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VÁLTER RAFAEL DA COSTA MATEUS

UTILIZAÇÃO DE BIOMASSA DE *CHLORELLA VULGARIS* UTILIZADA NA BIORREMEDIAÇÃO DE EFLUENTES VINÍCOLAS COMO BIOADITIVO AGRÍCOLA

Relatório de Projeto do Mestrado em Engenharia Biológica e Química

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Dezember, 2025

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JÚRI

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"*Abre a mente ao que digo e que baste/ e dentro o guarda; que não faz ciência/ tê-lo entendido, se o tu não guardaste*"
-Dante Alighieri, "A Divina Comédia"

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ABSTRACT

This master thesis aimed to study the effect of winery-effluent-grown C*hlorella vulgaris* biomass on the germination and plant growth of *Phaseolus vulgaris, Vicia lens* and *Zea may*s.

Microalgae, including *Chlorella vulgaris*, are very versatile organisms that produce different metabolites in different quantities based on the growth conditions. This capacity to grow under different conditions allows microalgae to be used for a vast number of uses, just like food, feed, the produced metabolites used in industries such as cosmetics and pharmaceuticals, as part of wastewater treatment and biofertilisers.

These last two, when combined, allow for the create a close circle of nutrients, where the nutrients that weren't transformed into the final product and resided in the wastewater are used by the microalgae, who can later be used as a biofertiliser. The phosphate, potassium and nitrogen present in the biomass can then be mineralised into phosphates, potassium ions and nitrates or ammonium, which are easily absorbed by plants. This availability of nutrients contributes to higher growth rates and/or better plant yield.

This thesis combined different scenarios, such as: The timing of biomass addition before sowing, the comparison between the application of wet biomass and freeze-dried biomass grown in conventional medium, and the effect of wet biomass and freeze-dried biomass grown on medium containing 10% (w/w) winery effluent. In all tests the verified growth difference was deemed not significant, however there is a measurable size difference.

Based on one-way ANOVA, the chemical fertiliser had a better performance. However, the use of effluent-grown biomass showed promising results when compared to the unamended soil.

Following these results, preliminary data from this thesis was presented in poster format at the "8° Simpósio Produção e Transformação de Alimentos em Ambiente Sustentável", organised by the Department of Earth Sciences of NOVA FCT in Almada, Portugal, on June 5, 2025, and at FAO Global Agrifood Biotechnologies conference organised by the Food and Agriculture Organization of the United Nations in Rome, Italy, between June 16 and 18, 2025.

Keywords: Winery effluent; *Chlorella vulgaris;* circular economy, biofertilisers, sustainability

RESUMO

Esta dissertação de mestrado teve como objetivo estudar o efeito da biomassa de *Chlorella vulgaris* cultivada em efluentes de vinicultura, na germinação e no crescimento das plantas: *Phaseolus vulgaris, Vicia lens* e *Zea mays*.

As microalgas, incluindo a *Chlorella vulgaris*, são organismos muito versáteis, que produzem diferentes metabolitos em diferentes quantidades, dependendo das condições de crescimento. Esta capacidade de crescer em diferentes condições permite que as microalgas sejam utilizadas para um vasto leque de aplicações, como a alimentação humana e animal, os seus metabolitos podem serem utilizados em indústrias como a cosmética e a farmacêutica, no tratamento de efluentes e como biofertilizantes.

A combinação destas duas últimas aplicações permite a criação de um ciclo fechado de nutrientes, no qual os nutrientes que não foram incorporados no produto final e permaneceram nos efluentes são assimilados pelas microalgas, que podem, posteriormente, ser utilizadas como biofertilizante. O fosfato, o potássio e o azoto presentes na biomassa podem então ser transformados em formas mineralizadas, como fosfatos, iões potássio e nitratos ou amónio, que são facilmente absorvidos pelas plantas. Esta disponibilização de nutrientes contribui para taxas de crescimento mais elevadas e/ou para um melhor rendimento vegetal

Esta tese aliou diferentes cenários: o período de adição de biomassa antes de semear, comparação entre a aplicação de biomassa húmida e de biomassa liofilizada produzida em meio de cultura convencional; e o efeito de biomassa húmida e de biomassa liofilizada produzida em meio de cultura contendo 10 % (v/v) de efluente agrícola. Em todos os ensaios, a diferença de crescimento verificada foi considerada não significativa, embora exista uma diferença de tamanho mensurável.

De acordo com a análise de variância simples, os fertilizantes químicos apresentaram um desempenho superior. Ainda, assim a aplicação de biomassa cultivada em meio com efluente revelou resultados promissores quando comparada com o solo sem adição de biomassa.

Em virtude destes resultados, dados preliminares desta tese foram apresentados em formato de poster no 8° Simpósio Produção e Transformação de Alimentos em Ambiente Sustentável, dinamizado pelo Departamento de Ciência da Terra da NOVA FCT em Almada, Portugal, no dia 5 de junho de 2025 e na “FAO Global Agrifood Biotechnologies” Conference, dinamizado pela Organização das Nações Unidas para Alimentação e Agricultura em Roma, Itália, entre 16 e 18 de junho de 2025.

Palavras-Chave: Efluente vinícola; *Chlorella vulgaris;* economia circular; biofertilizantes; sustentabilidade

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SYMBOLS AND ABBREVIATURES

ABA – Abscisic acid

ACC deaminase – 1-aminocyclopropane-1-carboxylate deaminase

C. vulgaris – *Chlorella vulgaris*

INIAV – Instituto Nacional de Investigação Agrária e Veterinária

NPK – Nitrogen, phosphorus and potassium fertiliser

P. vulgaris – *Phaseolus vulgaris*

PGPB – Plant growth-promoting bacteria

SDGs – Sustainable Development Goals

1. INTRODUCTION

1.1 HISTORICAL CONTEXT

Human development has always been linked with technological advances, from the Stone Age, where the *Homo* genus expanded through the world, to the Industrial Revolution where production was faster than ever before. The latter allowed humans to surpass theirs and animals' physical limitations, and with the power of coal and oil, it revolutionised transportation and energy use [1].

These Innovations through the 19th and 20th centuries lead to a drastic increase in the amount of carbon in the atmosphere. Nowadays, in the information age, modern lifestyle remains profoundly linked to technologies that are almost synonymous with carbon emissions [1], [2].

In 2023, over 36 billion metric tonnes of carbon dioxide were emitted just by fossil fuels, and now with over 420 ppm of carbon dioxide in the atmosphere, 52% over the levels observed in 1750 prior to the Industrial Revolution, and the likelihood of the global average temperature rising over 1.5ºC in the 2030s decade, the way we see the world needs to change [3], [4].

But this pollution doesn't come only in the form of carbon emissions. The most successful specimens of the *Homo* genus, *Homo sapiens*, have increased their presence substantially since the end of the Second World War. From under 3 thousand million in the late 1940s to over 8 thousand million achieved at the end of 2022 and advancing faster than projected. Though this growth came from the increase in quality of life, improvements in public health, access to food, hygiene, and medicine, as the population grows, how can the fertile lands produce food for all [5], [6]?

Plants need a bundle of nutrients to grow, with the three most important being nitrogen, phosphorus, and potassium, and as the crop grows, the amount available in the soil decreases, and a way to replenish it with these nutrients is the use of fertilisers, with chemical fertilisers being the most popular ones [7].

The application of fertilisers has become a crucial practice in today's agriculture to feed this increasing population. And even though chemical fertilisers helped overcome hunger and death by malnutrition, the application of chemical fertilisers also has negative effects on the soil, such as reducing the capacity to retain water, an imbalance of nutrients and water pollution due to leaching, and an accumulation of heavy metals that can be passed through the food chain [7], [8], [9].

The use of nitrogen fertilisers can lead to the acidification of soils and eutrophication with the leaching of nitrogen to nearby aquatic habitats, increasing the algae population and decreasing dissolved oxygen. The use of phosphorus fertilisers also affects the soil pH,

but to a minor extent. These acidic soils reduce the cation exchange capacity of the soil [7].

These impacts on soil health and surrounding ecosystems; combined with the increase in atmospheric carbon, highlight a broader issue: the current food production and natural resource management are not sustainable. As environmental pressure intensifies, with increasing numbers of heat waves, extinction and endangerment of many species and the thawing of the permafrost and Antarctic glaciers, it becomes ever more necessary to change, by rethinking and redesigning our practices to ensure long-term resilience. This need aligns closely with global efforts to create a more sustainable future, as reflected by the Sustainable Development Goals (SDGs) [10], [11], [12], [13].

The SDGs are ambitious objectives that have the mission to turn the world into a fair, peaceful, prosperous and sustainable one. From the 17 SDGs and 169 targets, the fight to reduce and capture these emissions and stop their effects involves some like [14]:

- Goal 12 - Responsible consumption and production.
- Goal 13 - Climate Action.
- Goal 14 - Life below water.
- Goal 15 - Life on Land.

One way we might change the path we are leading towards and achieve those goals is by using one of the first forms of living organisms that existed, microalgae.

1.2 MICROALGAE

Microalgae includes both eukaryotic and prokaryotic organisms, such as green microalgae and cyanobacteria, that have been widely studied since the late 2010s. Cyanobacteria have been present on earth since the Archean aeon, around 3 billion years ago, making them among the earliest forms of life. Eukaryotic microalgae appeared much later, during the Proterozoic aeon [15].

One of the first related uses for this organism comes from the Mesoamerican cultures, where populations like the Aztecs had some types of microalgae as an important food source, with current species like *Chlorella vulgaris* and *Arthrospira plantensis* being used as food supplements, but their importance to biotechnology scales further [15], [16].

Microalgae possess highly efficient and versatile metabolic pathways and can grow under different trophic modes. Many species are strictly autotrophic, relying exclusively on light and CO_2 through oxygenic photosynthesis. Others have exclusively heterotrophic growth, where there's absorption of nutrients especially those of organic carbon sources. Finally, there are mixotrophic organisms. These can combine photosynthesis with the uptake of organic carbon, these species can switch from autotrophic to heterotrophic growth when environmental conditions, such as light limitation or an increase in the availability of organic substrates, favour that grow regime. The autotrophic metabolism enables them to fix atmospheric carbon at rates significantly higher than most terrestrial

plants, at the same time, their ability to growth under heterotrophic conditions allows them to use metabolic pathways that support the production of compounds like lipids, pigments, proteins and carbohydrates. As their biochemical composition can be manipulated by changing the growth conditions, microalgae offer a broad range of applications, such as food and feed, biofuels, bioplastics, wastewater treatment and biofertilisers [15], [16].

Those last two applications can be acted upon simultaneously, using wastewater such as that coming from food scraps, which provides an excellent source of nutrients from both organic and inorganic matter that can be readily assimilated by microorganisms. When microalgae are cultivated in these conditions, they not only contribute to the bioremediation of said wastewater but also generate a high-value biomass [15].

This biomass contains key nutrient-rich molecules, such as phospholipids and nucleic acids, as major phosphorus-containing compounds, and proteins, amino acids, chlorophylls and nucleotides as major nitrogen-containing molecules. These molecules make microalgal biomass a promising biofertiliser capable of supporting crop growth. Additionally, some species produce bioactive compounds with antimicrobial properties, enabling them to act as natural biopesticides by inhibiting pathogen growth [17].

Table 1 provides a comparative overview of the main characteristics of traditional fertilisers and biofertiliser. By contrasting their composition, mode of action, environmental impact and potential benefits for plant growth.

Table 1 - Comparison between traditional fertilisers and biofertilisers' characteristics [17].

Characteristics	Traditional Fertilizers	Biofertilizers		
		Bacteria	Fungi	Microalgae/Cyanobacteria
Environmental damage by degrading the soil, water contamination, and eutrophication induction.	✔	x	x	x
Creation of symbiotic bonds with the plant roots and microorganisms within the soil.	x	✔	✔	✔
Role in the nitrogen cycle making it available to the plant.	x	✔	✔	✔
Promotion of the solubilization of phosphorus.	x	✔	✔	✔
Soil fertility improvement.	x	✔	✔	✔
The slow rate of nutrient release for the consumption of the plant	x	✔	✔	✔
N fixation by individual strains, P solubilization, and hormone production for promoting the growth of the plant.	x	x	x	✔
CO_2 capture and greenhouse emissions reduction capability during the addition of organic carbon to the soil.	x	x	x	✔
Industrial production and widespread used in the agriculture field.	✔	✔	✔	x

Despite the advantages associated with the use of biofertilisers, major challenges remain, particularly the bioavailability of nitrogen and phosphorus once the biomass is applied to the soil. The rate at which these nutrients become available to plants depends on the microbiota of the soil, environmental conditions and soil chemistry, which would promote the decomposition of the biomass, limiting the immediate effectiveness. Even

so, their potential to close nutrient cycles, reduce pollution, and enhance agricultural sustainability remains significant [17].

1.2.1 THE USE OF MICROALGAE AS BIOFERTILISERS

Worldwide, over 300 billion tonnes of cubic metres of wastewater are produced every year, and microalgae are capable of absorbing inorganic substances and synthesising organic substances, such as taking nitrates and producing proteins, and they can produce molecules that help in the growth of plants, such as amino acids, that can work as bio-stimulants and are beneficial for plants, stimulating fruit and root growth, and phytohormones such as auxins, which act as promoters of vegetal growth and enhance root development, making the plant's ability to absorb water and nutrients much higher. Some microalgae can also act as biopesticides, making the plant less susceptible to fungi and bacteria [15], [18].

The use of microalgae as a fertiliser has already been shown with good results and similar productivity when compared to chemical fertilisers [15].

An experiment by Garcia-Gonzales and Sommerfeld showed that the application of *Acutodesmus dimorphus* in Roma tomato cultivation 22 days prior to seed placement had a greater number of branches and flowers when compared to the control group (without biofertiliser) and to when the biofertiliser was added at the time of plantation [18].

Other experiments converge with the previous, the application of the biofertilisers prior to planting tends to be more efficient due to the time it takes for the organic substance to mineralise into a substance that can be absorbed by roots [15].

Even when a lower yield is obtained, the use of biofertilisers can increase the mineral content, the quantity of carotenoids and sugar levels [15].

Nitrogen is one of the most important elements for plant growth, not only is it present in the various enzymes and DNA that allow plants to have their activity, but it is also present in chlorophyll, which is vital for plants, as it allows the plant to capture light energy and convert it into chemical energy through photosynthesis [15].

Therefore, nitrogen-based fertilisers are usually used for the development of crops. Even though they can be produced from air, utilising the nitrogen present in it, they use a high amount of fossil fuel in the form on gas, making nitrogen fertilisers dependent on gas price, causing environmental impact and causing social and environmental impacts [15].

The other elements that can be found in large amounts in fertilisers, phosphorus and potassium, come from non-renewable sources. Phosphate fertilisers, another important element due to the metabolic use of phosphorus, are produced with phosphorus-rich rocks, normally coming from China, Morocco and the USA. With the deposits depleting and over half of the phosphorus being lost to wastewater, the price will tend to rise as the mineral reserves get lower [15].

Biofertilisers, substances containing living microorganisms which, when applied in agriculture, colonises the rhizosphere or the interior of the plant and enhance the availability or uptake of nutrients, promote growth and improve soil fertility. Being composed of organisms like nitrogen-fixing bacteria, phosphate-solubilising bacteria, mycorrhizal fungi and microalgae, among others, they can be used to face the weakness of traditional fertilisers (Figure 1), not only because microalgae have a similar plant production to NPK 20:0:20 fertilisers, but they can also act as a phosphorus recovery as they can grow and remediate wastewater [15], [19].

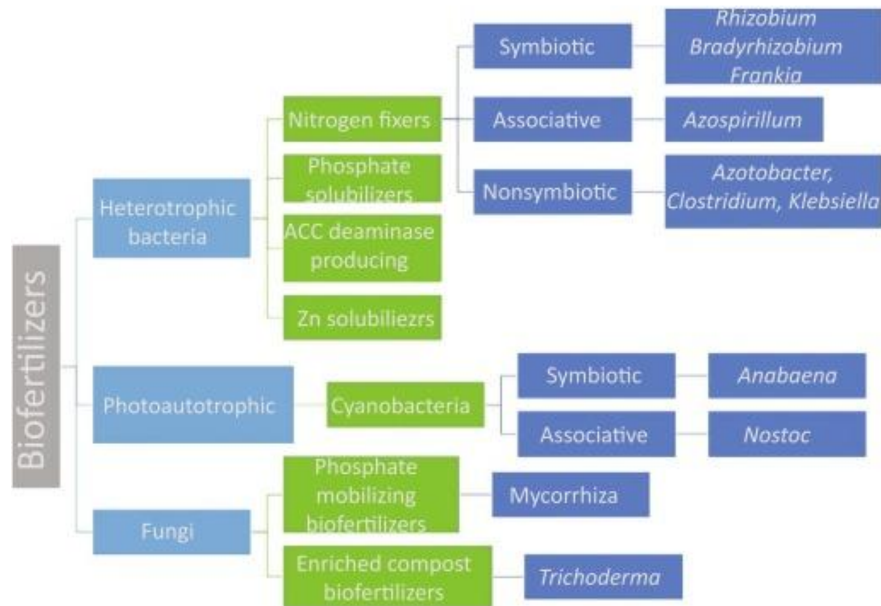

Figure 1 - Biofertiliser characterisation [19].

1.2.2 THE USE OF MICROALGAE IN WATER TREATMENT

Microalgae, being microorganisms that can be grown in a plethora of ways, allows them to be grown in mediums that stimulate the production of specific compounds or to be grown in non-optimal conditions, such as polluted water like wastewater [15], [18].

Wastewater have different compositions based on what industry it comes from but tend to be rich in at least one of the major elements needed for plant and algae growth. The carbon, nitrogen, phosphorus and potassium present there can be used efficiently by mixotrophic algae, creating a rich biomass and treated water. However, this biomass can't always be used as food, as the heavy metals present in the medium can be accumulated [15].

As microalgae can be grown without occupying fertile soils, a combination of using wastewater for growth and infertile soils to install the reactors makes microalgae growth cheaper and sustainable with a circular economy approach, in which resources are used efficiently, waste is minimised, and outputs from one process are reused as inputs for another (Figure 2). This interdependence between wastewater treatment and biomass production promotes resource reuse, reduces, environmental impact and enables the valorisation of flows that would otherwise be lost [15], [20].

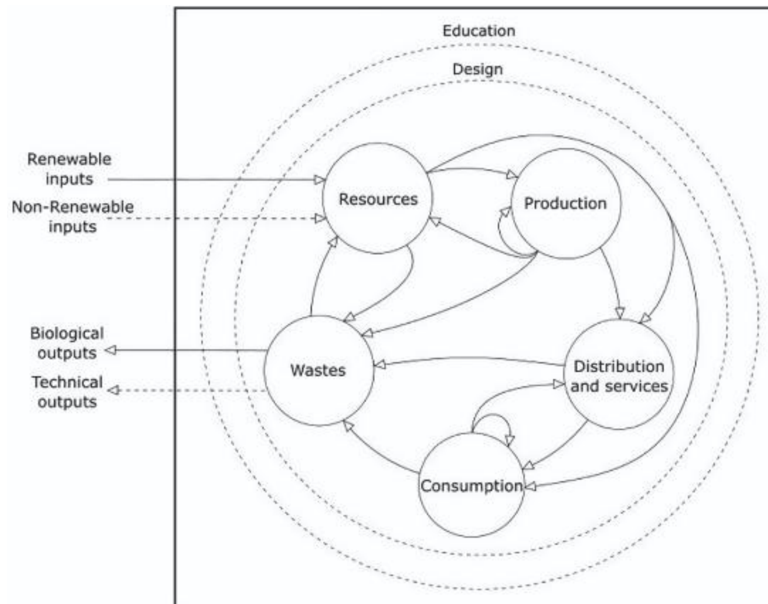

Figure 2 - Circular economy concept [20].

1.3 SYNTHETIC NITROGEN FERTILISERS AND ORGANIC NITROGEN FERTILISERS USE

While the lack of nutrients can limit the soil's maximum yield potential, an excess of nutrients can also pose risks, such as leaching into groundwater and surface water or contributing to the formation of greenhouse gases. These environmental risks highlight the importance of managing nutrient inputs carefully. Reflecting this challenge, the global use of synthetic fertilisers grew from 19 million tonnes in the 1960s to 106 million tonnes in the 2010s [21].

However, despite the increase in nutrients, the efficiency of nitrogen usage decreased by almost 10 points, from 53% in the 1960s to 44% in the 2010s, worldwide, in Europe, the efficiency went from 36% to 46% [21].

In the eurozone, Belgium has the highest nutrient budget, with 290 kg of nitrogen/ha, with 195 kg/ha being from synthetic fertilisers, while Spain is among the countries with the lowest budget, it being between 9 and 60 kg/ha in total. While data from Portugal is specified, it is between 60 kg/ha and 300 kg/ha (Figure 3) [21].

The figure below describes the global soil nutrient budget, which represent the difference between the nutrients removed from the soil, either by food production or due to the decline of soil fertility, and those added either by the natural increase of soil fertility or by the addition of organic or synthetic fertilisers. [21]

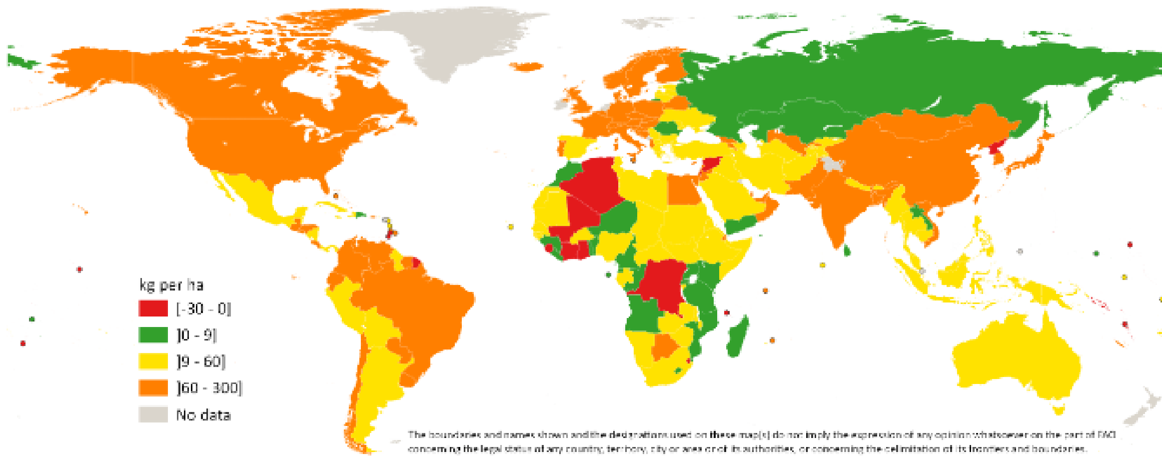

Figure 3 – Global soil nutrient budget, 2018 [21].

1.4 CHLORELLA VULGARIS

First discovered by the Dutch researcher Martinus Willem Beijerinck in 1890, *Chlorella vulgaris* is a unicellular species of microalgae present on earth since the Precambrian, circa 2.5 billion years ago. They have a spherical format, usually 2-10 µm in diameter. This genus can be found around the world in freshwater lakes, marine, brackish, soil and terrestrial habitats, with some species being symbionts for lichens and protozoa [16], [22].

They are asexual organisms and reproduce by self-sporulation, where the mother cell increases in size, an inner cell wall for the daughter cell is formed, followed by two duplications of the chloroplast, and it finalises with the formation and maturation of 4 daughter cells, followed by the rupture of the old cell wall (Figure 4) [23].

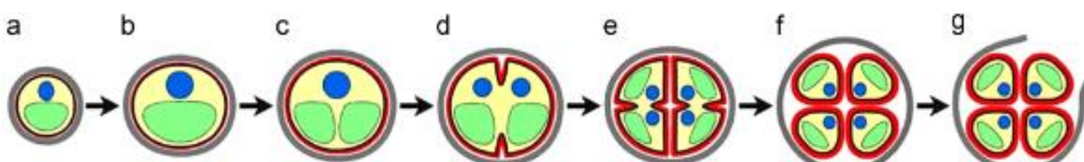

Figure 4 - Chlorella vulgaris life cycle. a) early cell-growth phase; b) late cell-growth phase; c) chloroplast dividing phase; d) early protoplast dividing phase; e) late protoplast dividing phase; f) daughter cell maturation phase; g) cell rupture [16].

1.4.1 MORPHOLOGY

Chlorella vulgaris is a small green spherical unicellular microorganism with a cell wall, the main defensive structure against abiotic and biotic factors, that gradually increases from the cell formation, around 2 nm, until cell maturation, 17-21 nm, with a microfibrillar layer composed of glucosamine. However, cell wall thickness and composition may vary due to environmental factors, such as light, temperature, and nutrient availability, and the growth stage of the cells. These variations can influence the cell's resistance to stress

factors, its ability to interact with other organisms, and the efficiency of processes like nutrient uptake or biomass extraction. Their structure is like plants cells, as they have a defined cell wall, mitochondria and chloroplasts (Figure 5) [16], [23].

They lack flagella that allow their mobility and can be found as individual cells or as a cluster of cells. These clusters can create a better environment for growth and facilitate the precipitation from the growth medium [23].

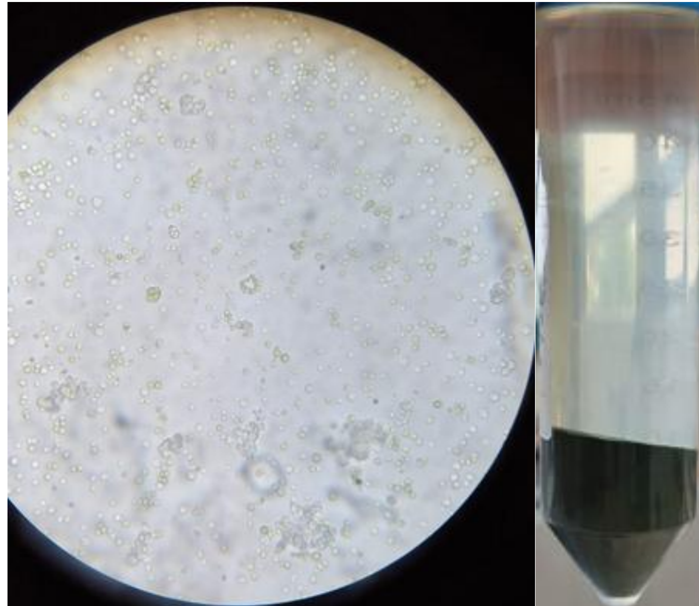

Figure 5 - Chlorella vulgaris 400x amplification (left) and centrifuged wet effluent-grown Chlorella vulgaris biomass (right). (Author's photography).

1.4.2 GROWTH

Chlorella vulgaris is a mixotrophic organism, able to grow both autotrophically and heterotrophically. Autotrophic growth, in which microalgae produce their own organic compounds using light energy and inorganic nutrients, is typically carried out in open pond systems or in closed photobioreactors (Figure 6) [16].

In open pond systems, one of the best ways to produce on a large scale, ponds with a depth of 15-50 cm are used to maximise both the sunlight exposure and atmospheric air absorption, usually helped by rotatory paddles, where the biomass can absorb CO_2 during the photosynthetic period. However, this type of system is more exposed to pollution, contamination by other organisms and water evaporation, and conditions like temperature, CO_2, sunlight exposure are difficult to manipulate. Other problems, such as bad vertical circularity, may decrease the yield [16].

In close photo-bioreactor systems most conditions can be manipulated to desire, allowing a higher concentration of cells or a higher concentration of specific subproducts for specific applications, however, this comes with a higher cost due to the construction, maintenance and utilisation of the reactor [16].

Heterotrophic growth doesn't need light to grow and relies on an organic carbon source, such as glucose, for energy and growth. Microalgae grown in fermenters have a higher yield and a low cost of harvesting, however, the cost and availability of the carbon source have a big impact on the cost of production [16].

Mixotrophic growth involves both the production of energy by photosynthesis during the day and by consuming organic carbon. This way cell growth is not limited by the photoperiod nor the carbon present in the medium, with the main advantage being reducing the impact of biomass loss during dark respiration and reducing the amount of organic carbon needed for growth [16].

Figure 6 - Flat-panel Photobioreactor. (Author's Photography)

1.4.3 MACRONUTRIENTS

1.4.3.1 PROTEIN

Proteins are key molecules for cell activity and function; they are involved in roles like growth and maintenance. In *C. vulgaris,* proteins represent 42-58% of the cell content, with 20% being linked with cell wall function, 50% with internal activity and 30% migrating between the interior and the exterior of the cell. The amino acid profile of these proteins is broad (Table 2), with high concentrations of some essential and non-essential amino acids, for humans, being rich in non-essentials like glutamic acid, aspartic acid and alanine, while having a concentration of essential amino acids, such as leucine and lysine, per 100 grams of protein that stands around the recommended consumption values [16].

Amino acids	*C. vulgaris* [b]	C. vulgaris [c]	C. vulgaris [d]
Aspartic acid	9.3	10.94	9.8
Threonine	5.3	6.09	5.15
Serine	5.8	7.77	4.32
Glutamic acid	13.7	9.08	12.66
Glycine	6.3	8.6	6.07
Alanine	9.4	10.9	8.33
Cysteine	n.d	0.19	1.28
Valine	7	3.09	6.61
Methionine	1.3	0.65	1.24
Isoleucine	3.2	0.09	4.44
Leucine	9.5	7.49	9.38
Tyrosine	2.8	8.44	3.14
Phenylalanine	5.5	5.81	5.51
Histidine	2	1.25	1.97
Lysine	6.4	6.83	6.68
Arginine	6.9	7.38	6.22
Tryptophan	n.d	2.21	2.3
Ornithine	n.d	0.13	n.d
Proline	5	2.97	4.9

Table 2 - Amino acid profile per 100 g of protein [16].

1.4.3.2 LIPIDS

Lipids are molecules insoluble in polar solvents like water and perform as a structural support and regulate the permeability of the cell wall. Their quantity in microalgae can vary considerably. The heterotrophic growth favours the formation of lipids in microalgae; therefore, they can go from as low as 5% to as high as 40%, and in unfavourable conditions for growth, they can reach 58%. The main lipid types include glycolipids, phospholipids, and, in smaller amounts, free fatty acids, within palmitic, stearic,

palmitoleic, and oleic acid being highly present in mixotrophic growth, whereas polyunsaturated fatty acids like linoleic acid and eicosapentaenoic acid are more concentrated when conditions are more favourable [16].

1.4.3.3 CARBOHYDRATES

Carbohydrates, composed of carbon, hydrogen and oxygen, serve as energy reserves and structural components in microalgae. Within these, sugars are simple carbohydrate molecules that provide readily available energy and can form part of structural polysaccharides (Table 3). In *Chlorella vulgaris*, carbohydrates represent 12% to 55% of dry weight, with the sugar profile being dominated by rhamnose, followed by galactose and xylose [16].

Table 3 - Simple sugar composition of the cell wall of Chlorella vulgaris [16].

Neutral Sugars	Percentage (%)
Rhamnose	45-54
Arabinose	2-9
Xylose	7-19
Mannose	2-7
Galactose	14-26
Glucose	1-4

1.4.4 MICRONUTRIENTS AND PIGMENTS

1.4.4.1 MINERALS

Minerals are inorganic substances required in small amounts by organisms for proper growth, development, and metabolic function. Essential minerals like calcium, potassium and magnesium play key roles in processes like enzyme activation, osmotic regulation, and structural support. In *Chlorella vulgaris*, concentrations of sodium, potassium, calcium, magnesium, phosphorus and iron can be found, along with trace amounts of minerals such as chromium, copper, zinc, manganese and iodine, contributing to its nutritional and functional value for diverse applications [16].

1.4.4.2 VITAMINS

Vitamins are essential organic compounds that play crucial roles in cell growth, differentiation, and metabolic processes across a wide range of organisms. Some vitamins function as cofactors in enzymatic reactions, while others have antioxidant properties that protect cells from oxidative stress. In *Chlorella vulgaris,* vitamin content (Table 4) is influenced by growth conditions, with a higher level typically observed under

heterotrophic growth, and a broad spectrum of vitamins, including A, B1, B2, B3, B5, B6, B7, B9, B12, C and E, present in the cell [16].

Table 4 - Vitamins present in Chlorella vulgaris [16].

	Content (mg 100 g^{-1})		
Vitamins	Maruyama et al.	Yeh et al.	Panahi et al.
B1 (Thiamine)	2.4	N/A	1.5
B2 (Riboflavin)	6	N/A	4.8
B3 (Niacin)	N/A	N/A	23.8
B5 (Pantothenic acid)	N/A	N/A	1.3
B6 (Pyridoxine)	1	N/A	1.7
B7 (Biotin)	N/A	N/A	191.6
B9 (Folic Acid)	N/A	N/A	26.9
B12 (Cobalamin)	tr	N/A	125.9
C (Ascorbic acid)	100	39	15.6
E (Tocopherol)	20	2787	N/A
A (Retinol)	N/A	13.2	N/A

1.4.4.3 Pigments

Pigments represent over 1% of the contents of *C. vulgaris*, the most common being chlorophyll. Other pigments like β-carotene, astaxanthin, canthaxanthin and pheophytin-a can also be found in *C. vulgaris,* however, their concentration varies based on growth condition [16].

1.4.5 Applications

Chlorella vulgaris is a very versatile microalga, and different growth conditions will optimise the characteristics for different applications. Biomass rich in lipids has a fatty acid profile that is appropriate to produce biofuel. Currently the production of algae-based biofuel isn’t competitive against fossil fuel refinement, the costs from infrastructure, harvesting, drying, transportation and oil extraction make this process have a low rentability, while commonly presenting a negative yield of energy [16], [24].

Another application is for human and animal consumption. Even though there are different safety requirements for the biomass being approved for consumption between animals and humans, contaminations would render the biomass unusable, however their protein content and antioxidant action make *C. vulgaris* a very popular product [16], [25].

In a bioremediation view, these microalgae have a big potential in wastewater treatment, studies show that *C. vulgaris* have a potential to fixate up to 74% of the carbon dioxide, 45-97% of the nitrogen, and 28-96% of phosphorus and reduce chemical oxygen demand by 61-86% from various wastewater types [16], [26].

Microalgae biomass can also be used for biofertilisation, algae extract rich in nitrogenase can be used as a foliar feed to increase the yield. Studies with *Triticum aestivum* showed a 140% increase in yield and a 40% increase in weight [16], [27].

1.5 AGRICULTURE

1.5.1 THE AGRICULTURAL REVOLUTION

For millions of years humans lived as hunter-gatherers, living from wild plants and animals until around 10000 years ago some groups of humans were able to domesticate plants and animals, changing the paradigm of human life [28].

The Neolithic, or New Stone Age, is marked by the agricultural revolution, where agriculture and animal domestication started. In the Levant region, human selection transformed some wild grasses into domesticated grains by 8000 BCE. these grains and other plants spread through the region, and by 6000 BCE, agriculture was present in Greece and around 4000 BCE in the central European regions along the Danube River. In different regions different techniques were used, like crop rotation in the Middle East, with lentils and grains, while in the Danube Valley fallowing was used, allowing the soil to maintain its fertility [28].

In other regions of the world, like the Tehuacan Valley in modern Mexico, with the decrease in game animals, the local populations became more dependent on wild plants around 8000 BCE, which led to a prominence of maize plantation by 3000 BCE. Around the same time in the Andean region, plants like potatoes and tomatoes became the staple before 1500 BCE before arriving in the nearby Caribbean islands. These advances allowed for the rise of civilisation along the fertile river valleys such as the Tigris, Euphrates, Indus and Nile [28].

1.5.2 THE SECOND AGRICULTURAL REVOLUTION

The second agricultural revolution, happening between the 18th and 19th centuries in Great Britain, improved crop yield and livestock production through selective breeding, better soil drainage and treatment, better tools and the use of livestock manure, leading to the development of the Norfolk system and ley-farming systems [29], [30].

Finally, the introduction of new species coming from the American and Asian continents and advances in fertilisation, with the use of guano and coprolite mixes with sulphuric acid, creating monocalcium phosphate, which enhanced soil nitrogen and phosphorus content, played a vital part in maintaining the soil's fertility [29], [30], [31].

1.5.3 THE THIRD AGRICULTURAL REVOLUTION

In the early 20th century a third agricultural revolution, also called the green revolution, brought high-yield varieties of cereal, widespread use of chemical fertilisers, controlled irrigation, pesticides, and mechanisation of labour, shaping the agriculture we know today [32], [33].

1.6 Maize

Maize are monoecious plants, during their development, they form separated male and female flowers. The tassel found on the top of the plant produces pollen that is shed from the tassels discontinuously in a period of 5 to 8 days. The pollen is transported by wind to the female flower, the ear, where the silks, attached to an ovary each, gather the pollen that would later develop into a maize grain [34], [35].

1.6.1 The origin and history of *Zea mays*

The earliest archaeological evidence is dated to 5000 BCE from Tehuacan caves in Puebla, modern Mexico, while in South America maize seems to be introduced before 3000 BCE in the Valdivia Site in modern Ecuador [36].

In what is the modern midwestern United states of America, maize was one of the first crops to be introduced, likely beginning in 300 BCE. However, the introduction of maize does not seem to have had a big impact initially, the archaeological record shows that the local population stayed as hunter-gatherers long after the introduction of maize. Only when irrigation systems were developed did the local cultures and economy change alongside the species of maize [36].

Nonetheless, maize was a staple of North American gastronomy and culture, and when the Europeans reached the Americas, a vast array of races, some of those still present to this day, had been cultivated, with different types used for different purposes. These left the Americas by the hands of the Portuguese and Spanish, and were gradually incorporated into local agriculture and diet [36].

1.6.2 Maize growth stages

Maize development is separated into two main stages: the vegetative stage and the reproductive stage, with the vegetative stage being composed of the emergence of the plant, root formation and leaf development, allowing the plant to capture light, water and nutrients [34].

1.6.2.1 Vegetative Stage

In the vegetative stage, starting with germination and emergence (VE, in Figure 7), the seed develops the coleoptile, a protective sheath surrounding the shoot, the mesocotyl, which connects the coleoptile to the remaining seed, the coleorhiza, a protective sheath surrounding the radicle, and the radicle, the primary root. When the coleoptile pushes out of the soil, the seedling is in the emergence stage. The seedling will continue to grow, and when the first true leaf emerges out of the coleoptile, usually with a rounded tip, forming the plant, it enters the one-leaf collar stage or V1 [34], [35].

This stage continues changing after the formation of every leaf collar. At V3, the growing point can be found below the soil surface, and nodal roots start to form. At V6, when the

growing point reaches the soil surface, the flowers start to form, and the nodal roots that characterise the previous stages are now dominant. At V9, the plant has increased their growth speed, with the following stages determining the characteristics of the flowers. At the VT (Figure 7), tasselling stage, the last branches of the tassel become visible. At the end of the vegetative stage occurs the transition or tasselling stage [34], [35].

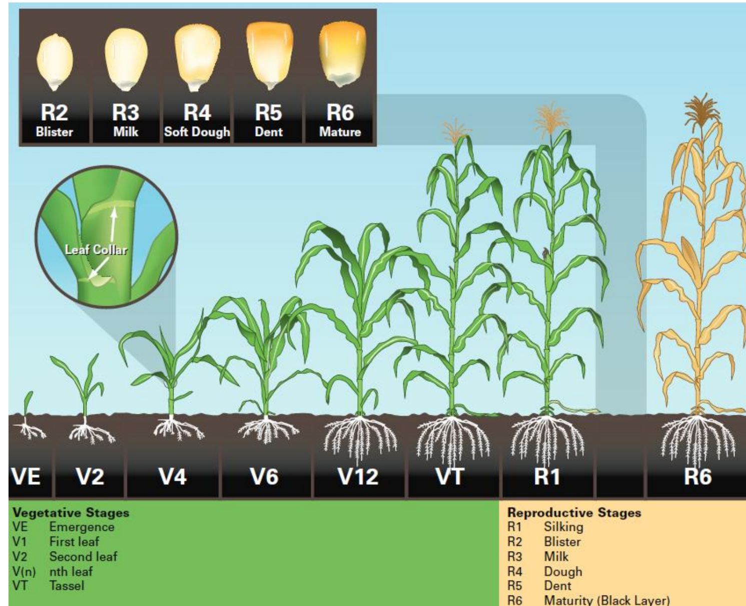

Figure 7 – Usual maize growth stages [35].

1.6.2.2 Reproductive Stage

In the reproductive stage (Rn, in Figure 7), starting at R1, the silks extend outside the leaves, and the tassel now releases pollen. The reproductive stage goes until R6, circa 2 months after R1, and is where the kernels develop, they start as a white colour present in R2 and reach the orange-yellow colour in R3 and R4. In the final stages of the reproductive stage, the kernel dries, reaching its final appearance [34], [35].

1.7 Bean

Phaseolus vulgaris is a grain legume species from the Fabaceae family that includes many types of beans, kidney beans, pinto beans, black beans and cranberry/borlotti beans, with borlotti beans being used in this study, that are cultivated in many countries. It is considered an important crop, as it can improve food security for populations vulnerable to malnutrition [36].

1.7.1 The origin and history of *Phaseolus vulgaris*

Beans originated in the American continent, ranging from the region of Chihuahua in modern-day Mexico to the region of Cordoba in modern Argentina, with morphologic variation between these regions, likely originating in the northern and central Andes [36].

The earliest seed dates to 5500 BCE, found in the inter-Andean valley, modern Northern Peru, before the introduction of maize and pottery in the region. Meanwhile, the early registers in Mesoamerica are from the Tehuacan caves in Puebla, Mexico, between 5000 BCE and 3500 BCE, making it likely that domestication was achieved independently in both regions [36].

However, unlike maize, beans seem to have spread slower, reaching the Atlantic region of the United States of America around the X century. Regardless, by the XV century beans had already become a part of the local diet, and “The three sisters”, squash, beans and maize, were a staple of the indigenous agriculture cultural. Beans rapidly spread to the rest of the world as the Europeans reached the Americas, with this type of “New World bean” being often considered better than those present in the “Old World” [36].

1.7.2 BEAN GROWTH STAGES

Bean growth can be divided into two main stages: the vegetative phase and the reproductive stage.

1.7.2.1 VEGETATIVE PHASE

The vegetative phase (Figure 8) starts from germination of the seed and goes until the first floral buds develop. Germination and emergence are characterised by the formation of the root and the emergence of the hypocotyl from the soil. The next stage, VC, is characterised by the unfolding of two cotyledons and the first true leaves on each node. The V1 stage marks the formation of the 1st trifoliolate leaf, until the end of the vegetative phase, In, the formation of the nth trifoliolate leaf at the n+2 node, after V4 there is an increase in growth speed [37].

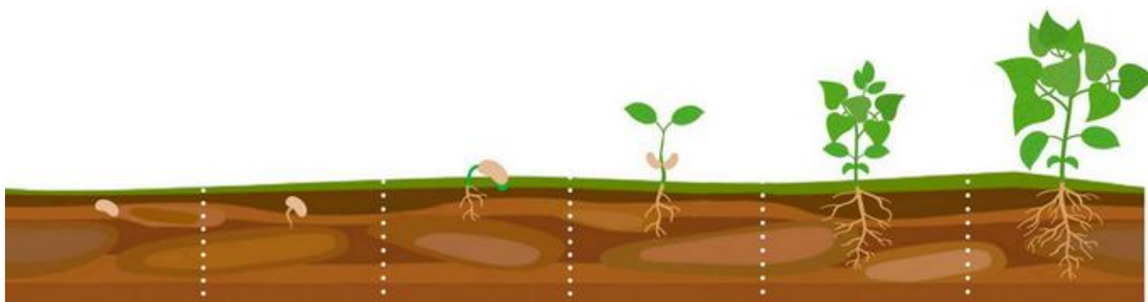

Figure 8 - Common bean vegetative phase [38].

1.7.2.2 Reproductive phase

In the reproductive phase the plant will flower and form the pod that, later, will be filled with the beans (Figure 9) [37].

The reproductive phase starts at R1, with the opening of the first flower of the plant, R2 occurs when 50% of the flowers are open, R3 is characterised by having one pod at its maximum length and R4 is when 50% of the pods reach maximum length [37].

On R5 and R6 there is the full development of the seed of one pod and of 50% of the pods, respectively. On R7 there is the visualisation of the first matured pod. The last stage is RH, where 80% of pods reach their mature colour and are ready to harvest [37].

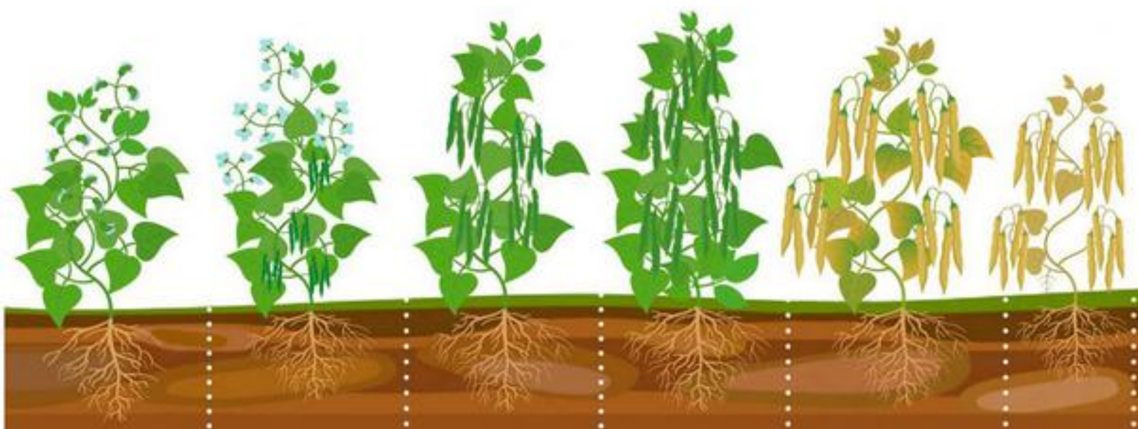

Figure 9 - Common bean reproductive stage [38].

1.8 Lentils

Lentil, *Vicia lens*, is a dicotyledonous plant from the Fabaceae family that has long ascending branches that can reach up to 45 cm. The leaves grow in alternate sites and are of an oblong-linear format and mucronate, the flowers have a pale blue-purple colour and are borne in the axil of the leaves. The seed has the shape of a convex lens, varies in uneven edges and is close to 0.5 cm long, it varies in colour, from red-orange, yellow, green, black or brown [39], [40], [41].

Lentils are common in the Indian and Middle Eastern diet, they have a high protein content, approx. 25%, and are a good source of calcium, phosphorous, iron and Vitamin B. After harvesting, the dried remaining can be used as animal feed [40], [41].

Lentils are a self-pollinating, diploid, annual grain legume adapted for warm, subtropical and high-altitude tropical regions, and nowadays planted worldwide. Lentil can be planted in bulk, with up to 100 plants per square metre, and can develop lateral branches due to poor emergence or thin stands and can grow in many types of soil and pH levels [42].

1.8.1 THE ORIGIN AND HISTORY OF *VICIA LENS*

Lentil plantation come from the start of the creation of agriculture, remains of lentils from 11000 BCE were found in Greece, smaller seeds then those commonly used nowadays, 2-3 mm, dated to around 8000 BCE were found in Syria, and a large storage of seeds found in an archaeological site, dated to 6800 BCE, show that by that time lentils have been part of the farmed plants. However, their origin does not only revolve around the Mediterranean and near east [40].

Lentils are native to the Near East, the Anatolian Peninsula, the Levantine region, and Central Asia, with domestication likely happening with the selection of plants that retain their seeds inside the pods before harvesting and are selected for larger seed sizes. Archaeological studies suggest that domestication started in today’s Turkey, Syria and Iraq, being flat on one side and convex on the other, and then expanding towards both the east, to South Asia, where its shape became convex on both sides, and the west, to Greece and Egypt [36], [40], [41].

1.8.2 LENTIL GROWTH STAGES

Lentil growth also can be divided into two stages, vegetative and reproductive, however, the start of the reproductive stage doesn’t stop the vegetative growth, with flowering being determined by temperature and photoperiod (Figure 10) [43].

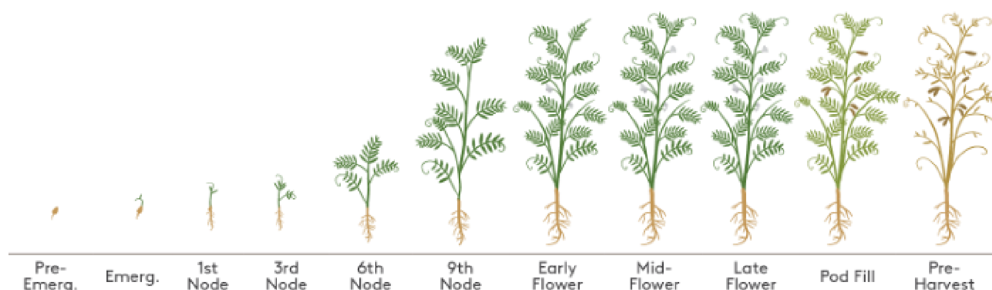

Figure 10 - Lentil growth stage [44].

1.8.2.1 VEGETATIVE STAGE

Unlike other leguminous plants, such as the common bean, lentils tend to branch instead of developing only along the main stem. This makes its vegetative stage being determined by the number of nodes [43].

The cotyledonary node formed during the germination is considered node 0, with its first pair of leaves being simple, followed by bifoliate leaves and finally multifoliate leaves. Based on what is the thickest branch, the number of nodes is counted, with this number being the representation of which growth stage the plant is in, following the logic of Vn, with n being the number of nodes [43].

1.8.2.2 Reproductive stage

As stated before, flowering is dependent on temperature and the photoperiod. The formation of these flowers followed by the development of the pods happens following the node number, with lower nodes developing flowers while upper nodes are being formed [43].

The reproductive stage starts with the blooming of the first flower, R1, followed by R2 with the development of the flowers in branches 10 to 13. R3 to R6 is related to the formation of the first pod to the development of the seeds inside the pods and finalises in R7 and R8 with the yellowing of flowers and browning of the pods, respectively [43].

1.8.3 N fixation in Lentils

The fixation of nitrogen in lentils is realised by a symbiotic relationship with *Rhizobium leguminosarum,* where this bacterium is enclosed inside root nodules. This nodule exists to enable the function of nitrogenase enzymes, which are sensitive to oxygen, and the rhizobia, an obligate aerobe bacterium, in a regulated environment, where oxygen is limited to a nanomolar concentration and oxygen diffusion is facilitated due to the intercellular spaces and leghemoglobin, a haemoprotein found in leguminous plants [45].

In this symbiotic relationship the plant provides a carbon source for the bacteria, while the bacteria consortium gives nitrogen in the form of ammonia, this ammonia is transformed into amides that are transported by the xylem [45].

For this relationship to happen, an infection by the bacteria is needed, however, this process can fail due to a low amount of rhizobia in the soil, inability to survive in the soil, inappropriate strains in the soil, competition by less efficient rhizobia, or failure to create the symbiosis due to lack of time, dependence on the growth stage, or an altered metabolism. Soils with high availability of nitrogen, limitations of boron, molybdenum, cobalt, iron and phosphorous and either a drought or waterlogged environment can also lead to a lower N fixation [45].

1.9 Nutrient needs

Plants can grow on soil rich or poor in nutrients, with yields varying based on the amount of nutrients present in the soil, and the presence of these nutrients in different stages also affecting the development of the plant. To ensure optimal plant performance, many of these nutrients, particularly nitrogen, phosphorus and potassium, are commonly supplied through chemical fertilisers, which supplement what may be deficient in the soil and support consistent crop production [46].

1.9.1 Nitrogen

Nitrogen can inhibit the production of the nodules, as previously stated, however, a higher nitrogen concentration when planting leads to a higher leaf area and a higher shoot and root amount [46].

1.9.2 Phosphorous

Phosphorus has a major role in plant development, with it being involved in storing and transferring energy, root growth, flowering, fruiting and seed formation stimulation and nodule development [46].

1.9.3 Potassium

Potassium doesn't seem to have a significant effect on seed yield, but the supplementation of potassium in soil can increase potassium content in the plant and its quality [46].

1.10 Soil

Soil is an unconsolidated mineral and organic material and is an essential resource for living organisms. It is formed from weathered rocks and minerals, mineral soils. However, if plants are submerged in water, their decay becomes slower, the accumulation of this organic matter creates the organic soils consisting of the peatland, muck land and fens [47].

1.10.1 Physical characteristics of soil

Soil is composed of stones, gravel, sands, silts and clays, varying from over 2 mm to under 0.002 mm in diameter, with sand-sized particles, 2 mm to 0.05 mm, that are visible and when rubbed between the fingers have a gritty feeling, silt-sized particles, 0.05 mm to 0.002 mm, are usually only visible with a microscope and have a smoother feeling, and finally, particles smaller than 0.002 mm are present, being majorly clays, that when wet form a sticky mass. The proportion of these particles can be used to classify the texture of the soil [47].

1.10.2 Soil microbiome

The soil microbiome is one of the most complex and diverse, with more than 50 000 species present in 1 gram of soil, however, less than 1% has been studied. The microbiota, consisting of bacteria, archaea, protists, fungi and viruses, is complex and

diverse and can present over 1 billion bacteria and archaea cells, however, most of this remains inactive for different periods of time [47], [48].

1.10.3 Soil microbiome-plant interactions

In traditional agriculture, different from hydroponic systems, the plant roots interact with the soil, not only can they absorb nutrients present in it, but they can also work with the organisms that surround the roots. This region, the rhizosphere, is affected by the activity of the plant root, and the interactions between the plant and the soil microbiota are essential for the functioning of the ecosystem [49].

1.10.3.1 Phytohormones

Phytohormones are hormones produced by plants, non-exclusively, that have multiple functions. These hormones are divided into abscisic acid (ABA), involved in plant development, abiotic stress response and regulating root growth and water conductivity, cytokinin, involved with cell division, chloroplast differentiation and leaf expansion and nutrient mobilisation, auxins, which promote cell differentiation, induce ethylene production and disease resistance, gibberellin, which stimulates shoot growth and flowering, stem growth and induces seed germination, and ethylene, involved in fruit ripening, breaking seed dormancy and regulating leaf growth. Certain microorganisms, including microalgae, can produce these hormones. For instance, *Chlorella vulgaris* as potential for auxin production, reaching concentrations of approximately 1.56 µg/mL [50], [51].

1.10.3.2 Growth-promoting bacteria

Plant growth-promoting bacteria, PGPB, also known as rhizobacteria, are a group of beneficial bacteria that colonises the root of the plant. They are described as free-living bacteria that can act as symbiotic bacteria by inhabiting the roots, however, unlike endophytic bacteria, they can also act as PGPBs. PGPB are sensitive to changes in the soil conditions, such as temperature, pH, moisture and the presence of competing microorganisms, which can affect their function. These bacteria assist plant growth through multiple mechanisms, including nutrient uptake, nitrogen fixation, phosphorus solubilisation, phytohormone synthesis, increasing root volume, and enhancing stress resistance by producing metabolites and antioxidants [49], [52].

One key enzyme produced by some PGPBs is ACC deaminase, which regulates ethylene levels in plants. By breaking down 1-aminocyclopropane-1-carboxylic acid (ACC), the immediate precursor of ethylene, ACC deaminase reduces excessive ethylene production that would otherwise inhibit root elongation. This activity promotes root development, improves tolerance to biotic and abiotic stress such as salinity or drought, and facilitates better nutrient absorption, ultimately supporting healthier and more vigorous plant growth [49], [52].

2. METHODS

2.1 SOIL SAMPLING

The soil was obtained using the zigzag method to ensure that a representative sample of the soil was taken from a small residential garden in Alcochete (38.7487°N; -8.96811°W), at a depth of 15 cm to 25 cm. 15 samples were taken from 2 regions, one which supports a pomegranate tree and some decorative flowers, while the other supports a madrone and an orange tree, while occasionally supporting some crops in small quantities (Figure 11). The individual samples from each region were homogenised to form a single representative soil sample for analysis.

Figure 11 - Sampling location. (Author's Photography)

2.2 SOIL MOISTURE AND PARTICLE SIZING

It was weight 230 g of saturated soil and dried over the weekend at 70ºC in an oven.

The dried soil and sieves (Figure 12) were individually weighed; the soil was then sieved until each sieve reached a consistent weight.

Figure 12 - Sieves with mesh pores ranging from 2 mm (top) to 0.075 mm (second to las), and collection tray (Author's Photography).

2.3 SOIL ORGANIC MATTER

It was weighed 100 g of soil and added 150 mL of 20V (6%) hydrogen peroxide and left it soaking overnight. Then, the soil and hydrogen peroxide solution were mixed and stirred until it stopped reacting and most of its liquid content was evaporated. This solution was dried in an oven at 105ºC until a consistent weight was reached.

2.4 SOIL ASH CONTENT

In a pre-weighed porcelain crucible, 4 g of soil, dried at 70ºC overnight, were analytically weighted. The crucible then was placed inside a muffle furnace and heated to 550ºC for 3 h. After incineration, the crucible with the ash content was cooled inside a desiccator until achieving room temperature and then weighed.

2.5 SOIL NITROGEN QUANTIFICATION

The nitrogen quantification of the soil sample was done utilising the Kjeldahl method. The Kjeldahl method is a method where the sample is digested with sulphuric acid at high temperatures, followed by a distillation where the ammonium sulphate is converted to ammonia gas utilising sodium hydroxide. Finally, the ammonia collected from the distillation is titrated with an acid solution. The method used was the following:

To Kjeldahl digestion tubes was added 1 g of dried soil, 2 VCM catalyst tablets (3.5 g K_2SO_4 + 0.1 g $CuSo_4.5H_2O$), 12 mL of concentrated sulphuric acid and ebullition regulation spheres. Tube contents were digested in a Velp DK 8 Digestion unit (in Figure

13), for 1 hour at 420ºC (in later analysis changed to 400ºC as literature suggests that above 410ºC there might happen some losses) on an automated digestion heating system. After the digestion the tubes were air cooled to, at least, 60ºC before proceeding with the distillation.

Figure 13 - Velp DK 8 Digestion unit. (Author's Photography)

The distillation was done on an automatic distillation unit (Velp Scientifica, UDK 139 Semi-Automatic Distillation Unit) with 50 mL of H_2O and 50 mL of NaOH 30% (w/w) on full steam power. The distillate was then collected in a flask with 30 mL of H_3BO_3, methyl red and bromocresol green.

2.6 SOIL MICROBIOTA

Due to the interest in verifying the presence of root nodule-forming bacteria, the presence of Rhizobia bacteria was analysed. For this, YEM-agar medium (Table 5) and Congo red YEM-agar medium (Table 6) were used. Inoculation was done using consecutive dilutions followed by selection using a smear loop to transfer to a new Petri dish with medium.

Table 5 - YEM-Agar medium composition [53].

Reagent	Concentration
Yeast Extract	1 g/L
Mannitol ($C_6H_{14}O_6$)	10 g/L
Dipotassium Phosphate (K_2HPO_4)	0.5 g/L
Magnesium sulphate ($MgSO_4$)	0.2 g/L
Sodium Chloride (NaCl)	0.1 g/L
Calcium Carbonate ($CaCO_3$)	1 g/L
Agar	15 g/L

Table 6 - YEM-Agar Congo red medium composition [54].

Reagent	Concentration
Yeast Extract	1 g/L
Mannitol ($C_6H_{14}O_6$)	10 g/L
Dipotassium Phosphate (K_2HPO_4)	0.5 g/L
Magnesium sulphate ($MgSO_4$)	0.2 g/L
Sodium Chloride (NaCl)	0.1 g/L
Congo Red ($C_{32}H_{22}N_6Na_2O_6S_2$)	0.025 g/L
Agar	20 g/L

2.7 *EFFLUENT-GROWN CHLORELLA VULGARIS* BIOMASS

Chlorella vulgaris was grown using a 2.65 L vertical flat panel photo-bioreactor (later changed to a 4 L column reactor) using 50% (v/v) MCV medium (Table 7), 10% (v/v) winery effluent (Table 10), 10% (v/v) pre-inoculum and 30% (v/v) water. Both winery effluent and growth conditions were previously studied in the institution.

Table 7 - MCV medium.

Reagent	Concentration
Potassium nitrate (KNO_3)	1.25 g/L
Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	1 g/L
Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.11 g/L
Monopotassium phosphate (KH_2PO_4)	1.25 g/L
Sodium Bicarbonate ($NaHCO_3$)	0.5 g/L
Chu solution (Table 8)	10 mL/L
Fe-EDTA Solution (Table 9)	10 mL/L

Table 8 - Chu solution.

Reagent	Concentration
Boric acid (H_3BO_3)	286 mg/L
Manganese (II) sulphate monohydrate ($MnSO_4.H_2O$)	154 mg/L
Zinc sulphate heptahydrate ($ZnSO_4.7H_2O$)	22 mg/L
Copper (II) Sulphate ($CuSO_4$)	5 mg/L
Sodium Molybdate dihydrate ($NaMoO_4.2H_2O$)	6 mg/L
Cobalt (II) chloride hexahydrate ($CoCl_2.6H_2O$)	8 mg/L

Table 9 - Fe-EDTA solution.

Reagent	Concentration
Ferric EDTA (Fe-edta)	0.9 g/L

Table 10 - Winery effluent Characterisation.

Effluent Characteristics	
pH	4.39
Conductivity (mS/cm)	2.43
CQO (g O_2/L)	53.08
Total Solids (g/L)	7.738
Organic matter (%)	75.68
Polyphenols	0.222
Sugars (g/L)	5.501

2.8 LYOPHILISATION

Lyophilisation was performed in an Alpha 1-2 LDplus freeze dryer. Biomass was spread in glass dishes and frozen at -20ºC until ready for lyophilisation. Lyophilization happens overnight or until the biomass looked fully dried.

2.9 SEED SELECTION

From store-bought beans, maize and lentils, similar-sized, coloured and shaped seeds were selected and stored. Seeds with indentations, partial breakage or splitting, irregularities on the outer layer or signs of sunburn were discarded.

2.10 Seed sterilisation

The seeds were soaked in a 5% sodium hypochlorite solution for 10 minutes on the same day they were used and then rinsed twice with water. The wet seeds were dried with paper towels before being used. This procedure was performed to surface-sterilise the seeds, eliminating potential microbial contaminants that could interfere with the experiment. The rinsing steps ensured the removal of residual sodium hypochlorite, while drying prevented dilution or alteration of the experimental conditions.

2.11 Soil preparation and conditions test

On Petri dishes were weighed 30 g of sieved soil and 1.414 g of biomass. The soil and biomass were mixed with the addition of 4 ml of tap water to facilitate the mixing.

On each dish, 10 seeds were added on the day of plantation (0, 10 or 20 days after the soil preparation).

Tap water was added when needed until it was wet but not waterlogged.

This test was realised under constant temperature (20ºC) and constant light (1.5 m, 620 lumen/m neutral white 4000K LED strip).

2.12 Seed viability test

A seed viability study was realised, where the seeds were placed in wet cotton at a constant 20ºC in the presence of light.

2.13 Germination test

The germination test was realised to verify if the newly selected conditions supported germination of each type of seed.

On perforated Petri dishes with 20 mL of tap water, a wet round cotton pad and 40 g of soil were added. On each dish, 9 seeds were added.

The Petri dish was covered with a plastic film; this should allow it to maintain a moist atmosphere. Tap water was added when needed until the soil was wet but not waterlogged.

This test was realised under constant temperature (25ºC) and constant light (1.5 m, 620 lumen/m neutral white 4000K LED strip).

The growth of the seedlings was measured with a tailor’s tape.

2.14 WET BIOMASS INFLUENCE TEST

On small gardening pots, 60 g of unamended soil was added over cotton pads to facilitate water absorption through the soil. To test the effect of microalgae on plant growth, 3.2 g of *C. vulgaris* biomass was added.

To prepare the biomass, 80 g of centrifuge biomass was mixed with 30 ml of distilled water to facilitate its handling and application.

To further study the effect of the biomass, the effect of adding biomass 10 and 20 days before planting was studied.

For each treatment, no algae, adding algae the same day as planting, adding algae 10 days before planting and 20 days before planting, 28 bean seeds, divided into 7 pots, and 27 maize and lentil seeds, divided into 3 pots each, were used.

Water was added as needed while avoiding waterlogging.

This test was performed inside a greenhouse-like structure while inside the building, with temperatures and luminosity registered as possible.

2.15 2ND WET BIOMASS INFLUENCE TEST

In small gardening pots, 100 g of soil, either unamended soil or amended soil with wet biomass, was placed over cotton pads to facilitate water absorption. To study the effect of the biomass, it was thoroughly mixed with the soil to obtain a 5% biomass concentration before being distributed into the pots.

To further study the effect of the biomass, its addition was tested either 10 days before planting or on the day of planting. For each parameter, no microalgae, adding microalgae the same day as planting and adding microalgae 10 days before planting, 18 bean seeds were used, divided into 3 pots, and 18 maize and lentil seeds were used, divided into 2 pots each. Water was added as needed while avoiding waterlogging.

This test was performed inside a greenhouse-like structure while inside the building, with temperatures and luminosity registered as possible.

2.16 WET BIOMASS AND FREEZE-DRIED BIOMASS INFLUENCE COMPARISON

In small gardening pots, 60 g of unamended soil, soil amended with wet biomass or soil amended with freeze-dried biomass over cotton pads to facilitate water absorption. On this growth test amended soils were prepared 10 days before sowing to obtain a 5% wet biomass concentration or a 1.4% lyophilised biomass concentration.

For each parameter, unamended soil, soil amended with wet biomass and soil amended with freeze-dried biomass, 16 previously germinated lentil seeds divided in 2 pots, 15 germinated maize kernels divided in 3 pots, and 12 germinated bean seeds divided in 4 pots were used (Figure 14).

The remaining conditions were performed as stated before.

Figure 14 - Growth setup (left) and grown plants (right). (Author's Photography)

2.17 COMPARISON OF THE EFFECTS OF REGULAR BIOMASS AND EFFLUENT-GROWN BIOMASS

On 6 small aluminium trays was added 400 g of unamended soil, soil amended with wet biomass, soil amended with freeze-dried biomass, soil amended with wet effluent-grown biomass, soil amended with freeze-dried effluent-grown biomass and soil amended with chemical nitrogen-based fertilisers. This soil was prepared 10 days before sowing to obtain a 5% wet biomass concentration or a 1.4% lyophilised biomass concentration.

On each tray were added 17 ungerminated sterilised bean seeds, with this test focusing on the growth success.

The remaining conditions were performed as stated before, however, due to the need to grow enough effluent-grown biomass, this growth test was realised in early autumn instead of spring/early summer like the remaining tests.

3. RESULTS ANS DISCUSSION

3.1 SOIL MOISTURE

The soil moisture content was determined to be 11.96% (Table 11). However, this can be easily manipulated to desire by watering. This value may vary a lot due to evaporation and drainage, over time, and precipitation, therefore, it is important to obtain a sample of a deeper layer.

Furthermore, the obtained value may not be representative due to the time between sampling and analysis, as water could have been evaporated while the sample was stored, and precipitation happened during the week of sampling.

Table 11 - Soil moisture results.

Saturated soil	**231.6 g**
Dry soil	203.9 g
Moisture content	11.96%

3.2 SOIL PARTICLE SIZE

The soil particle size distribution provides important information about soil texture and its suitability for agricultural practices. In this study, the proportions of sand, silt and clay were determined and are presented in Table 12.

The smallest sieve used had a pore size of 0.075 mm, corresponding to sand-size particles, which represent 93.53% of the soil particles. Silts and clays, ranging from 0.05 mm to 0.002 mm and below, were gathered in the same tray, representing a total of 6.47%.

Therefore, using the United States Department of Agriculture, Textural classification triangle (Figure 15), this soil would be classified as sand. This result may not be true representative, as conglomerates of clay and silts would be retained on sieves with higher pore diameters.

Compared with data obtained in the Portuguese National Institute for Agrarian and Veterinary Research (Instituto Nacional de Investigação Agrária e veterinária, INIAV), clay concentration ranges between 5% and 14%, and silt concentration ranges between 12% and 18%. While this still makes the sand content high, this would classify the soil as loamy sand or as sandy loam. Soil with a lower sand concentration, a maximum of 52%, clay between 8% and 27%, and a silt between 27% and 50%, typically a loam, would be more suitable for cultivation. However, this soil can still be treated to support better plant growth [55], [56].

The addition of organic matter, like compost, and techniques to increase soil moisture retention would make this soil more viable for plant growth. The analysed conditions show a high hydraulic conductivity, infiltration and drainage, the latter two show that the soil conditions allow more water to enter the soil but quickly drain away. INIAV soil characteristics show a slight decrease in infiltration and drainage ability, however, the retainability of water in the soil is almost 4 times greater, making the soil moister [57].

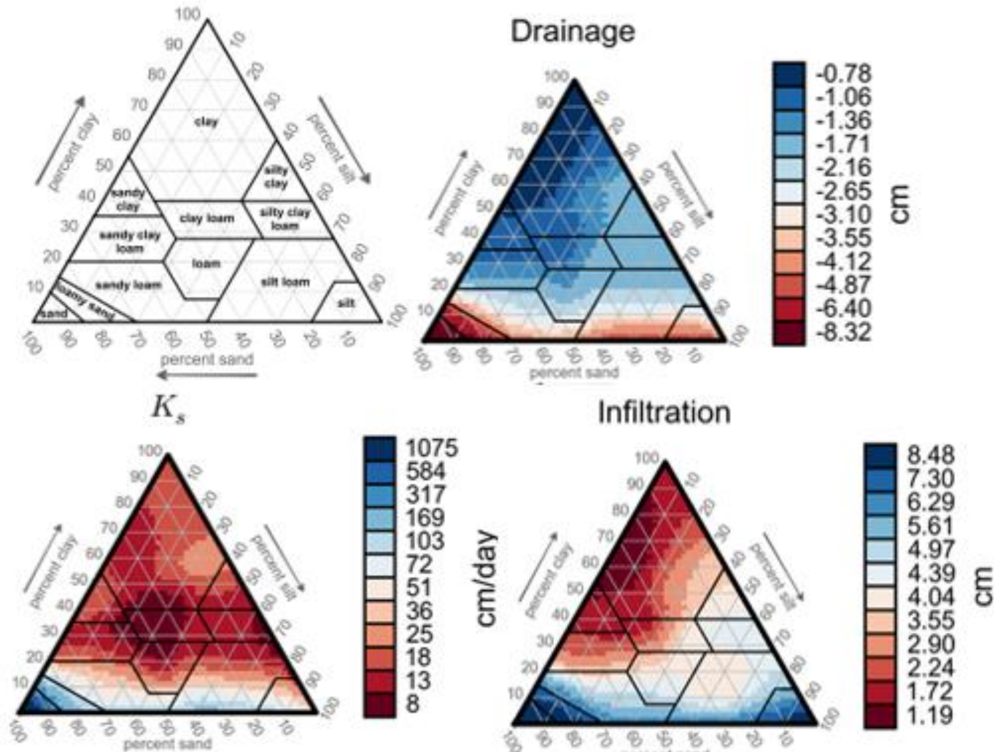

Figure 15 - Soil composition triangle and characteristics change by soil components [57].

Table 12 - Soil particle sizes.

Sieve (mm)	Sieve weight (g)	Sieve + soil (g)	soil (g)	%	Particle Classification
>2	592.7	610.3	17.6	8.62%	Sand
0.85	508.1	539	30.9	15.14%	Sand
0.425	473.9	534.6	60.7	29.74%	Sand
0.25	441.2	491.6	50.4	24.69%	Sand
0.106	428.8	454	25.2	12.35%	Sand
0.075	409.2	415.3	6.1	2.99%	Sand
<0.075	359.2	372.4	13.2	6.47%	Silts and Clays

3.3 Soil organic matter

The determination of soil organic matter is essential for assessing soil fertility, nutrient availability and overall soil health. In the study, the soil was dried and then treated with hydrogen peroxide (H_2O_2) to remove inorganic components and facilitate the measurement of organic matter content (as per Figure 16). The percentage of organic matter was subsequently determined to be 1.49% (w/w), as shown in Table 13.Table 13 - Soil organic matter results

Table 13 - Soil organic matter results

Wet soil	**101.1 g**
Dried soil after H_2O_2 treatment	87.5 g
Organic matter	1.5 g
Organic matter (%)	1.49%

This value is slightly higher than those available for the same region in INIAV, ranging from 0.30% to 1.33%. This higher value may come from the continuous use of the soil for light agricultural practices combined with the incorporation of fallen vegetation [55].

Although this value is relatively low, it is typical of soils under extensive cultivation and indicates that the soil could benefit from the addition of organic amendments, such as the incorporation of biomass, to improve fertility and water retention.

Figure 16 - Soil organic matter reaction with H_2O_2 (left) and soil after reaction with H_2O_2 (right). (Author's Photography)

3.4 Soil ash content

Determining the soil ash content is important for assessing the mineral fraction of the soil, which includes essential nutrients such as calcium, potassium, magnesium and other inorganic compounds. Soil ash content was 97.17% (DW), corresponding to 85.55% of wet soil weight (Table 14), this value corresponds to inorganic matter, sand, clay and silt particles, and inorganic nutrients like phosphorus and potassium. Due to the limitation of the method, it isn't possible to distinguish between soil particles and inorganic nutrients, but most of this value is composed of the soil particles.

Table 14 - Ash content matter.

soil ashes	Crucible (g)	Dry soil weight (g)	Crucible + ash (g)	Ash (g)	DW %
C1	39.5059	4.5448	43.9102	4.4043	96.91%
C2	42.0567	4.0862	46.0068	3.9501	96.67%
C3	31.105	4.3212	35.3367	4.2317	97.93%
				average	97.17%

3.5 Soil nitrogen content

The Kjeldahl method was done 3 times, the third being done after both pieces of equipment's maintenance, and with a reduced temperature, 400ºC instead of 420ºC since the second attempt.

On the first attempt, the analyte showed a purple/pink colour after distillation, while it should have shown a green hue.

On the second attempt, the analyte showed a purple colour half of the time including in control samples of ammonium sulphate (Figure 17), where maximum recuperation was under 50%, ranging from 5% to 40%.

On the third attempt, most of the analytes showed a green hue after distillation, however, one blank sample showed a purple colour after distillation. The recuperation was also under 50% in control samples, ranging from 10% to 20%.

Due to time constraints no more attempts were made after the performance of this method was considered unfit.

Therefore, data publish on the European Commission website will be taken into consideration. That data shows a concentration of under 1.2 g of nitrogen/kg of soil in the area where the sample was collected, it also shows a concentration of under 14 mg of phosphorous and between 140 mg and 185 mg of potassium per kilogram of soil. This study also showed that soil pH is between 7.2 and 8.8, and a C:N ratio is between 10.05 and 11.72 [55], [56], [58].

These values are closely related to those present in INIAV and INFOSOLO, who show lower values for every parameter, however, most of INIAV's values for the region come from samples outside of active agricultural soils [55].

Figure 17 - Analyte after distillation (left) and analyte after titration (Right). (Author's Photography)

3.6 Soil microbiota

The growth of specific soil microorganisms was evaluated using two selective media: YEM-Agar and YEM-Agar Congo Red. YEM-Agar supports the growth of organisms such as *Rhizobium leguminosarum, Rhizobium meliloti* and *Agrobacterium tumefaciens*, while YEM-Agar Congo red favours the growth of *Rhizobium japonicum* and *Rhizobium meliloti* [53], [54].

Figure 18 shows the obtained results, where microbial growth can be observed.

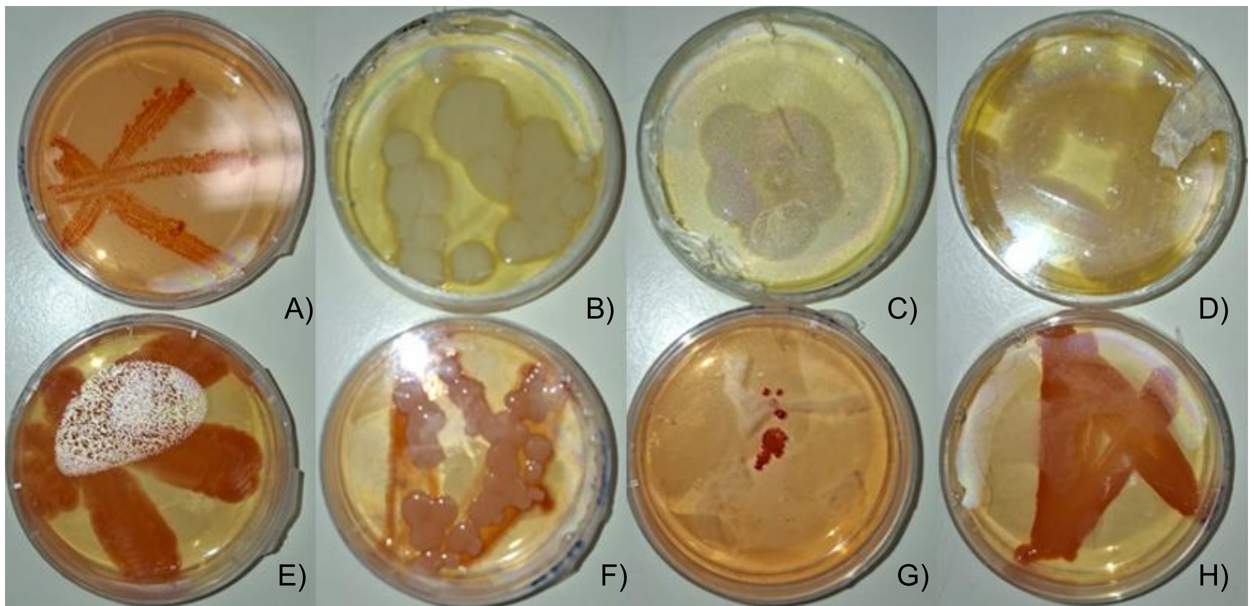

Figure 18 - Isolated colonies grown in YEM-Agar medium (Petri Dish B), C), D) and E)) and YEM-Agar Congo red medium (Petri dish A), F), G) and H)). (Author's photography)

Petri dishes A) and E) were from the same colony however, E) presents a more vigorous growth, being the only one where growth is visually bigger in the YEM-Agar medium. It is most likely *Agrobacterium tumefaciens* or *Rhizobium leguminosarum*. The same is true for dishes C) and G), which are isolated from the same colony between themselves.

Morphologically the first two look opaque and when isolated had a filament-like structure, the second has a creamy, white and soft texture when in YEM-Agar medium, that changes to a hard texture when grown in YEM-Agar Congo red medium.

Petri dishes B) and F) were isolated from the same colony and formed creamy and soft colonies in both mediums, with those grown in YEM-Agar Congo red medium had a pink colour with a red ring around them, while those in YEM-Agar medium had a white and yellow ring delimiting their space. Both have a shiny colour. This would likely be *Rhizobium meliloti* due to their vigorous growth in both mediums.

Lastly, Petri dishes D) and H) showed a similar growth in both mediums, in both, they showed opaque and didn't form a domed colony. This is probably another species of *agrobacterium* or *Rhizobium* that wasn't specified by the medium's technical sheet.

This study demonstrates the presence of root nodule-forming bacteria, however, it fails to specify what type or quantity, for the later consecutive dilutions would allow the counting of colonies reaching a colony-forming unit value that would be representative of the number of bacteria present in soil. However, due to the high number of different microorganisms present in soil, competition could lead to non-representative results.

3.7 *EFFLUENT-GROWN CHLORELLA VULGARIS* BIOMASS

Biomass growth was done under the previously mentioned conditions (2.7 - *Effluent-grown Chlorella vulgaris* Biomass). After three days, fungal contamination became visible, making the conditions unsuitable for algal growth. However, in sequent repetitions, by adjusting the pH of the effluent to around pH 8, this would make the growth conditions for *Chlorella* more viable, allowing the growth of effluent-grown biomass (Figure 20).

The pre-inoculum was grown (Table 15) without effluent, with the optical density being measured by a UV-Vis spectrophotometer before its addition to the reactor, this value after points to a value of 0.6 in dilution factors 1 and 2, while being over 1 in dilution factors of 4 and 1.0.

Table 15 - Inoculum optical density.

Day	Dilution Factor	λ (nm)	sample	O.D.		
				A	B	C
10/9/2025	1	600	pre-inoculum	0.648	0.636	0.635
10/9/2025	2	600	pre-inoculum	0.341	0.352	0.34
10/9/2025	4	600	pre-inoculum	0.218	0.23	0.227
10/9/2025	10	600	pre-inoculum	0.13	0.134	0.153
10/9/2025	1	600	Reactor	0.12	0.119	0.124

The higher the optical density is, the higher the amount of cells present in the reactor at the start, this allows for a faster growth of biomass, as fewer reproduction cycles are necessary to reach the desired amount, while ensuring the same conditions are present at the start.

On the reactor the optical density steadily increased on the four days, Tuesday to Friday, over the weekend the value rapidly decreased, meaning that, between September 24th and September 25th, the microalgae when from the lag phase to the exponential phase, and over the weekend it reached the death phase, maintaining a constant value until potassium nitrate was added. This led to a small increase in optical density, however, this value had a slower increase.

Table 16 and Figure 19 show the evolution of the optical density over time.

Table 16 – Reactor 1 optical density.

Day	Dilution Factor	λ (nm)	O.D.		
			A	B	C
23/09	1	600	0.135	0.118	0.136
24/09	1	600	0.175	0.197	0.244
25/09	1	600	0.643	0.64	0.669
26/09	5	600	0.178	0.172	0.154
29/09	1	600	0.162	0.166	0.167
30/09	1	600	0.152	0.166	0.198
01/10	1	600	0.263	0.281	0.238
02/10	1	600	0.232	0.262	0.262
03/10	1	600	0.307	0.376	0.307

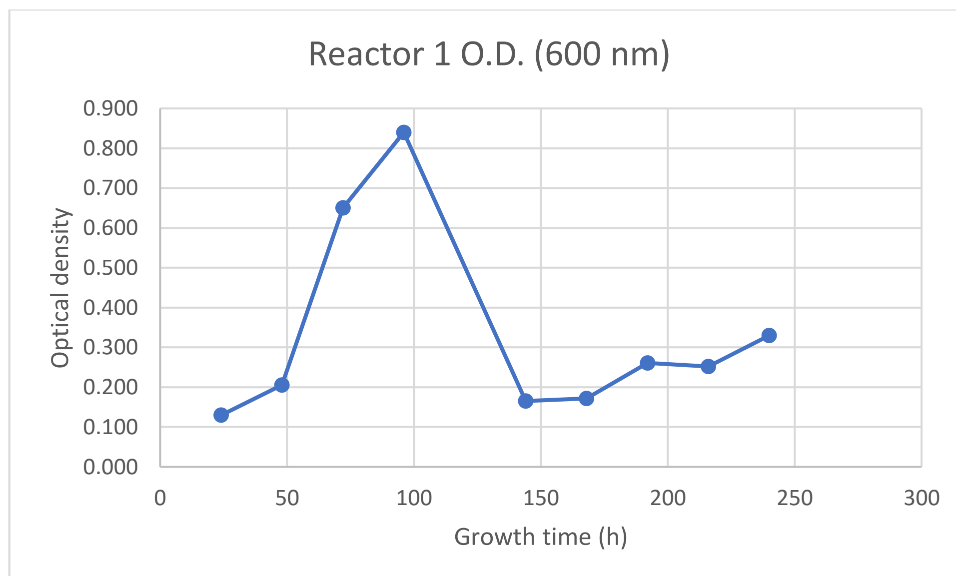

Figure 19 - Reactor 1 optical density evolution.

Figure 20 - Filtrated winery-effluent (top-right), Chlorella vulgaris pre-inoculum (top-left) and Column reactors 1 and 2 (bottom). (Author's photography)

3.8 Soil amendment

Soil amendment was done by an approximation of the value published by FAO for Belgium, of 195 kg/ha of nitrogen-based fertiliser. This value represented a wet biomass in soil concentration of almost 5% in the gardening pots (Table 17). For the last experiment where the pots were changed to aluminium trays, the same concentration was used, however, due to its smaller height profile, this represented a 155 kg/ha nitrogen amendment (Table 18). After lyophilisation a 5:1.389 ratio between the concentrated biomass and freeze-dried biomass was found [21].

Table 17 - Soil amendment calculation for gardening pots.

Wet Biomass (g)	**5**
Freeze-dried biomass	1.389
%N DW	9%
N per pot (g)	0.125
soil per pot (g)	100
N% in soil	0.125%
Pot area (cm^2)	63.61
kg N/ha	196.5

Table 18 - Soil amendment calculations for trays.

Wet Biomass (g)	20.088
Freeze-dried biomass	5.58
%N DW	9%
N per tray (g)	0.5022
soil per tray (g)	401.76
N% in soil	0.125%
Pot area (cm^2)	324
kg N/ha	155

3.9 Soil preparation and conditions test

After 10 days under the mentioned conditions, the experiment was stopped due to the conditions not meeting with the expected results. The soil was completely dry after 24 h of watering with 10, 15 or 20 ml, the soil quantity also didn't allow covering most seeds, and when it was possible to cover the seeds, the water would displace the top layer of sediments, revealing the seed once again. Using almost double the amount of soil would allow the seed to be covered, but adding water without waterlogging the soil would still result in completely dry soil on the next day. The sterilisation could have also impacted the growth of the seed but wasn't seen as a key factor in the obtained results. The soil after this experiment had a different texture based on if it had or had not been enhanced with biomass, the dishes without added biomass presented loose small conglomerates of soil. Meanwhile, the dishes where biomass was added to the soil had a more compacted texture, and biomass and soil particle agglomerates would form chips.

3.10 Seed viability test

The viability of the seed was also evaluated after this phase. After 4 days, maize and bean seeds had not germinated, while lentil seeds had already developed small roots of approximately 4 mm. By day 6, all lentil seeds had germinated, with some already showing the first true leaves, maize seeds had developed roots of approximately 2 cm and coleoptiles of at least 4 mm, and bean seeds reached a germination rate of 50% by the end of the observation period. These results suggest that water availability may have limited germination, highlighting the importance of maintaining adequate moisture to optimise seed development.

3.11 Germination test

The setup using the plastic film positioned as shown in Figure 21 and Figure 22 created a more humid environment, preventing soil dryness and allowing the seeds to germinate and develop.

Figure 21 - Lentil, bean and maize seeds (top to bottom) planted inside the indoors greenhouse. (Author's photography)

Figure 22 - Maize, lentil and bean seeds (left to right) inside the incubator. (Author's Photography)

Germination was monitored daily through directed observation of the cotyledon status. Unlike the previous test, at day 2, there was visible root/coleorhiza development in at least one seed of each species. On bean seeds, one had a visible root coming out of the bean, and two seeds split open with a visible radicle inside (3/27). On maize seeds, six of them showed a small coleorhiza that didn't surpass the 5 mm mark (6/27). On lentil seeds, only those inside the greenhouse had the coleorhiza not visualised in every seed, with all of those inside the incubator showing a coleoptile. The coleoptile was bent partially inside the seed on every seed that showed germination. Data was collected until the 10th day of growth, with germination varying between seed type and location, with the final values being present in Table 19.

Table 19 – Germination rate 10 days after sowing.

		Germinated seeds	Nº Seeds	Percentage
Bean	Greenhouse	6	9	66.7%
	Incubator	9	18	50.0%
Maize	Greenhouse	7	9	77.8%
	Incubator	13	18	72.2%
Lentil	Greenhouse	9	9	100.0%
	Incubator	16	18	88.9%
Total	Greenhouse	22	27	81.5%
	Incubator	39	54	72.2%
	Overall	61	81	75.3%

All types of seeds had a better germination percentage in the greenhouse, as seen in Table 19, with all lentil seeds germinating when inside the greenhouse. Maize seed showed a small difference between those inside the greenhouse and those inside the incubator. The biggest difference is in bean seeds, with 66.7% germination inside the greenhouse and 50.0% inside the incubator. Overall, 75.3% of seeds germinated, this was seen as a great value due to these seeds being sold for consumption and not for production. Due to the great result obtained inside the greenhouse, an 81.5% germination rate, it was determined that the following test would be done inside the greenhouse.

Size-wise (Figure 21), it is possible to see a big difference between seeds in the same location, with the standard deviation being between 14 mm and 50 mm. For beans and maize, the tallest plant was seen inside the greenhouse, however, beans had a higher deviation inside the greenhouse, while maize had a higher deviation inside the incubator.

Lentils, on the other hand, had their tallest plant observed inside the incubator, having the overall smallest deviation.

These findings showed that the sunlight exposure was better than the constant LED light exposure for both beans and maize. Lentils showed better growth inside the incubator, this may be due to a more constant temperature while needing a lower temperature to grow, making it more efficient inside the incubator. Due to these results, growth inside the greenhouse was deemed a better option because not only did it had a better result

for two out of the three seed types, but it also better represented the real growth conditions while removing the need to use electricity during growth.

Table 20 - Growth results observed 10 days after sowing.

		Average (mm)	Maximum Size (mm)	Minimum Size (mm)	Deviation (mm)
Bean	Greenhouse	134.7	174	70	45.2
	Incubator	102.4	133	68	19.3
Maize	Greenhouse	116.4	176	67	36.6
	Incubator	84.8	151	21	48.4
Lentil	Greenhouse	63.6	97	37	34.2
	Incubator	104.4	131	83	14.9

3.12 WET BIOMASS INFLUENCE TEST

Some practical constraints prevented temperature measurements inside the greenhouse on the first day, as well as during holidays and weekends. Ideally, all planting should have occurred simultaneously for every condition, however, this was not feasible due to logistical limitations. For the treatment involving the addition of microalgae biomass 20 days before planting, the setup began slightly later than the remaining tests due to scheduling constraints.

The same water quantity was added in each pot on the first day, however, some of them, those with algae mixed with the soil, got waterlogged.

On the 5th day of the experiment fungus was visible on the soil, the affected area was removed in an attempt to save the remaining area.

On the 10th day the test was stopped as fungus growth affected most pots, after the removal of the pots' contents it was possible to see that the seed had rotted during the test.
This may be a result of human fault, as the soil may have been waterlogged on the starting day (Friday), followed by a rainy and cold weekend, allowing the fungus present in the non-sterile environment to thrive while rotting the seeds inside the pots.

3.13 2ND WET BIOMASS INFLUENCE TEST

On this growth test, temperature and light intensity were measured every Monday, Wednesday and Friday during the 14 days of growth.

Following the wet biomass influence test result and criticism of growth momentum, this test was performed with all seeds being planted on the same day and more caution when watering.

This test was performed during a shorter time window and without the “biomass added 20 days prior to ploughing” due to time constraints.

The average minimum temperature during this experiment was 14.1ºC, with the lowest minimum temperature being 12.0ºC, the highest minimum temperature being 16.0ºC with a mode temperature of 15.0ºC, the average maximum temperature was 25.2ºC, with the lowest maximum temperature being 20.0ºC, the highest maximum temperature being 31.ºC with a mode temperature of 26.0ºC.

Inside the incubator the temperature was able to rise above the maximum atmospheric temperature, reaching temperatures as high as 44.8ºC. Light intensity changed between the time of day and shelf of the greenhouse, having a variation of up to 50%, to avoid discrepancies due to light exposure, the trays changed locations with every watering.

The germination results for the three conditions, biomass added 10 days before planting, biomass added on the day of planting, and no biomass added, are presented in Table 21.

Table 21 - Growth results 14 days after planting.

	Biomass added 10 days before planting			Biomass added in the days of planting			No Biomass added		
Plant	Bean	Lentil	Maize	Bean	Lentil	Maize	Bean	Lentil	Maize
Germination rate	83.3%	0.0%	55.6%	38.9%	0.0%	33.3%	88.9%	66.7%	100.0%
Average (mm)	165.1	0	203.5	170.7	0	209.8	184.2	139.4	240.7
Deviation (mm)	37.4	0	50.7	17.9	0	37.6	45.8	15.3	51.9

Plant size values were determined by comparison with the control assay, in which no biomass was added to the soil (Table 22). On average, plants showed reduced growth when microalgal biomass was incorporated into the substrate.

A possible explanation for these effects is that freshly applied microalgal or cyanobacterial biomass may undergo rapid microbial decomposition, temporarily alter nutrient availability and increase oxygen demand in the soil. The breakdown of untreated or insufficiently stabilised biomass can lead to short-term imbalances in nutrient mineralisation and to the release of ammonium and other intermediate compounds that may inhibit early seedling growth. These transient effects can slow root development during the initial stages, resulting in smaller plants compared with the control condition [59].

Table 22 - Size ration comparison between plants grown in amended soil with biomass and unamended soil.

Plant	Unamended Soil	Soil amended with biomass 10 days prior to planting	Soil amended with biomass added in planting day
bean	100%	89.6%	92.6%
maize	100%	84.6%	87.2%

Growth-wise, as seen in Table 21, there was no germination of lentil seeds in any pot that had biomass in it, this may have happened due to many diverse motifs, with waterlogging being one of the most probable ones. However, in the pots without biomass, there was a germination rate of 66.7%, significantly lower than the 100% in the previous test, this may be, once again, due to some level of waterlogging and different weather conditions.

For maize and bean seeds, both of them had worse results in pots with biomass than without biomass. In both cases, the germination rate was higher in the pots with no biomass, followed by the pots with biomass added 10 days before ploughing, with the biggest difference being in maize, where only 55.6% of seeds germinated in the pots with biomass added 10 days before compared to the 100% germination rate in the no-biomass pots (83.3% vs 88.9% in beans, respectively). The germination rate in the pots with biomass added on the day of planting was the lowest (below 40%).

Size-wise, plants in the pots with biomass added 10 days before ploughing had the lowest average visible growth, with 165.1 ± 37.8 mm above soil level for bean seeds and 203.5 ± 50.7 mm for maize seeds. On the pots with biomass added at the day of ploughing, the average growth was 170.7 ± 17.9 mm and 209.8 ± 37.6 mm, respectively, and in the pots without biomass, it was 184.2 ± 45.8 mm and 240.7 ± 51.9 mm, respectively.

This represents a size variation between 7% and 19% (Table 22), however, one-way ANOVA (α=0.05) showed no statistical difference between results. Standard deviation was around 20% to 25% in the pots with biomass added 10 days before ploughing and pots without biomass, and between 10% and 18% in the pots with biomass added on the day of ploughing, this difference may be due to a lower number of plants grown in the latter. The biggest individual plants were found in the pots with no biomass added (307 mm for maize and 255 mm for bean) (Figure 23).

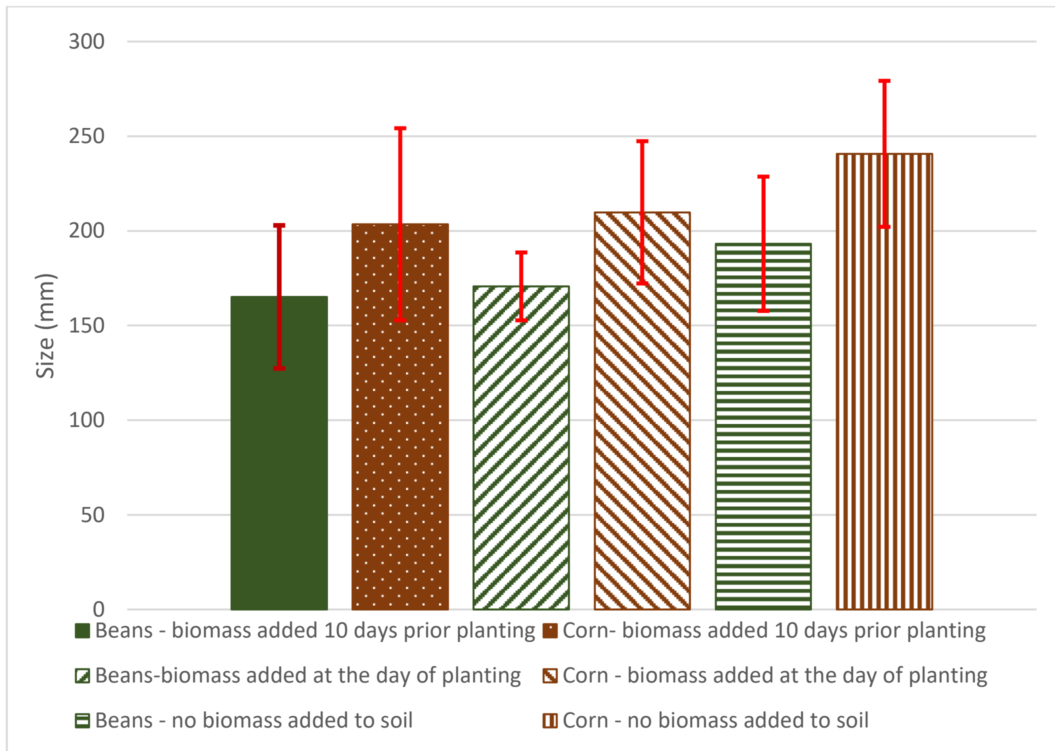

Figure 23 - Average size of plants in different soil amendment conditions.

3.14 WET BIOMASS AND FREEZE-DRIED BIOMASS INFLUENCE COMPARISON

In this study, germination was realised outside the pots using the conditions mentioned in the germination test for 5 days, and then they were transplanted to the pots with soil, due to this reason, the growth was analysed for only 7 days. During the 7 days of growth, the daily temperature was registered using the data from IPMA (Portuguese Institute for the Sea and Atmosphere). During that time the average minimum temperature was 17.9ºC and ranged from 17ºC to 19ºC with a mode minimum temperature of 18ºC, the average maximum temperature was 28.3ºC and ranged from 25ºC to 34ºC with a mode maximum temperature of 26ºC.

Table 23 presents the germination rate of seed on wet cotton pads inside an indoor greenhouse.

Table 23 - Germination rate, on wet cotton pads inside indoors greenhouse.

	Maize	Lentils	Bean
total seeds	60	63	76
viable seeds	52	58	44
Germination rate	86.7%	92.1%	57.9%

The emergence rate refers to the proportion of seeds that successfully emerge above the surface of the soil of after germination. While germination indicates that the seed embryo has started to grow, emersion reflects the seedling's ability to break through the soil and establish a visible plant. A high emersion rate indicates that the conditions of the substrate, moisture and handling were suitable to support the early seedling development, whereas a low emersion rate may reveal problems such as soil compaction, insufficient water availability or damage during transplantation. Therefore, analysing emersion rate allows us to evaluate not only germination success but also the practical viability of seedlings under specific growth conditions. Table 24 shows, both growth data and emersion rate.

Emergence has the best emergence rate with soil amended with wet biomass showing a 100% emergence rate, compared with 83.3% in soil amended with freeze-dried biomass and 58.3% from unamended soil. As per the previous study, each seed showed a combination of results, with lentils having its maximum emergence rate on both soil amended with wet biomass and unamended soil, 75.0%, with soil amended with freeze-dried biomass not even reaching half of that value, 31.2%. Maize has its best emergence rate on soil amended with freeze-dried biomass, 66.7%, while soil amended with wet biomass and unamended soil reach 53.3% and 26.7%, respectively.

There was no single condition that consistently resulted in the best performance for transplantation. Soil amended with freeze-dried biomass performed better with beans and the worst with lentils. Soil amended with wet biomass also performed better with beans, while maize had the worst performance, although still above 50%, and unamended soil performed better with lentils and the worst with maize. Consequently, no clear pattern can be observed. This variation is likely due to handling during transplantation, where some plants could have suffered damages to their structures, such as broken roots or stems and bad soil water absorption, making them unavailable to support further growth.

Size-wise (Table 24), growth was visualised in all conditions for all plant types. Beans had a maximum size of 348 mm, present in soil amended with freeze-dried biomass, and a minimum size of 56 mm, present in soil amended with wet biomass. After excluding outliers, any plant with a size lower than 70 mm, beans grown in soil amended with freeze-dried biomass had the highest average size, with an average size of 229.3 mm and a standard deviation of 71.7 mm, equivalent to 31.3%, those planted in soil amended with wet biomass had the lowest average, 186.1 mm, with a standard deviation of 69.3

mm (37.2%), and beans grown in unamended soil showed a size average of 226.4 mm with a standard deviation of 41.8 mm (18.5%).

Maize had a maximum size of 250 mm, present in soil amended with wet biomass, and a minimum size of 70 mm, present in soil amended with freeze-dried biomass. After excluding outliers, any plant smaller than 60 mm, maize has the highest average size, 171.9 mm, in soil amended with wet biomass, with a standard deviation of 41.9 mm (24.4%), while the lowest average, 115.5 mm, was visualised in unamended soil, with a standard deviation of 39.4 mm (34.2%), maize grown in soil amended with freeze-dried biomass had an average size of 138.4 mm, and the standard deviation was 49.6 mm (35.8%).

Table 24 – Average, range standard deviation for plant size (mm).

	Freeze Dry			Wet biomass			No biomass		
	Beans	Maize	Lentils	Beans	Maize	Lentils	Beans	Maize	Lentils
Plant size (mm)	160	98	41	208	130	140	209	86	44
	0	70	0	125	173	63	0	113	36
	0	172	0	146	0	32	0	0	24
	180	0	0	256	0	40	266	0	40
	315	0	0	162	0	0	0	0	63
	265	147	0	243	159	0	0	172	0
	348	205	0	125	221	0	230	91	0
	287	215	0	280	144	0	160	0	0
	143	86	10	280	250	79	0	0	161
	240	159	95	91	0	119	280	0	138
	185	124	62	131	148	56	245	0	107
	170	108	61	56	150	66	195	0	134
		0	0		0	86		0	98
		0	0		0	57		0	113
		0	0		0	30		0	58
			0			165			0
Maximum growth (mm)	348	215	95	280	250	165	280	172	161
Minimum non 0 growth (mm)	143	70	10	56	130	30	160	86	24
Emersion rate	83.3%	66.7%	31.2%	100.0%	53.3%	75.0%	58.3%	26.7%	75.0%
Average (mm)	229.3	138.4	72.7	186.1	171.9	102.6	226.4	115.5	116.3
Standard deviation (mm)	71.7	49.6	19.4	69.3	41.9	47.3	41.8	39.5	31.8

The largest lentil individual reached 165 mm in soil amended with wet biomass, while the smallest reached 10 mm in soil amended with freeze-dried biomass. After excluding outliers, any plant smaller than 60 mm, the highest average size was from unamended soil, 116.3 mm with a standard deviation of 31.8 mm (27.3%), and the smallest was from soil amended with freeze-dried biomass, with an average size of 72.7 mm and a standard deviation of 19.3 mm (26.6%). Lentil plants grown in soil amended with wet biomass had an average size of 105.6 mm and a standard deviation of 47.3 mm (46.1%).

Due to the reduced number of grown specimens for maize and lentils in some conditions, unamended soil for maize, and soil amended with freeze-dried biomass, it was only possible to compare the growth between the other parameters. For maize, one-way ANOVA ($\alpha=0.05$) showed a p-value of 0.147, making the growth difference not significant, and for lentils, it showed a p-value of 0.49, making the growth difference not significant.

For beans, there were enough grown specimens in each condition to compare the three. Using ANOVA ($\alpha=0.05$) between the three conditions, the p-value was 0.26, making the growth differences between the three scenarios not significant.

As no definitive conclusion could be drawn regarding the optimal conditions, all treatments were selected for analysis for the next test.

3.15 REGULAR BIOMASS AND EFFLUENT-GROWN BIOMASS INFLUENCE COMPARISON

On this growth test it wasn't possible to measure greenhouse temperature and lighting conditions, however, outside temperature was resisted using the data obtained by IPMA.

This test went from November 5th to November 27th, during this time, on 10 of the 21 growth days, rainy weather was observed, with only 7 days of clear or partially clear weather, with a bigger incidence in the last week of the experiment. Temperatures ranged from 15ºC to 20ºC on maximum temperature and 6ºC to 16ºC on minimum temperature, average maximum temperature was 17.9º and the average minimum temperature was 11.0º C. It is expected to have higher temperatures inside the greenhouse, however, these low temperatures and less solar exposition, fewer sunny days and smaller sunlight exposition periods have negatively affected the growth.

For this test only bean seeds were used, as they had the better germination rate of the previous tests, while biomass, both effluent and non-effluent grown, had

After 7 days, 2 trays didn't show any growth: one with soil amended with freeze-dried effluent-grown biomass and the other with soil amended with wet biomass, the latter with a mass fungal contamination making the conditions incompatible with plant growth. The remaining trays had 2 to 3 grown seedlings, except the tray with soil amended with chemical fertilisers, which had 7 grown seedlings.

After 14 days, unamended soil showed the same number of grown specimens, soil amended with freeze-dried effluent-grown biomass increased from no growth to 2 grown specimens, soil amended with effluent-grown wet biomass decreased from 3 to 1 specimens due to a fungus infestation. Soil amended with freeze-dried biomass increased from 3 to 7 specimens, and soil amended with chemical fertilisers Increased from 7 to 13 specimens.

Table 25 - Bean growth based on soil amendment type.

Soil type	maximum size (mm)	minimum size (mm)	Emerging rate	Average (mm)	Deviation (mm)
Unamended soil	318	36	11.8%		
Soil amended with freeze-dried effluent-grown biomass	243	160	11.8%		
Soil amended with wet effluent-grown biomass	404	404	5.9%		
Soil amended with freeze-dried biomass	330	24	41.2%	289.8	30.2
Soil amended with wet biomass	0	0	0.0%		
Soil amended with chemical fertiliser.	385	98	70.6%	238.4	74.4

After 21 days (Table 25), there was no change in the number of grown specimens, and size was measured one last time. Excluding specimens smaller than 100 mm, unamended soil had one specimen with 318 mm, and soil amended with freeze-dried effluent grown biomass has two specimens with 243 mm and 160 mm, soil amended with wet effluent-grown biomass had one specimen with 404 mm. This specimen was able to grow despite a large fungal infection. This growth could indicate that the plant is fungus resistant or that its growth had reached a state less dependent on soil nutrients when the infection happened.

Soil amended with freeze-dried biomass had 5 specimens ranging from 251 mm to 330 mm, an average of 289.8 mm and a standard deviation of 30.21 mm. Finally, soil amended with chemical fertilisers had the best number of germinated individuals, with 10 being over 100 mm, ranging between 125 mm and 323 mm, this growth being represented with an average of 238.4 mm and a standard deviation of 74.4 mm.

Using one-way ANOVA (α=0.05), the p-value is equal to 0.16, therefore being not significant.

The plants' appearance after 21 days can be seen in Figure 24, Figure 25, Figure 26 and Figure 27.

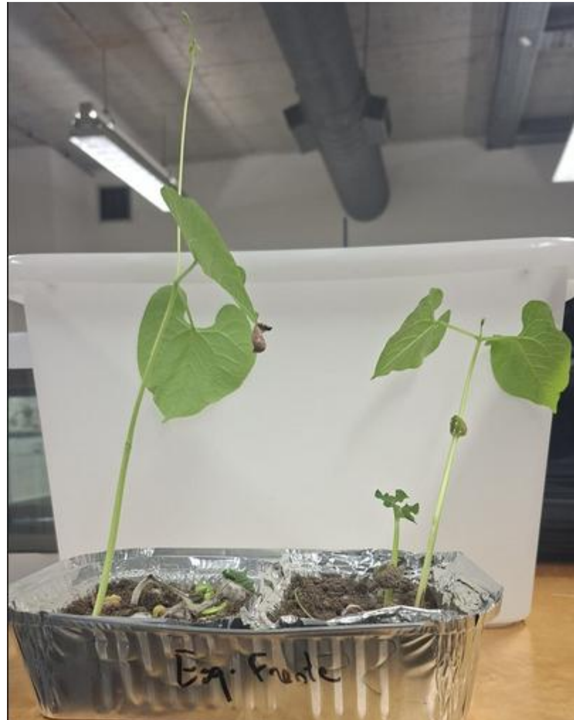

Figure 24 - Beans grown in: Soil amended with wet effluent-grown biomass (left); soil amended with freeze-dried effluent-grown biomass (right). (Author's photography)

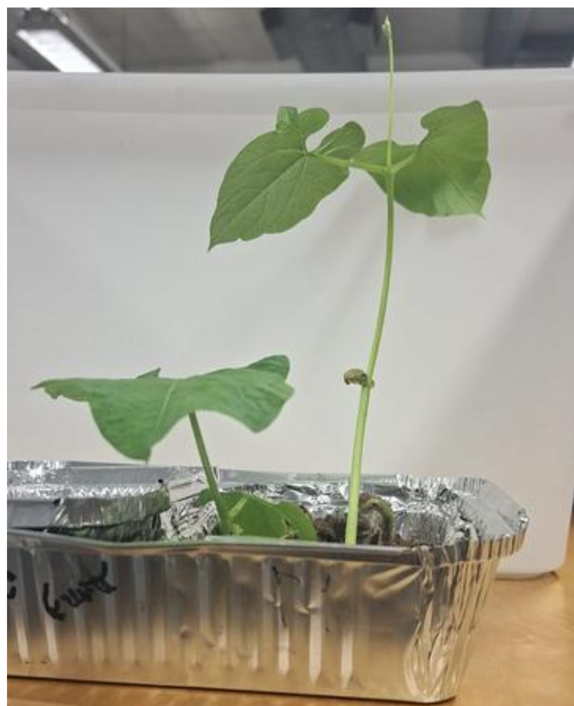

Figure 25 - Beans grown in unamended soil. (Author’s photography)

Figure 26 - Beans grown in soil amended with wet biomass. (Author's photography)

Figure 27 - Beans grown in soil amended with chemical fertiliser. (Author's photography)

4. CONCLUSION

This thesis had as its objective to study the effects of *Chlorella vulgaris* biomass grown in winery effluent on plant growth. Through three tests where the effects of wet biomass, freeze-dried biomass, effluent-grown wet biomass and effluent-grown freeze-dried biomass were tested, after a test to determine the optimum time for biomass addition.

The addition of biomass on the day of planting and 10 days before doesn't show a significant difference in the early stages of growth, however, the best result was obtained without biomass, with size decreases between 7% and 15% and 11% and 18%, respectively. These values contradict the expectation of a better result with biomass, however, this may come from prejudicial metabolites released during the deterioration of the biomass.

When comparing wet biomass and freeze-dried biomass, we can find better growth in each soil condition for each seed, including unamended soil. Bean seeds performed better with freeze-dried amended soil, maize with wet biomass-amended soil and lentils with unamended soil. Once again, these values don't represent a significant difference between conditions. Finally, adding effluent-grown biomass, either freeze-dried or wet biomass, freeze-dried non-effluent-grown biomass had the better result, which once again doesn't represent a significant difference. This last test also added a soil with chemical fertiliser parameter.

Throughout the whole experiment soil amended with biomass, especially those amended with wet biomass, showed a bigger prevalence in fungous growth on the surface of the soil and usually a decrease in the number of emerged specimens. This made the number of subjects per condition inconstant, with this fact affecting the success of specimens.

Another factor that may have influenced the results is the plant characteristics, themselves, with plants growing with different stalk diameters. Making some plants grow tall but with a skinnier stalk or be smaller while having a stalk of a larger diameter.

In the last experiment, even with soil amended with freeze-dried biomass presenting a better average size, it comes from a lower number of grown specimens, with soil amended with chemical fertiliser showing a greater number of grown specimens.

Finally, the effluent-grown biomass showed around the same number of emerging specimens, except for soil amended with chemical fertiliser, which had one specimen that outgrew the remaining while being in a tray with a visible fungal infection.

Although this experiment did not reveal significant differences in plant growth due to the use of effluent-grown biomass, as indicated by one-way ANOVA, these preliminary results are valuable for guiding future research. The findings suggest that the biomass affects plant growth positively and may provide useful insights into optimising growth conditions. An observed increase in fungal growth, likely due to the higher water content of the biomass, highlights aspects that can be managed in subsequent studies. Overall, these results lay a foundation for further investigations into the potential of effluent-grown biomass as a sustainable growth substrate.

5. WAY FORWARD

Further study on this matter is needed with the characterisation of winery effluent-grown biomass, focusing on the determination of the nitrogen, phosphorous and potassium content, followed by studies about the phytohormone produced by the microalgae. These analyses would provide essential information to optimise experimental conditions and better understand the mechanism through which biomass may influence plant growth.

Regarding the soil, a full characterisation of it would allow us to better understand its conditions, a complete analysis of nitrogen, phosphorous and potassium content, C:N ratio, organic and inorganic matter, soil type, soil moisture and soil microbiota characterisation.

On soil amendment conditions, a further study about the optimal moment for the addition of biomass and type of biomass is needed. This includes evaluating how the stage of biomass decomposition, moisture content, and physical form (e.g., Wet vs freeze-dried) influence nutrient availability, microbial activity, and early seedling establishment. Understanding these factors will help optimise the application strategy to maximise plant growth while minimising potential negative effects, such as waterlogging or fungal proliferation.

Expanding the experiment to analyse the entire growth cycle and in conditions closer to those that would be used in agricultural practices, such as realising the experiment in a greenhouse with raised beds allowing for a bigger space for root development. Studying the effects of other types of biofertilisers, such as foliar fertilisers made from microalgal byproducts.

6. BIBLIOGRAPHY

[1] X. Yuan *et al.*, "Global impacts of land use on terrestrial carbon emissions since 1850," *Science of The Total Environment*, vol. 963, p. 178358, Feb. 2025, doi: 10.1016/j.scitotenv.2024.178358.

[2] L. J. R. Nunes, "The Rising Threat of Atmospheric CO2: A Review on the Causes, Impacts, and Mitigation Strategies," *Environments*, vol. 10, no. 4, p. 66, Apr. 2023, doi: 10.3390/environments10040066.

[3] "As pollution increases, world falls further behind climate targets." Accessed: Mar. 03, 2025. [Online]. Available: https://research.noaa.gov/no-sign-of-fossil-fuel-pollution-peak-as-the-world-falls-further-behind-climate-targets/

[4] R. Lindsey, "Climate Change: Atmospheric Carbon Dioxide." Accessed: Mar. 03, 2025. [Online]. Available: https://www.climate.gov/news-features/understanding-climate/climate-change-atmospheric-carbon-dioxide

[5] J. N. O'Sullivan, "Demographic Delusions: World Population Growth Is Exceeding Most Projections and Jeopardising Scenarios for Sustainable Futures," *World*, vol. 4, no. 3, pp. 545–568, Sep. 2023, doi: 10.3390/world4030034.

[6] "Population growth, environmental degradation and climate change." Accessed: Jul. 23, 2025. [Online]. Available: https://desapublications.un.org/un-desa-voice/feature/february-2022/population-growth-environmental-degradation-and-climate-change

[7] S. Iqbal *et al.*, "Chemical Fertilizers, Formulation, and Their Influence on Soil Health," in *Microbiota and Biofertilizers*, Cham: Springer International Publishing, 2021, pp. 1–15. doi: 10.1007/978-3-030-48771-3_1.

[8] S. Nosheen, I. Ajmal, and Y. Song, "Microbes as Biofertilizers, a Potential Approach for Sustainable Crop Production," *Sustainability*, vol. 13, no. 4, p. 1868, Feb. 2021, doi: 10.3390/su13041868.

[9] Z. Atafar *et al.*, "Effect of fertilizer application on soil heavy metal concentration," *Environ Monit Assess*, vol. 160, no. 1–4, pp. 83–89, Jan. 2010, doi: 10.1007/s10661-008-0659-x.

[10] S. B. Guerreiro, R. J. Dawson, C. Kilsby, E. Lewis, and A. Ford, "Future heat-waves, droughts and floods in 571 European cities," *Environmental Research Letters*, vol. 13, no. 3, p. 034009, Mar. 2018, doi: 10.1088/1748-9326/aaaad3.

[11] J. Peñuelas *et al.*, "Assessment of the impacts of climate change on Mediterranean terrestrial ecosystems based on data from field experiments and long-term monitored field gradients in Catalonia," *Environ Exp Bot*, vol. 152, pp. 49–59, Aug. 2018, doi: 10.1016/j.envexpbot.2017.05.012.

[12] S. Wilkenskjeld, F. Miesner, P. P. Overduin, M. Puglini, and V. Brovkin, "Strong increase in thawing of subsea permafrost in the 22nd century caused by anthropogenic climate change," *Cryosphere*, vol. 16, no. 3, pp. 1057–1069, Mar. 2022, doi: 10.5194/tc-16-1057-2022.

[13] C. Amaral *et al.*, "Abrupt greening observed since 2020 at Admiralty Bay, King George Island, Antarctica," *Polar Biol*, vol. 48, no. 2, p. 40, Jun. 2025, doi: 10.1007/s00300-025-03364-y.

[14] "THE 17 GOALS." Accessed: Mar. 03, 2025. [Online]. Available: https://sdgs.un.org/goals

[15] L. S. Dagnaisser, M. G. B. dos Santos, A. V. S. Rita, J. Chaves Cardoso, D. F. de Carvalho, and H. V. de Mendonça, "Microalgae as Bio-fertilizer: a New Strategy for Advancing Modern Agriculture, Wastewater Bioremediation, and Atmospheric Carbon Mitigation," *Water Air Soil Pollut*, vol. 233, no. 11, p. 477, Nov. 2022, doi: 10.1007/s11270-022-05917-x.

[16] C. Safi, B. Zebib, O. Merah, P. Y. Pontalier, and C. Vaca-Garcia, "Morphology, composition, production, processing and applications of Chlorella vulgaris: A review," *Renewable and Sustainable Energy Reviews*, vol. 35, pp. 265–278, 2014, doi: 10.1016/j.rser.2014.04.007.

[17] J. G. Osorio-Reyes *et al.*, "Microalgae-Based Biotechnology as Alternative Biofertilizers for Soil Enhancement and Carbon Footprint Reduction: Advantages and Implications," *Mar Drugs*, vol. 21, no. 2, p. 93, Jan. 2023, doi: 10.3390/md21020093.

[18] J. Garcia-Gonzalez and M. Sommerfeld, "Biofertilizer and biostimulant properties of the microalga Acutodesmus dimorphus," *J Appl Phycol*, vol. 28, no. 2, pp. 1051–1061, Apr. 2016, doi: 10.1007/s10811-015-0625-2.

[19] N. Bharti and M. Suryavanshi, "Quality control and regulations of biofertilizers: Current scenario and future prospects," in *Biofertilizers*, Elsevier, 2021, pp. 133–141. doi: 10.1016/B978-0-12-821667-5.00018-X.

[20] B. Suárez-Eiroa, E. Fernández, G. Méndez-Martínez, and D. Soto-Oñate, "Operational principles of circular economy for sustainable development: Linking theory and practice," *J Clean Prod*, vol. 214, pp. 952–961, Mar. 2019, doi: 10.1016/j.jclepro.2018.12.271.

[21] F. Tubiello and N. Wanner, "Soil nutrient budget Global, regional and country trends, 1961-2018," *FAO Food Nutr Ser*, May 2021.

[22] S. Aigner *et al.*, "Adaptation to Aquatic and Terrestrial Environments in Chlorella vulgaris (Chlorophyta)," *Front Microbiol*, vol. 11, p. 585836, Oct. 2020, doi: 10.3389/FMICB.2020.585836/FULL.

[23] J. Alberto CORONADO-Reyes, J. Salazar Torres, B. Juárez-Campos, and J. González-Hernández, "Chlorella vulgaris, a microalgae important to be used in Biotechnology: a review," *Ciência e Tecnologia de Alimentos*, vol. 42, Aug. 2020, doi: 10.1590/fst.37320.

[24] M. Al-Hammadi and M. Güngörmüşler, "New insights into *Chlorella vulgaris* applications," *Biotechnol Bioeng*, vol. 121, no. 5, pp. 1486–1502, May 2024, doi: 10.1002/bit.28666.

[25] A. R. Mendes, M. P. Spínola, M. Lordelo, and J. A. M. Prates, "Chemical Compounds, Bioactivities, and Applications of Chlorella vulgaris in Food, Feed

and Medicine," *Applied Sciences*, vol. 14, no. 23, p. 10810, Nov. 2024, doi: 10.3390/app142310810.

[26] H. S. Kusuma, N. Illiyanasafa, D. E. C. Jaya, H. Darmokoesoemo, and N. R. Putra, "Utilization of the microalga Chlorella vulgaris for mercury bioremediation from wastewater and biomass production," *Sustain Chem Pharm*, vol. 37, p. 101346, Feb. 2024, doi: 10.1016/j.scp.2023.101346.

[27] K. Pooja, V. Priyanka, B. C. S. Rao, and V. Raghavender, "Cost-effective treatment of sewage wastewater using microalgae Chlorella vulgaris and its application as bio-fertilizer," *Energy Nexus*, vol. 7, p. 100122, Sep. 2022, doi: 10.1016/j.nexus.2022.100122.

[28] R. W. Bulliet, *The earth and its peoples : a global history*. Recording for the Blind & Dyslexic, 2005.

[29] G. E. . Mingay, *The Agricultural revolution : changes in agriculture, 1650-1880*. Adam & Charles Black, 1977.

[30] M. Overton, "Agricultural Revolution in England 1500-1850," BBC. Accessed: Sep. 18, 2025. [Online]. Available: https://www.bbc.co.uk/history/british/empire_seapower/agricultural_revolution_01.shtml

[31] B. O'Connor, "The Origins and development of Great Britain's Coprolite Industry," *Mining Today*, vol. 14, no. 5, 2001.

[32] W. S. Gaud, "The Green Revolution: Accomplishments and Apprehensions," Mar. 08, 1968.

[33] B. H. Farmer, "Perspectives on the 'Green Revolution' in South Asia," *Mod Asian Stud*, vol. 20, no. 1, pp. 175–199, Feb. 1986, doi: 10.1017/S0026749X00013627.

[34] S. Endicott *et al.*, "Corn Growth and Development," 2015.

[35] D. E. Clay, G. G. Carlson, S. A. Clay, and E. Byamukama, "Corn Growth and Development," in *iGrow Corn: Best Management Practices*, 2016, ch. 5.

[36] Jonathan D. Sauer, *Historical Geography of Crop Plants*. Boca Raton: Lewis Publishers, 1993.

[37] K. Lamkey, *Crop Improvement*. Iowa State University Digital Press, 2023. doi: 10.31274/isudp.2023.138.

[38] Fertiglobal, "Common bean program." Accessed: Dec. 11, 2025. [Online]. Available: https://www.fertiglobal.com/cmp/common-bean/

[39] S. S. Yadav, D. L. McNeil, and P. C. Stevenson, Eds., *Lentil*. Dordrecht: Springer Netherlands, 2007. doi: 10.1007/978-1-4020-6313-8.

[40] J. S. Sandhu and S. Singh, "History and Origin," in *Lentil*, Dordrecht: Springer Netherlands, 2007, pp. 1–9. doi: 10.1007/978-1-4020-6313-8_1.

[41] B. Redden, N. Maxted, B. Furman, and C. Coyne, "Lens Biodiversity," in *Lentil*, Dordrecht: Springer Netherlands, 2007, pp. 11–22. doi: 10.1007/978-1-4020-6313-8_2.

[42] M. Andrews and B. A. McKenzie, "Adaptation and Ecology," in *Lentil*, Dordrecht: Springer Netherlands, pp. 23–32. doi: 10.1007/978-1-4020-6313-8_3.

[43] W. Erskine, F. J. Muehlbauer, and R. W. Short, "Stages of Development in Lentil," *Exp Agric*, vol. 26, no. 3, pp. 297–302, Jul. 1990, doi: 10.1017/S0014479700018457.

[44] ADAMA, "Upgrade to Maxentis." Accessed: Dec. 11, 2025. [Online]. Available: https://simplicity.adama.com/all-in-on-fungicides/maxentis

[45] D. L. McNeil and M. Materne, "Rhizobium Management and Nitrogen Fixation," in *Lentil*, Dordrecht: Springer Netherlands, 2007, pp. 127–143. doi: 10.1007/978-1-4020-6313-8_8.

[46] B. A. McKenzie, M. Andrews, and G. D. Hill, "Nutrient and Irrigation Management," in *Lentil*, Dordrecht: Springer Netherlands, 2007, pp. 145–158. doi: 10.1007/978-1-4020-6313-8_9.

[47] R. P. Voroney and R. J. Heck, "The Soil Habitat," in *Soil Microbiology, Ecology and Biochemistry*, Elsevier, 2015, pp. 15–39. doi: 10.1016/B978-0-12-415955-6.00002-5.

[48] E. Nadeu, R. van Dijk, and N. Hiller, "The Soil Microbiome: its contribution to soil health and One Health," Brussels, Dec. 2023.

[49] P. Chauhan *et al.*, "Soil Microbiome: Diversity, Benefits and Interactions with Plants," *Sustainability*, vol. 15, no. 19, p. 14643, Oct. 2023, doi: 10.3390/su151914643.

[50] A. Mukherjee *et al.*, "The bioactive potential of phytohormones: A review.," *Biotechnol Rep (Amst)*, vol. 35, p. e00748, Sep. 2022, doi: 10.1016/j.btre.2022.e00748.

[51] F. Minaoui, Z. Hakkoum, A. Chabili, M. Douma, K. Mouhri, and M. Loudiki, "Biostimulant effect of green soil microalgae Chlorella vulgaris suspensions on germination and growth of wheat (Triticum aestivum var. Achtar) and soil fertility," *Algal Res*, vol. 82, p. 103655, Aug. 2024, doi: 10.1016/j.algal.2024.103655.

[52] A. Fiodor, N. Ajijah, L. Dziewit, and K. Pranaw, "Biopriming of seed with plant growth-promoting bacteria for improved germination and seedling growth," *Front Microbiol*, vol. 14, Feb. 2023, doi: 10.3389/fmicb.2023.1142966.

[53] Himedia, "YEM Agar," https://www.himedialabs.com/media/TD/M1853.pdf.

[54] Himedia, "Yeast MAnnitol Agar w/ Congo Red," https://exodocientifica.com.br/_technical-data/M721.pdf.

[55] M. Gonçalves, T. Ramos, J. Martins, M. horta, and F. Pires, "INFOSOLO," INIAV. Accessed: Dec. 07, 2025. [Online]. Available: https://portalgeo.iniav.pt/portal/apps/webappviewer/index.html?id=956291022483413893a70c22505ac2ac

[56] C. Ballabio *et al.*, "Mapping LUCAS topsoil chemical properties at European scale using Gaussian process regression," *Geoderma*, vol. 355, p. 113912, Dec. 2019, doi: 10.1016/j.geoderma.2019.113912.

[57] D. G. Groenendyk, T. P. A. Ferré, K. R. Thorp, and A. K. Rice, "Hydrologic-Process-Based Soil Texture Classifications for Improved Visualization of Landscape Function," *PLoS One*, vol. 10, no. 6, Jun. 2015, doi: 10.1371/journal.pone.0131299.

[58] "NPK in European soils," https://esdac.jrc.ec.europa.eu/themes/npk-european-soils.

[59] P. Renganathan, L. A. Gaysina, R. J. Holguín-Peña, J. C. Sainz-Hernández, J. Ortega-García, and E. O. Rueda-Puente, "Phycoremediated Microalgae and Cyanobacteria Biomass as Biofertilizer for Sustainable Agriculture: A Holistic Biorefinery Approach to Promote Circular Bioeconomy," *Biomass*, vol. 4, no. 4, pp. 1047–1077, Sep. 2024, doi: 10.3390/biomass4040059.