) of 100 mg.kg^{-1} and 1.19 mg.kg^{-1}, respectively.

For the 2nd Trial, no statistically significant differences were observed among the treatments, however the same cannot be said when those were compared to the reference values. Ntotal, Fe, Na, Cu and Zn had values statistically significantly lower than the reference values with p-values below the significance level of 0.05. The lowest value for Ntotal corresponded to those of T2 (541.1 mg.kg^{-1}) fruits, which were was almost two times lower than reference values (1024 mg.kg^{-1}Negative and positive controls as well as T4 (*Cv* 36 g.L^{-1}) fruits had P contents significantly lower than reference values. All treatment fruits showed Ca contents higher than the reference value (120 mg.Kg^{-1}), even though Ca levels in positive (232.2 mg.Kg^{-1}) and negative (226.8 mg.Kg^{-1}) control fruits were statistically similar to reference values. For magnesium, T2 fruits, with a concentration of 191.5 mg.Kg^{-1}, as compared to the reference number, with 118 mg.Kg^{-1}, presented statistically differences. For Mn, only negative control (3.50 mg.Kg^{-1}), T1 (3.57 mg.Kg^{-1}) and T4 (3.50 mg.Kg^{-1}) fruits were statistically similar to reference fruit values.

Table 11: Macro- and micronutrients content of 500g of dried fruits for Trials 1 and 2, as well as the reference values for comparison.

	mg.kg^{-1}									
Treatment	**N_{total}**	**P**	**K**	**Ca**	**Mg**	**Fe**	**Mn**	**Na**	**Cu**	**Zn**
	Trial 1									
C+	609.2	186.8	1916.0	212.6	132.0	2.1	3.8	18.2	0.2	1.6
C-	569.4	209.0	2173.6	218.3	146.6	2.1	4.7	19.8	0.2	2.2
T1	556.8	180.2	1872.2	225.7	138.0	1.4	3.9	21.2	0.1	1.4
T2	681.8	200.1	2219.6	248.6	153.8	2.4	4.4	18.1	0.1	1.9
T3	645.4	192.1	1962.5	211.1	134.4	2.1	3.8	20.0	0.2	2.3
T4	696.5	195.8	2105.3	227.2	143.2	2.9	4.5	9.4	0.1	1.1
	Trial 2									
C+	667.9	151.4	2064.2	232.2	150.0	0.7	3.9	7.5	0.1	0.1
C-	575.4	144.0	2062.1	226.8	155.8	0.6	3.5	6.2	0.1	0.1
T1	575.8	146.1	1969.0	275.4	174.6	0.7	3.6	7.4	0.2	0.1
T2	541.1	186.1	2088.5	359.6	191.5	0.7	3.9	39.9	0.2	0.1
T3	592.8	145.4	1942.6	312.6	157.0	0.6	4.1	8.3	0.5	0.1
T4	597.8	174.1	1980.2	350.3	184.3	0.6	3.5	41.9	0.3	0.0
	Reference									
	1024	200	890	120	118	2.8	2.8	100	1.19	1.8

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

2.3.3.5 Organoleptic test

The results for the appearance of the fruits are seen in Table 12. It was observed that T3 (*Cv* 3.6 g.L^{-1}) had the best appearance with 29.0% classifying the fruits as "Excellent", followed by T2 (*Cv* 0.4 g.L^{-1}) with 26.3% of fruits getting the classification of "Excellent", while T1 fruits (*Cv* 0.04 g.L^{-1}) showed the worst appearance with 7.7% of testers marking them as "Very Bad".

Table 12: Fruit appearance on organoleptic test for Trial 1, the values are given in percentage (%).

	C+	C-	T1	T2	T3	T4
Excellent	18.4	18.4	11.5	26.3	29.0	18.4
Very Good	44.7	57.9	53.9	34.2	47.4	55.3
Good	26.3	21.1	15.4	21.1	18.4	18.4
Bad	5.3	0.0	11.5	13.2	2.6	7.89
Very Bad	5.3	2.6	7.7	5.3	2.6	0.0

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

The analysis for the fruit odor is seen in Table 13. Exactly half of the people said the T3 (*Cv* 3.6 g.L^{-1}) fruits had an excellent odor, while T1 (*Cv* 0.04 g.L^{-1}) had the least liked smell among the treatments with 3.8% of testers classifying it as very bad.

Table 13: Fruit odor on organoleptic test for Trial 1, the values are given in percentage (%).

	C+	C-	T1	T2	T3	T4
Excellent	28.9	15.8	7.7	23.7	50.0	15.8
Very Good	23.7	21.1	26.9	36.8	18.4	21.1
Good	31.6	44.7	50.0	31.6	26.3	42.1
Bad	13.2	18.4	11.5	7.9	5.3	21.1
Very Bad	2.6	0.0	3.8	0.0	0.0	0.0

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

The fruits with the most pleasant flavor were from T2 (*Cv* 0.4 g.L^{-1}) plants with 36.8% classified as "Excellent", followed by T3 (*Cv* 3.6 g.L^{-1}) with 26.3%, as can be seen in Table 14. The fruit with the least favorite flavor were those of the negative control with 5.3% of the testers classifying them as "very bad".

Table 14: Fruit flavor on organoleptic test for Trial 1, the values are given in percentage (%).

	C+	C-	T1	T2	T3	T4
Excellent	10.5	5.3	7.7	36.8	26.3	18.4
Very Good	52.6	28.9	42.3	26.3	36.8	36.8
Good	26.3	34.2	38.5	18.4	28.9	31.6
Bad	10.5	26.3	11.5	15.8	7.9	13.2
Very Bad	0.0	5.3	0.0	2.6	0.0	0.0

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

The test for the texture of the fruit pointed to T3 (*Cv* 3.6 g.L^{-1}), obtaining the best classification in terms of texture, with 47.4% of the respondents marking them as "Excellent". However, they also presented the highest value (10.5%) for a classification of bad texture, suggesting some contradictory perceptions among testers (Table 15). T2 (*Cv* 0.4 g.L^{-1}) fruits had the second-best texture with 42.1% classified as "Excellent".

Table 15: Fruit texture on organoleptic test for Trial 1, the values are given in percentage (%).

	C+	C-	T1	T2	T3	T4
Excellent	34.2	23.7	23.1	42.1	47.4	21.1
Very Good	52.6	42.1	42.3	31.6	21.1	44.7
Good	10.5	26.3	30.8	23.7	21.1	28.9
Bad	2.6	7.9	3.8	2.6	10.5	5.3
Very Bad	0.0	0.0	0.0	0.0	0.0	0.0

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

Positive control fruits were qualified as "sweet enough" by half of the tasters, while negative control fruits were qualified as "little sweet" by the same number of tasters. T1 (*Cv* 0.04 g.L^{-1}) and T3 (*Cv* 3.6 g.L^{-1}) fruits were more on the spectrum of "sweet enough" as judged by over half of the responses, while T4 (*Cv* 36 g.L^{-1}) fruits were just below half. T2 (*Cv* 0.4 g.L^{-1}) fruits were defined as having an optimal sweetness, however, closer to responses classifying them as "little sweet" (Table 16).

Table 16: Fruit sweetness on organoleptic test for Trial 1, the values are given in percentage (%).

	C+	C-	T1	T2	T3	T4
Little Sweet	26.3	50.0	30.8	36.8	21.1	36.8
Sweet Enough	50.0	36.8	53.8	23.7	55.3	42.1
Optimal Sweetness	18.4	13.2	15.4	39.5	21.1	21.1
Too sweet	5.3	0.0	0.0	0.0	2.6	0.0

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

The acidity analyses are displayed in Table 17. Over half of the tasters indicated that all treatments fruits had an "Adequate Acidity", except for the negative control fruits which exactly half of the testers marked with this classification. Negative control fruits showed that 26.3% of the tasters found its flavor to be too acid.

Table 17: Fruit acidity on organoleptic test for Trial 1, the values are given in percentage (%).

	C+	C-	T1	T2	T3	T4
Little Acidity	23.7	23.7	15.4	18.4	7.9	10.5
Adequate Acidity	63.2	50.0	80.8	68.4	76.3	71.1
Too Much Acidity	13.2	26.3	3.9	13.2	15.8	18.4

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

The final question asks if any tasters would have this fruit again in the future. The only treatments with a positive answer were T2 (*Cv* 0.4 g.L^{-1}), T3 (*Cv* 3.6 g.L^{-1}) and T4 (*Cv* 36 g.L^{-1}) fruits. Positive and negative controls, and T1 (*Cv* 0.04 g.L^{-1}) were qualified as "maybe". However, negative control had a highest percentage (34.2%) of tasters that would not want to consume this fruit in the future (Table 18).

Table 18: Would you consume this fruit again? Organoleptic test for Trial 1, the values are given in percentage (%).

	C+	C-	T1	T2	T3	T4
No	13.2	34.2	7.7	23.7	7.9	18.4
Maybe	44.7	39.5	57.7	23.7	42.1	36.8
Yes	42.1	26.3	34.6	52.6	50.0	44.7

C+ (Algaman B), C- (water), T1 (*C. vulgaris* 0.04g.L^{-1}), T2 (*C. vulgaris* 0.4 g.L^{-1}), T3 (*C. vulgaris* 3.6 g.L^{-1}) and T4 (*C. vulgaris* 36 g.L^{-1}).

2.4. Discussion

The vast majority of research on agricultural applications of algae focus on the use of cyanobacteria for many reasons, one of them being their ability to fix atmospheric nitrogen to plant-available forms (Sharma et al., 2010), or on macroalgae, since they can be harvested from coastal areas and are easier to process compared to microalgae (Zodape, 2001). The use of microalgae-based biostimulants has become a global approach for obtaining environmentally friendly and high yield crops with good qualities that are safe for humans and the oceans (Refaay et al., 2021). The interest in developing human consumption products made from microalgae has led to an increase in research for potential product or by-product applications that can make cultivation and production more economically achievable. Thus, the potential of *C. vulgaris* as a plant biostimulant on the growth and yield production of *Fragaria x ananassa* was investigated in the present study.

In this study, foliar application treatment T3, of *C. vulgaris* suspension with a concentration of 3.6 g.L^{-1}, significantly enhanced the plants growth development of leaves, trusses, flowers, fruits, as well as the number of fruits and the weight of the fruit, compared with the controls. Many studies have revealed that foliar application technique is an advantageous tool in enriching plant nutrients; the nutrient uptake via leaf surface is significantly quicker than through roots (Dineshkumar et al., 2020), where they can penetrate via cuticular cracks, stomata, trichomes, or lenticles, reaching target cells where nutrients are required (Fernández & Brown, 2013). Strawberries prefer slightly acidic soils, with a pH between 5.5 and 6.5. In this project, pH remained close to neutrality, although slightly above pH 7. Interestingly, good vigor has also been obtained on slightly alkaline soils (Fennimore et al., 2013), with an optimal EC value of about 1.5 dS.m^{-1}. The values of EC were above or below this value for the 1st and

2nd Trial, respectively. However, there were no statistically differences when compared among themselves.

Compared to both positive and negative control, the *C. vulgaris* 3.6 g.L^{-1} treated plants showed an increase in leaves per plant by 16-20 %, thus the number of fruits per plant showed a 10.6 – 26.2% increase. According to Garcia-Senin's (2013), similar results were also seen on the growth and production of tomato. The obvious positive changes in both vegetative growth, yield and *F. x ananassa* development can be the outcome to the effect on the plant of rich contents in terms of microalgal protein containing essential amino acids, combined with high amount of micro- and macronutrients, being also rich in vitamins and minerals, iron, potassium, phosphorus and calcium (Safi et al., 2014). Additionally, the phytohormones, auxins and cytokines, presented in *C. vulgaris* have been reported to play a crucial role in cell division, cell elongation, and root development (Refaay et al., 2021). The use of *C. vulgaris* extract as a biostimulant is in agreement with similar results reported for lettuce in terms of better yield and growth as well as enhanced plant metabolism (La Bella et al., 2021).

The treatment with 3.6g.L^{-1} of microalgae showed an increase in development on the number of flowers per plant, by 12.5 – 33%, compared to the controls. High zinc contents in plants under this treatment (T3) might have promoted a higher production of flowers. Zinc is responsible for greater numbers of flowers per plant and with larger sizes and, thus, a greater number of fruits per plant (Stoyanova & Doncheva, 2002). Potassium could have also played an important role in this, as it directly affects the quantity of fruits. It also possible that the high levels of this nutrient also affected the sugar content, yielding fruits with a brighter coloration and better flavor (Prajapati & Modi, 2012). Although organoleptic tests are qualitative and subjective to change depending on the tastes of the consumers (and on the geographical areas of the world), T3 fruits, treated with *C. vulgaris* at a concentration of 3.6 g.L^{-1} , were very attractive, gathering the best marks among the consumers, which were significantly higher than the controls.

Since this work is innovative, the objective is to bring knowledge and information. So far, no literature has produced anything similar where microalgae were applied to improve the production and the organoleptic properties of strawberries. Currently, we live in times when human health and pollution are something that concerns society. This study is a contribution and an alternative to improve this preoccupation. It confirms that microalgae might improve the growth and yield of strawberry, and can be used as a biostimulant, and with this reducing the number of chemical stimulants and biofertilizers being used in agriculture. Lower amounts of nutrients in run-off waters will certainly help decrease the number and extension of dead zones in the oceans.

2.5. Conclusion

The foliar spray application of microalgae-based biostimulant in agriculture practice is to be considered as a promising and innovative agricultural technique, as it is safe to the environment, eases agricultural sustainability, and achieves high yield in crop production. Indeed, taking all the results together, *Fragaria x ananassa* plants treated with *C. vulgaris* 3.6 g.L^{-1} showed a higher productivity, enhancing its yield and growth. In this regard, these results represent the first study about a foliar application of *Chlorella vulgaris* dry extract on strawberry production, reporting a successful biostimulant effect. For future studies, it would be interesting to investigate concentrations between 3.6 g.L^{-1} and 36 g.L^{-1}.

2.6. References

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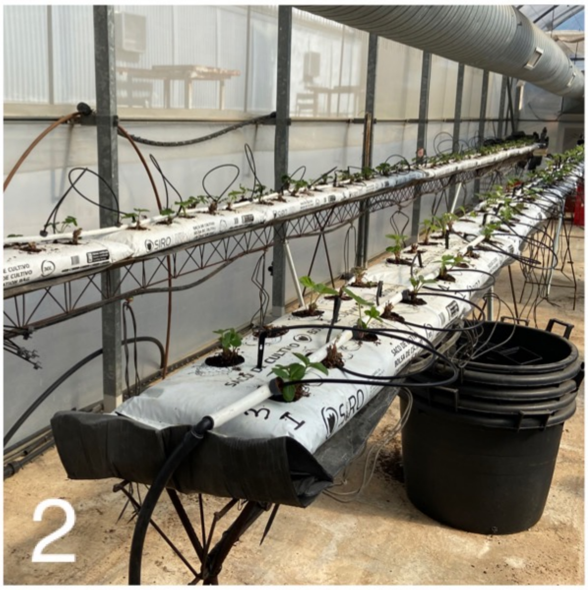
2.7. Appendix

Appendix 1: Leaves development throughout the project


1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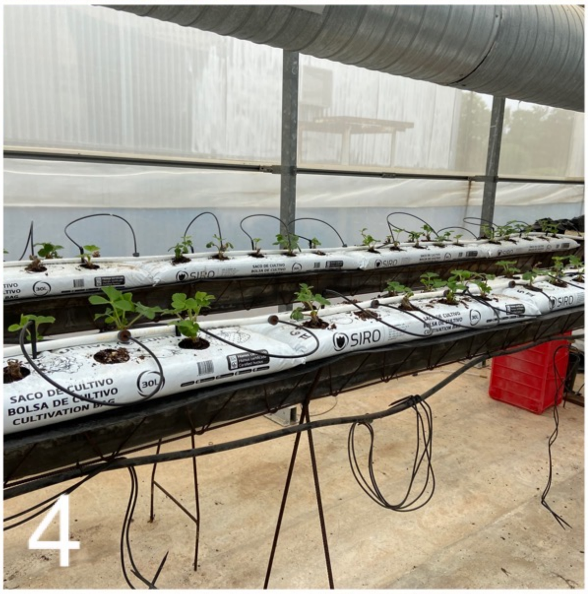
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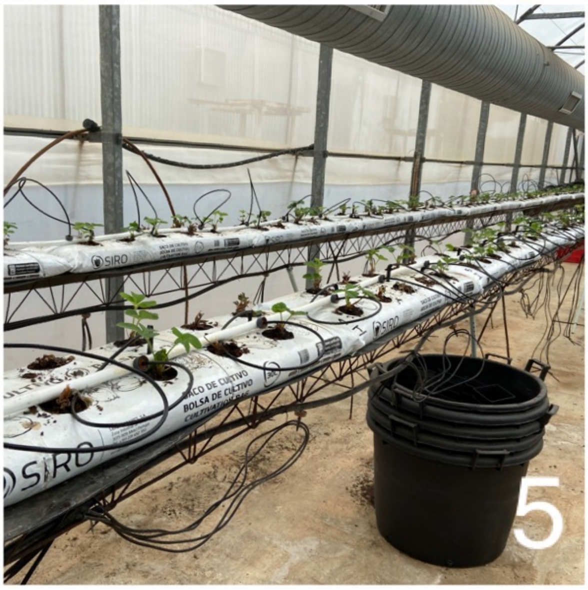
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