Entry to the Stockholm Junior Water Prize 2024

Genetic Modification of Plants for Increased Salt Tolerance

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Abstract

The purpose of this project is to explore research on developing a genetically modified crop that can be irrigated with seawater, thereby conserving groundwater. The study summarizes scientific evidence regarding the challenges facing groundwater and agriculture, including purification assessments and the placement of groundwater boreholes. It also examines the impact of climate change on agriculture, such as flooding and drought. The evolution of plants, particularly the development of salt-tolerant mutants known as halophytes, is discussed. Methods for genetic modification of plants are reviewed, focusing on the use of *Agrobacterium Tumefaciens* for gene transfer and the role of the particle gun in transferring selected genes to bacteria. Legal considerations and soil impacts of salinity are also explored, with an examination of regulations and historical causes of soil structure issues. Relevant quantitative and qualitative experiments are included. Experiment 1 demonstrates osmosis in plant cells using cucumbers. Experiment 2 challenges the theory that glycophytic plants cannot tolerate salt by irrigating Pilea plants with saltwater. Experiment 3 confirms that salt accumulates in soil after irrigation. Despite challenges and sources of error, these experiments support the potential of genetically modified crops to reduce resource waste. Future work will involve gathering more empirical data and developing our own halophytic crop.

Acknowledgements

We want to thank Sara Miller from Aarhus University, for the tour at their labs and greenhouses in Flakkebjerg. Furthermore, we want to thank our teachers Jesper Melchjorsen, Nils Hedehus and Pia Møller for their lessons and help in our journey to Unge Forskere. Besides a thanks to Claus Gammelmark Therkildsen from Danish environment consultancy (DMR) for the interview on soil and soil structure. Nonetheless, a thank to Flemming Dyring from Nordic Harvest, for the talk about vertical farming. Lastly a huge thanks to Poul Due Jensen's Fond, for choosing our project for SJWP.

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Abbreviations

GMO: Genetically modified organisms

PFAS: Poly- and perfluoroalkyl substances

PCR: Polymerase chain reaction

BAM: 2,6-dichlorbenzamid (A degradation product of the pesticides dichlobenil and chlorthiamid)

Introduction

We live in a world where climate change is causing floods that submerge fields with saltwater, creating significant problems for farmers who lose their crops. Additionally, our groundwater quality is under strict surveillance due to threats from nutrients in fertilizers and hazardous pollutants like PFAS and pesticides. As pollution escalates, we may need to conserve groundwater as more wells are closed. This concerns us as the new generation. We aim to tackle these issues through plant genetic modification to possibly irrigate crops with saltwater.

Our research question is: How can we genetically modify plants to tolerate saltwater, and what impact will this have on nature? We also aim to explore the considerations of genetic modification and conduct our own experiments.

The project views the problem from a scientific perspective. Our data comes from scientific works and websites like videnskab.dk and SEGES.dk. Given the scientific approach, we included experimental work with results evaluated both qualitatively and quantitatively. We used an idiographic approach by examining a specific plant's response to salt exposure and a diachronic approach to study soil changes over time.

The paper is structured with an introduction, followed by theory and related experiments. It includes a discussion challenging our views on genetic modification, a conclusion, and perspectives for future work.

Hypothesis

We expect that genetic modification of plants can improve their salt tolerance. Additionally, this method can conserve groundwater and enhance soil quality in vulnerable areas. This expectation is based on research showing that genetic modification can successfully enhance desired traits or suppress unwanted ones.

The Threat of Climate Change to Agriculture and Groundwater

Most people have heard about climate change and its impacts on our planet. Specifically, it has severe consequences for agriculture, with up to 1.5 million hectares of farmland being destroyed annually due to salinization[1]. Rising sea levels, driven by more frequent seawater flooding, increase soil salinity, which damages crops and reduces yields[2]. This is especially concerning given the growing global population. Prolonged droughts exacerbate soil salinity by reducing water to flush out salts[3].

This highlights the need for genetically modifying plants to tolerate higher salinity levels[4]. Increasing salinity in oceans, primarily from eroded rocks and volcanic activity, contributes to this problem[5].

Climate change also impacts groundwater sources. Rising sea levels risk contaminating groundwater with salt, leading to well closures[6]. Over-extraction of groundwater can lower groundwater levels, harming local ecosystems and biodiversity. Additionally, groundwater is threatened by nutrient runoff from agriculture and pollutants like PFAS and pesticides. If contamination levels rise, wells are shut down. In Denmark, simple groundwater treatments might not suffice, necessitating costly advanced treatments[7].

Therefore, genetically modifying plants to thrive in saline environments and use saltwater for irrigation is crucial for future sustainability and conserving threatened groundwater resources.

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[1] (Thomsen M. H., 2022)
[2] (SalFar - Saline Farming., Juni 2022) (Biotech Academy, Unknown date of publication)
[3] (Koszyczarek, 2023)
[4] (Røde Kors, 2016)
[5] (Skou, 2022)
[6] (Vandets vej, Forurening af grundvand, Unknown date of publication)
[7] (Vandets vej, Udvidet vandrensning, Unknown date of publication)

The Effect of Salt on Plants

To understand how plants are affected by saltwater, we first need to explain plant structure and the mechanisms influenced by salt. Plant cells, like animal cells, are eukaryotic, meaning their DNA is housed in a nucleus. Unlike bacterial cells, which have DNA floating in the cytoplasm, plant cells contain large vacuoles that store fluids and nutrients, impacting cell turgor and pressure. The vacuole takes up most of the plant cell's volume. To prevent bursting, plant cells have a semipermeable membrane and a rigid cell wall made of cellulose and pectin, providing structural support[8]. When the external environment is hypertonic[9] (higher salt concentration than inside the cell), water exits the vacuole through osmosis, causing the plant to dehydrate. This process, known as osmotic pressure, drives water from areas of high to low concentration, leading to plant dehydration under salt stress. We have conducted an experiment with the plant pilea pepperomoides, where we watered them with different saltwater ratios. This experiment clearly substantiated the theory as the plants thrived without salt and died because of salt.

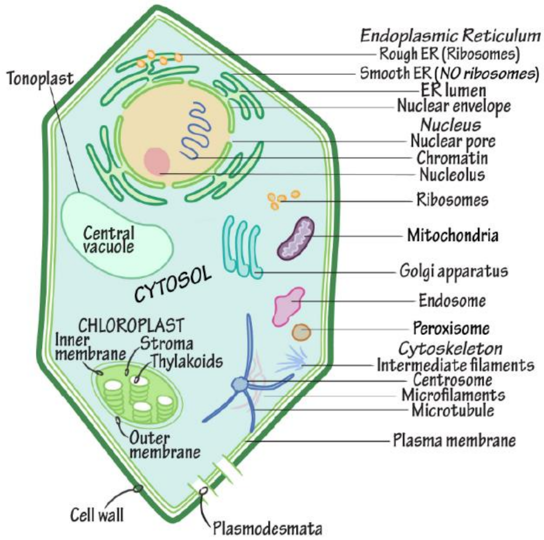

Figure 1 The structure of a plant cell[10]

Evolution

Why can plants live on the beach while others die from the slightest amount of salt? To understand this, we need to revisit an old concept: Charles Darwin's theory of evolution. Organisms are sorted by selection, with only the strongest or those with beneficial mutations surviving. As biologist Theodosius Dobzhansky said, "Nothing in biology makes sense except in the light of evolution." [11] We follow this line of thought.

[8] (Halkier & Skibsted, 2023)
[9] (Biotech Academy, Unknown date of publication)
[10] (Ditki, Unknown date of publication)
[11] (Brendborg, 2021)

Many years ago, a small plant called *Aster Amellus*[12] slowly moved into new territory, spreading to the beach. The closer it got, the more salt it absorbed with the water, harming the plant. At some point, one of its offspring mutated to avoid salt stress, while another mutation in a different offspring didn't help. The plant with the beneficial mutation had better survival and reproduction chances, eventually outcompeting the others. This mutated plant, now known as sea aster or *Aster Hybridus*[13], developed a method to deposit absorbed salt into older parts of the plant, like leaves, which would eventually be shed[14]. Plants that evolved to handle high salt concentrations are called halophytes, while those that haven't are known as glycophytes[15].

Figure 2, Beach asters[17]

Genetical modification

GMO involves altering the DNA of living organisms to introduce, change, or enhance specific traits. DNA, contains the genetic material in cells[18]. For example, crops can be modified by inserting genes from other plants to improve their usefulness. This involves cutting the organism's DNA and inserting a new gene, a process known as transgenic technology when genes are transferred between different species. Humans have been altering plant and animal genetics since the beginning of agriculture by selecting the best seeds[19]. Modern genetic modification goes beyond traditional breeding by transferring traits between different species, including plants, animals, and microorganisms, because DNA is fundamentally the same across all life forms[20].

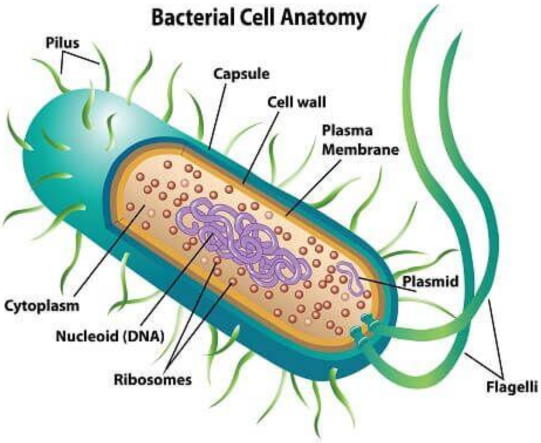

Figure 3 The structure of a prokaryotic cell[18]

[12] (Plantorama, 2023)
[13] (Plantorama, 2023)
[14] (Arvedlund, Ravnsted-Larsen, & Vire, 2012)
[15] (Arvedlund, Ravnsted-Larsen, & Vire, 2012)
[16] (PlanteABC, Unknown date of publication)
[17] (Biology dictionary, Unknown date of publication)
[18] (Biotech Academy, Unknown date of publication)
[19] (Gade, 2014)
[20] (Fødevarestyrelsen, 2005)

How to genetically modify

The following section is based on an article from Biotech Academy[21], which explains step-by-step how to genetically modify plants. Further details were explored on various sections of the Biotech Academy website.

To genetically modify plants, start by isolating the gene of interest from the plant containing it. If the gene is already known, this step is easier. Otherwise, preliminary research is necessary to identify the desired gene. Once the gene is identified, use an enzyme called DNA polymerase to perform PCR to make copies of the DNA. This process doubles the DNA sequence each time the enzyme reacts with it, ensuring enough copies are available for experimentation, as not all attempts at genetic modification will be successful. Next, the gene must be transferred into the plant using a bacterium. Bacteria contain plasmids, which are circular DNA strands that can serve as vectors. A commonly used bacterium is *Agrobacterium Tumefaciens*, which contains the Ti-plasmid. This bacterium can insert a piece of DNA into plant root cells, exploiting plant injuries to do so. Scientists can insert the gene of interest into the Ti-plasmid, creating recombinant DNA.

Restriction enzymes are then used to cut both the donor plant DNA and the Ti-plasmid at specific sequences, allowing the insertion of the desired gene into the plasmid. This recombinant Ti-plasmid can then be used to transfer the gene into the plant cells. One of the most widely used methods to insert a gene into a plant is the particle gun or gene gun[22]. This method physically shoots DNA-coated gold particles into plant cells using a burst of helium, facilitating the gene transfer[23].

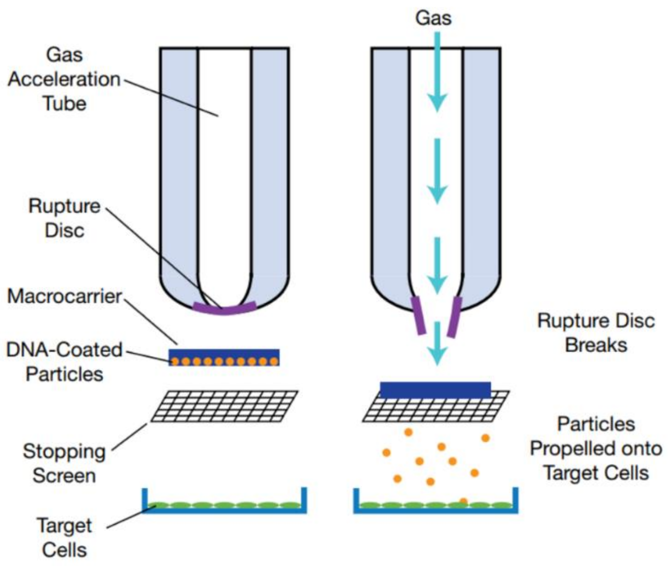

Figure 4, a particle cannon that can deliver a transgene to a cell[25]

[21] (Biotech Academy, 2021)
[22] (Cretive Biolabs gene therapy, Unknown date of publication)
[23] (Turanek & Raska, 2015)
[24] (Cretive Biolabs gene therapy, Unknown date of publication)

After introducing the gene into the bacterium and letting it grow, scientists use antibiotics to isolate successful transformations, eliminating bacteria where the gene insertion failed. The successful bacteria are then introduced to the plant roots, where they transfer the gene into the plant cells.

The plant is then allowed to grow, and its genome is analyzed to confirm the presence of the inserted gene. This results in a genetically modified plant. This method can be used to modify plants to tolerate saltwater, among other applications.

Salt tolerance

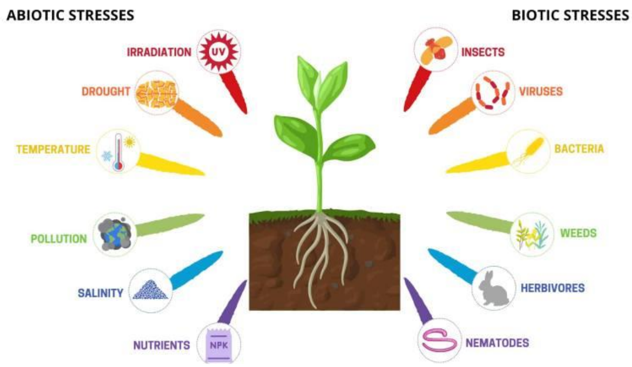

Figure 5 Biotic and abiotic stress[30]

The following section is based on an article from videnskab.dk[25] explaining how to create salt-tolerant plants using a specific gene. We aim to explore whether we can genetically modify crops to withstand saltwater in the future. There are several genetic modification methods relevant here, but we will focus on two that have shown promise in similar cases. As previously described, crops suffer from salt stress, which hampers growth and can lead to plant death[26]. Plants expend significant energy to expel salt, resulting in lower crop yields[27]. The required energy varies by plant type, soil salinity, and environmental conditions. We want to investigate if genetic modification can solve this problem. Researchers have studied a method using the model plant *Arabidopsis thaliana* to enhance salt tolerance. This method involves the HKT gene, which regulates the transport of sodium ions (Na^+) and maintains the balance between sodium and potassium ions (K^+), crucial for plant growth, development, and response to abiotic stress[28]. Abiotic stress is stress caused by non-living factors such as high heat, drought, cold, and salt imbalance, which weakens plants and makes them more susceptible to diseases and pests. The HKT gene family helps maintain ionic homeostasis by excluding excess salt from plant cells, balancing growth and stress tolerance to improve plant survival[29]. The HKT gene codes for a Na^+ transporter protein, and the challenge is to express this gene strongly in the right part of the plant to ensure its proper function.

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25 _(Thomsen R. P., 2009)
26 (Vejdirektoratet, september 2001)
27 (Thomsen R. P., 2009)
28 (Huayang, Guangzhao, Chao, Long, & Zhenchang, 2019)
29 (Fu & Yongqing, 2023)
30 (Osnato, 2023)

Another method for genetically modifying plants to increase salt tolerance is by using the mannitol enzyme Mannitol Dehydrogenase[31]. It belongs to the alcohol dehydrogenase (ADH) family, which functions to convert alcohols to aldehydes and ketones[32]. The Mannitol Dehydrogenase enzyme is coded by the MDH gene[33] and plays a significant role in the metabolism of mannitol, which is widespread in several plants, fungi, and bacteria[34]. This enzyme has been studied for its potential to enhance salt tolerance in plants.

Since Mannitol Dehydrogenase is an osmotically active substance, meaning it attracts water, it enables the plant to absorb water even from dry and salty soil[35]. It has been investigated as an osmoprotective agent, possibly aiding plants in coping with salt stress. Osmoprotectants are substances that accumulate in cells in response to various environmental factors such as high salt content. Their function is to protect cells from damage, including protecting cellular structures under stressful conditions like salt stress[36]. Mannitol dehydrogenase assists in the accumulation of mannitol and fructose. Mannitol acts as an osmoprotective agent, safeguarding plant cells against osmotic stress. By using the MDH gene in plants, we can genetically modify them to better tolerate saltwater.

These two genes, the HKT gene and the MDH gene, could be options for enhancing salt tolerance in plants, something we would like to explore further in the future. However, before determining which plant species to insert the gene into, we will investigate various crops and their ability to express the different genes. We are inclined to work with the MDH gene as we have the gene sequence in *Arabidopsis Thaliana*, allowing us to design restriction enzymes specific to this gene and extract it from this plant.

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[31] (Creative Enzymes, Unknown date of publication)
[32] (Wikipedia, 2021)
[33] (Creative Enzymes, Unknown date of publication)
[34] (Creative Enzymes, Unknown date of publication)
[35] (Møller, 2023)
[36] (Creative Enzymes, Unknown date of publication)

Legislation

If we successfully grow genetically modified salt-tolerant plants, understanding the relevant legislation is crucial for broader implementation. Danish law includes about 44 regulations concerning GMOs to ensure public and environmental safety[37]. GMOs can spread unpredictably, so criteria are needed for their management. Most actions require approval rather than prohibition, but causing harm can lead to penalties, including up to two years in prison.

Legal considerations are essential when working with GMOs. Production should occur in contained environments like laboratories and greenhouses to prevent environmental contact. Approval from the Environmental Protection Agency and the Danish Working Environment Authority is required before starting GMO production[38]. Planting GMOs in nature requires approved soil and a pollen-proof environment, such as a greenhouse.

Using seawater for irrigation has fewer regulations. SEGES-Innovation states that no permission is needed to use seawater for coastal irrigation[39]. SEGES-Innovation states that they are a company focusing on protecting the Earth's resources and ensuring balance, so we assess them to be a credible source. However, according to the section on groundwater, consideration must be given to where seawater is being used for irrigation.

The Influence of Chloride

Chloride (Cl^-), along with nitrogen and calcium, is one of the 16 essential elements for plant growth[40]. Despite this, excessive chloride can cause toxicity. Chloride is a micronutrient needed in small amounts for healthy growth and is crucial for photosynthesis, specifically in Photosystem II

[37] (Petersen J. , 2017)
[38] (Petersen J. , 2017)
[39] (SEGES, 2018)
[40] (Colmenero-Flores, Franco-Navarro, Cubero-Font, & Peinado-Torrubia, 2019)

located in the thylakoid membrane[41]. This system requires two chloride molecules to maintain its structure[42]. However, plants can suffer from chloride toxicity more quickly than sodium toxicity under salt stress, leading to symptoms like leaf burn and defoliation[43]. Therefore, it is crucial to manage chloride levels carefully. In our project, we must ensure that genetically modified plants do not exclude chloride entirely, as it is vital for photosynthesis, while still being able to manage high salt levels to avoid salt stress.

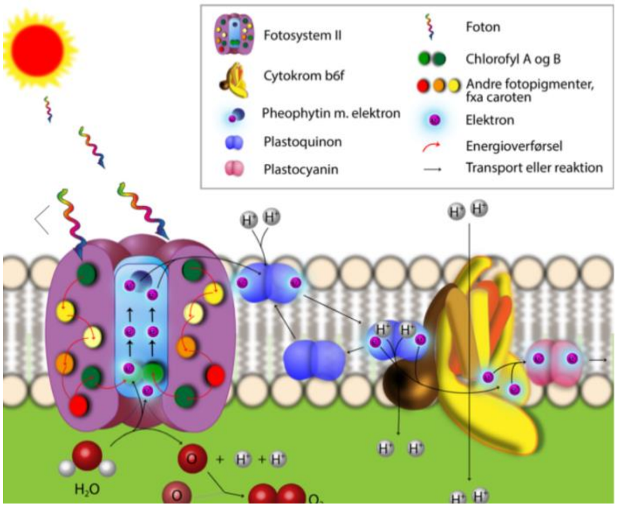

Figure 6, Photosystem II (the purple complex)[44]

Salt's Influence on Soil

When watering plants, not all the water is absorbed. Some evaporates, some is taken up by non-crop plants, and some seeps into the soil. Soil type affects water infiltration; sand allows better flow than clay due to its structure[45]. We wanted to prove that the salt seeped through the different soil types, by which we conducted a titration. This titration proved our theory. However, watering with seawater damages soil structure, particularly through the leaching of salt[46]. Sodium ions (Na^+) harm soil colloids, which are negatively charged particles in clay and humus that can absorb water[47]. This damage affects both the rhizosphere and deeper soil layers, potentially impacting groundwater[48].

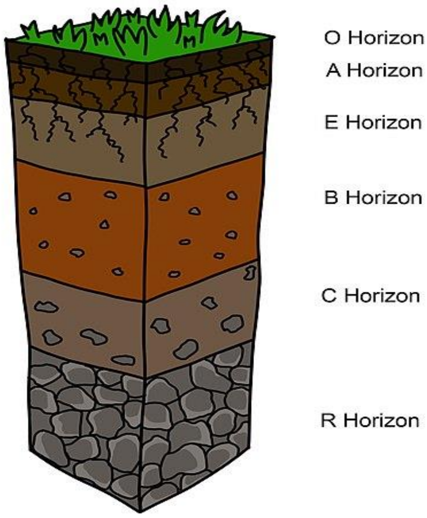

Figure 7 Soil Horizons[49]

[41] (Miljøstyrelsen, Unknown date of publication)
[42] (Colmenero-Flores, Franco-Navarro, Cubero-Font, & Peinado-Torrubia, 2019)
[43] (Miljøstyrelsen, Unknown date of publication)
[44] (PederGasbjerg, Unknown date of publication)
[45] (Therkilden, 2023)
[46] (SEGES, 2018)
[47] (Plantetorvet, Unknown date of publication)
[48] (Andersen, et al., Unknown date of publication)
[49] (Wikimedia, Unknown date of publication)

Cultivated soils, typically a mix of loam, sand, and humus, are found in the A horizon, close to the surface[50]. Soil composition varies regionally in Denmark due to the last ice age, which left different soil types like sand and gravel in certain areas[51]. In Denmark, this variation is evident in the soil's colloid content, influenced by glacial movements during the Weichsel Ice Age[52].

In Norway, a type of clay called "quick clay" becomes unstable when salt is leached out by rain. While watering with seawater might stabilize such clay, the risks to soil structure and groundwater contamination are too high. Therefore, genetically modified plants are best used in controlled environments like vertical farming setups.

Microbiology

Not only are we composed of organisms, but we are also constantly surrounded by them. All living things, whether unicellular or multicellular, are made up of organisms. The Earth is teeming with organisms, including the soil we walk on. According to Denstoredanske.lex.dk[53], there can be up to 100 million bacteria per gram of soil. These soil bacteria are crucial for a healthy ecosystem, helping to purify water by breaking down organic material[54]. One important bacterium, *Aminobacter,* can degrade harmful pesticide residues like BAM[55], a common contaminant responsible for many well closures[56]. *Aminobacter* converts BAM into water and CO_2, improving groundwater quality. As previously mentioned in our section on osmosis, plant cells struggle with salt-induced osmotic stress, and so do bacteria. When exposed to high salt concentrations, bacteria lose water through their cell walls, leading to dehydration and death.

[50] (Petersen, Vejre, & Callesen, 2016)
[51] (Therkilden, 2023)
[52] (Krüger, Binderup, & Jakobsen, 2022)
[53] (Struwe, 2014)
[54] (Jørgensen K. R., Unknown date of publication)
[55] (Holtze & Aamand, 2021)
[56] (Vandguiden.dk, Unknown date of publication)

Vertical farming

Vertical farming is an alternative cultivation method where plants are grown indoors under controlled conditions. This method often uses artificial lighting and hydroponic systems, allowing plants to grow in multiple layers, maximizing space and yield[57]. Vertical farming presents significant potential for cultivating genetically modified plants without the risk of spreading modified genetic material to the external environment. Saltwater can be integrated into the system, utilizing vast ocean resources to grow food for the increasing population. This method avoids soil contamination as water remains in pipes, preventing seepage. We believe vertical farming is a promising, sustainable method that can replace extensive agricultural land, freeing it for forest and biodiversity projects.

Figure 8, Vertical farming[58]

Discussion

In our experiments, we tested various theories, including the salt tolerance of Pilea peperomioides. We aimed to see if its mineral glands could expel salt, as some halophytes do. The experiment ran for 129 days, but both plants, watered with tap water and 3.5% saltwater, struggled. This may be due to damage from insufficient chloride. We concluded that Pilea lacks halophytic properties to withstand salt stress. We also examined soil's ability to retain sodium ions. Results suggest that soil retains salt when watered with saline water, though there were many sources of error. We plan to further explore gypsum's effect on soil. Chloride, rather than sodium, was a significant factor in plant and groundwater health. Protecting our groundwater is crucial. Denmark is fortunate to have abundant groundwater, but maintaining it is essential. Excessive groundwater extraction harms local ecosystems. Salt-tolerant plants could help conserve groundwater, especially in vertical farming where soil conditions are controlled, and water sources can be managed. One challenge is societal acceptance of genetically modified crops. While some oppose this technology, younger generations are more supportive, recognizing the urgency of climate change. Our primary goal is to determine if

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[57] (Therkilden, 2023)
[58] (Solberg, 2023)

genetic modification can make plants salt-tolerant. We consulted with experts at Aarhus University and learned about particle guns and PCR processes in genetic modification. Following further research, we've understood the importance of not completely excluding chloride from plants, as it is vital for photosynthesis.

Conclusion

We can conclude that the consequences of climate change have a significant impact, particularly in Denmark where we face significant water pollution problems. Through plant genetic modification, we aim to create halophytic plants capable of surviving and even improving conditions in future challenges. By isolating the desired gene and using DNA polymerase for PCR to replicate genes, we can insert them into the bacterium Agrobacterium Tumefaciens, which naturally inserts the plasmid DNA into the plant's genome. However, before insertion, the gene must be isolated and cut from the original genome using restriction enzymes. Despite the expense of particle cannons, we explored alternative methods and discovered CRISPR, simplifying gene insertion into bacteria. Once inserted, bacteria transfer the genes to plants, initiating growth. This process, though lengthy and costly, represents a significant project.

Hopefully, this will make plants resistant to salt stress, allowing them to thrive in areas too saline for other plants due to climate change. In practices like vertical farming, utilizing seawater instead of groundwater could conserve resources. However, considerations must be made for soil impact and relevant legislation. While saltwater irrigation alters soil structure, which can be beneficial in some areas lacking salt, legislation on genetically modified organisms (GMOs) is stricter. GMO plants must not come into contact with other plants for civic and environmental safety. Qualitative and quantitative experiments have been conducted to understand soil's response to salt and how glycophytic plants react, illustrating the project's complexity.

Reflection

Throughout this process, our group has delved into the world of GMO's. We've explored, learned, and conducted numerous experiments, gaining valuable new insights. As we consider our next steps, we reflect on the problems we want to further investigate. During the "Unge Forskere" program, we reached out to Professor Henrik Brinch-Pedersen, Section Leader at the Department of Agroecology, Crop Genetics, and Biotechnology. He directed us to Sara Miller, who graciously showed us around and answered our questions in Flakkebjerg, Slagelse, at Aarhus University. This visit was a tremendous opportunity to acquire high-quality knowledge relevant to our project, including observing particle guns and genetically modified plants. In the section explaining the process of genetic modification, it is mentioned that not all gene transfers are successful, and failed recombinant bacteria must be killed with antibiotics. We're interested in exploring and understanding which antibiotics are used for this purpose, possibly drawing from previous projects like the Tiny Earth Project. Additionally, we're keen to investigate other methods such as sequencing. We also aim to further explore which plants are suitable for genetic modification, drawing inspiration from the Department of Agroecology, Crop Genetics, and Biotechnology, where successful plant modifications have been achieved. Furthermore, we'll explore the necessary contacts to scale up our idea. As mentioned earlier, the climate crisis is a pressing issue, and based on our research and gathered knowledge, we believe that the concept of irrigating with seawater could benefit our land and contribute to the fight against natural disasters and extinction events.

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