Protoplast isolation and transformation in sweetpotato

Establishing the optimal parameters for protoplast isolation, culture and transient gene expression to enable efficient gene editing in sweetpotato

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Abstract

In the pursuit of a less restrictive technique to be used for the purpose of gene editing in sweetpotato (*Ipomoea batatas*), both in terms of economic limitations as well as the enormous time and energy inevitably sunk into such a project, we have worked hard to establish optimized parameters for protoplast isolation, and furthermore investigated promising protocols for transformation and regeneration of these protoplasts, to ultimately yield new genotypes.

With this in mind, an extensive literary review of existing protocols, using a wide selection of explants from sweetpotato but also closely related species within the *Solanales* order was conducted, and the data analyzed through multiple advanced statistical models. This has in turn allowed us to investigate possible and even probable correlations between qualitative parameters, for instance protoplast yield and viability, in relation to digestive enzyme concentrations such as cellulase and macerozyme as well as incubation time.

Armed with this theoretical knowledge, our task has then been to validate these findings in practical experiments and ultimately conclude which parameters are most crucial for use in future research into the topic.

Abstrakt

Med målet att utvärdera en mindre restriktiv teknik för genmodifiering i sötpotatis (*Ipomoea batatas*), både gällande ekonomiska begränsningar såväl som det tid och energi bemödande som ett sådant projekt oundvikligen påbrår, har vi arbetat flitigt med att etablera optimerade parameters för protoplast isolation och fortsättningsvis också utforskat lovande protokoll för transformation och regeneration av dessa protoplaster, för att slutligen framställa nya genotyper.

Således har en extensiv litteraturundersökning av tidigare publicerade protokoll, varav explantor från sötpotatis men också relaterade arter inom *Solanales* ordningen har diskuterats, utfärdades varefter datan analyserades via diverse avancerade statistiska modeller. Detta har vidare lett oss till att undersöka möjliga och till och med troliga korrelationer mellan kvalitativa parametrar, exempelvis protoplast utbyte och viabilitet, relativt till nedbrytnings-aktiva enzym koncentrationer såsom cellulas och macerozym samt inkubationstid.

Utifrån denna teoretiska kunskap har vårt uppdrag varit att validera dessa resultat genom praktiska experiment och slutgiltigen fastslå vilka parametrar är av störst vikt för framtida studier inom området.

Preface

This project was carried out between February and May of 2022 as a collaboration between Lunds University and the Inland Norway University of Applied Sciences (INN University) located in Hamar, Norway to further scientific progress in a relatively unexplored yet promising field of research. I want to thank all of those who helped me during this project's active phase but especially Dennis Eriksson (from INN University) who has proven to be a very valuable source of expertise while designing and performing the practical work. I also want to express my gratitude towards Nelida Levia Eriksson who has been my main supervisor from Lund University as well as Rui Guan from the Swedish University of Agricultural Sciences (SLU) for providing me with guidance and support during the course of this project.

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1. Introduction

1.1 General background

The CRISPR-Cas endonuclease system has provided an exceptionally simple, selective and moreover versatile tool for nucleic acid cleavage, for the purpose of gene editing, since its popularization. This is achieved through the CRISPR associated (Cas) endonuclease catalytic function at DNA sites complementary to the designed crispr RNA (crRNA) sequence allowing for introduction of precise double strand breaks. Following this, presuming your goal is the introduction of a new sequence specifically designed for expression in a secondary host, the cell's own DNA-repair machinery can be utilized for homologous recombination with your favorite sequence. While this methodology is fairly standard, multiple alternatives exist for translocation and transformation of the Cas-carrying plasmid (Yue *et al*, 2021). This study will make use of protoplast culture as it is highly suitable in regards to time-constraints.

Protoplast culture refers to cultivation of plant cells which lack cell walls, either through mechanical or chemical intervention. This characteristic of the cells enables direct transfection of genomic material, making extensive as well as oftentimes expensive transformation protocols extraneous. These protoplasts can be regenerated into plants following transfection, thus generating genetically modified plants without necessitating use of tumor inducing plasmids or biolistics for transformation.

Sweetpotato, *Ipomoea batatas*, is a highly nutritious root rich in starch (Medical News Today, 2019, Gurmu *et al*, 2014), and while it is, per its name, very linguistically similar to the common potato they are only distantly related as they both belong to the *Solanales* order. The nutritional value however, as well as the appetizing taste of the sweetpotato, has in recent years made it an attractive candidate crop for cultivation in the Scandinavian peninsula. This is supported by local media, reporting an increase in consumption of sweetpotato exceeding 1600% over 8 years (2010-2018) with comparable numbers originating from Norway (Germundsson, 2020).

Furthermore, according to SCB, a Swedish government agency responsible for producing statistics, the annual import of sweetpotato has seen a sharp increase from 359 in 2010 to 5800 tonnes in 2018. Despite this, the price of sweetpotato actually increased by on average 3.84 Swedish kronor per kilogram over this period, clearly demonstrating the still growing demand for the product (SCB, 2021). Today, all of the sweetpotato on the market is imported, regulating consumental sales of this root vegetable to an import-based market. With this in mind, field trials for cultivation of sweetpotato have been conducted on both sides of the border, at the Swedish University of Agricultural Sciences (SLU) in southern Sweden and by the company Bjertnaes & Hoel at Nøtterøy in southern Norway. Enterprising individuals have also attempted cultivation of sweetpotato in the Scania province of Sweden with mixed results (Martinsson, 2020; NIBIO, 2017).

Problematically, sweetpotato has been extensively documented to be difficult to cultivate in Scandinavian countries at latitudes with long daylight (LD) conditions and relatively low temperatures during the cultivation season. Whereas sweetpotato is native to subtropical environments with short day lengths (SD), this suboptimal climate leads to comparatively slow growth rates as well as poor yields. This could possibly be attributed to photoinhibition, which manifests similar symptoms and has also been shown to develop when sweetpotato is subjected to prolonged periods of relative cold and or irradiation. In fact cultivation in colder climates has been linked to chronic photoinhibition (K. H. Lin *et al*, 2007, Ohnishi *et al*, 2005). However, no matter the underlying cause, this poses a problem for cultivation of sweetpotato in the Scandinavian countries, as this root vegetable is not adapted to survive periods of frost, sustained cultivation in substantially lower average temperatures, between 0 - 15 °C, nor well adapted to the long summer days of the local cultivation season. Instead, an average of 24 °C has been established as optimal for growth (Wijewardana *et al*, 2018).

Thus, genetically modified sweetpotato cultivation with regards to higher chilling stress tolerance, adaptation to prolonged daylight exposure and overall more timely maturation (within 90-100 days after initial seeding) could prove a promising solution to this otherwise diverse and complicated dilemma. However, not much has yet been done in terms of tissue culture and transformation in sweetpotato. Although a few rudimentary protoplast culture protocols are available (e.g. Sihachakr & Ducreux, 1987; Dhir *et al*, 1998; Guo *et al*, 2006). There is to our knowledge also only one published report on CRISPR/Cas9-based mutagenesis in sweetpotato (Wang *et al*, 2019).

1.2 Aim of the project

The ultimate goal of this master thesis project is to establish optimized parameters in protoplast culture protocols as a first step to enable gene editing through CRISPR/Cas in sweetpotato. This will facilitate the development of more adapted and hardy crops. Furthermore this work will further studies with the aim of adapting sweetpotato to the unfavorable climatic and geographic conditions native to Sweden and Norway.

The experimental framework for this project will consist of an investigation of; how parameters such as enzyme concentration and incubation time affect the efficiency of viable protoplast isolation, optimize the parameters for protoplast transfection, such as polyethylene glycol (PEG) concentration, and optimize the parameters of the protoplast-to-microcalli regeneration protocol. Primarily focus was placed on two sweetpotato genotypes, Mira and Rosa, from Elitplantstationen in the Scania region of Sweden, with the goal of testing further genotypes and different tissues such as leaf and petiole - depending on the available time frame.

2. Theoretical background

2.1 Growth regulators

To facilitate and support an enhanced growth rate both in terms of *in-vitro* propagation and protoplast regeneration usage of plant growth regulators (PGR) is crucial. PGRs included in the auxin and cytokinin classes are widely used for these purposes as all propagation and regeneration protocols consulted in the literature research have demonstrated (see Appendix A through D).

As is made quite clear by the terminology, cytokinin functions through promoting cytokinesis and thus cell division. This is achieved through the phosphorylation of a histidine kinase (HK) receptor, which may then act on type B response regulators (RR). RR may in turn function as transcription factors, regulating the transcription of various genes (Hutchison & Keiber, 2002). Specifically RR10, which is part of the cytokinin-mediated response pathway, has been shown to promote and act upstream of genes involved in callus formation, primarily root development, carbohydrate (anthocyanin) metabolism, synthesis of chlorophyll, root meristem growth, seed growth and shoot development (Argyros *et al*, 2008, Meng *et al*, 2017, Hill *et al*, 2013).

There are two types of cytokinins; the phenylurea type which are not naturally occurring in plants and the adenine-related cytokinins which are typically produced and stored in roots. Though it should be noted the latter are not exclusive to plants as kinetin, an adenine cytokinin, has been positively identified in human cell cultures as well (Naseem *et al*, 2020).

Another exception to adenine type cytokinins being produced and naturally occurring within plants is 6-benzylaminopurine or rather benzyl adenine (BAP) which is a synthetic PGR (ACS, 2016). In addition to promoting shoot elongation and propagation BAP actively inhibits vertical growth terms of both rooting and branching, thus focusing resources and development vertically. This is a lasting effect as BAP may accumulate in the tissue in the form of conjugated chains, therefore prolonging the prioritization of shoot development over accretion of a root system (Podwyszynska, 2003). For this reason several protocols for protoplast regeneration recommend gradually reducing the BAP concentration of the medium to be more conducive to rooting as the propagation proceeds (see Appendix D).

Alternatively, inclusion of auxins may also stimulate and promote rooting. In fact, natural differential concentration of auxin stored within the plant tissue serves to directly influence and guide hydro-, geo-, and phototropism, which refer to how the plant growth is affected by its immediate environment and factors such as water availability, gravity and light intensity respectively. In short, variability in auxin concentration can be considered the plant's way of recognizing critical resources and promoting growth towards such resources. This growth includes development, lateral branching and accretion of root structures in general. The effect is

further strengthened by the presence of gibberellins such as gibberellic acid (GA_3) whereas combination with cytokinin stimulates cell division (Benková *et al*. 2003).

A popular auxin that has seen widespread use in experimental explant propagation as well as callus regeneration from protoplasts is 1-Naphthaleneacetic acid (NAA), which is another synthetic PGR (Flasinski & Hac-Wydro, 2014).

2.2 Enzymatic cell wall digestion

The composition of the cell wall inherent to plants found on land has a general structure shared among the vast majority of species, sweetpotato included. This structure is predominantly made up of different polysaccharides such as cellulose, hemicellulose and pectin, as well as smaller compounds functioning as anchoring points for the superstructure. While this is a massive simplification, to go into more detail, the outermost section consisting of “cellulose-fibers”, classically referred to as microfibrils, are anchored through hemicellulose strands to the underlying pectin matrix. Thus, while a multitude of viable and effective techniques for removal and or degradation of the cell wall exist, generally categorized as either mechanical or enzymatic, the latter tend to focus on digesting either cellulose, hemicellulose, pectin or a combination of the three. For this purpose there are three classes of enzymes that are regularly employed, corresponding to cellulases, hemicellulases and pectinases (see Appendix B & C).

Cellulases are a class of hydrolytic enzymes that catalyze cellulolysis or hydrolysis of the polysaccharide cellulose into monosaccharides such as β-glucose, or shorter polysaccharides and oligosaccharides (Jayasekara & Ratnayake, 2018). As such cellulases are enzymes that break down cellulose. Similar to these, hemicellulases are enzymes that break down material typically associated with or attached to cellulose. This category includes a multitude of digestive enzymes. (Yi, 2021). Pectinase is also an umbrella term, including enzymes such as pectolyase, pectozyme and polygalacturonase and work through the hydrolysis, transelimination and deesterification of pectin. (Singh, 2019).

While a number of protoplast isolation protocols also reference commercially available digestion enzyme mixtures such as driselase and carbohydrase viscozyme in addition to cellulase, hemicellulase and pectinase, the active chemicals in these products remain some combination of these groups of enzymes (Creative Enzymes, 2022, Garcia de Figueiredo *et al*, 2018).

2.3 Plasmolysis

The effects of pre-treatment via incubation in a plasmolysis solution has been found to be effective in separating the plasma membrane from the cell wall, thus making protoplast isolation more effective (Yue *et al*, 2021). In fact, many articles on protoplast isolation seem to include a pre-digestion step where most commonly the explant is sliced and subjected to hypo-osmotic pressure, with some variation as to the exact specifications of the solution. According to Nicolia

et al (2021), who optimized protoplast isolation for potatoes, the material should be incubated in the plasmolysis solution for 30 minutes.

Thus, this will also be included in the protoplast isolation protocol in sweetpotato.

2.4 FDA staining

A fluorescein diacetate (FDA) hydrolysis assay is a popular viability assay used to measure both enzymatic activity and microbial viability in solution. In short, FDA is a membrane-permeant molecule which functions as a substrate for esterases present in the cytoplasm of viable cells, the product of which is a dissociated fluorescein with excitation and emission wavelengths at 498 and 517 nm respectively (Fontvieille *et al*, 2015, Vitecek *et al*, 2007, AAT Bioquest, 2022). The latter wavelength corresponds, in practice, to a light green fluorescence which makes viable cells quite simple to distinguish from their non-viable counterparts. It should however be noted that the FDA staining alone does not allow for differentiation between protoplasts and intact cells in general.

2.5 YFP plasmid

A pGEM® T-based vector from ProMega, encoding for a yellow flourescent protein (YFP), will be used as a visible marker for successful transfection of viable protoplasts (Sainsbury *et al*, 2009). For this purpose the bacterium *Escherichia coli* (*E. coli*) carrying the plasmid has been made available for our use. While these bacteria have been stored in the freezer at -80 °C for an extended period of time, these will be easy to regenerate to the required densities over a few hours. Beyond the YFP marker, the plasmid is equipped with an AmpR gene, enabling selection through ampicillin resistance. (Sutcliffe, 1978, McLean, 2018).

Beyond this the plasmid is also equipped with a ubiquitin promoter allowing for constitutive expression of the YFP protein in all successfully transformed cells. This protein can then be excited by light with a wavelength of 513 nm which then allows detection of fluorescence emission at 530 nm (AAT Bioquest, 2022). Figure 1 below showcases a graphic representation of the plasmid for clarity.

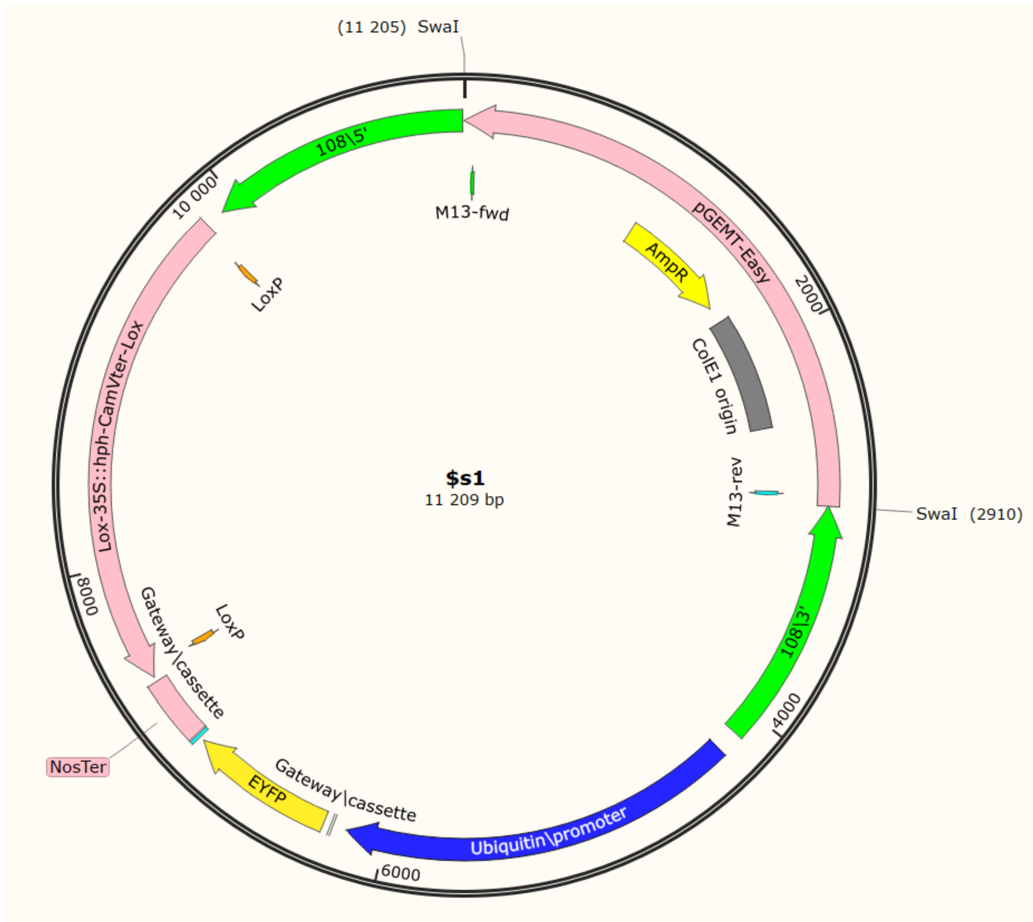

Figure 2.1. *A graphical representation of the YFP carrying plasmid discussed above.*

Incubation of the isolated protoplasts within a medium containing both the discussed plasmid as well as polyethylene glycol (PEG), allows for transportation of the plasmid across the intact cellular membrane, thus enabling expression of YFP from within the cytoplasm.

3. Methodology

3.1 General overview

The general methodology consists of; *in vitro* propagation of sweetpotato shoots, protoplast isolation of propagated leaf material, transfection of isolated protoplasts and ultimately callus regeneration of transfected material.

3.1.1 Materials of special note

Growth cabinet:
The growth cabinet referenced in various protocols is the Versatile Environmental Test Chamber by SANYO Electric Co., Ltd. (MLR-351H model) supplied, installed and operated in accordance with IEC 1010-1 (General Safety Requirements) (SANYO, 2022).

Sterile workbench:
The sterile workbench referenced in various protocols is the Heraguard ECO Clean Bench by Thermo Fisher Scientific (Heraguard ECO 50138900 model) (Thermo Fisher Scientific, 2013).

Microscope:
The microscope referenced in the protoplast isolation protocols is an inverted light microscope with fluorescence filter capabilities by Nikon (T*i*-U model) (Nikon, 2016).

Propagation containers:
The propagation containers referenced in the propagation protocols are Sterivent High Containers (107 x 94 x 96 mm) by Duchefa.

Cell strainer:
The cell strainer referenced in the protoplast isolation protocols is the VMR Cell Strainer (100 µm pore size) by Avantor.

Plasmid extraction kit:
The extraction kit referenced in the inoculation of plasmid for transfection protocol is the Qiagen® Plasmid Midi Kit (100) by Qiagen.

Based on the preliminary literature study as well as statistical and mathematical analysis of relevant data collected from contemporary and comparable protocols, these standard solutions were established for use in all protocols;

BAP stock solution:
The standardized BAP stock solution contained 0.1% BAP in autoclaved distilled water.

NAA stock solution:
The standardized NAA stock solution contained 0.1% NAA in autoclaved distilled water.

Propagation media:
The standardized propagation media contained 3% sucrose and 0.6% agar in autoclaved distilled water.

Plasmolysis solution:
The standardized propagation media contained 9% sorbitol in autoclaved distilled water.

Isolation media:
The standardized isolation media contained 0.44% MS, 3% sucrose and digestion enzymes in accordance with the experimental plan in autoclaved distilled water. pH set at 5.8.

Cellulase R-10:
The cellulase used for digestion in the protoplast isolation protocols is "Cellulase Onozuka R-10" with an enzyme activity estimated to exceed 10000 units per gram by Duchefa.

Macerozyme R-10:
The macerozyme used for digestion in the protoplast isolation protocols is a mixture of enzymes including pectinase with an estimated activity of 500 units per gram, cellulase with an estimated activity of 100 units per gram and hemicellulase with an estimated activity of 250 units per gram by Duchefa.

Regeneration media:
The standardized regeneration media contained 0.44% MS, 3% sucrose, 0.00004% BAP and 0.0001% NAA in autoclaved distilled water. pH set at 5.8.

Washing solution:
The standardized washing solution contained 0.44% MS, 9% sucrose and 0.5% calcium chloride in autoclaved distilled water. pH set at 5.8.

Alginate solution:
The standardized alginate solution contained 2.8% alginic acid sodium salt and 7.5% sorbitol in autoclaved distilled water.

Setting gel:
The standardized setting gel contained 0.6% bactoagar, 7.3% sorbitol and 0.735% calcium chloride in autoclaved distilled water.

Ampicillin stock solution:
The standardized ampicillin stock solution contained 10% ampicillin sodium salt in autoclaved distilled water.

LB agar plates:
The standardized LB agar plates are sterile agar plates coated in a solidified solution of 1% tryptone, 0.5% yeast extract, 1% sodium chloride, 1.5% bactoagar and 0.01% ampicillin sodium salt in autoclaved distilled water. pH set at 7.0.

LB medium:
The standardized LB medium contained 1% tryptone, 0.5% yeast extract and 1% sodium chloride in autoclaved distilled water. pH set at 7.0.

Transformation medium:
The standardized transformation medium contained 25% PEG, 7.3% mannitol and 2.4% Calcium nitrate tetrahydrate in autoclaved distilled water.

3.2 *In-vitro* propagation

3.2.1 Initial *in-vitro* propagation

The propagation media was autoclaved 121 °C for 30 minutes to sterilize the media after which it was allowed to cool at 4 °C for approximately 30 minutes. After transferring the propagation media to the sterile workbench, 1 milliliter BAP stock solution and 0.01 milliliter NAA stock solution into the propagation media per 1 liter. The media was mixed thoroughly by gently shaking the flask. Following this approximately 150 milliliters of propagation media was transferred to each propagation container and allowed to solidify over the course of upwards to 60 minutes.

Nodes were isolated from the plant material using a sterilized (1% sodium hypochlorite, 70% ethanol) sharp razor blade. The nodes were then washed in sodium hypochlorite (1%) for 15 minutes and ethanol (70%) for 1 minute, followed by distilled water three consecutive times. Concluding this washing process the nodes were transplanted in the solidified propagation media and incubated in the growth cabinet at light exposure ratio 16:8 hours, 35 µmol m^{-2} s^{-1}, 25 °C and 70% relative humidity.

In case of fungal or otherwise microbial infection, the affected nodes were discarded and the remaining nodes subjected to the same washing procedure as described above.

The nodes were then replanted into fresh propagation medium on a monthly basis. In conjunction with this replantation the nodes were washed in sodium hypochlorite (1%) for 15 minutes and

ethanol (70%) for 1 minute, followed by distilled water three consecutive times before being transferred to new propagation containers.

3.2.2 Adjusted *in-vitro* propagation

This adjusted protocol includes the same procedures and steps as described in 3.2.1, with the exception of the exclusion of washing of the nodes in conjunction with replantation every month interval, as well as using petri dishes instead of propagation containers.

3.3 Protoplast isolation

3.3.1 Isolation experiment 1

Leaf and petiole material was gathered from the shootings set in soil, whereafter the weight of each instance was noted. The material was then thoroughly washed with autoclaved distilled water, after which it was cut into pieces between 1-2 millimeter in diameter in fresh petri dishes using a sterilized (70% ethanol, 1% sodium hypochlorite) sharp razor. The leaf material was then allowed to soak in autoclaved distilled water for 24 hours before continuing with the experiment.

The material was allowed to soak in 15 milliliters plasmolysis solution for 30 minutes at room temperature while covered with aluminum foil. The plasmolysis solution was then removed through pipettation and the material was further incubated for 4 and 8 hours at 25 °C in isolation media while covered in aluminum foil. The isolation media tested at this point pertained to four combinations of cellulase R-10 (at 1% and 2%) and macerozyme R-10 (at 0.2% and 0.8%) for leaf and petiole material, see Table 3.1.

Table 3.1. *Overview of experimental treatment ID's and the parameters tested in isolation experiment 1, where L and P refers to leaf or petiole material respectively.*

Experimental treatment ID	Incubation time, hours	Cellulase R-10 concentration, %	Macerozyme R-10 concentration, %
L111	4	1	0.2
L112	4	1	0.8
L121	4	2	0.2
L122	4	2	0.8
L211	8	1	0.2
L212	8	1	0.8
L221	8	2	0.2

L222	8	2	0.8
P111	4	1	0.2
P112	4	1	0.8
P121	4	2	0.2
P122	4	2	0.8
P211	8	1	0.2
P212	8	1	0.8
P221	8	2	0.2
P222	8	2	0.8

The petri dish was incubated for a further 30 minutes with shaking at 50 rpm and room temperature. At this point a 100 microliter aliquot (aliquot 1) was taken and stored at 4 °C for analysis at a later time.

The protoplast suspensions were filtered through a sterilized nylon cell strainer with 100 µm pore size into 50 milliliter centrifuge tubes and diluted up to 20 milliliters with washing solution. A second 100 microliter aliquot (aliquot 2) was taken and stored at 4 °C for analysis at a later time.

The tubes were then centrifuged at 50 g for 5 minutes, following which the supernatant was discarded and the pellet resuspended in 1 milliliters regeneration media. A 100 microliter third aliquot (aliquot 3) and a quaternary 100 microliter aliquot (aliquot 4) was taken off the supernatant and resuspended pellet respectively and stored at 4 °C for analysis at a later time.

900 microliter of alginate solution was pipetted into each tube and mixed by gently pipetting up and down. This alginate mixture was transferred to the surface of setting gel petri dishes and allowed to solidify for 2 hours at room temperature.

Approximately 1 milliliter of floating solution was used to remove the intact solidified alginate lens from the setting gel and the alginate lens was transferred to a fresh sterile petri dish. An approximate 10 milliliters of regeneration media was poured into the petri dish, after which the petri dish was covered in aluminum foil and incubated for 5 days in the growth cabinet.

The light intensity was then gradually increased by replacing the aluminum foil with white tissue paper and subsequently removing it entirely when microcalli visible to the naked eye were observed. Following this, fresh regeneration media was provided every week.

The aliquots were stained by fluorescein diacetate (FDA) in a protoplast suspension to FDA standard solution ratio of 100:2, and allowed to incubate at room temperature for 30 minutes in the dark. The cell density was then determined by counting the average number of cells distributed throughout 5 separate 1000x1000 micrometer squares in a hemocytometer. In addition to this, the average number of viable cells, cells corresponding to protoplasts and viable protoplasts were also noted for the same 5 squares for each aliquot and treatment.

3.3.2 Isolation experiment 2

Following the previous isolation experiment (see 3.3.1) and subsequent statistical analysis a secondary isolation experiment was carried out with the aim of further optimizing the incubation time within the isolation media for leaf tissue. The enzyme concentration was held constant at 2% cellulase R-10 and 0.8% macerozyme R-10, whereas the tested incubation times are shown in Table 3.2 below.

Table 3.2. *Overview of experimental treatment ID's and the parameters tested in isolation experiment 2.*

Experimental treatment ID	Incubation time, hours
L2	2
L3	3
L4	4
L5	5

As for the methodology, a conscious effort was made to carry out isolation experiment 2 according to the same protocol as the previous isolation optimization experiment as described in subsection 3.3.1.

3.3.3 Isolation experiment 3

To investigate the validity and reliability of the conclusions drawn from the results of isolation experiment 2 (see 3.3.2) the same experimental methodology was repeated wíth the aim of reproducing the same results.

3.3.4 Isolation experiment 4

This experiment, while not planned, was reappropriated with the aim of investigating the effect of no plasmolysis treatment prior to isolation of the leaf material. Protoplasts from two leaves were incubated in the standardized isolated medium containing 2% cellulase R-10 and 0.8% macerozyme R-10, for 2 hours.

As for the methodology, a conscious effort was made to carry out isolation experiment 5 according to the same protocol as the previous isolation optimization experiment as described in subsection 3.3.1. However, due to a laboratory error no plasmolysis treatment was conducted prior to the isolation. This deviation from the established method did however offer us valuable insight into the effectiveness of this particular step of the methodology.

3.4 Inoculation of plasmid for transfection

A sterile pipette tip was used to retrieve a smaller amount of *E. coli* culture containing the YFP plasmid structure from the frozen stock (at -80 °C), and this was then smeared out over an LB agar plate and incubated at 37 °C overnight. A single colony was extracted and inoculated into approximately 40 ml of LB medium with ampicillin sodium salt (1 milliliter per 1 liter medium). The inoculate was then incubated for several hours until the internal OD value had reached a point deemed satisfactory for isolation of a sufficient amount of material to be used for transfection. At this point the bacterium was separated from the medium through centrifugation. The plasmid was then extracted using the plasmid extraction kit from Qiagen.

Approximately 100 000 protoplasts in total were then exposed to the purified plasmid within the specially prepared transformation medium as described in subsection 3.1.1 for 3 minutes and the resulting transformation efficiency determined via cell counting under a fluorescence filter for 530 nm light.

3.5 Callus regeneration

As explained in subsection 3.3.1 the callus regeneration protocol simply corresponds to carefully increasing the light intensity (up to 35 µmol m^{-2} s^{-1}) over several weeks while providing a constant 16:8 hour light exposure ratio at 25 °C. The regeneration medium was also changed on a weekly basis.

Due to the limited time allotted to the project and severe delay in the delivery of materials due to the *Covid* pandemic, no actual experiments were conducted on callus regeneration beyond these attempts.

4. Results & Discussion

4.1 Establishing parameters and interval of interest

4.1.1 *In-vitro* propagation

Based on the literature research alone, some parameters for *in-vitro* propagation protocols could be established. For instance, the 55 protocols consulted for sweetpotato propagation all used MS (Murashige and Skoog) plant cultivation base medium, indicating this a quite popular, well documented and more importantly effective base medium, suggesting it provides sufficient quantities of all essential vitamins, macro- and micronutrients (see Appendix A). Furthermore, although using half-strength MS remains a viable option, Dewar *et al* (2020) demonstrated that the seemingly only advantage to this was an increase in tuber length at the expense of the number of nodes and shoots, which would be counterproductive to our purposes. That is, continuous generation of petiole and leaf material for protoplast isolation and regeneration.

In a similar vein to base cultivation medium; gel composition, propagation pH, temperature, light exposure ratio, light intensity, humidity and carbon source concentration could also be established without deeper analysis. See subsection 3.2 for exact parameters and values.

In an attempt to further summarize and identify more patterns in the information gathered from a multitude of articles, a multivariate analysis was performed using Matlab. However due to a vast variation in how articles report the degree of success of different protocols, it was challenging to find common qualitative parameters to compare quality. For example, tuber length, fresh weight, quantity of nodes, quantity of shoots and percent of shoots over different time frames are merely the most common parameters used in an attempt by different researchers to estimate effectiveness of a given propagation protocol. To make this even more complicated, a surprising amount of data was rendered unusable as the authors simply did not report the time span over which the data was collected - making it impossible to compare to results from other articles.

Despite these complications a preliminary analysis was conducted using 7 different protocols (Abubakar *et al*, 2018) through a basic partial least squares regression to investigate the relation between the quantity of shoots and shoot length versus different growth regulators such as BAP and NAA. The results of which confirmed what several independent articles had already concluded. That being, while both the quantity of shoots and shoot length is positively correlated with BAP concentration in the propagation medium, growth abnormalities may result at concentrations around 2.5 mg/l and higher (Beyene *et al* 2020, Dewar *et al* 2020, Buko 2019). In other words, while growth regulators quite obviously have a positive effect on the growth of the plants, at a certain point an increase in concentration may actually have a negative correlation with growth.

Investigation of a further 38 protocols for propagation of sweetpotato nodes through a statistical analysis (at 95% significance) concluded that the optimal concentration of BAP and NAA in terms of percent of shoots was 1 mg/l and 0.01 mg/l respectively (Dugassa & Feyissa 2011). Based on these findings, the standard propagation medium solution was established as can be found described in 3.1.1.

4.1.2 Protoplast isolation

Based on the literature research of protoplast isolation protocols using different tissue origins, parameters of interest were isolated to carbon-source concentration of the isolation media, incubation temperature, incubation time and digestion enzyme concentration in 19 individual protocols (see Appendix B). As for the latter, specifically Cellulase R-10 in combination with macerozyme R-10 and pectolyase Y-23 were noted as the most abundant and widespread in use.

Performing a preliminary partial least square regression to investigate the relation between the protoplast yield and viability versus these parameters indicated a positive correlation between hemicellulase concentration and protoplast viability (80% significance) and a positive correlation between macerozyme R-10 concentration and protoplast yield (95%). Positive correlations between incubation time and yield as well as a corresponding negative correlation between incubation time and viability was also identified, though at a low significance.

In an effort to increase the significance between identified relations and investigate further, a secondary regression was performed using data collected from articles only pertaining to isolation protocols using plant material originating from the *Solanales* order. For this purpose an additional 36 protocols were consulted (see Appendix C). In addition to the parameters previously discussed, explants besides nodes, shaking during incubation and sieve pore size were also included in this analysis.

Incubation time was confirmed to be positively related to protoplast yield at 92.5% significance, though the hypothesized effect on viability could not be ascertained. In addition, sieve pore size was found to be strongly correlated with an increase in yield and a decrease in viability at 95 and 90% significance respectively. This would be expected as predictably less extreme filtration should lead to a direct decrease in loss of protoplasts while also letting more cellular debris through into the protoplast suspension. Beyond this pectolyase Y-23 concentration and temperature during incubation were identified as factors strongly associated with protoplast yield (at 95% significance), though pectolyase Y-23, temperature as well as cellulase R-10 concentration were connected to a decrease in protoplast viability - likely dependent on incubation time (at 90% and 80% respectively). Unfortunately no significant effect was observed based on the material used for protoplast isolation, though it was noted the most common explants remained leaf and petiole tissue.

While these are decidedly not conclusive results, ignoring the inherent differences between species within the *Solanales* order to not mention differences between genotypes within individual species, it does serve to indicate which parameters are of interest to investigate further in experiments to be conducted. Based on these findings, digestion enzyme concentrations and incubation time were identified as the most important parameters to optimize. Furthermore, shaking during enzymatic digestion should be avoided.

4.1.3 YFP transfection

As for the transfection of isolated protoplasts, the relevant parameters could quite easily be isolated to just plasmid concentration, PEG concentration and incubation time within the transfection medium - based on the literature review. Ideally, another parameter of interest would have been the CRISPR-Cas module concentration, which would in theory have enabled targeted mutagenesis of the cells, though this was never investigated due to material- as well as time constraints placed on the practical work.

4.1.4 Callus regeneration

Based on the literature research of protoplast regeneration protocols using different tissue origins, parameters of interest were isolated to starter culture density, base medium, carbon-source concentration, auxin (specifically NAA) concentration as well as cytokinin (BAP) concentration in 30 individual protocols (see Appendix D).

Performing a preliminary partial least square regression to investigate the relation between the time to division and time to microcalli versus these parameters indicated time to microcalli is negatively dependent on NAA concentration at 60% significance. Unfortunately the poor comparability and quality of the data did not allow for any more insight into any further factors correlated to regeneration rate. However, this does serve to demonstrate that growth regulating hormone concentration should be optimized in further experiments.

While the idea of conducting a more in-depth analysis on only regeneration protocols relating to the *Solanales* order or even specifically sweetpotato, this was deemed unnecessary. Based on the available literature, the parameters of interest could be isolated to PGR concentrations and starting density of protoplasts.

4.2 *In-vitro* propagation trials

4.2.1 Initial *in-vitro* propagation

As part of the *in-vitro* propagation, plant material corresponding to the Rosa genotype and the Mira genotypes were used. While the initial propagation attempt was successful, it was noted that the growth of the nodes was slower than literature research of comparatively similar propagation attempts had indicated should be expected (see Figure 4.1a). Despite this, it is easy

to see the root structure developing and shooting off of the nodes in the figure below. In addition to this, abnormal growth of the submerged nodular stem was observed in all surviving cases (see Figure 4.1b).

Figure 4.1. *Pictures taken of the nodes in a Rosa in-vitro propagation attempt following; a) 18 days and b) 21 days of cultivation.*

The latter could be an indication of an overabundance of growth regulators, more specifically BAP, but also possibly NAA as the latter is more readily responsible for root development. On the other hand the literature research conducted would indicate concentrations of 1 mg/l of BAP and 0.01 mg/l of NAA should not result in abnormal growth. Thus we were uncertain as to the cause of these nodes and it is entirely possible this is normal for propagation of sweetpotato nodes.

Unfortunately, all containers containing nodes of the Mira genotype were inevitably affected by fungal growth and had to be discarded (see Figure 4.2). As such, this phenomenon was only observed in relation to the Rosa genotype.

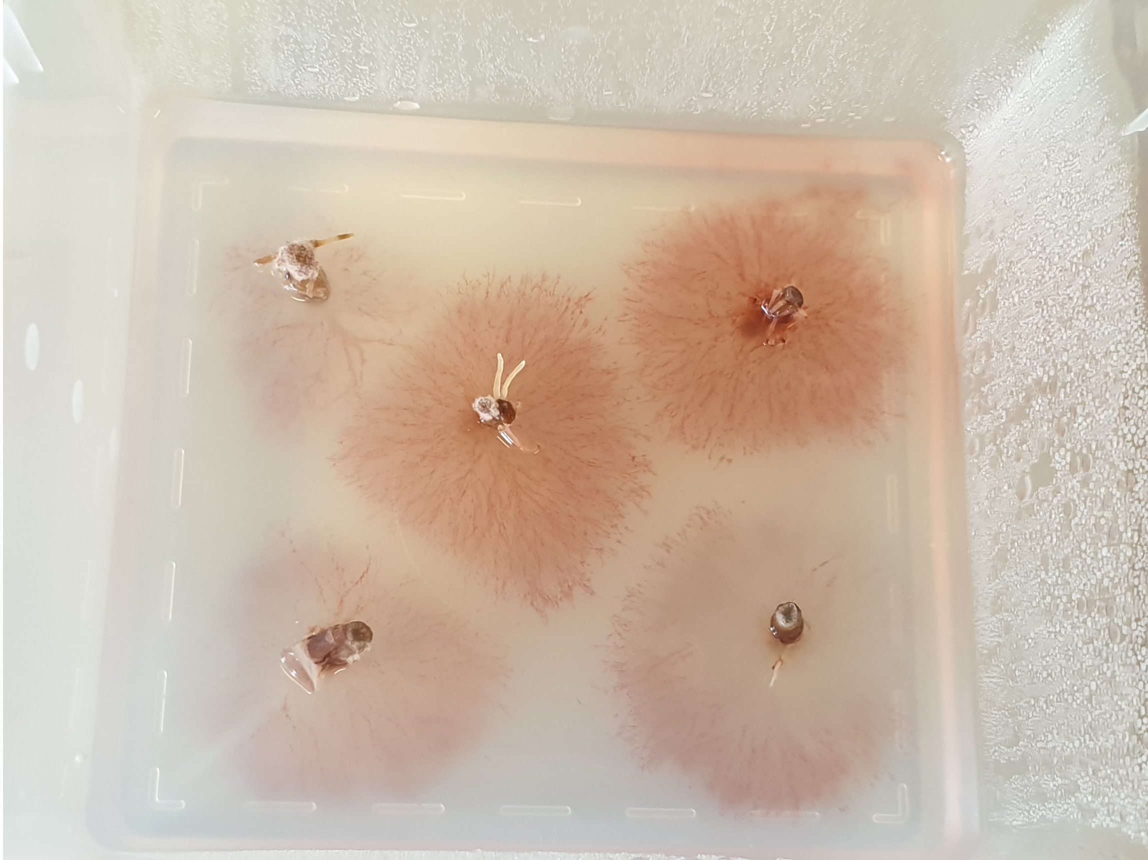

Figure 4.2. *Pictures taken of the nodes in an in-vitro propagation attempt infected with fungal growth.*

Another factorial worth of note is the negative effect of washing the nodes in ethanol and sodium hypochlorite in conjunction with the replantation into fresh medium. While such extreme treatment may indeed be necessary for sterilization and removal of fungal infections and alike, this washing attempt seemed to effectively kill the previously thriving nodes as well. Thus it would be ideal to avoid such rough treatment in the future unless the nodes are actually infected (see 3.2.2 for the adjusted propagation protocol). Below is a figure demonstrating the long term effects of this washing procedure.

Figure 4.3 *Pictures taken of the nodes in a Rosa in-vitro propagation attempt following 75 days after washing. No noticeable growth has been observed during this time frame.*

It should also be noted that washing of infected nodes had a similar effect on the growth rate of these nodes, as a very clear distinction could be made between nodes that had been washed and then replanted and nodes that had been left undisturbed since the initial planting. In the latter cases, the root systems were expansive and some fresh shoots were beginning to take form, while in the other cases no new growth could be identified with certainty following the washing procedures.

It was later realized that the BAP that was readily available and used in the propagation medium was likely old and thus of limited effectiveness. This then would have directly led to a lower effective activity of the PGR on the node's vertical growth. In addition to not providing enough focus to development vertically, this could have been a contributing factor to the previously noted abnormal growths of the root system (see Figure 4.1b). For this reason, a new *in-vitro* propagation attempt was initialized using fresh BAP with the hope of speeding up the time-line as well as possibly avoiding these undesired nodular stem growths.

4.2.2 Adjusted *in-vitro* propagation

As part of the adjusted *in-vitro* propagation, plant material corresponding to the Rosa genotype and the Mira genotypes were also used, though no distinction was made for either origin.

It was quickly discovered that the progression rate of the propagation trial had improved considerably as compared to the initial propagation attempt, although it is difficult to estimate by exactly how much. Nevertheless this led credence to the theory of BAP's effectiveness being affected by prolonged storage as using newly aquisted BAP was the only major difference between these propagation attempts.

Figure 4.4. *Pictures taken of the in-vitro propagation attempt using the adjusted protocol following; a) 13 days and b) 27 days of cultivation.*

However, the same nodular stem growths were observed in all instances despite these differences, as can be seen both in Figure 4.4a and 4.4b. Thus this rules out the age of the BAP being the cause for this phenomenon and instead lending support to the idea of this simply being part of the development of the root system for nodes.

4.3 Isolation experiments

4.3.1 Isolation experiment 1

The cell counting following the first isolation experiment as presented in Table 3.1 was collected in a spreadsheet after which the average viable cell yield as well as average viable protoplast yield could be calculated in regards to each treatment and plant material origin, normalized as an amount of cells per and milliliter and gram of fresh weigh. Table 4.1 below includes these normalized values inherent to aliquot 4 for each treatment.

Table 4.1. *Overview of experimental treatment ID's and the resulting total viable cell yields and viable protoplast yields in aliquot 4 based on isolation experiment 1. The experimental ID's relate to the experimental parameters represented in Table 3.1.*

Experimental treatment ID	Total viable cell yield, cells ml^{-1} gfw^{-1}	Viable protoplast yield, cells ml^{-1} gfw^{-1}
L111	81301	10163
L112	110497	33149
L121	107643	43057
L122	289505	48251
L211	8760	0
L212	9551	0
L221	11230	0
L222	124224	20704
P111	32026	16013
P112	167625	0
P121	39894	17730
P122	25790	0
P211	0	0
P212	161435	0
P221	94062	141093
P222	195122	0

While it could be argued that counting all aliquots (especially aliquot 1 through 3) should not give any additional information than simply focusing our efforts on aliquot 4, this was done primarily to ascertain that no major loss of cells occurred in the washing process described in the 3.3.1 methodology. Through this effort it was indeed discovered that a significant amount of cells were present in aliquot 3 which relates to the discarded supernatant before resuspension of the pellet relating to aliquot 4. Thus in the future, additional centrifugation steps should be employed to hopefully decrease the loss of yield in this step. However no further conclusions could be made based solely on the collected data. Instead, an extensive mathematical analysis was conducted in MATLAB.

The differentiation of whole cells versus protoplasts, as mentioned when discussing the underlying theory behind FDA staining, could not be achieved objectively - but were thus instead judged on an individual basis based on the sphericality of the cells. In theory, the osmotic pressure of the cytosol of any cell lacking the otherwise topographically limiting cell wall should force the cell to take on a more round appearance, allowing for easy identification. An example of this effect can be seen below in Figure 4.5.

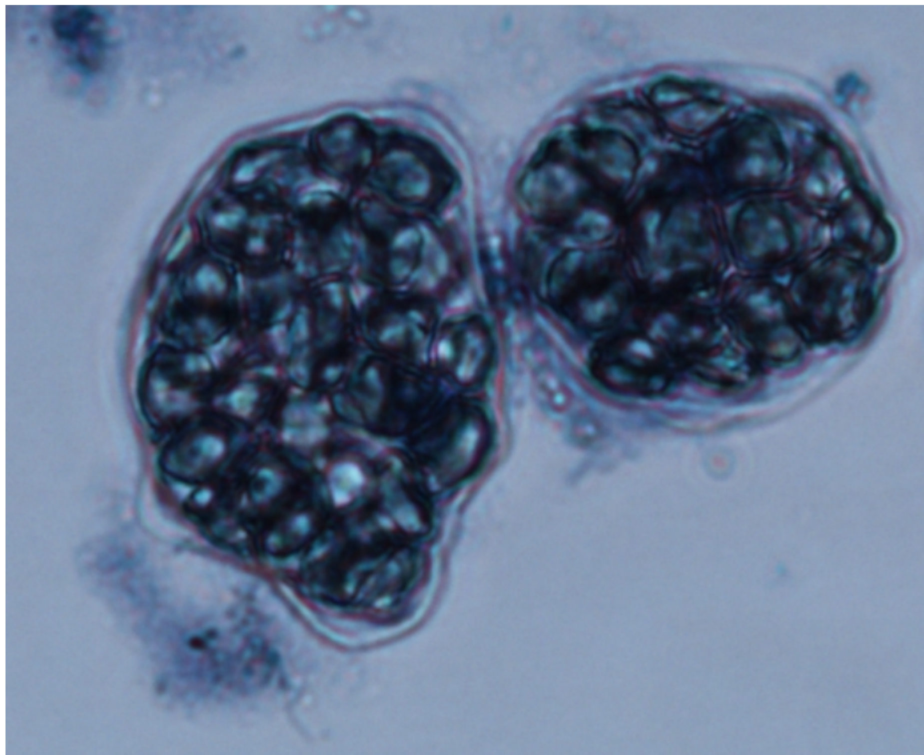

Figure 4.5. *Picture of a supposed protoplast (to the right) and a cell with a relatively intact cell wall (to the left) as seen under an inverted light microscope with 60 times magnification.*

As is made quite obvious from this example, the differences between cells with most of their cell wall intact and protoplasts are made obvious under this degree of magnification without

requiring further staining reagents. However, such a subjective identification methodology also means that the numbers presented in Table 4.1 are approximate in their character. In regards to the FDA staining itself, identification of viable cells were made surprisingly straightforward.

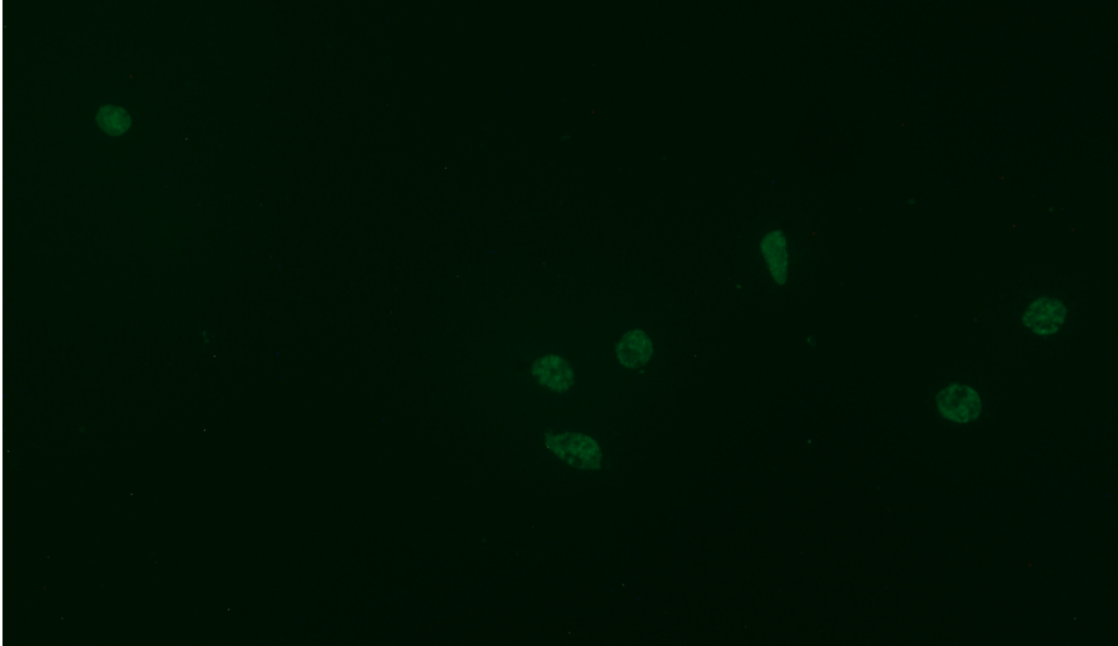

Figure 4.6. *Picture of cells stained with FDA as seen under an inverted light microscope with 20 times magnification.*

It was determined early on that treatments 121, 112 and 122 represented a significantly higher viable protoplast yields for leaf material, while treatment 221 correlated to a significantly higher viable protoplast yields for petiole material, both at 95% significance. While not being conclusive in and of itself, this indicated early on in the analysis that incubation time is negatively correlated to viable protoplast yield in the case of leaf material, while positively correlated for petiole based treatments. Which would signify that an optimized isolation protocol would not only necessitate optimization in terms of sweetpotato but also the exact origin of the material, despite no significant relation between explant and protoplast yield being found in the preliminary literature study.

Performing a PLS analysis, the four quantitative parameters were found to explain 96.884% of the variance within the incubation time and digestive enzyme concentrations, and resulted in Figure 4.7 below.

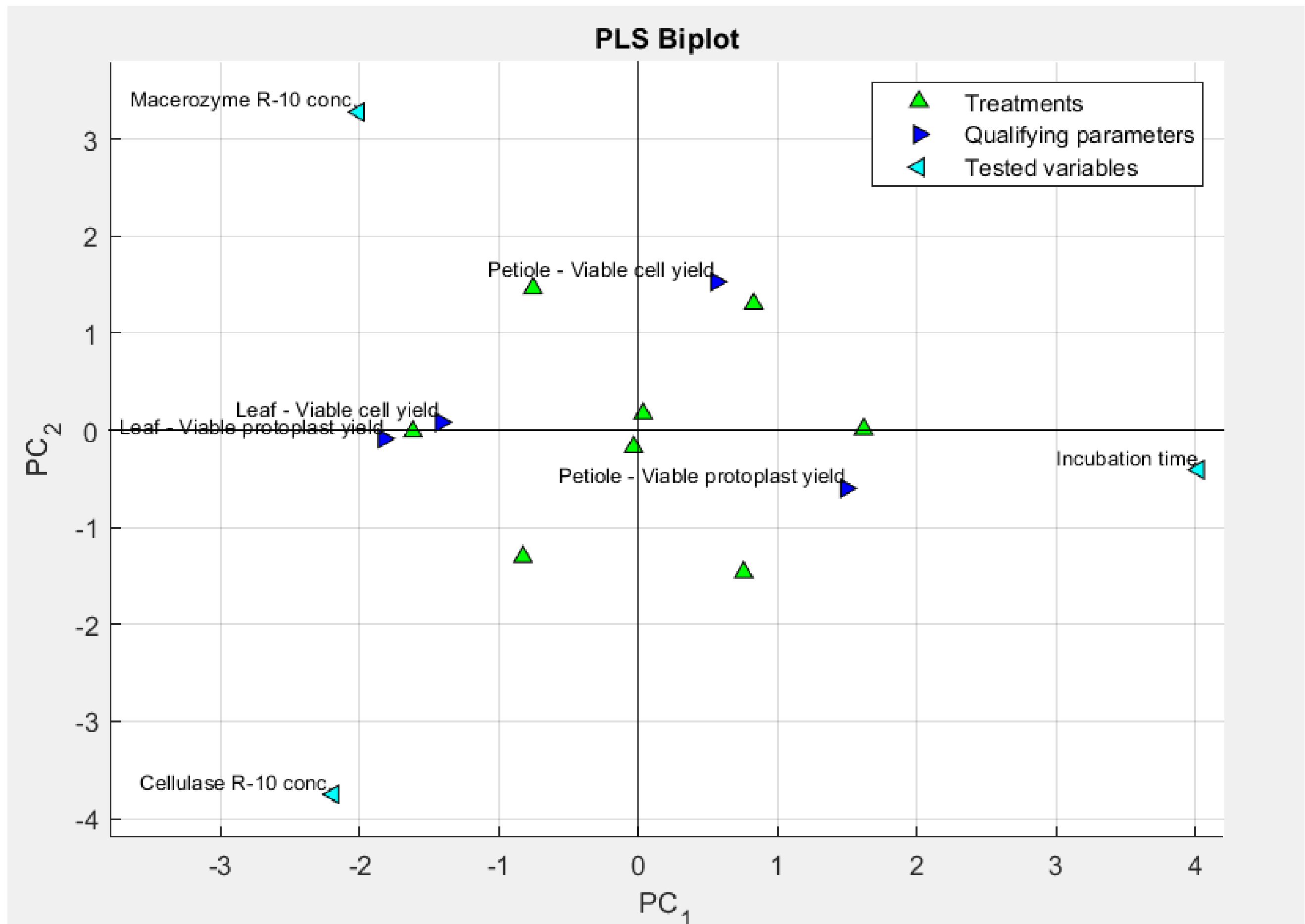

Figure 4.7. *PLS biplot generated using the complete dataset constructed based on isolation experiment 1. Showcasing the distribution and correlation between qualifying parameters in blue (Leaf - Viable cell yield, Leaf - Viable protoplast yield, Petiole - Viable cell yield and Petiole - Viable protoplast yield), Tested variables in cyan (Cellulase R-10 concentration, Macerozyme R-10 concentration, Incubation time) and the different treatments in green.*

This clearly demonstrated the previously noted negative correlation between viable protoplast yield in leaf and incubation time and vice versa for petiole. More specifically, it was found that incubation time is positively correlated with viable protoplast yield in petiole treatments at 74% and negatively correlated with the same for leaf treatments at 92% significance respectively. In addition to this, macerozyme R-10 concentration was shown to negatively affect viable protoplast yield in petioles at 76% significance.

Obviously, at this level of certainty these findings are questionable at best and for this reason the same analysis was conducted using data only pertaining to aliquot 4 for all treatments in the hope of illuminating more significantly relevant correlations, and further narrowing down the optimal protocol for isolation when using both petiole and leaf material.

Thus performing another PLS analysis, the four quantitative parameters were found to explain 96.961% of the variance within the incubation time and digestive enzyme concentrations, and resulted in Figure 4.8 below. It should be noted that this was a minor though obvious improvement in explanational power despite a drastic decrease in data points.

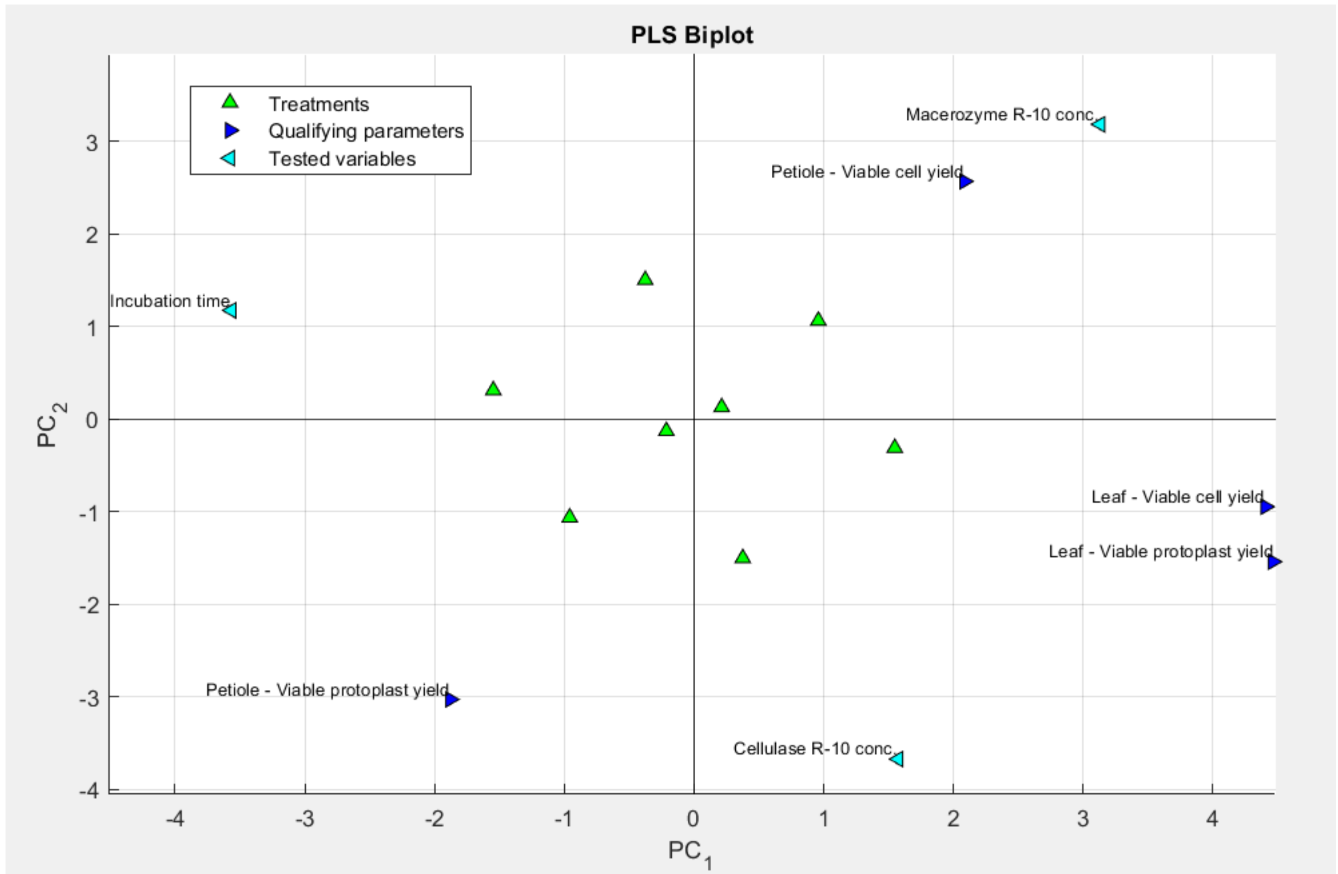

Figure 4.8. *PLS biplot generated using only data from aliquot 4 constructed based on isolation experiment 1. Showcasing the distribution and correlation between qualifying parameters in blue (Leaf - Viable cell yield, Leaf - Viable protoplast yield, Petiole - Viable cell yield and Petiole - Viable protoplast yield), Tested variables in cyan (Cellulase R-10 concentration, Macerozyme R-10 concentration, Incubation time) and the different treatments in green.*

In addition to the relations theorized based on Figure 4.1, cellulase R-10 concentration can be seen to be negatively correlated to viable cell yield in petiole material, while macerozyme R-10 concentration seems to be closely clustered with this parameter. Furthermore macerozyme R-10 concentration seems to be negatively correlated with viable protoplast yield in petioles.

Investigating this further it seems like the exclusion of the data collected from aliquots 1 through 3 weakened the relation between viable protoplast yield in petiole material and the tested variables as no significant relation could be found to incubation time and the previously observed relation to macerozyme R-10 concentration was lowered to 71% significance, from the previous

76. However, incubation time could now be negatively correlated at 97.5%, while cellulase R-10 and macerozyme R-10 concentration were found to be significantly positively correlated to viable protoplast yield in leaf treatments at 90% and 80% respectively.

Based on this, the optimal treatment out of those tested would correspond to 122 for leaf material and likely either 211 or 221 for petiole material. In other words, low incubation time with high digestion enzyme concentrations seem optimal for viable protoplast yield when using leaf material, while longer incubation time and low macerozyme R-10 concentration seem more effective when using petiole material. Thus any future experiments should use these standardized treatments as a basis for optimizing these isolation protocol parameters further.
Interestingly, these results are not supported by the preliminary literature research and subsequent PLS regressions. For instance, incubation time was found to be positively correlated to protoplast yield at 92.5% significance, which is directly contradicted by the experimental findings for protoplast isolation in leaf tissue discussed above. Beyond this, the positive correlation relates to a range of 2 to 18 hours of incubation, indicating a decidedly extreme difference in the theoretical optimal protoplast isolation incubation time in the *Solanales* order in general, and sweet potato leafs specifically. Where isolation in regards to sweetpotato leaf tissue is most effective at lower incubation times (at 4 as opposed to 8 hours), incubation conditions should be kept for upwards to 18 hours in general. Thus it might be the case that the analysis in relation to the preliminary literature research did not procure correlations between incubation time and protoplast yields that are representative of any one protoplast isolation instance. The same could be said for any other parameter we had decided to investigate. But rather is a gross generalization.

As the former data analysis stretched to include multiple species from within the *Solanales* order as well as different tissues used in the isolation protocols, this discrepancy does not come as a surprise. In fact, based on the clear differences in effectiveness between the same treatment for different tissue types (that is leaf and petiole tissue for sweetpotato), a mathematical analysis of a so obviously non-homogeneous dataset may be difficult to relate to protoplast isolation in any singular species of plant. Ideally, such an analysis should be carried out using information that is directly comparable to each individual instance.

This does not however mean that our literature study was inherently fruitless, as it served to demonstrate which parameters have a major impact on protoplast yield in general, which could still be used to isolate which parameters we were interested in optimizing.

Performing an analysis of variance (anova) it was further demonstrated that any treatment of leaf tissue with a lower incubation time at 4 hours resulted in significant increase in viable protoplast yield as opposed to the treatments incorporating a longer incubation time with a p-value of 2.81%. Note that this variance does not account for the varying levels of enzymatic concentration

and merely denotes how impactful the variance in incubation time affects the result. Thus, it was decided a second protoplast isolation experiment with the aim of further optimizing this parameter should be conducted, specifically targeting leaf tissue.

4.3.2 Isolation experiment 2

Carrying out the experiment in accordance with the parameters described in Table 3.2 and the same standardized methodology as previously employed, it was determined that protoplast yield was significantly heightened with an incubation time of 2 hours, as opposed to 3, 4 or 5 hours. In Table 4.2 below is a summarization of the viable yield as determined in aliquot 4 for each isolation treatment.

Table 4.2. *Overview of experimental treatment ID's and the resulting total viable cell yields and viable protoplast yields in aliquot 4 based on isolation experiment 2.*

Experimental treatment ID	Total viable yield, cells ml^{-1} gfw^{-1}	Viable protoplast yield, cells ml^{-1} gfw^{-1}
L2	192540	119098
L3	46893	7816
L4	15306	8163
L5	105570	0

As can be seen in Table 4.2, it appears 2 hours incubation in the isolation medium is optimal in terms of yield for both protoplasts and cells in general. Interestingly, the cell yield falls off at higher incubation times while increasing drastically again at 5 hours. This trend is surprising, however could be explained by enzymatic breakdown of cellular wall related structures accumulating in the epidermal layer of the leaf material and at roughly 5 hours reaches a turning point where the underlying cells may also be isolated. Thus, the exposure time to the isolation medium could differ between cellular layers within the starting material, which may have led to a sudden spike in isolated cells at higher incubation times.

Assuming this hypothesis were accurate, this would mean that optimal conditions for isolation of protoplasts are widely different for different layers of cells, since we observe no such spike in protoplast yield accompanying the cell yield spike at 5 hours incubation. This would make sense, though this phenomenon could also just as easily be explained by the spike being lost due to only investigating incubation times' effect on yield with 1 hour intervals. Alternatively, these results could be the result of poor data quality and or other sources of error.

A factorial that supports this latter hypothesis is that in isolation experiment 1, the same treatment and incubation time as seen in L4 (Table 4.2) resulted in a roughly 6 times higher

viable protoplast yield, which is obviously a extreme variance that can not be explained by simply pointing to minor differences in the leaf tissue used or laboratory technique. Instead, this signifies that there exists additional parameters that have not been controlled or accounted for in these experiments. For instance, a difference in the health of the leaves used for isolation of protoplasts could have had a major effect. Much like other factors such as age and water content when weighing the leaf tissue could've artificially inflated the fresh weight and thus negatively affected the estimated yield.

Thus, a follow up experiment with the goal of replicating these findings should be conducted to ascertain the accuracy and reliability of the collected data. However, given the information gathered from isolation experiment 1 and 2 collectively, the optimal protoplast isolation protocol has been indicated to include an incubation of leaf material in isolation medium (2% Cellulase R-10 and 0.8% Macerozyme) for 2 hours - leading to a ratio of viable protoplasts to viable cells of 0.6.

4.3.3 Isolation experiment 3

Yet again conducting isolation experiment 2 (see 3.2.2), with two replicate treatments in regards to each incubation time corresponding to 2, 3, 4 and 5 hours respectively yielded the results represented in Table 4.3 below.

Table 4.3. *Overview of experimental treatment ID's and the resulting total viable cell yields and viable protoplast yields in aliquot 4 based on isolation experiment 3.*

Experimental treatment ID	Total viable yield, cells ml^{-1} gfw^{-1}	Viable protoplast yield, cells ml^{-1} gfw^{-1}
L2-A	62162	17552
L2-B	124956	0
L3-A	-	-
L3-B	37597	16871
L4-A	0	0
L4-B	0	0
L5-A	176615	16653
L5-B	141377	33223

It should be noted that unfortunate laboratory error led to the L3-A treatment being lost, and due to the time constraints placed upon the project no actions could be taken to rectify this. Despite this, the collected data is certainly quite illuminating as there seems to be significant variance in the yield of both whole cells and protoplasts irrespective of incubation time. This then suggests that there indeed exists other parameters that are perhaps even more important to account for and optimize.

The validity of this hypothesis is clearly demonstrated in how not only does the 5 hour incubation treatment result in the highest cell yields period - despite the previous experiment indicating that a lower incubation time of 2 hours was preferential. But also in how the 4 hour incubation treatment in both replicates resulted in no isolated cells at all, which brings the validity of the results represented in Table 4.1 from isolation experiment 1 into question as well. Ultimately it is clear that more experiments are required to enable any robust theories yet a higher quantity of replicates for future experimental treatments should generate more robust data.

4.3.4 Isolation experiment 4

Carrying out the experiment in accordance with the parameters described, it was determined that relative to the isolation attempt L2 in subsection 3.2.2, the total viable yield was on average 37 times lower, which quite obviously is an extremely pronounced effect. The underlying theory would then dictate that the effect on viable protoplast yield should be even more pronounced as plasmolysis is conducted primarily for the purpose of separating the plasma membrane from the cell wall, thus making protoplast isolation more effective. This is indeed supported by the relative viable yield where the L2 isolation attempt resulted in a quantity of viable protoplasts on average 142 times greater than the comparable attempts which exclude plasmolysis treatment.

While this mathematical comparison includes relatively few replicates and should thus be taken with a healthy amount of skepticism, this does serve to underline how important plasmolysis treatment is for attaining reliably high viable protoplast yields. Moreover the essence of this result could be easily logically predicted as it is supported by the theory discussed in subsection 2.3.

4.4 YFP transfection

A very promising surprise at this point of the practical application of what would end up being the sole transfection experiment, was the methodology described in subsection 3.4 resulting in an approximate 89,17% transformation efficiency. Meaning a vast majority of all viable protoplasts were successfully transformed with the YFP carrying plasmid without any degree of optimization on our part. For future experiments, controlling the plasmid concentration as well as varying the PEG concentration within the transformation medium could very well lead to a further refined transformation efficiency, although this remains to be seen.

4.5 Callus regeneration

While no major variation or experiment was carried out in regards to optimizing callus regeneration, it was noted that the protocol as described in subsection 3.3, resulted in visible colonies developing approximately 2 weeks (15 days) following the isolation of cells from leaf material taken from the Mira genotype.

4.9. *Pictures taken of a callus regeneration attempt using the standard protocol described in subsection 3.3 following 15 days. Note the green dots spread throughout the gel lens.*

Though this parameter likely varies depending on a multitude of factors, it does serve to show what can be expected from future regeneration attempts.

5. Conclusions

While further endeavors to optimize the parameters such as enzyme concentration and incubation time in regards to viable protoplast yield will undoubtedly be required to establish robust generating reproducible results, this project has achieved much by laying down the foundation for such studies in the near future. That being said, due to limitations in materials and a definite overestimation of the quantity of time allotted to the project, have led to less focus being placed on the optimization of the protocols regarding protoplast transfection and protoplast-to-microcalli regeneration. While such a situation is regrettable, it should be noted that even so a basic framework for future research has been established and many pitfalls have through trial and error been uncovered - hopefully allowing future studies to proceed more effectively.

Thus, despite the setbacks, this project has been very fruitful in establishing what parameters are most crucial for optimizing protoplast isolation protocols in regards to viable protoplast yield and has resulted in useful data that may be appropriated for further studies into protoplast culture, transformation and regeneration.

Starting with the protoplast isolation experiments, these have concluded that while more research is required to ascertain and validate the results found in this study, cellulase R-10 and macerozyme R-10 concentrations at relatively high levels combined with shorter incubation times have proved more effective in terms of yielding larger quantities of viable protoplasts per gram fresh weight. However, the inconsistencies inherent in these results also indicate that parameters not controlled for in the proposed protocol (see subsection 3.3) are present and have a significant effect on the yields of not only protoplasts but also whole cells. Thus it would be of interest to investigate what these might be and in future studies attempt to normalize all tissue to be used for isolation purposes as much as feasible. For instance, the age of the tissue or the size of the pieces after extraction from the plant are parameters that could prove important in monitoring.

Regarding the transformation protocol, while exceedingly simple, it does show remarkable promise as far as efficiency goes already at this stage, and so with any future improvements and optimizations it should only be a matter of time before a higher transformation efficiency is reached reliably. With this in mind it should be noted that a substantial amount of work will have to be dedicated to regeneration of the transformed protoplasts, as even though the results seen in subsection 4.5 demonstrate the validity of the proposed protocol, this should ideally be judged on a month-to-month basis which this project has objectively failed to do. It is thus my hope that future research into the topic builds upon the protocols I have established here and improve them further.

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7. Appendices

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