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Strategies for Improving the Work Cycle in a Potato *In vitro* Culture Bank: Medium, Meristem Culturing and RPA Diagnosis.

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Summary

The goal of this thesis was to enhance the effectiveness of the conservation of the potato *in vitro* bank, by finding more efficient ways to maintain the bank and to introduce new varieties. A different method for virus detection was examined (Recombinase Polymerase Amplification on potato skin cDNA samples), slow growth mediums were tested on different potato varieties, and meristem culturing was carried out to eradicate viruses from infected plant material.

First, an initial slow growth test experiment with ten potato varieties was executed. Apical and axillary shoot cuttings were subjected to mediums with 0 mg/L, 0,5 mg/L, 1 mg/L, 3 mg/L or 5 mg/L Abscisic acid (ABA). The apical shoot cuttings had a lower survival rate. The ABA successfully reduced growth, but the high ABA mediums (3 mg/L and 5 mg/L ABA) had a lower survival rate. After nine months on the mediums the survival rate was higher on the 3 mg/L and 5 mg/L ABA mediums.

Second, a bigger slow growth experiment was done including ten potato varieties and 12 different mediums. The mediums both varied in sucrose and ABA concentration. The experiment showed that sucrose and ABA both stunt the growth of potatoes *in vitro.* The medium with the best characteristics seemed to be 3% sucrose and 1 mg/L ABA, as this medium gave no abnormal growth (contrary to what is seen on the high sucrose mediums), no fall in early survival (compared to what was seen on high ABA medium), low variance in growth between varieties (compared to the other sucrose treatments) and gave considerable growth reduction. This medium would allow the varieties to be transferred to a new medium after more than four months in the potato *in vitro* bank, lessening the work needed to maintain the potato *in vitro* bank.

Four virus-infected varieties were meristem cultivated to virus free material. Only one of these, Blåpotet, was completely cleaned of viruses. The potato virus Y (PVY) (Species *Potato virus Y*, genus *Potyvirus*, Family *Potyviridae*) was cleaned from Kvit Rund Kvam. Åkerøy was not successfully cleaned for viruses, but some meristems survived and grew into plants. Only the shoot tip *in vitro* cultures of Early Rose survived.

Primers were designed to test for PVY using Recombinase Polymerase amplification (RPA). Three different primer pairs were tested; “PVY1” which used primers from two different papers (Cassedy et al., 2022; Y. Wang et al., 2020), “PVY2” from a paper by Ying Wang and

Ruhao Chen (Y. Wang et al., 2020), and “PVY3”, a primer pair designed by me. The primer pair that gave the least extra bands and streaks of nucleic acid when visualized on gel was PVY1. The RNA samples extracted from Potato skins were however of too low quality to be used for regular PVY testing. Better sampling of potato skins and improvement of the RNA extraction is needed. RPA on potato skin cDNA samples cannot be reliably used for virus testing, until the protocol is changed. This could be done by using a RNA extraction kit optimized for tuber samples.

Preface

This thesis was conducted at NIBIO Division Biotechnology and plant health, located in Ås, from August 2023 to May of 2024. The master supervisors were Dr. Dag-Ragnar Blystad and Dr. Zhibo Hamborg. Sissel Haugslien offered great advice and help during the lab work and Dr. Stacy Hammond facilitated the preparation of the slow growth test experiment.

Early in my studies I had taken courses where *in vitro* cultures of plants were a central theme. The topic interests me and I wanted to learn more and get more hands-on experience in the field of biotechnology. Potato plants are peculiar as they are vegetatively propagated and susceptible to some devastating diseases.

I want to also thank my family, Eva, Svein and Mari, for being there for me and my boyfriend Roald.

Definitions

Term	Abbreviation	Definition
Abscisic Acid	ABA	Plant growth hormone that regulates response to abiotic stresses and reduces growth.
Recombinase Polymerase Amplification	RPA	An isothermal reaction that exponentially copies duplexed DNA by use of enzymes called recombinases.
Enzyme-Linked Immunosorbent Assay	ELISA	A virus test that uses antibodies to detect specific proteins, such as a virus coat protein. The test is done on a grid of wells, making it possible to test many samples at a time.
Polymerase Chain reaction	PCR	A reaction that exponentially copies single stranded DNA. The duplex DNA is split by heat for the polymerase to be able to bind.
Gene bank		Storage of either seeds, tissue cultures or other plant material. They are kept virus, bacteria and fungi free. This is not as important for seeds. Its purpose is to preserve varieties and genetic diversity.
Meristem		Tissues of undifferentiated cells divide and push out new tissue on a plant.
Apical		Used to describe top of or end of. In this context, the end of the plant.
Axial		In botany denotes growth that is on the stem rather than the top of the plant. Examples are axillary shoots, and axillary buds which come from the space between leaf stalk and stem.
In vitro		Means “in glass” and refers to in this context the cultivation of plants sterilely in closed containers on a gel nutrient medium.
Potato virus Y	PVY	A virus that infects potatoes, giving yield and quality loss. It can be transmitted through cell juice being in contact with a mechanical abrasion or non-persistently with aphids.

Potato virus S	PVS	A virus that infects potatoes and only gives moderate losses but is very common in farmers' fields.
Potato virus X	PVX	A virus that infects potatoes and increases the severity of symptoms when in a plant also infected with PVY. The virus is mechanically spread and survives the winter on potatoes left in the field.
Potato virus M	PVM	A virus that infects potatoes and has very varying symptoms depending on the potato variety it infects making it hard to identify visually in the field.
Tris-borate-EDTA	TBE	A buffer solution often used for stabilizing nucleic acids during electrophoresis.

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1. Introduction

Root and tuber crops have been the third most produced carbohydrate source in the world, and one of the major contributors in this category is potato (*Solanum tuberosum* L.) (Food and Agriculture Organization, 1997). Around ⅔ of the world eat predominantly potatoes inn their meals (Dr. Qu Dongyu Director-general, FAO, 2022). As Europe and North America are cutting down production of potatoes, countries in Asia, Africa and Latin America are increasing their area used for potato production. The total amount of area in the world used for potato production has been decreasing, but the total amount of potatoes produced is increasing.

Potatoes also have some health benefits as food. The potato is a source for vitamins and minerals such as potassium, folate, vitamin C and vitamin B6 (Atli Arnarson BSc, PhD & Alyssa Northrop, MPH, RD, LMT, 2019; Dr. Qu Dongyu Director-general, FAO, 2022). Red or purple tuber varieties have the highest polyphenol amount in their tubers. These compounds are antioxidants which are beneficial for human health. Compared to other carbohydrate rich foods, potatoes are more filling. This might help in weight management.

The wholesale value of potato in Norway is around 2,4 billion according to the directory of agriculture (Landbruksdirektoratet, 2023; Statistics Norway, 2023). Christian Gartner's book "Horticultura" (Gartner & Winding, 1694) is the first documented example of growing potatoes in Norway. On page 21 "Horticultura" the tubers are referred to as "Jord Æbler" which is the same name used today in the northern part of Gudbrandsdalen. According to oral tales, one of the first instances of potato cultivation was done in Austmark Kongsvinger in the 1730s at the Karterud farm (Veteläinen & Bjor, 2001, p. 50). The first concrete evidence of potato cultivation stems from the 1750s, and from 1755 farming of potatoes for self-sufficiency became more widespread at Sørlandet and Sør-Vestlandet. The priests spread knowledge about the potato around the country. In the 1770s to the end of the century the potato was just seen as an emergency food source, the farming on Vestlandet being an exception. Times of war and scarcity from 1800 to 1814 changed this notion, and gave villagers better nutrition, as the potato served as a source of C-vitamin. The year 1846 was the first time late blight scoured Norway. The varieties at that time were very susceptible to this disease (Veteläinen & Bjor, 2001, p. 51).

Under both the first and second world war the production of potatoes increased, with a dip in amount between the wars (Veteläinen & Bjor, 2001, p. 50). The use of potatoes as animal feed has sunk continuously since 1960. The use of potatoes in food has also fallen. The production of potato has become mostly specialist work, with the farming being done by just a few farmers. Shortly after the turn of the 18th century the first Norwegian made potato variety was released, but the use of this variety was of small scale and short lived. As the Norwegian climate is very different to the south of Europe, where many potato varieties are bred, there are government funded potato breeding programs in Norway run by Graminor AS.

For the Norwegian climate long-day potato plants are the most ideal, but the potato lines are also selected for quality traits and resistance. Examples of this are the starch content of the line, the amount of crop, the ease of production, or their resistance to late blight. The strongest selective pressure is put on the line's inherent resistance to important pathogens. In 1998 55% of the area used for commercially farming potatoes grew Norwegian varieties (Veteläinen & Bjor, 2001, p. 52).

In Norway we have a long history of growing potatoes. The potato has been a staple food under times of crisis and been an invaluable source for vitamins. As varieties are made, some of the genetic resources are lost along the way as breeding lines are selected and other lines are discarded. Conservation is important to preserve landraces for their later use in breeding for new varieties, but also for their own inherent cultural and historical value. This is what the potato clone bank exists for. It is a reserve of pathogen clean varieties, ready to be propagated for use in breeding or to be used as primary stock for seed potato production. This is why my master thesis has purpose and value.

The *in vitro* culture bank of potatoes at NIBIO is a national potato bank for long-term preservation of Norwegian potato genetic resources. All preserved potato accessions in the bank should be disease free, without infection of pathogenic bacteria or fungi, or any known viruses. Establishing disease-free materials is a time-consuming process that requires extensive virus cleaning and testing.

The current preservation methods involve maintaining accessions on media that is optimized for growth over a period of three months. The plants then require transfer to new media. The plants are held at 8°C to reduce growth. The transfer is labor-intensive, leading to high operational costs. Furthermore, unrestricted micropropagation can induce genetic variations in the materials. This undermines the long-term preservation of the potato gene resources.

My research question is then:

How can the work cycle at the Norwegian in vitro potato gene bank be improved?

This would be done by testing potato varieties' reactions to growth-deterring mediums, cleaning new varieties of viruses, and testing a new method for diagnosing PVY infections.

2. Literature

2.1. Slow growth

When the potato varieties are in the bank, they are kept on medium *in vitro.* Normally, after a period of three months, the *in vitro* potato plants are moved to a new medium. This is done as they grow too long for their tubes, use up their nutrients and die. Finding a medium that permits the plants to stay longer on the same medium would reduce the work hours delegated to the bank, which might cut costs.

2.1.1. *In vitro* cultivation of plants

Haberlandt (1902) thought that plants were totipotent, and by that could regenerate a plant from just a few cells. This idea initiated the cultivation of plants on nutrient medium in sterile containers, called *in vitro* propagation of plants. The possibility of having plants *in vitro* made it easier to study plant physiology and development. The most commonly used medium mixture for *in vitro* plants is called the Murashige and Skoog medium (Murashige & Skoog, 1962). They had analyzed tobacco leaf ashes to formulate the medium. The optimization of *in vitro* growth led to development of micropropagation. This is the practice of multiplying plants by using cuttings or small pieces of tissue from a mother plant and growing them *in vitro.* This is often followed by reintroducing the plants to growing on soil. Some plants need exogenous hormones (auxin and cytokinin balance) to determine their cells into growing roots or shoots *in vitro* (Slater et al., 2008). Potato plants are easy to handle in this regard and produce both shoots and roots on their own *in vitro* as long as there are nutrients available to them.

2.1.2. Gene banks

At NIBIO preservation of potato varieties is done *in vitro,* but there are other practices to preserve gene resources of potato. One way is to cryopreserve shoot tips of potato plants (Vollmer, R. et al., 2022). The shoot tips are in cryopreservation preserved in liquid nitrogen, by first cooling the shoots controlled down with anti-frost liquid inside and outside of the shoot tips. This method of preservation is very machine reliant, and the equipment needed is costly. A lot of competence is needed for this type of preservation. Shifting to a totally new

protocol would take too much time and money for my master thesis to encompass the change (and might not even be profitable or practical), and so the existing protocol was chosen to be improved upon instead.

De Oliveira tested the effect of Sorbitol for reducing the growth of *Hancornia speciosa in vitro* (de Oliveira et al., 2022). The addition induced water stress and stunted the plants growth. Sorbitol can cause skin irritation and irritate when inhaled. Du Yung-peng tested ABA or high sucrose concentrations in the medium of *in vitro* wild lilies (Yun-peng et al., 2012). The purpose was to slow their growth to be able to keep them on the same medium for longer. They found the most effective to be ¼ MS with either 9% sucrose or 3 mg/L ABA. These were the highest concentrations of sucrose and ABA that they tested. Du Yung-peng were able to keep the lily seedlings on the same medium for 15 months. ABA has been used by many others to reduce growth of in vitro plant cultures (Jakobsone et al., 2006; Kamińska et al., 2016; Pan et al., 2013).

2.1.3. Abscisic acid

ABA was first observed to inhibit growth in dormant buds of ash and potatoes in 1949 by Paul F. wareing (Evert & Eichhorn, 2017, p. 649). He called the hormone *dormin*. In 1972 Frederick T. Addicot found a hormone that accelerated the abscission of leaves and fruits on the cotton plant (*Gossypium hirsutum*) (Davis & Addicott, 1972). He called this hormone *abscisin. Dormin* and *abscisin* turned out to be of identical chemical composition leading to the adoption of the name “abscisic acid”.

The cells make ABA in their plastids from carotenoid pigments (Bidlack & Jansky, 2017, p. 198). The hormone is ubiquitous to vascular plants but can be found in many other clades such as mosses or metazoans (Taiz et al., 2018, p. 419).

The signaling of ABA starts by its binding to the receptor PYR/PYL/RCAR (Taiz et al., 2018, p. 436). The binding changes the confirmation of the receptor. This lets the receptors interact with the phosphatase PP2C or enhances their interaction. The PYR/PYL/RCAR receptor makes the PP2C inactive. As PP2C inhibits the SnRK2 kinases, SnRK2 is active when this signaling path is completed by ABA. SnRK2 phosphorylates proteins, for example ion channels and transcription factors that bind ABA Responsive Elements to promoters.

ABA regulates growth by inhibiting cell division, and steers plant tissue development (Slater et al., 2008, p. 43). ABA for example regulates the heterophylly of the shoots and roots (Taiz et al., 2018). ABA helps other promoters of senescence, giving faster yellowing of leaves in cut flowers (Ferrante et al., 2004). It as well down-regulates growth by inhibiting cell growth hormones (Bidlack & Jansky, 2017, p. 198). ABA regulates the action of gibberellin, auxin and cytokinin, together with ethylene (Wien & Stützel, 2020, p. 74). ABA down regulates enzymes important in photosynthesis, so the plants produce less sugar (Rook et al., 2006, p. 430).

ABA gives increased tolerance to thermal stress, drought, salinity, heavy metal stress and radiation (Vishwakarma et al., 2017, p. 1). ABA closes stomata by signaling to lower the osmotic pressure of guard cells (Rook et al., 2006, p. 426). This lowers the water loss of the leaves, lessening water stress, but also hinders the entry of pathogens through the stomata (Evert & Eichhorn, 2017, p. 650). A fall in transpiration increases the plant's resilience to salinity.

In the root tips of plants under prolonged drought stress, there is an increase in ABA (Sharp et al., 2004). This makes the root's growth less affected by the drought, than the shoot. The ABA is sent though both the xylem and the phloem and are thus transported nonpolar (Bidlack & Jansky, 2017, p. 198). There is often a higher amount of ABA in the phloem than the xylem (Evert & Eichhorn, 2017, p. 649).

ABA keeps potato tubers from sprouting during the winter (Suttle & Hultstrand, 1994). Gibberellin is antagonistic in tuber dormancy towards ABA, increasing and ending the dormancy as the potato tubers are subjected to cold storage (Haider et al., 2022). Zeatin and cytokinin play a role in breaking dormancy.

As ABA is a growth inhibitor, photosynthesis down regulator and because it lowers the transpiration of the plants, the hormone was chosen to be tested as a growth inhibitor in the *in vitro* potato medium. This was done by adding ABA to the slow growth medium.

2.1.4. Sucrose

High sucrose amount in the plant is in a negative feedback loop with photosynthesis, reducing the production of enzymes important for photosynthesis (Rook et al., 2006). This was shown *in vitro* on coconut (Fuentes et al., 2005). It also gave a higher survival rate of coconut plants

when transferred to soil and enhanced growth after transfer. Giving exogenous sucrose lowers the transport of sucrose (Yoon et al., 2021, p. 3). Other sugars have the same effect, but to a lesser degree. The carbohydrate accumulation, in the phloem and mesophyll cells, give down regulation of photosynthesis.

Sucrose shares signaling pathways with ABA. High sucrose amounts in plants promote the making of starch molecules and increased plant growth (Rook et al., 2006). Plant growth is modified by sucrose's influence on cell division, ribosome synthesis, tuber development and far-red light signaling (Yoon et al., 2021, p. 1). Sucrose also regulates the synthesis of starch, storage proteins, and anthocyanin. Starch synthesis has been shown in many plants to not be regulated by other sugars. How sucrose influences these processes molecularly as a signal is not well understood. This lack in knowledge is stemming from sucrose being broken down into other sugars in plants (Yoon et al., 2021, p. 1).

When sweet potatoes were subjected to high amounts of sucrose, they expressed a gene for ADP-glucose pyrophosphorylase (Yoon et al., 2021, p. 2). This is a limiting factor in starch biosynthesis. Sucrose also induces expression of an enzyme that helps in the elongation of amylose. The *patatin* gene in potatoes is induced by sucrose, and codes for the protein making up 30%-40% of the soluble proteins in the tubers.

As high amounts of sucrose in plants lower sucrose transport, down regulates photosynthesis and signals the plant to make storage protein and starch, sucrose was chosen as the second variable to make the medium growth inhibiting. Increasing sucrose concentration in mediums to down regulate growth is not normal practice. Many rather use other sugars that give comparatively the same effect, such as sorbitol or mannitol (Charoensub & Phansiri, 2004; de Oliveira et al., 2022).

2.2. Virus eradication

As stated in "Forskrift om settepoteter" § 7 all breeding of seed potatoes in Norway must be based upon disease free meristems (Forskrift om settepoteter, 1996). All the potato varieties in the bank are virus free and without pathogenic bacteria or fungi, after rigorous testing and cleaning in accordance with this law. Only potatoes certified by the Norwegian Food Authority can be legally planted and grown. This is to protect against the spread of pathogens and also against introduction of new diseases into Norway. The work of cleaning new potato

varieties of pathogenic viruses is important for a continued high food production, and low economic loss to virus infection.

The most important way to avoid losses from pathogens, is to plant out pathogen free seeds or vegetative propagules. This is even more important for viruses, as pesticides do not work. To be sure the material is clean, different diagnostic tests can be done. If no clean plant material is available, then meristems are often used to establish virus-free *in vitro* culture nuclear stock.

2.2.1. Virus control

Before a new variety is put into the *in vitro* potato bank, the plant material must be cleaned of bacteria, fungi and viruses. After cleaning the material, there can still be endobacteria in the plants, but these are normally not a hindrance to growth and seldom appear on medium. Not bringing virus infected material into the bank is important, as the bank gives out potato material for propagation of seed potatoes, and most virus infections can lower the yield or quality of the plant significantly (Wilson, 2014, p. 8). This in turn leads to economic loss for the farmers of the potatoes. In a longer scope this heightens price and can give food shortages. Viruses cannot be eradicated in the field by applying chemicals as many other pathogens can, strengthening the need for virus free seed potatoes and by that virus free bank material (M.-R. Wang et al., 2018).

Import control and quarantine legislations help to stop the introduction and spread of new viruses. To lessen the spread of a newly settled virus, infected plants can be removed and destroyed, which is called roguing. It is least labor intensive and most effective when there are few infected plants, and the primary source of inoculum comes from the infected plants (Wilson, 2014, p. 133). There are no pesticides that work against viruses. Virus infection can also come with vectors such as insects and nematodes. These can be sprayed against, if the vectors have to stay on the plant for an extended period to become an infectious carrier. The date of sowing can be altered so vector arrival doesn't coincide with the plants being young. Keeping the machinery and tools clean is also important. Very hardy viruses or viroids can survive on objects for a long time.

Rotation of crops can help to take away the susceptible crops from the virus, but this does not work when there are naturally occurring volunteer plants (host plants for the viruses) in the

surrounding area. Farmers in the vicinity may also have volunteer plants making rotation farming less effective. The volunteers can also come from inside the field. PVY and *Potato leafroll virus* give smaller tubes that are easily left behind in the field during harvesting. These are then volunteer plants the next season. Killing volunteer plants with pesticides can help against virus spread.

It can take up to 15 years of selection and testing of a line before it can be released on the market as a new variety (Veteläinen & Bjor, 2001, p. 51). This is to test and choose the lines with the best attributes, such as pathogen resistance to specific disease. It can backfire, as it takes such a long time to make a new variety, that the pathogens might themselves evolve new ways to bypass the plant's resistance before the variety has even been marketed.

Dealing with a virus pandemic after it has started is costly, and it's never enough to use one control measure. This is why certified seed potatoes are so important, and why all varieties entering the potato variety *in vitro* bank must be clean. When all available plant material is found to be infected with viruses, then the infections must be eradicated. Eradicating virus infections are difficult as the virus particles are inside the plant's cells.

2.2.2. Meristem culture

The oldest and most used way to clean plants of viruses, is to regrow plants from extracted meristems. The fastest way for viruses to move systemically is via vascular tissues (Wilson, 2014, p. 48). Meristems are therefore often devoid of viruses because there are no vascular tissues going into the meristem. This is because they are composed of layers of undifferentiated dividing cells, surrounded by differentiating cell tissues. The differentiating cells were made in the meristem and pushed out by new cells. The virus can therefore only move into the meristem cell-to-cell through plasmodesmata, which is a much slower way of transport than moving in xylem or phloem (Wilson, 2014, p. 44). This gives the plant often enough time to react to the infection and initiate systemic antiviral RNA silencing, before the virus reaches the meristem (Wilson, 2014, p. 138). The meristem is by that mostly clean of viruses, but this depends on the virus in question, as some spread more aggressively and/or suppress the plant's silencing (Wilson, 2014, p. 51).

It is possible to use chemotherapy and thermotherapy to lessen the virus content before doing meristem extraction, increasing the likelihood of the part extracted being virus free. The

chemotherapy works by reducing the virus replication (Wilson, 2014, p. 138). Ribavirin is one such chemical that hinders replication. With thermotherapy the temperature is raised so high that the replication of the virus is slowed, but not so high that the virus particle is inactivated. Both these treatments need a month or so to work, when used on herbaceous plants. The waiting period needed and logistics of setting up the treatments, made this option not viable.

When meristem cutting, sprouts and shoot tips are used as plant material. The leaves and leaf primordia covering the meristem must be bent away, and only the undifferentiated part of the meristem must be cut out. The process of cutting them out is difficult, as meristems are small. Becoming competent in extracting meristems takes time. Such a skill is harbored by few and is important for preservation of plants.

2.2.3. Important viruses on potatoes in Norway

Some important potyviruses in Norway are PVY and potato virus A (PVA) (Species *Potato virus A*, genus *Potyvirus*, Family *Potyviridae*). Only 10% of a certified seed potato batch is allowed to test positive for these two viruses.

Potato virus X (PVX) *(Potato virus X, Potexvirus, Alphaflexiviridae)*, potato virus S (PVS) *(Potato virus S, Carlavirus, Betaflexiviridae)* and potato virus M *(Potato virus M, Carlavirus, Betaflexiviridae)* are all in the order *Tymovirales*. These viruses have no limit to their presence in certified seed potato batches, but have a limit to percent infected potatoes allowed in the first propagations (pre-basis and basis).

Potato leafroll virus is a quarantine species for seed potato growers, and it's in the *Polerovirus* genus, in the family *Solemoviridae.* This means if a seed potato farm finds out they have potato leafroll virus in their potato plants, they are obligated to disclose this to the right governmental authorities in Norway and stop any further seed potato production until certified again.

These laws are made to protect the potato farmers from yield loss, by preventing the introduction of these viruses into the field. The official seed potato testing is organized by the Norwegian Food Authority, but NIBIO also offers a testing service to farmers who want to know their own seed-potatoes infection status.

2.3. Virus detection by Recombinase Polymerase Amplification

At NIBIO leaf samples are taken to test for viruses. Potato samples they get from farmers for routine testing are often given as potato tubers. This means the tubers have to be grown into plants in a greenhouse before detecting viruses. This takes extra time and money. Finding a method where a potato tuber can be tested for virus, would rid the test of any lengthy waiting period. This is why RPA was tried out on potato tuber skins, as testing on stored tuber skins shorten the testing process, and because of RPA's properties. Being able to test multiple samples at a time was also paramount when choosing a test method.

The experiment testing for PVY with RPA started with RNA extraction, then continued with reverse transcription into cDNA and then copying via RPA. Gel electrophoresis was used to visualize the copied DNA. Q-RPA and RT-RPA are a lot more expensive, and there were fewer producers of primers for these methods. This is why we opted for just an RPA reaction with a Basic RPA kit from TwistDx (TwistDx Limited, 2018).

2.3.1. Virus diagnostics

Why was RPA chosen to test the tubers? There are many other ways to diagnose a virus infection. Viruses can be tested immunologically. One such test is Enzyme-Linked Immunosorbent Assay (ELISA), where antibodies are made to specifically bind to one virus species. First the antibodies are fastened to wells in a plastic grid (Wilson, 2014, pp. 118–119). A sample of leaf to be tested is grinded up with a buffer and pipetted into the wells. After the virus particles have bound to the antibodies in the wells, the rest of the sample is washed out. A second antibody, bound to an enzyme, is added. It binds to the virus particle, and the bound enzyme shows which well is positive for example by producing a colored product.

The bonding between virus particles and antibodies takes time. The grid is often left overnight. The benefit of using ELISA is that many samples can be tested at once. There exists other variants of ELISA and other immunological tests. All these tests are antibody based. The faster immunological tests are often more expensive, as they are prepared in advance for the user. These can be in test-strip (lateral flow) form (Wilson, 2014, p. 120). Test-strips are used at NIBIO for a rapid test of individual leaf samples.

Another way to check for virus particles is by transmission electron microscopy. A sample of crushed cells are then thinned out with a buffer and put on an electron microscope lattice (Robin Harris, 1999). The lattice is negatively stained, and so virus particles will be outlined by heavy metals. The virus particle's structure, length and size is then used to categorize the virus. This method is however time consuming, has a high equipment cost and is heavily skill based.

One can also test for viruses by checking for virus nucleotides in the plant's cytoplasm. With enzyme amplification techniques, nucleotides are extracted from the plant tissue and only selected segments of the virus genome (target sequences) are copied. The most widely used copying reaction is polymerase chain reaction (PCR) (Wilson, 2014, p. 122). When testing for RNA viruses, a reverse transcription into cDNA must happen before the target sequence can be copied. The product from the PCR is run through gel electrophoresis, to visualize it. A negative and positive control sample is often used to show how a negative or positive result should look, and to show if the test was faulty. A faulty test would show no bands of the right nucleotide size for the positive control. PCR followed by gel electrophoresis is labor intensive. On microarrays the presence of many hundreds or thousands of different virus nucleotides can be tested for all at once. The matrix of nucleotides homologous to different target sequences are stuck to a slide. These binds select virus nucleotide sequences. This test is only fit for a few samples at a time, as one array is needed for every sample. As the microarrays have pre-made matrixes of virus homologous nucleotides, the test is expensive.

Symptoms on the potato plant can also be used for virus detection. In most cases a virus sensitive test plant (for example quinoa or tobacco plant) is inoculated with cell juice from the plant. After a few weeks the test plant should show symptoms associated with the virus infection. Some viruses are not transferred by simple wounding, and for them juice inoculation is not practical. Grafting of sick plant material onto test plants or use of vectors for inoculation can be used to test such viruses (Wilson, 2014, p. 112). Symptoms produced by a virus vary from plant species to plant species (Wilson, 2014, p. 104). This is why it is important to observe all the symptoms of the test plant. Symptoms can also come from non-virus sources and the virus species should not be solely confirmed symptomatically. Rellying on symptoms to choose which viruses to test for, cuts down on the number of tests needed. Virus specific tests, such as nucleotide testing and immunological tests are both time consuming and expensive.

2.3.2. Recombinase polymerase amplification

RPA is an isothermal DNA amplification method (Piepenburg et al., 2010). The amplification is exponential as PCR, but with the use of different enzymes that ensure a stable strand displacement under the synthesis of new homologous DNA strands. The reaction works without a change in temperature, because of these enzymes. The RPA does not demand special machinery, other than a stable heating source for incubation and a -20℃ freezer for keeping reagents.

The RPA reaction is normally optimal at around 37℃ to 42℃ (TwistDx Limited, 2018, p. 6). RPA can make a detectable amount of amplified DNA after just 10-12 minutes of reaction time. Amplifying a few (1-10 DNA) targets requires more time, but should still take less than 20 minutes (Lobato & O'Sullivan, 2018). This is much less time than the reaction time for PCR, which falls between 45 and 60 minutes. The primers used in RPA reactions are optimal at other specifications than under PCR. As the primers are used under low temperatures it is important for the RPA primers to be long, have a low chance of bonding with itself and be a sequence unique to the target sequence. Specific primer melting temperature (Tm) is not a factor when designing primers for RPA, as the reaction temperature is low. For PCR primers the melting temperature is important as temperature fluctuates during the reaction to separate template strands from newly synthesized strands, and to start the polymerization again.

The quality and quantity of RNA is still as important in PCR as in RPA. To test quality and quantity a nanodrop machine is used. It sends a broad spectrum of light through a drop of sample. The sensor picks up the transmitted light, and an absorption spectrum for medium is made. For RNA samples to be considered pure the A260/A280 ratio should be 2.0. This comes from the nucleic acids having a characteristic peak at 260 nm in their absorption spectrum (Thermo Fisher Scientific, n.d.). Because of this the A260/A230 ratio should also be 2.0-2.2 for RNA to call it a pure sample. A sample with ratios as low as 1.5 is still possible to work with, but might give varying results when amplifying as contaminants can interfere with the reaction. Extraction of RNA from leaves gives consistently much higher quality and quantity of RNA than extraction from tubers. However, if the copying method (RPA) is sensitive enough, the tuber skin's low quality and quantity RNA might show results on the gel.

The RPA reaction starts with recombinase binding to the primers. This complex finds the homologous DNA target (TwistDx Limited, 2018, p. 8). The single-stranded DNA binding (SSB) protein binds to the other strand stabilizing the D-loop. The recombinase lets go of the DNA to make space for the polymerase (Lobato & O'Sullivan, 2018). The strand displacing polymerase then binds to the primer 3' end and starts synthesis of a new DNA strand. More SSB proteins keep being added behind the polymerase on the parent strand that's being displaced and not used as a template. When the strand displacing polymerases meet, the two parental strands detach completely from each other. The polymerase then displase the SSB proteins in front of it as it moves up the strand towards the 5' end. When they reach their 5' ends, then two new homologous strands should have been made from the double stranded DNA (Lobato & O'Sullivan, 2018). This keeps happening as long as the temperature is kept and there are enough substrates for the reaction.

NAD is a housekeeping gene, this means it is present in all of the plant's cells, as it is vital to the cell's survival (Joshi et al., 2022). A target sequence from NAD should then always be possible to detect in a plant sample. To have an internal control of the virus testing experiment, a nucleic acid sequence in NAD was copied during RPA. This NAD target sequence was 188 base pairs (bp) long. This is within the range of lengths that a RPA reaction can handle (TwistDx limited, 2018). The NAD primers were originally designed for PCR reactions, and might not be optimal for the RPA reaction.

2.3.3. Potyviridae

The Potyviruses have rod-shaped virus particles that can bend (Wilson, 2014, p. 14). They are only 11-13 nm in diameter and have no visible core. Their genome is single stranded sense RNA (Wilson, 2014, p. 23). Plant viruses mutate very often, with an estimated base pair mutation every $2 \cdot 10^{-3}$to $2 \cdot 10^{-5}$ replication cycle (Wilson, 2014, p. 29). Conserved sites in the genome are often important for viral protein's function (Wilson, 2014, p. 30). The sequence coding for cylindrical inclusion protein seems to be the most conserved regions of the potyviruses (Kekarainen et al., 1999). The genome has one large open reading frame, giving a polyprotein when translated (Wilson, 2014, p. 37). The polyprotein is cleaved to make the working proteins by the viruses' own proteases or autoproteolyticaly. This strategy often leads to accumulation of the virus's proteins inside the cells.

Potyviruses have proteins that are dual purpose, both involved in movement and other virus functions (Wilson, 2014, p. 47). The coat protein, the P3N-PIPO protein, the helper companion protein, virus genome linked protein (VPg) and the cylindrical inclusion protein all help with movement cell-to-cell. VPg has been shown to be essential for phloem movement of potyvirus particles (Wilson, 2014, p. 49).

Some viruses interact with the plant's gene silencing to limit its silencing of the virus genome (Wilson, 2014, p. 51). This happens often through the manipulation of the host's kinases. A potato plant infected with PVY and PVX makes the plant's symptoms more severe. This stems from the two viruses affecting different silencing stages of the plant's virus silencing.

The cylindrical inclusion was used to find primer pairs for RPA, as it is a conserved region. This region was chosen as it would be conserved between variations of PVY, but would vary in sequence between the potyviruses.

2.3.4. Gel electrophoresis

To visualize the product of the amplification it can go through gel electrophoresis (Department of Biosciences - University of Oslo, 2016). The samples with nucleic acid are pipetted into holes called wells in the gel. An electrical current is run through the gel. This drags the positively charged nucleic acid towards the anode. Shorter lengths of nucleic acid chains travel faster than the larger ones, because of the porous gel. Filling a well with a ruler of known sequence lengths, makes it possible to read the sequence length of a band of nucleic acids. The nucleic acids are often stained with UV-fluorescent dyes, making the results possible to safely photograph with an imaging machine.

3. Materials and methods

3.1. Basis medium

The potato growth medium used in the potato bank is made with: 4.4 g/L Murashige and Skoog mix with vitamins, 30 g/L of crystalline sucrose, pH 5.7-6.0, 9 g/L. The liquid is then dispensed, 5.7 mL into each tube, and autoclaved at 110°C for 25 minutes minimum (the highest pressure during autoclaving being 2 bar) and put in cool storage. When the medium is poured after autoclaving, then the medium is autoclaved at 121 °C for 20 minutes.

This medium was used to propagate the plants needed for the start of the slow growth experiment, and was the basis medium for the test experiment, slow growth experiment and the meristem medium.

3.2. Slow growth

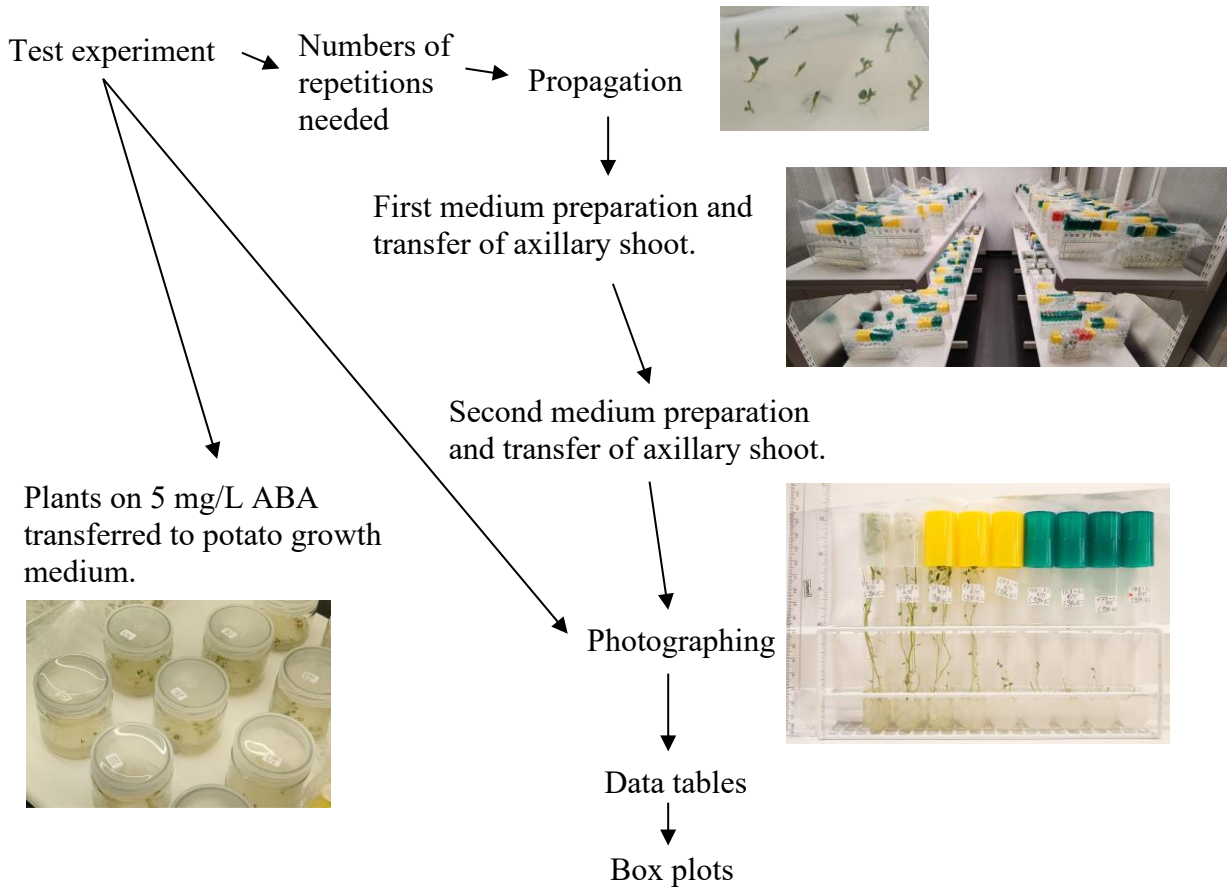


Figure 1. A flow chart for the slow growth experiment.

3.2.1. Material

All the plant material was from extra *in vitro* cultures (3 months old) from the potato bank at NIBIO.

For the test experiment the varieties were intake number 234 (Y-67-20-40-18), 238 (Bruse), 241 (Gullauge P2), 377 (Eggeplumme), 393 (Raude from Sjkåk), 449 (Rigneriks pot.), 458 (Purple Peruvian), 508 (Iverpotet/Smaragd), 562 (Liverpool) and 636 (Asparges potato from Hvaler). Apical and Axillary shoots of the ten potato varieties were used.

For the slow growth experiment the varieties were: N-94-30-22 (173), Y-67-20-40 (231), Beate (236), Bruse (238), Gullauge P2 (241), Innovator P3 (243), Kerrs Pink (244), Laila (246), Oleva (250), Peik (253). Axillary shoots of the ten potato varieties were used.

Other resources used were sterile laminar flow bench, autoclave, ABA, crystalline sucrose, 1440 test tubes, pH-meter, precision dispenser, growth room (18°C and 40-45 $\mu mol/m^2/s$ lighting), cold storage and R-studio (2023.06.1 Build 524) (Posit, 2023) for statistical validity tests.

3.2.2. Method

Table 1. The different mediums used in the slow growth experiment.

Mediums and tubes used

<table>
<tr><td>Use</td><td>Test experiment</td><td colspan="4">Slow growth</td></tr>
<tr><td>Variable(s)</td><td>ABA</td><td colspan="4">ABA and sucrose</td></tr>
<tr><td rowspan="5">Added to basis medium</td><td>0 mg/L</td><td rowspan="2">0 mg/L ABA and 3% sucrose</td><td rowspan="2">0.5 mg/L ABA and 3% sucrose</td><td rowspan="2">1 mg/L ABA and 3% sucrose</td><td rowspan="2">3 mg/L ABA and 3% sucrose</td></tr>
<tr><td>0.1 mg/L</td></tr>
<tr><td>0.5 mg/L</td><td rowspan="2">0 mg/L ABA and 7% sucrose</td><td rowspan="2">0.5 mg/L ABA and 7% sucrose</td><td rowspan="2">1 mg/L ABA and 7% sucrose</td><td rowspan="2">3 mg/L ABA and 7% sucrose</td></tr>
<tr><td>3 mg/L</td></tr>
<tr><td>5 mg/L</td><td>0 mg/L ABA and 15% sucrose</td><td>0.5 mg/L ABA and 15% sucrose</td><td>1 mg/L ABA and 15% sucrose</td><td>3 mg/L ABA and 15% sucrose</td></tr>
<tr><td>Medium tubes of same treatment and variety</td><td>3</td><td colspan="4">12</td></tr>
</table>

3.2.2.1. Test experiment

For the test experiment ten potato varieties were put on nutrient medium with differing amounts of ABA added. Five different mediums based upon the potato growth medium were made with differing ABA content; 0 mg/L for the control group, 0.5 mg/L, 1 mg/L, 3 mg/L

and 5 mg/L (see table 1). For each variety and medium combination three test tube *in vitro* cultures were made. One tube had two apical shoot cuttings in them, and two tubes had two axillary shoots with two nodes in them. One node was put in the medium and the other node was left over it. The shoot segments were cut to a length of 2 cm. Cutting and transfer of shoots were done under sterile conditions in a laminar flow bench. The tubes were put in racks of 10x3 tubes. After this, the tubes were photographed every month, and the photos were used to measure the length of the potato shoot, survival percent and count their leaves.

3.2.2.2. T-test

These test measurements were used to do a power test to find the number of repetitions needed to have statistical power behind the difference in height and number of leaves. This was calculated with R-studio (Posit, 2023) using T-test power. The slow growth experiment itself varied in sucrose and ABA concentration. Ideally an ANOVA test should have been done, as this allows for two parameter input. Using the T-test and adding some extra margin of error, by using the standard deviation in height of the two treatments with the most variance (control and 0,5 mg/L) in the T-test code. The code became:

```
power.t.test(power=0.80, delta=1, sd=1.15)
```

The result was n = 21.7. The slow growth experiment ended up needing 24 test plants per variety and treatment combination.

3.2.2.3. Propagation

For there to be enough plant material from every variety in the slow growth experiment, *in vitro* propagation of the ten varieties had to be done. The medium used for this was the potato growth medium (no adjustments). It took a month or so for the cuttings to grow enough to be used in the experiment. Around 55 plants with 3 to 5 nodes were needed.

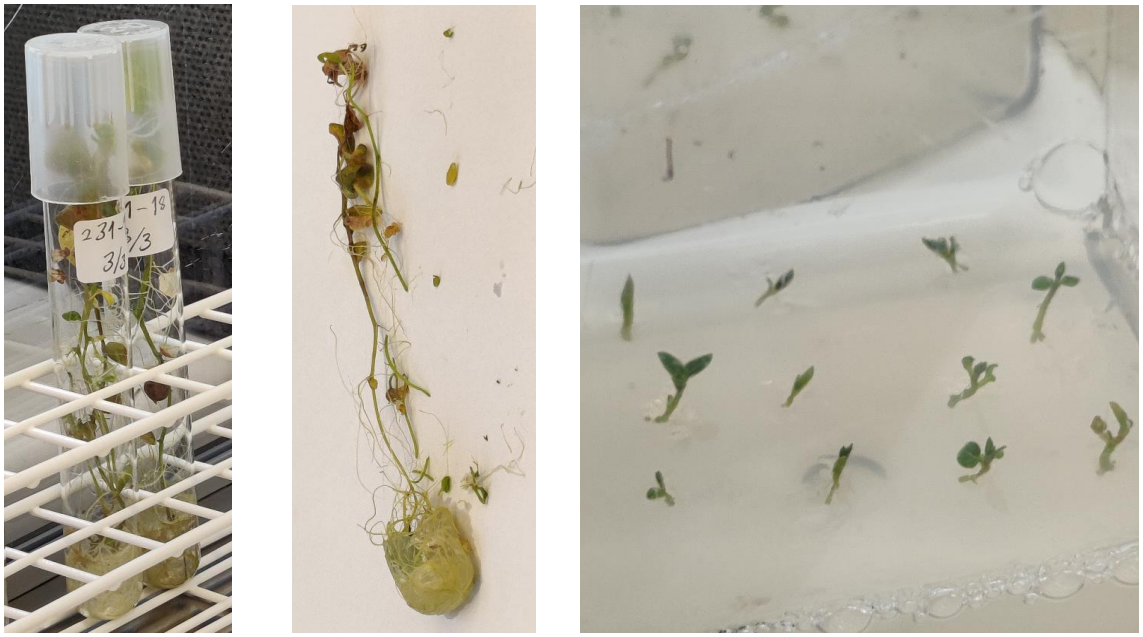


Figure 2. Tubes with *in vitro* cultures of the variety 231 to the left, a potato plant after cutting segments for propagation in the middle and cuttings of variety 173-52 three days after start of propagation (picture 26.06.2023) to the right.

3.2.2.4. Experiment setup

In the slow growth experiment 12 different mediums/treatments were tested on 10 potato varieties. These mediums varied in amount of sucrose added and in ABA concentration. Three different sucrose concentrations (3%, 7% and 15% sucrose) and four different ABA concentrations (0 mg/L, 0.5 mg/L, 1 mg/L, 3 mg/L) were tested (see table 1). The basis for the mediums were the potato growth medium just adjusting the sucrose and adding ABA. See table 1 for the slow growth mediums. The mediums for the slow growth were made in two separate repetitions. This was done to be able to catch faulty mediums. As such the transfer of cuttings also was done at two different times.

For every combination of treatment and variety 12 tubes were made with two cuttings in each. The axillary shoot cuttings were only a stem segment with one node. The axillary shoots were cut to a length of 1-2 cm under sterile conditions in a laminar flow bench. All the leaves on the axillary shoots were removed. The stem was put obliquely into the medium with the node just touching the medium, with the node facing up.

Figure 3. How the axillary shoot cuttings were positioned on the slow growth medium. This picture was taken sometime after transfer, but these cuttings have changed very little in that time.

3.2.2.5. Data collection

Every second month the racks of tubes were photographed. These photos were used to measure height, and the data was put into tables in Excel. The mean heights of plants on the same medium and of the same variety were used as data points in boxplots.

3.3. Virus eradication

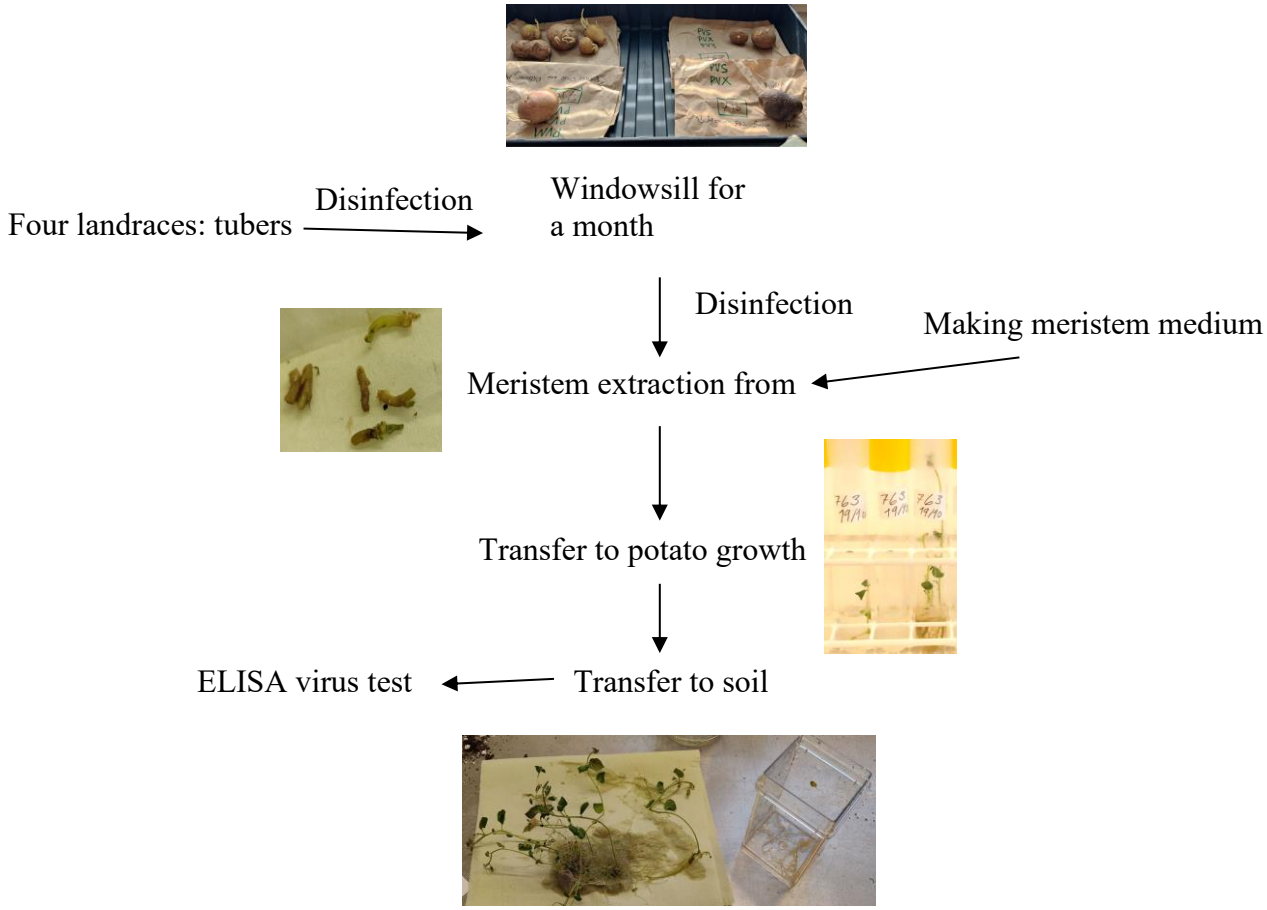


Figure 4. A flow chart for the virus eradication.

3.3.1. Material

Potato tubers of variety: Åkerøy nr. 707 (PVS and PVX infected), Early Rose from Arendal nr. 714 (PVM, PVX and PVY infected), Blåpotet from Snåsa nr. 715 (PVS and PVX infected), and Kvit Rund Kvam nr. 763 (PVS, PVX and PVY infected). Tuberes of these varieties originated from different parts of Norway, as part of the effort to incorporate old landraces into the potato genebank. The tubers I was given had been grown in a greenhouse by NIBIO.

Other materials needed were deconex, 1% sodium hypochlorite and 75% ethanol for the disinfection. A sterile laminar flow bench, an autoclave and growth chamber (18°C and 40-45 $\mu mol/m^2/s$) was also needed.

The meristem medium was made of potato growth medium with only 2% sucrose and added 1 g/L Casein hydrolysate, 0.1 g Indole-3-butyric acid, 40 mg/L adenine hemisulphate, 1 mg/L gibberellic acid. Half of the medium had no agar added.

3.3.2. Method

Tubers from four varieties infected with viruses were given to me for virus eradication; Åkerøy, Early Rose from Arendal, Blåpotet from Snåsa, and Kvit Rund Kvam. The eyes of the potatoes had sprouted in cold storage. After washing the tubers clean, they were marked and disinfected in 5% deconex for an hour.

3.3.2.1. Shoot tip preparation

The potato tubers were then put in a windowsill for a month (September 2023) at room temperature. For variety Kvit Rund Kvam *in vitro* shoot tip cultures were already established in June 2023, so no disinfection or sprout growth was needed. In October 2023, sprouts were cut off the tubers and disinfected. The sprouts were disinfected in jars with up to six shoots in each. The shots were cut into 2 cm segments, put in the jars, and the jars were marked with the intake number of the tubers. 75% ethanol was added, and the jars were swirled for 30 seconds. The jars were decanted, and 1% sodium hypochlorite was added. The jars were then swirled for 10 minutes, before the jars were decanted again. Lastly, the shoots were washed in autoclaved water two times.

3.3.2.2. Meristem culturing

From the disinfected sprouts and the *in vitro* shoots of Kvit Rund Kvam, 20 meristems were cut under sterile conditions for each variety. A part of the meristem and one or two leaf primordia was cut out. The tissue extracted was 2 µm in diameter. They were put on liquid and hard mediums optimized for meristems (see materials). Two *in vitro* shoot tip cultures were also made for every variety. After two months the plants that had grown from the meristems were propagated on potato growth medium. The plants originating from different meristems were propagated separate from each other. Five months after putting the meristems on medium, the plants that grew were transferred to soil and acclimatized. After one month on soil, leaves from the plants were tested for PVA, PVS, PVY, PVM, PVX, PLRV with ELISA.

3.3.2.3. ELISA test

Into the wells of the ELISA grid there was pipetted 100 µL of diluted antiserum (1:1000 dilution in coating buffer). The grid was left to incubate overnight at 4°C. The wells were then washed 3 times for 3 minutes with a wash buffer. 0,5 mg of leaf sample was crushed in 4,5 mL of sample buffer and 100 µL of this was pipetted into a well for every sample. The wells were left overnight at 4°C. The wells were washed again. 100 µL of conjugate (alkaline phosphatase, AP) diluted 1:1000 in sample buffer was added to each well and incubated overnight at 4°C. The wells were then washed. A tablet of substrate 4- nitrophenyl phosphate dinatrium salt hexahydrate was dissolved in 40 mL of substrate buffer and pippetted in the wells. After 30 minutes in the dark at room temperature, the grid was checked for positive yellow wells.

Figure 5. Tubers just after being disinfected and put on a windowsill (first: September 2023) and tubers that have grown for a month on the windowsill (second: October 2023).

Figure 6. Left: Plant of variety Kvit Rund Kvam used for establishment of *in vitro* culture before meristem culturing. **Right:** Spouts that have been cut off tubers before disinfection.

Figure 7. Removal of medium off a subculture (from a single meristem) for soil transfer, five months after meristem cutting.

3.4. Virus detection: RPA

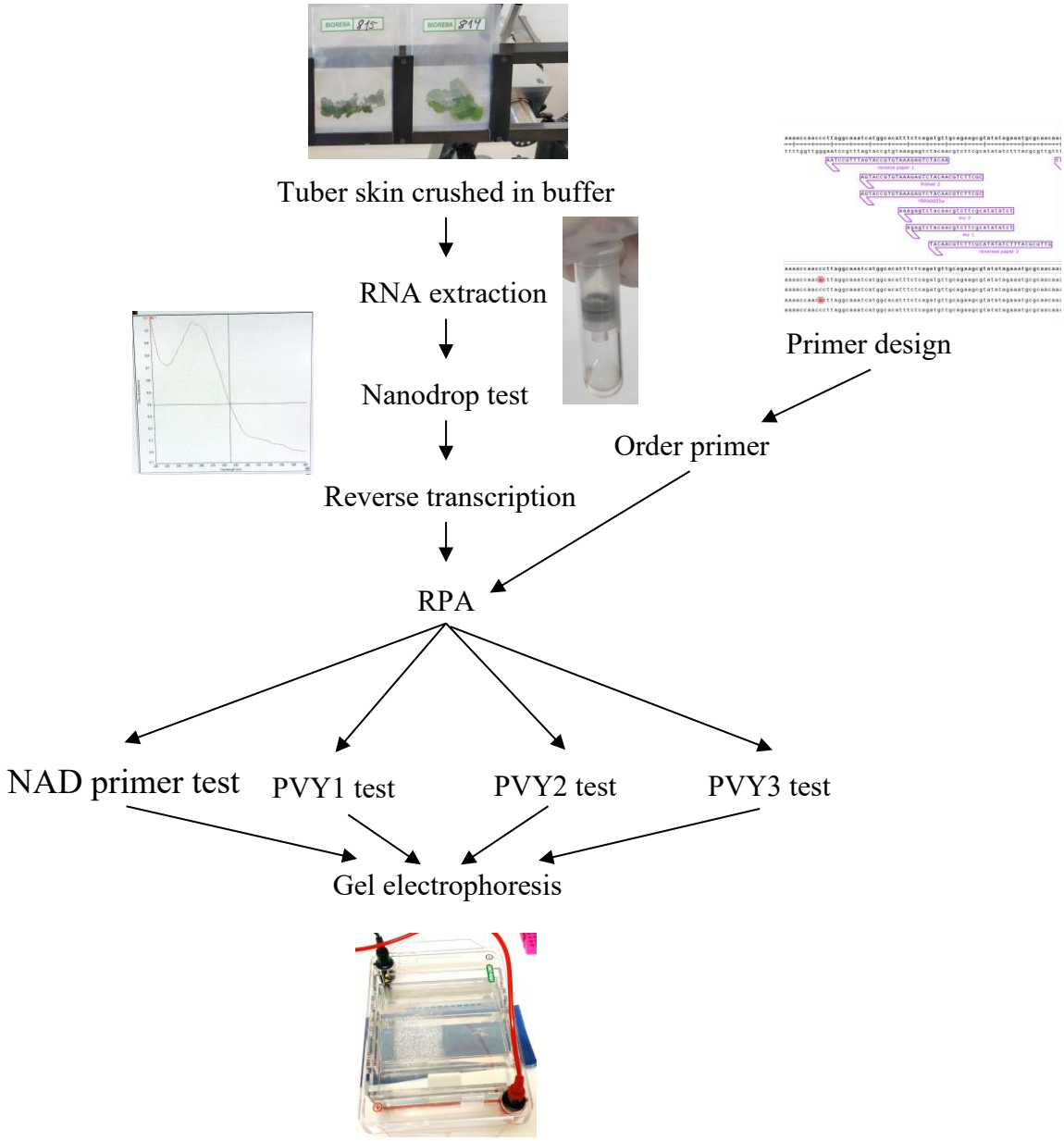


Figure 8. A flow chart for the virus detection: RPA.

3.4.1. Materials

A hundred Mandel (nr.82) potato tubers. When ELISA tested more than 20% of the samples from these tubers tested positive. Four Jacky (nr.86) potato tubers negative for PVY when ELISA tested were also used. These tubers were sent to NIBIO by potato farmers for ELISA virus testing. The bud ends of the potato tubers had been cut out and used for the ELISA tests, before they were handed over for this experiment.

A primer design tool (snapGene (GSL Biotech LLC, n.d.)) and nucleotide database BLASTN tool (National Library of Medicine, n.d.) were needed for primer design.

For the sample preparation: extraction bags (BIOREBA, n.d.), a -20°C freezer and Phosphate-buffered saline (PBS) based buffer with bovine serum albumin (BSA) made of 100 mL/L 10x phosphate-buffered saline (PBS), 50 mL/L Tween 20, 5 g/L polyvinylpyrrolydon (PVP), and 2 g/L bovine serum albumin (BSA) dissolved in distilled water.

For RNA extraction beta-mercaptoethanol and Norgen Plant/Fungi Total Purification kit (Norgen Biotek Corp., n.d.) were used.

A Nanodrop machine (Thermo Fisher Scientific, n.d.) and M-MLV Reverse Transcriptase (Thermo Fisher Scientific Inc., 2022) were used to prepare for RPA.

TwistAmp Basic kit (TwistDx limited, n.d.), Sub Cell GT System (BIO RAD, n.d.-b), SYBR-safe (Thermo Fisher Scientific Inc., n.d.), 100 bp DNA ladder, and an imaging machine (ChemiDoc (BIO RAD, n.d.-a)) were needed for the copying of cDNA and imaging of the result.

3.4.2. Methods

3.4.2.1. Primer design

The primer design was done first. Five whole genome sequences were aligned to look for sequences conserved between the strains. This was the basis for finding segments to copy. The primers were designed using the software SnapGene (GSL Biotech LLC, n.d.) and according to instructions from the primer design manual from TwistDx (TwistDx limited, 2018).

All the RPA primers were between 30-35 nucleotides long as recommended by the supplier (TwistDx limited, 2018). None of the primers chosen had repetitive sequences or sequences that had a lot of the same nucleotides. Low or high amounts of GC (over or under 30%-70%) and primer on primer interactions were evaded when choosing primers. The chosen target sequences were 100-200 bp long and no bigger than 500bp. The GC contents of the target sequences were from 40% to 60% and the targets were not repetitive or had any palindromes (TwistDx limited, 2018).

After finding some candidate primers, the primers were screened for secondary structures and hairpins made by the primers bonding with themselves. SnapGene software (GSL Biotech LLC, n.d.) was used for this, as it shows a map of the primers most likely structure. The ones with any high chance of secondary structures were scrapped. The target sequences were also run through a BLASTN tool on NLM's website (National Library of Medicine, n.d.) to check for sequence similarities in other genomes, as the target sequence needs to be specific only to PVY.

Three primer pairs were chosen to test if it was possible to find PVY infections using RPA. The first pair of primers (PVY1) were primers from two different papers (Cassedy et al., 2022; Y. Wang et al., 2020). The target sequence was 174bp long and both primers were 32bp long (see table 2). The second primer pair (PVY2) was taken from a single paper (Y. Wang et al., 2020), and the target sequence was 183 bp long with both primers 32bp long. The last primer pair (PVY3) was designed by me, and the target was 182bp long with 30bp long primers.

Table 2. Table with information on primer pairs.

Primer pair	**PVY1**	**PVY2**	**PVY3**
Target sequence	ccaactgtgatgaatgggcttatggt ttggtgcattgaaaatggaacctcgc caaatgtcaacggagtttgggttatg atggatgggaatgaacaagttgagt acccgttgaaaccaatcgttgagaat gcaaaaccaaccccttaggcaaatca tggcacatttctcagatgtt 174bp, 43%GC	ccaactgtgatgaatgggcttatggtttg gtgcattgaaaatggaacctcgccaaat gtcaacggagtttgggttatgatggatg ggaatgaacaagttgagtacccgttga aaccaatcgttgagaatgcaaaaccaa cccttaggcaaatcatggcacatttctca gatgttgcagaagcg 183bp, 44%GC	atgaatgggcttatggtttggtg cattgaaaatggaacctcgcc aaatgtcaacggagtttgggtt atgatggatgggaatgaacaa gttgagtacccgttgaaaccaa tcgttgagaatgcaaaaccaa cccttaggcaaatcatggcac atttctcagatgttgcagaagc gtatataga 182bp, 42%GC
Forward primer	ccaactgtgatgaatgggcttatggt ttggtg 32bp, 47%GC	ccaactgtgatgaatgggcttatggtttg gtg 32bp, 47%GC	atgaatgggcttatggtttggtg cattgaa 30bp, 40%GC
Reverse primer	aacatctgagaaatgtgccatgattt gcctaa 32bp, 38%GC	cgcttctgcaacatctgagaaatgtgcc atga 32bp, 47%GC	tctatatacgcttctgcaacatc tgagaaa 30pb, 37%GC

3.4.2.2. Experiment setup

The RPA primer pairs were tested on 26 samples. Each sample contained tuber skins from four potato tubers. Det skin was peeled from around the bud end. The batch of Jacky potatoes (nr. 86) had tested mostly negative with ELISA. The test had shown no positives for PVA and PVY, after testing 100 tubers by testing them together in fours, as I did. The single sample taken from four Jacky tubers, was used as a negative control when running the gel electrophoresis. The Mandel (nr. 82) tubers had a high number of potato tubers infected with PVA and PVY (16/25 and over 20% infected). 25 Mandel samples were used to test the three primer pairs, against the result from the ELISA.

3.4.2.3. Sample preparation

The tuber samples were prepared by grinding 0,5-1 mg of skins in sample bags with 4,5 mL of PBS buffer containing BSA. The solution was pipetted into centrifuge tubes, after going through the sample bag mesh.

3.4.2.4. RNA extraction

The samples were stored at -20℃ between the protocol steps. RNA was then extracted using the Norgen Plant/Fungi Total purification kit (Norgen Biotek Corp., n.d.). The protocol from the manufacturer was mostly followed. 100 µL of each freezed sample had its RNA extracted. The optional addition of beta-mercaptoethanol was done, “Protocol for Optional On-Column DNA Removal” was skipped and only 30 µL of the Elution Solution A was added to the spin column. This was done to increase the RNA concentration. The Elution solution was also run through the spin column two times to extract more of the RNA from the filter. After extraction the samples RNA quality and quantity were tested using a Nanodrop machine (Thermo Fisher Scientific, n.d.).

3.4.2.5. Reverse transcription

The RNA was then converted to cDNA by reverse transcription with M-MLV Reverse Transcriptase (200 U/µL) (Thermo Fisher Scientific Inc., 2022). It was carried out as

instructed in the product sheet by Thermo Fisher with some slight deviations. 8 µL of the RNA samples were added, random primers were used and RNaseOUT was not used. The optional removal of template was not done either.

3.4.2.6. RPA

The cDNA target sequence was exponentially copied with RPA using one of the primer pairs with the TwistAmp Basic kit (TwistDx limited, n.d.). The instructions in the kit were followed. The incubation temperature was set to 39°C, 11,2 µL of nuclease free water was added to the master mix per test sample, and 2 µL of each cDNA sample was used in the RPA reaction. 4 minutes into incubation the tubes were agitated, spun down and put back to incubate further. This was done because of the poor quantity and quality of the original RNA samples.

First the RPA procedure itself was tested using NIBIO's standard NAD PCR primers in the RPA reaction. The three different primer pairs (PVY1, PVY2, PVY3) were then tested on the samples that had the highest RNA quality and quantity (82-4 and 82-5) before reverse transcription into cDNA. The most promising primer set was then tested on 10 more samples. All the three primer pairs were tested on 10 samples. To test the method used, total RNA was extracted from leaves from four plants, and ran though RPA with the best primer pair. Two with symptoms and two without. This was done as the extraction from leaves usually gives higher quality and quantity of RNA.

3.4.2.7. Gel electrophoresis

To visualize the resulting RPA product the samples were run through gel electrophoresis using the Sub Cell GT System and using the protocol in its instruction manual (BIO RAD, n.d.-b). SYBR-safe (Thermo Fisher Scientific Inc., n.d.) was added to the gel and the buffer used was Tris-borate-EDTA (TBE). The gel was hardened for 20-40 minutes. In the leftmost well a 100 bp ladder was added. The wells to the right of this, had a positive and a negative control sample, followed by the rest of the samples. 10 µL of RPA product was pipetted in these wells. The electrical current over the gel was driven by a voltage of 90 V, and when the loading dye had traveled ⅔ of the gel imaging was done. To get a gel image a ChemiDoc imaging machine was used with the Blot/UV/Stain-Free Tray (BIO RAD, n.d.-a)). All three

primer pairs were tested in this way, to check if any of the pairs gave clear results when the samples were negative or positive.

4. Results

4.1. Slow growth test experiment

In the test experiment, shoot cuttings were put on medium the 05.06.2023. The control plants were omitted from the data gathering after four months, as all the control plants had overgrown the tubes. It was hard to distinguish any height or number of leaves. The 0,5 mg/L ABA treated plants were not measured after 8 months as they had reached the top of the tubes and withered by then. After nearly six months the control plants from the test experiment still had green shoot ends, with many varieties having formed mini-tubers (see figure 9).

Figure 9. Test experiment control after six months of growing (28. November). The red arrows point out minitubers

As figure 10 and 11 show, the control and 0,5 mg/L ABA treated plants had a logarithmic growth, as they reached the top of the tube (12,5 cm) early and died off. The 0,5 mg/L treated plants grew slower and lost viability approximately two months later than the control.

The highest ABA concentration (5 mg/L) kept the plants very short (see figure 10 and 11), but some plants had died at five months of growth on the higher (3 mg/L and 5 mg/L) ABA concentration mediums (see figure 12). When comparing the boxplots of the apical shoot cuttings (figure 10) and the axillary shoot cuttings (figure 11), the apical shoot cuttings seemed to grow faster on every treatment, except 5 mg/L ABA treatment, which grew too little to tell.

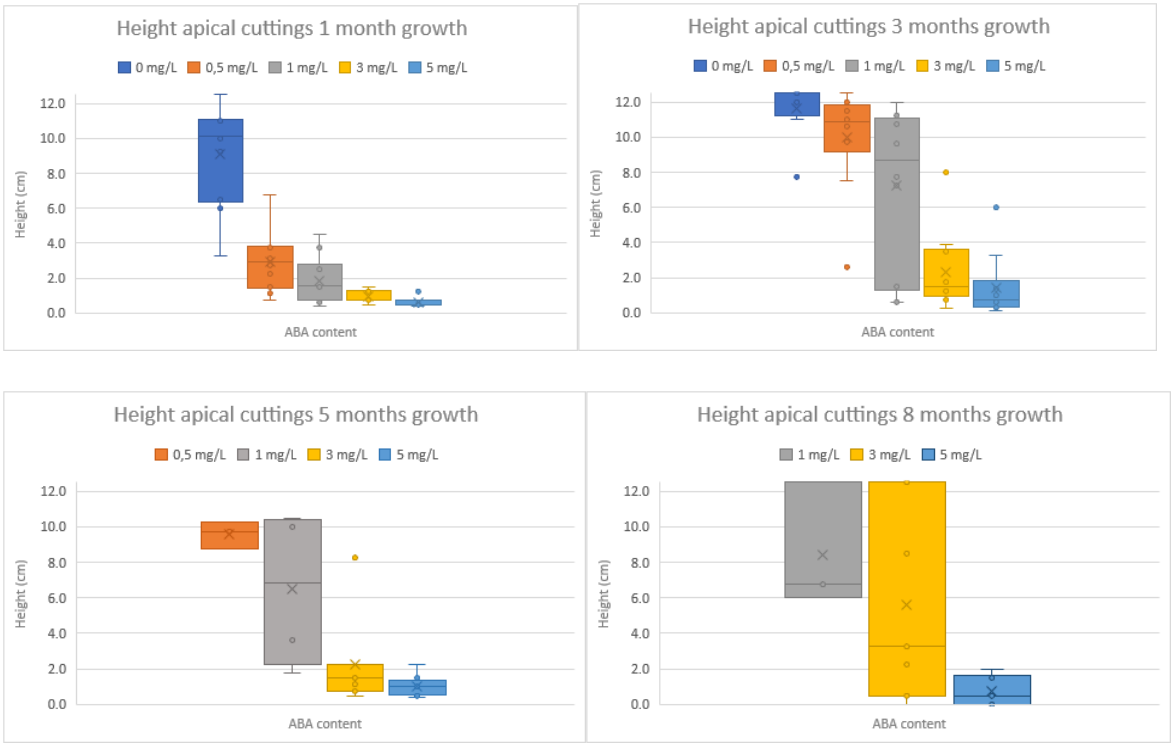


Figure 10. Boxplot of mean height of all ten varieties apical shoot cuttings 1, 3, 5 and 8 months after they were put on medium. The data points used for the boxes were based on the mean height of the ten varieties. The X marks mean and the line over the box shows the median.

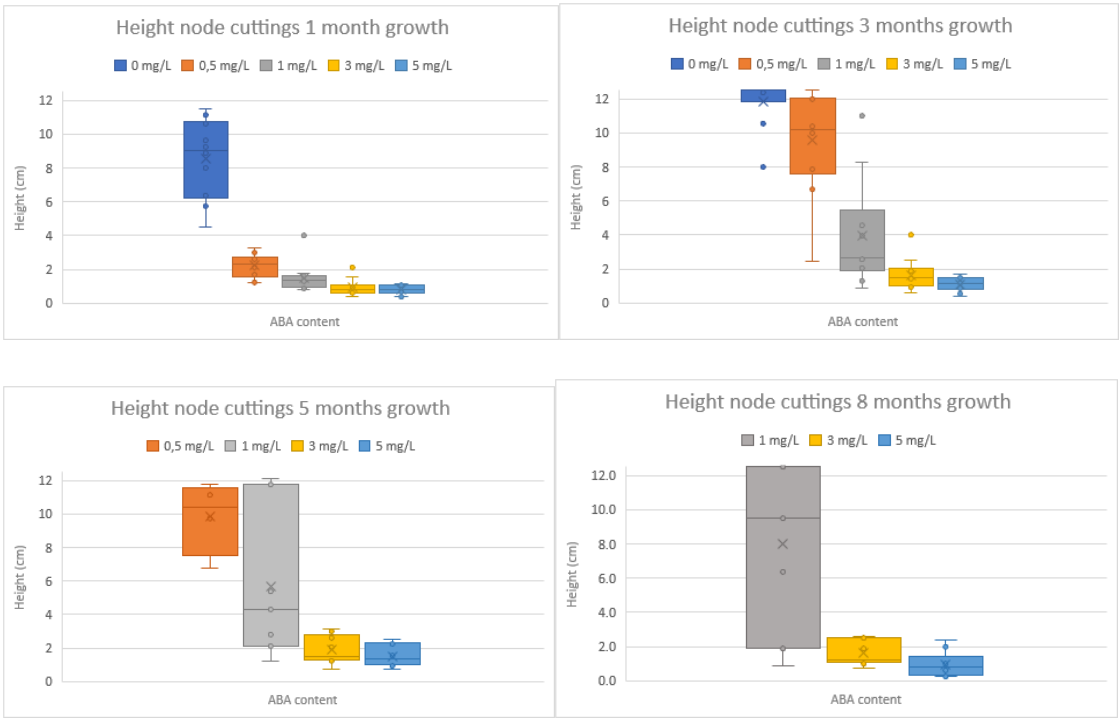


Figure 11. Boxplot of mean height of all ten varieties axillary shoot cuttings 1, 3, 5 and 8 months after they were put on medium. The data points used for the boxes were based on the mean height of the ten varieties. The X marks mean and the line over the box shows the median.

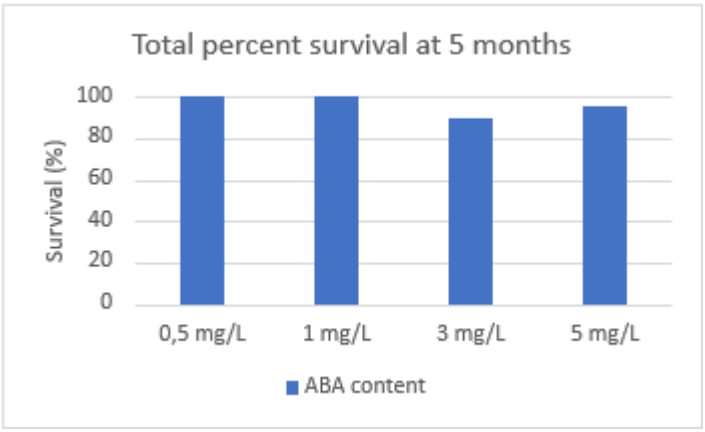


Figure 12. Diagram of percent plants that had survived on the different ABA concentration after five months. The diagram was based on both apical shoot and axillary shoot cuttings.

At 9 months of growth the treatments with lower content of ABA (control, 0,5 mg/L and 1 mg/L) had the highest death percentages (figure 13). The control and 0,5 mg/L ABA plants were all unviable and so omitted from the diagrams. Figure 13 shows a higher survival rate for the axillary shoot cuttings than the apical shoot cuttings, if we compare all the remaining treatments (1 mg/L, 3 mg/L and 5 mg/L).

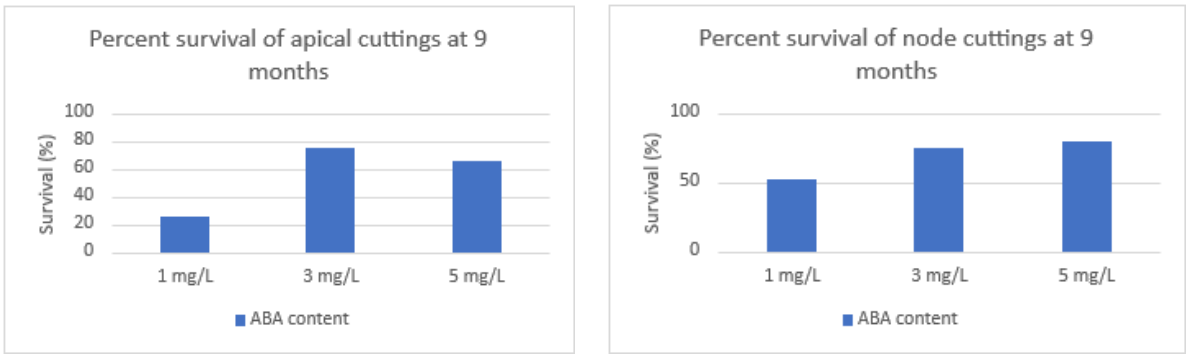


Figure 13. Diagrams showing percent of plants that had survived, against the ABA concentration they had stood on. The left diagram shows apical shoot cutting survival and the right shows axillary shoot cutting survival. Data was gathered after 9 months on the medium.

To see if the still green 5 mg/L ABA plants were viable and alive they were transferred to a new medium without ABA after 9 months on medium (5/3-2024). The plants were put together according to their variety number.

14 days after the 5 mg/L ABA treated plants were put on medium, most of the plants had grown considerably (see figure 15). The apical shoot cuttings had markedly grown less than the axillary shoot cuttings, some apical shoots not growing at all.

From right, variety: 234 (Y-67-20-40-18), 238 (Bruse), 241 (Gullauge P2).	
From right, variety: 377 (Eggeplumme), 393 (Raude from Skjåk), 449 (Rigneriks pot.) and 458 (Purple Peruvian).	
From right, variety: 508 (Iverpotet), 562 (Liverpool) and 636 (Asparges potato).	

Figure 15. Shows the length of the 5 mg/L ABA treated plants 14 days after transfer to potato growth medium (19/3-24). The plants had stayed on 5 mg/L ABA medium for 9 months.

4.2. Slow growth

The plants in the bigger slow growth experiment were observed over a four-month period. Every month all the tubes were photographed to gather data (see figure 16). The height data was plotted into a table in Excel (see table 3), and the mean heights (cm) of the varieties were used to make boxplots (see figure 17).

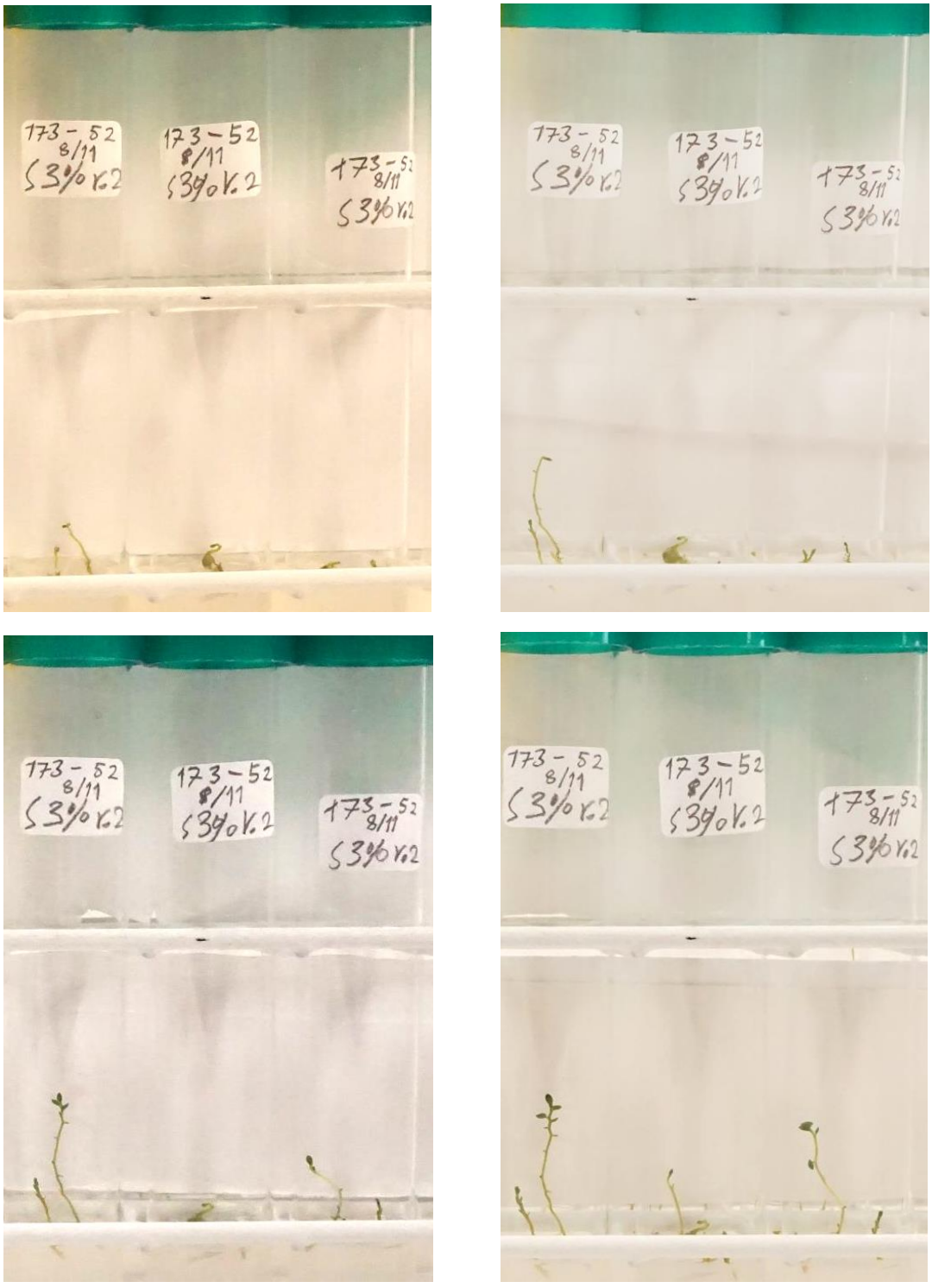


Figure 16. Photographs taken of variety 173 on medium with 3% sucrose and 1 mg/L ABA after 1-, 2-, 3- and 4-months growth.

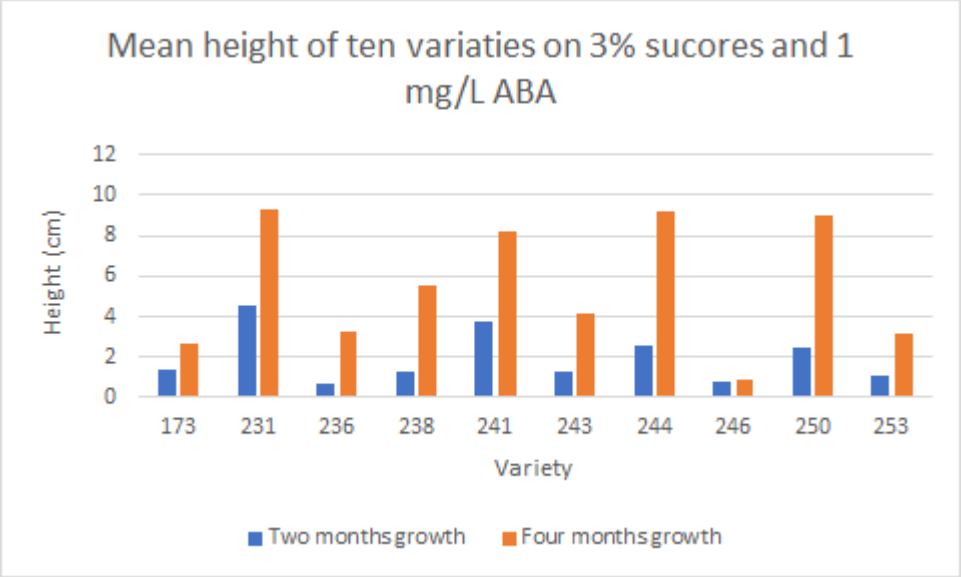
When looking at the boxplots for mean variety height after two and four months there is a marked difference between the heights of the 3%, 7% and 15% sucrose treated varieties (See figures 17). A higher sucrose content correlated with a lower growth rate. An increase in ABA and sucrose exacerbated the growth reduction. Notably, 15% sucrose gave only growth in the control group. The high sucrose also gave some unordinary shoot growth. Plants grew down into the medium and there was a tendency for early development of mini-tubers.

Figure 17. Boxplots of the mean heights of all ten potato varieties compared to the ABA treatment after two months (left) and four months (right) on the medium. The data points used for the boxes were based on the mean height of the ten varieties. The X marks mean and the line over the box shows the median.

Table 3. Data table with mean height (cm) after four months sorted by varieties and medium.

		N-94-30-22	Y-67-20-40	Beate	Bruse	Gull-auge P2	Inno-vator P3	Kerrs Pink	Laila	Oleva	Peik
Sucrose [%]	**ABA [mg/L]**	**173**	**231**	**236**	**238**	**241**	**243**	**244**	**246**	**250**	**253**
3	**0**	11.4	12.3	8.1	11.6	12.0	10.8	12.1	10.9	11.7	10.2
7	**0**	8.8	9.3	7.7	10.5	9.7	9.3	11.8	6.7	10.7	7.4
15	**0**	6.8	2.8	1.8	2.3	4.4	4.3	7.6	1.7	1.5	0.9
3	**0.5**	7.1	10.3	4.3	10.5	11.5	5.0	12.5	6.5	9.5	5.0
7	**0.5**	1.3	5.6	2.4	8.0	3.1	4.8	12.5	0.5	7.7	0.6
15	**0.5**	0.2	0.4	0.2	0.3	0.2	0.4	0.3	0.3	0.1	0.1
3	**1**	2.7	9.3	3.3	5.5	8.2	4.1	9.2	0.9	9.0	3.2
7	**1**	0.6	6.7	1.1	0.6	3.8	0.9	7.7	0.3	3.0	0.2
15	**1**	0.1	0.1	0.1	0.2	0.1	0.2	0.1	0.3	0.3	0.0
3	**3**	0.4	2.8	1.2	0.4	1.4	1.2	2.0	0.3	2.3	0.8
7	**3**	0.3	0.4	0.4	0.2	0.2	0.3	0.7	0.2	0.3	0.1
15	**3**	0.1	0.1	0.1	0.3	0.2	0.1	0.1	0.3	0.2	0.1

Comparing the mean heights on the medium with 3% sucrose and 1 mg/L ABA against the mean heights on the medium with 7% sucrose and 0,5 mg/L ABA shows that there is a higher variance between the varieties on the 7% sucrose and 0,5 mg/L ABA (see figure 17 and 18). Calculating variance in Excel using =VARIANS.S(mean of varieties height) gave a variance of 9,86 for 3% sucrose and 1 mg/L ABA and a variance of 15,04 for 7% sucrose and 0,5 mg/L ABA.



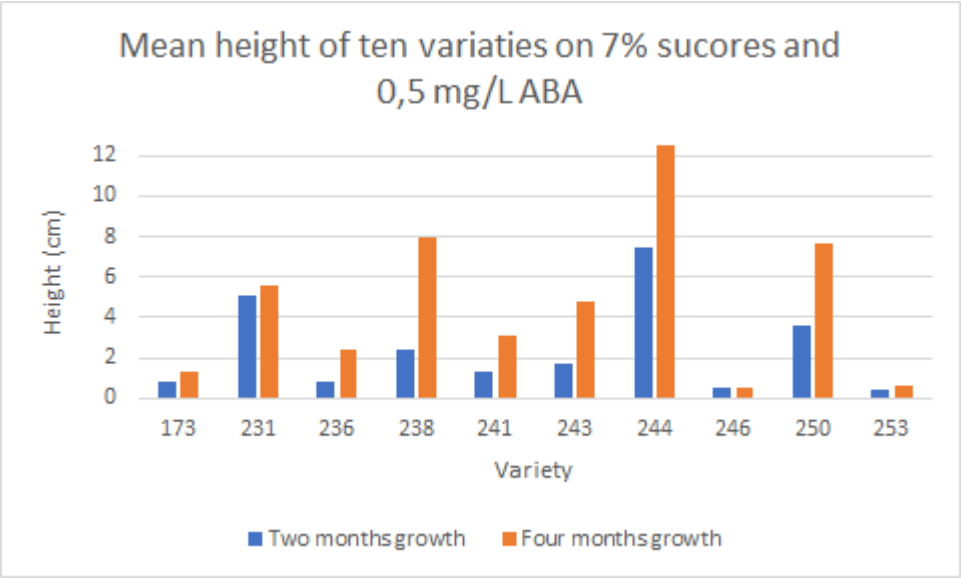


Figure 18. Bar charts showing mean heights of the ten varieties on the medium with 7% sucrose and 0,5 mg/L ABA (over) and on the medium with 3% sucrose and 1 mg/L ABA (Under).

4.3. Virus eradication

The survival of the meristems was very low, and many only grew into a leaf, a root, or became undifferentiated callus lumps (see figure 19 for callus). Some of the calluses later grew shots and roots that were usable. Figure 19 shows some of the plants that grew normally. The variety Early Rose was therefore not successfully grown from meristem. An *in vitro* culture grown from shoot tips was established for Early Rose however, making further attempts at meristem cultivation easier.

Figure 19. To the left is meristem derived Kvit Rund Kvam plant material after 6 weeks on meristem medium (6/11-2023), and in the middle are the same tubes with regenerated plants 22 days later flipped. The picture to the right is a callus grown from a meristem of the variety Åkerøy.

Five months after meristem culturing, five plants were transferred to soil. The plants originated from different meristems. Two Kvit Rund Kvam plants were transferred. Two plants of Blåpotet were also put on soil and one åkerøy plant. All the plants survived the first week in the greenhouse (see figure 20).

Figure 20. To the left are the mersitem derived plants right after being planted in soil after five months *in vitro* (07.03.2024), to the right the same plants a week later (14.03.2024).

When the plants had grown in the greenhouse for a month, an ELISA test for PVA, PVM, PVS, PVX, PVY and PLRV was done on leaf samples from them. This showed that both the Blåpotet clones were virus free, and that both the 736 clones were clean of PVY virus (see table 4). The clone Kvit Rund Kvam-1 seemed to have contracted PVM oddly enough. The Åkerøy clone had the same virus infections before and after meristem cutting.

Table 4. Virus infections detected before and after meristem culture.

Clone	Virus infection before meristem culture	Virus infection after meristem culture
Åkerøy-1	PVS, PVX	PVS, PVX
Blåpotet-1	PVS, PVX	None
Blåpotet-2	PVS, PVX	None
Kvit Rund Kvam-1	PVS, PVX, PVY	PVM, PVS, PVX
Kvit Rund Kvam-2	PVS, PVX, PVY	PVS, PVX

4.4. Virus detection: RPA

Starch from the potato tubers left a white residue in the spin column when extracting the total RNA (see figure 21). There was a varying concentration and purity of RNA measured in the samples from tuber skins (see sample 82-1 to 86-25 in table 5). Most of the potato tuber-skin samples yielded low quality and very low quantity of RNA from the total RNA extraction. The total RNA extraction from potato leaves all yielded high quality and quantity of RNA (see the last four rows of table 5).

Figure 21. Shows yellow-white starch (red box) stuck in the spin column filter after centrifugation.

Table 5. Table showing RNA sample concentration and quality. The results marked with green are acceptable for reverse transcription and PCR. The stronger colored results are more optimal than the pale green. The last four samples were taken from leaves (1, 813, 814 and 815).

RNA sample	RNA concentration (ng/µL)	260/280	260/230
86-1	82.2	1.96	1.76
82-1	27.15	1.895	1.135
82-2	23.55	1.895	1.145
82-3	27.5	1.71	0.96
82-4	89.5	1.99	1.81
82-5	108	2.02	1.92
82-6	51.8	1.93	1.51
82-7	38.8	1.87	1.34
82-8	23	1.9	1.13
82-9	22	1.78	1.05
82-10	18.1	1.84	0.97
82-11	75.3	2.01	1.87
82-12	2.64	1.86	1.33
82-13	25.3	1.91	1.21
82-14	29	1.89	1.25
82-15	14.3	1.65	0.97
82-16	18.3	1.78	1.12
82-17	29.5	1.9	1.42
82-18	11.2	1.54	0.89
82-19	10	1.55	0.6
82-20	21.4	1.87	0.99
82-21	20.3	1.78	0.94
82-22	37	1.9	1.26
82-23	81.2	1.99	1.74
82-24	16.3	1.77	0.93
1	312.2	2.07	2.26
813	570	2.1	2.43
814	487.7	2.09	2.25
815	664.4	2.1	2.47

The RPA procedure itself was tested by amplifying the housekeeping gene NAD in the test samples 82-1 and 82-2. Gel electrophoresis of the product gave streaks of DNA fragments

longer than the target sequence, a band around 190 bp (which is near the length of the target sequence) and a band around 150 bp long (Figure 22 left picture).

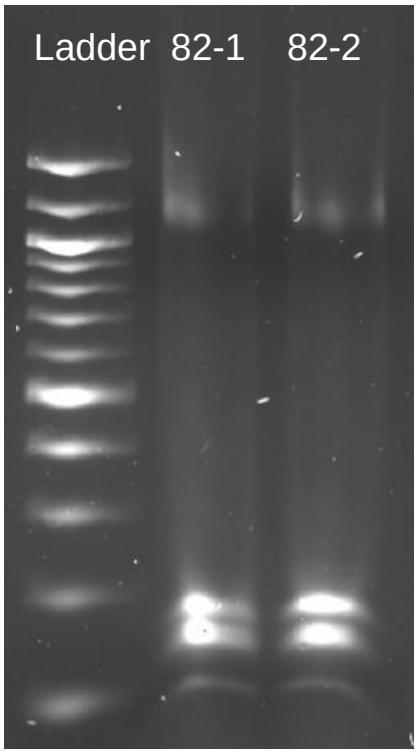


Figure 22. Test gel electrophoresis of RPA and NAD primer product (target sequence 188 bp). From left to right; 100 bp ladder, test sample 82-1, and then test sample 82-2.

The samples that had the highest RNA quantity and quality (82-4 and 82-5) were used to see if the primer pairs amplify the target virus gene sequences. NAD was also amplified in the two samples to use as positive control, but was lacking in signal strength, and (as earlier seen) gave two bands where it should have been just one at 180 bp (see figure 23).

82-4 gave a good positive signal with all the primer pairs, and was after this used for positive control, as NAD was not viable as a positive control. The clearest band was produced by the PVY3 primer pair with sample 82-4 (figure 23), and so PVY3 was chosen to be tested on more samples first. The strongest signal came from 82-4 amplified with PVY2 . All of the primer pairs gave diffuse streaks of gene sequences when used on sample 82-5. The lanes were somewhat distorted and so the voltage got lowered for the next runs to 90 V.

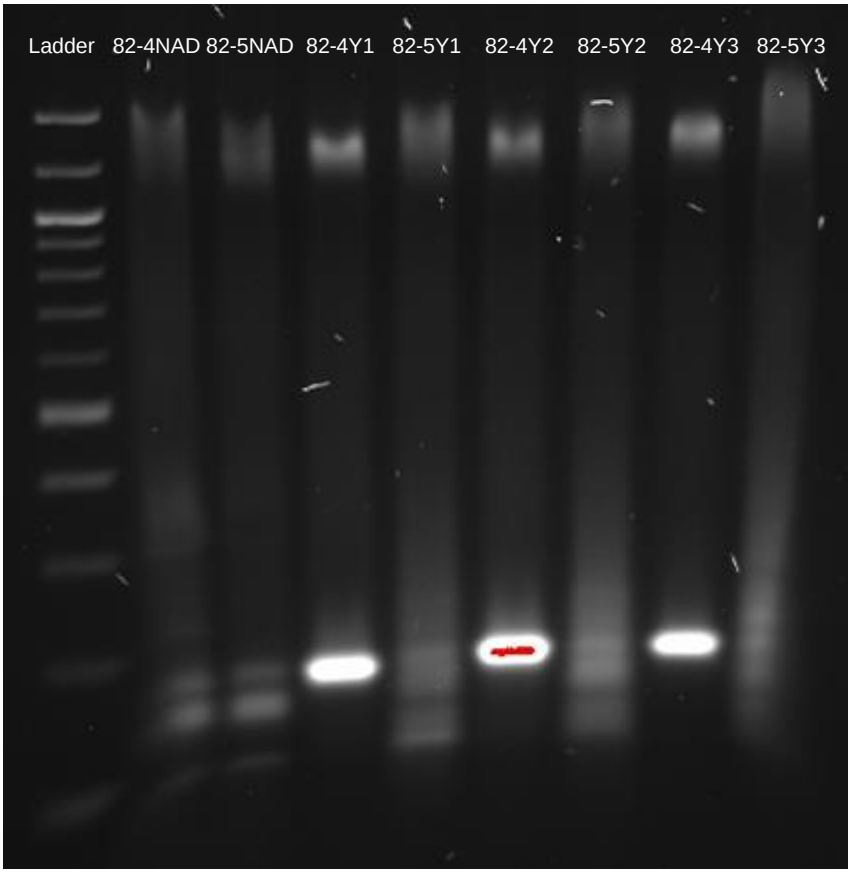


Figure 23. From left: 100bp ladder, 82-4 NAD, 82-5 NAD, 82-4 PVY1, 82-5 PVY1, 82-4 PVY2, 82-5 PVY2, 82-4 PVY3, 82-5 PVY3.

Primer pair PVY3 were tested on 10 other potato skin samples (82-1, -2, -3, -6, -7, -8, -9, -10, -11, -12) (see figure 24). The 82-4 sample used as positive control gave a single strong band. The other samples had in addition some diffuse bands around the stronger band, of the same theoretical length as the target sequence. Because of this PVY2 was tested on the same samples, to check if it would give clearer results.

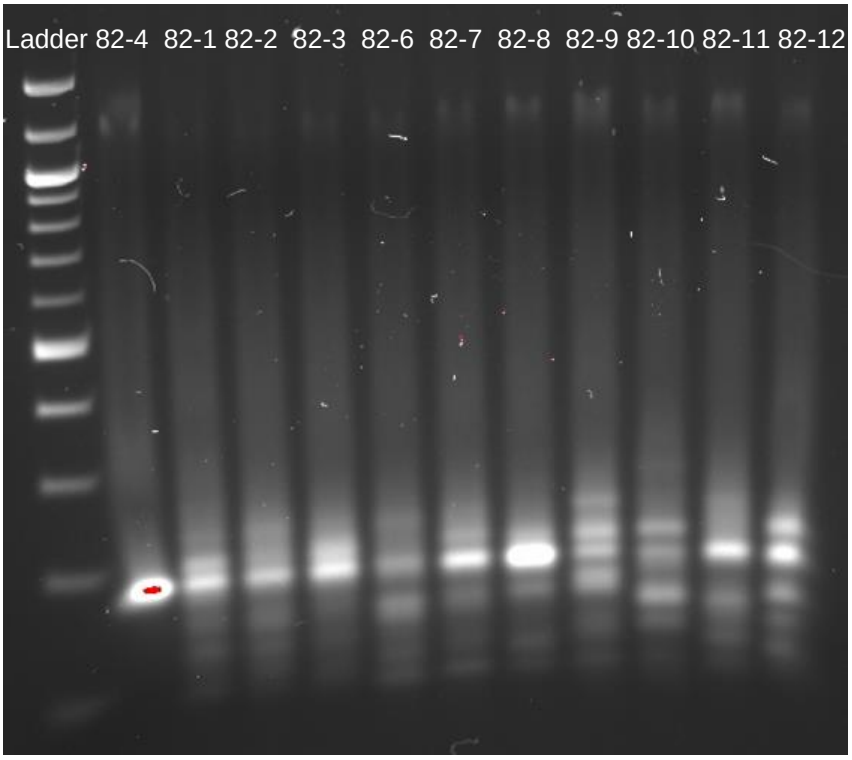


Figure 24. A gel showing fluorescence of PVY3 primer pair products. From left: 100 bp ladder, positive sample (82-4), 82-1, 82-2, 82-3, 82-6, 82-7, 82-8, 82-9, 82-10, 82-11, 82-12.

The only clear band that appeared using PVY2 on samples 82-1 to 82-12 came from the positive control sample (82-4) (see figure 25). All of the other samples had no bands, or a very diffuse band in the same spot as the positive, in addition to a stronger band of shorter nucleic acids. The primer pair PVY1 was then tested on the same samples to see if it would yield clearer test results for PVY.

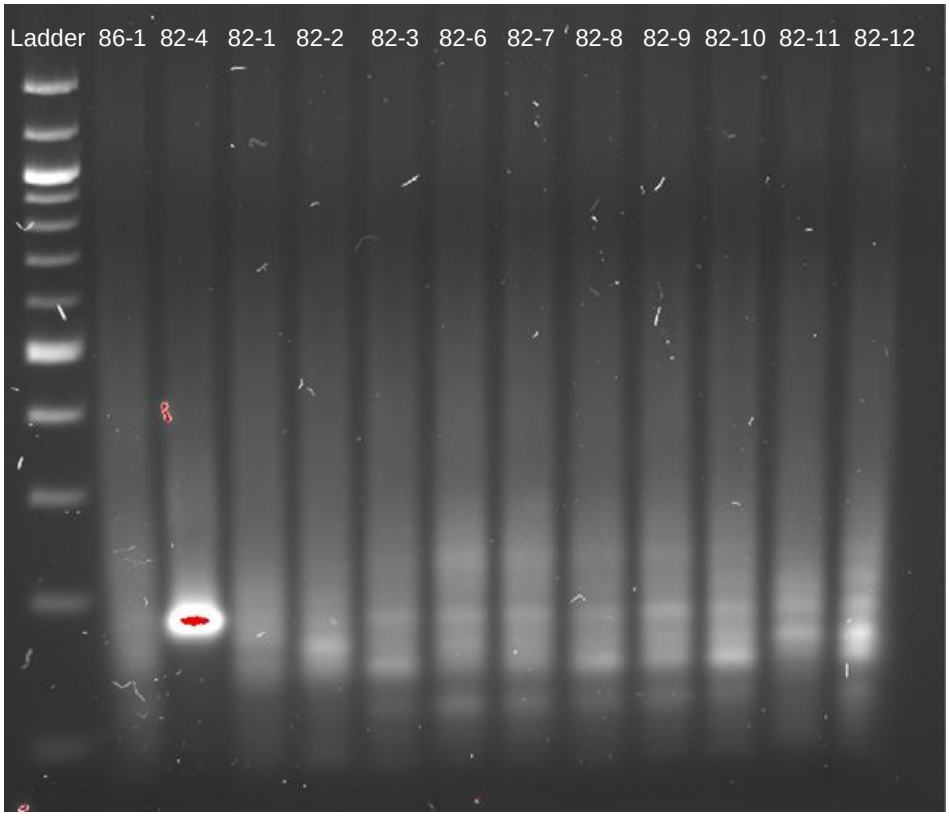


Figure 25. A gel showing fluorescence from PVY2 primer pair products. From the left: 100 bp ladder, negative 86-1, positive sample (82-4), 82-1, 82-3, 82-6, 82-7, 82-8, 82-9, 82-10, 82-11, 82-12.

To try and make the primer bindings more specific to the sequences designed for them, the PVY1 RPA was repeated with an incubation temperature increased to 42℃ according to the tips on optimizing the RPA amplification written in the Twist amp instruction manual (TwistDx Limited, 2018, p. 6). Mixing under incubation was also skipped to reduce the amount of shorter DNA fragments produced during RPA. The second picture in figure 26 shows the results of this change in method. The second gel run was done on a bigger gel to get a more readable result. The bigger gel gave more space between the wells and that meant less distortion.

Running PVY1 products gave clear bands at the expected length for most of the samples, but there are longer nucleic acid sequence bands visible on all samples (see figure 26). The positive control sample 82-4 gave a strong positive band. However, three very diffuse bands on the negative control (86-1), are also visible on all the samples except for the positive control (82-4). The results are clearer when using PVY1 instead of using PVY2 or PVY3.

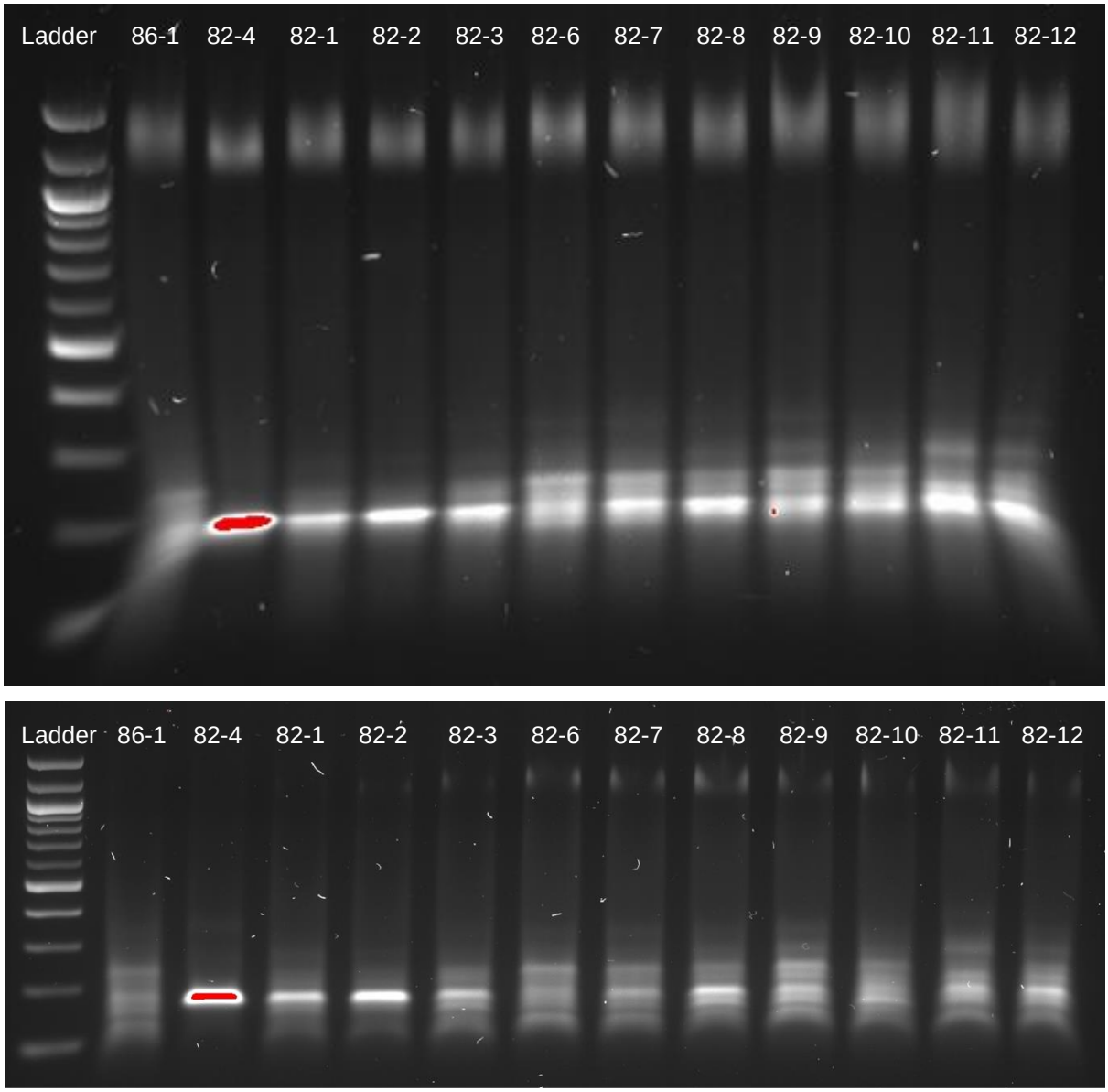


Figure 26. Electrophoresis ran for about 56 minutes and all the samples went through RPA using the PVY1 primer pair (primers from two different papers). From the left to the right of both pictures: 100 bp ladder, negative control 86-1, positive control 82-4, 82-1, 82-2, 82-3, 82-6, 82-7, 82-8, 82-9, 82-10, 82-11, 82-12.

When the PVY1 primer pair was tested on leaves from two symptomless plants and two plants with symptoms for PVY, the strong bands corresponded with the symptoms (see figure 27 and 28). Both 814 and 815 had symptoms and a bright band around the right length (the amplified DNA should be 183 bp) (see figure 28). The negative control (shallot DNA sample), looks the same as the samples from the two symptomless plants (1 and 813). RPA incubation was done at 42°C and no mixing was done during incubation.

Figure 27. Plants from which leaves were taken for testing the PVY1 primer pair. Two plants with PVY symptoms (814 and 815) and two plants without symptoms (1 and 813).

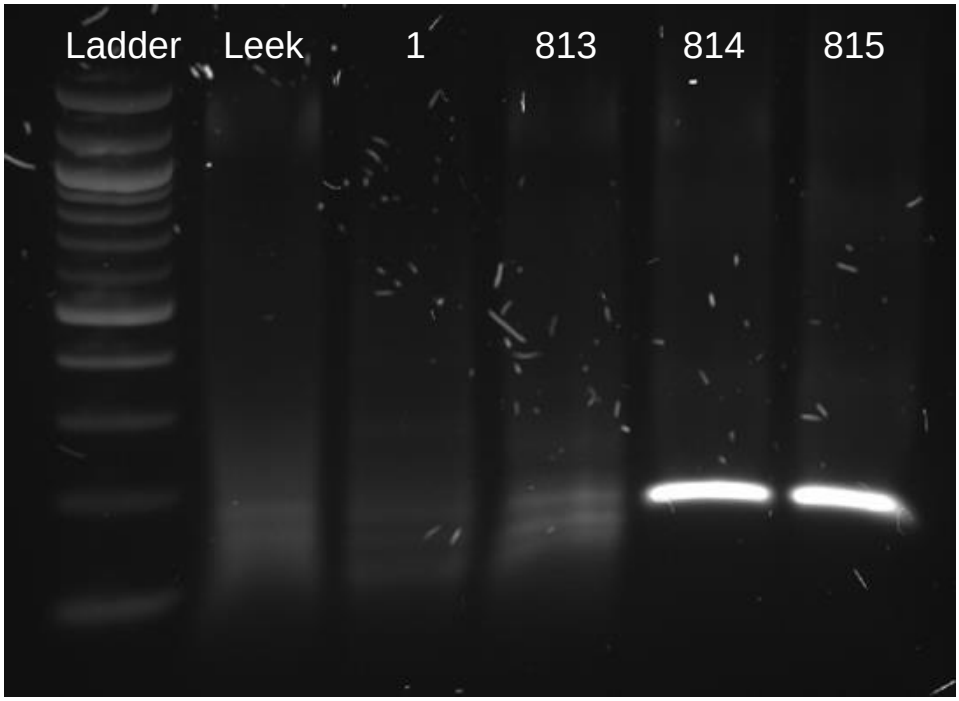


Figure 28. Electrophoresis ran for about 54 minutes and all the samples went through RPA using the PVY1 primer pair (primers from two different papers). From left to right: 100bp ladder, negative control of shallot leek DNA, nr. 1, 813, 814, 815.

4.5. Sources of error

In the slow growth test experiment the middle row of tubes were under less intense light than the other two rows. The row in question consisted of one of the tubes with axillary shoot cuttings from each of the varieties. This may skew the relations between the axillary shoot and apical shoot results.

As stated under "T-test" the best test of statistical power would have been ANOVA, but as the test experiment only has one variable (ABA), only a T-test was possible. That is why the standard deviation in the T-test was based upon the treatments with most variance at that time, the groups subjected to the least ABA (control and 0,5 mg/L ABA). This T-test should have given the highest number of repetitions necessary. The survival percent was not tested in statistical power, but was only registered for the test experiment.

In some instances, acetic acid had to be used to lower the pH of the slow growth mediums, as the addition of ABA made the mediums more basic. There were also times where too much 1M NaOH was used for increasing the pH and acetic acid was used to lower it again. This

happened when making the 3% sucrose with no ABA medium for repetition 1 and inn repetition 2 for 3% sucrose with 0.5mg/L ABA medium.

In repetition 1 there were just enough medium tubes for the setup of the experiment, but too much or too little of the medium was dispensed in some of the tubes. Extra tubes were therefore made in repetition 2 to mediate the lack of tubes. The tubes that were missing were four 3% sucrose with 0.5mg/L ABA tubes, one 7% sucrose 0.5 mg/L ABA tube, two 7% sucrose 3 mg/L ABA tubes and two 15% sucrose 0.5 mg/L ABA tubes. All of the missing tubes were for variety 253. All the missing tubes, except one 15% sucrose 0.5mg/L ABA tube, were reproduced in repetition 2.

Some tubes were lost to infections by bacteria or fungi (see figure 29). Occasionally it was hard to distinguish between fungi infection and root hair growth. The tubes that were visibly infected in repetition 1 were thrown out, and duplicates were made in repetition 2. There were some infected tubes in repetition 2 as well. These were also thrown away, as the infection most likely would weaken the plants and influence the results.

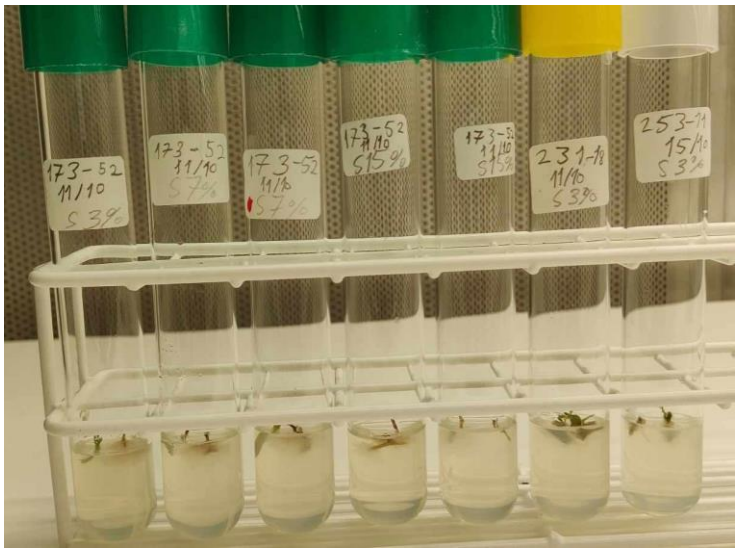


Figure 29. A few of the tubes that were lost to infection.

The light in the growth chamber was uneven from shelf to shelf, with repetition 2 plants standing under less illumination until 18.12.2023.

I might have, in some instances, counted the shoot tip as a node when there were no unfolded leaves. This is an error on my part. After four weeks the test experiments control plants became unreadable as all the plants had reached the top of their tubes, and leaves had begun to wilt. The counting of leaves was too difficult and imprecise, giving very varying results. This is why boxplots for leaf numbers had to be scrapped, as these results are not to be trusted. The data on the height of the plants and survival percent was kept.

5. Discussion

5.1. Test experiment

The test experiment was primarily done to find the number of repetitions needed for the large-scale experiment. It was also used to see the survival over a longer period and to gauge which ABA concentrations to use in the slow growth mediums of the large-scale experiment.

The apical shoots seemed to grow faster on every treatment. This might stem from the axillary shoot cuttings having to induce the node sticking over the surface of the medium to grow the shoot. This slowed the growth independent of the medium. However, the middle standing row of axillary shoots were subjected to less light as they were shadowed by the other tubes. This might have skewed the results, and we cannot say if the axillary shoot cuttings themselves grow slower or if it was because of the tight spacing.

Five months into the test experiment some 3 mg/L and 5 mg/L ABA treated plants died. These most likely died from the high amount of ABA in the medium. ABA signals to close the stomata lessening the loss of water (Evert & Eichhorn, 2017, p. 650), but as a side effect this may affect the uptake of nutrients negatively. This could have killed the plants. ABA is also commonly known to be a growth inhibitor by inhibiting cell division (Slater et al., 2008, p. 43). This might have hindered the development of the cuttings roots so much that it died before establishing on the medium. To reiterate, it used up the nutrients in the medium near it as it had no roots. After nine months of growth the treatments with 3 mg/L and 5 mg/L ABA had the highest survival percent from 80% to 60%.

The axillary shoot cuttings survived better on the 5 mg/L ABA medium, growing more after transfer to normal medium than the apical shoot cuttings. Looking at the survival percentage also shows a higher overall survival of the axillary shoot cuttings at nine months of growth, compared to the apical shoot cuttings. This could stem from their slowed growth, as the fast-

growing plants will use up the nutrients that are available to them faster. The higher axillary shoot survival can also come from a difference in uptake of nutrients or ABA.

5.2. Slow growth

As 15% sucrose had given only growth in the control group at four months and made many shoots grow downwards, this amount of sucrose is too high for use in the potato bank. The plants on the high sucrose mediums also seemed to make mini-tubers earlier than the others. This might have happened because the *patatin* gene encoding for 40%-30% of the soluble protein in tubers are induced by sucrose (Yoon et al., 2021, p. 2), and sucrose amount influences tuber development (Yoon et al., 2021, p. 1).

As can be seen in the literature part on sucrose and ABA, they work together to slow the growth. The high sucrose down regulates transport of sucrose and promotes synthesis of starch (Rook et al., 2006; Yoon et al., 2021, p. 3). Both ABA and sucrose down regulate the making of enzymes important for photosynthesis (Rook et al., 2006). This is most likely why we see a decrease in growth on the high sucrose and ABA mediums. The osmotic pressure of the medium might also have been modified by the sheer amount of sucrose added, stressing the plants to grow less. The sucrose amounts might also have been so high as to be toxic for the plants, resulting in slower growth and plant death.

The medium with 7% sucrose and 0,5 mg/L ABA could be a good candidate for use in the potato bank. This medium however gives very varied height after four months (see figures 17 and 18)). The medium with 3% sucrose and 1 mg/L ABA also gives a slow growth, without having a deadly amount of ABA. If the amount of early axillary shoot death is acceptable, then the 3% sucrose and 3 mg/L ABA medium might be considered for use in the potato *in vitro* bank.

The tubes could have been observed over a longer period of time than just four months, but the start of the experiment took longer than expected. The tubes were handed over to NIBIO for further observation, after my experiment was terminated. It would have been interesting to see if the 15% sucrose and 0 mg/L treated plants would grow normally on potato growth medium when transferred.

5.3. Virus eradication

Few meristems survived the meristem cutting. This might have happened because they dried out during the cutting. Too much time passed. For many plants I failed to cut out the meristem, rather cutting out cell tissue determined to become a leaf or root.

In the end five meristems grew into plants that were tested for viruses. There were two meristem derived plants of variety Kvit Rund Kvam, two of variety Blåpotet and one meristem derived plant of Åkerøy that survived the transition to soil. No plants of the variety Early Rose were produced from meristem.

Blåpotet-1 and Blåpotet-2 gave negative ELISA results for all the viruses (see table 4). This could stem from cutting meristems off of a random virus free tuber. This could also be the result of two successful meristem extractions. The PVY infection in Kvit Rund Kvam was successfully cut away from Kvit Rund Kvam-1 and Kvit Rund Kvam-2, but Kvit Rund Kvam-1 was infected by PVM. The infection could have happened during transfer to soil, when transferring between mediums or under meristem extraction. The infection most likely came from the Early rose plant material. I learned how to extract meristems, but need more practical experience for higher survival rate of the meristems, increasing the chance to produce virus free meristems.

5.4. Virus testing: RPA

The NAD primers that were used in the RPA at the start of the experiment gave two bands. One of the bands lay around the sequence length expected (188bp), while the other was shorter than this (see figure 22). The primers might have bonded on another sequence giving a second band or the shorter lengths of sequence came from fragmentation of the copied target sequence. This means that the primer pair was not sequence specific enough when using it for RPA or the copied sequences got damaged. The primer pair was originally designed for PCR and was not optimal for RPA.

When testing the three different primer pairs that amplified a conserved sequence in the PVY's genes, the best result came from primer pair PVY1. It gave the least number of extra bands or defuse streaks of nucleic acid. It seems these diffuse bands correlate with low quality and quantity of RNA after extraction of total RNA (see table 5 and figure 26). It seems the diffuse bands also showed up for PVY negative plants, as the shallot leek sample as well as symptom free plants 1 and 813 got diffuse bands on the gel. This means the bands are made

when the PVY1 primers don't have target sequences to bind to, and then bind to other sequences. The amount of RNA and the quality of the samples from the potato skins seemed to be too low for the PVY1 test to work.

A faster method to test for PVY by using RPA on potato skin samples, was not possible to achieve during my thesis work. The idea was to drop the step involving waiting for a tuber to grow into a plant. The problem lies in the RNA sampling.

The sampling of potato skin or extraction of RNA might be improved to get a higher RNA quality and quantity, for example by taking less of the starchy inside of the potato when sampling by filing off the skin instead of peeling. Not using guanidium salts and instead using a borate-tris buffer with high amounts of NaCl, sodium dodecyl sulfate and Na_2SO_3 for the extraction solution might help (Kumar et al., 2007). The guanidium salts make a viscous substance with the starch. Improvements to the method must be made for the RPA with PVY1 to work as a test for PVY. Using DNase as the optional step of the extraction protocol suggests during extraction of RNA would help increase the quality of the RNA sample. The optional step of removing RNA template after reverse transcription into cDNA, might also give less background noise on the gel.

Switching to using a kit optimized for RNA extraction from tubers might yield the best and fastest result. BIOREBA's Potato DNA/RNA rapid extraction set is meant for pathogen detection and only has a five-step protocol (*Potato DNA / RNA Rapid Extraction Set*, n.d.). BIOREBA states that with their kit it only takes 30 minutes to complete extraction. The kit utilizes extraction bags in the same way I did prior to RNA extraction. Testing these changes in method should give a more reliable diagnosis. Now testing for viruses with RPA in the manner described in the methods of this thesis on tuber skin samples is unreliable.

6. Conclusion

The slow growth test experiment showed that the axillary shoot cuttings seemed to have a higher survival percent than the apical shoot cuttings. The experiment itself went on for only a short period of four months. One month more than the time the *in vitro* potato plants usually stand on medium. 15% sucrose gave no growth with ABA and malformation of plants in the control group, all things we do not want. ABA of 3 mg/L gave lower survival percent in the test experiment, but the plants started growing as normal when transferred to potato growth

medium. 3 mg/L ABA mediums would be viable to use in the potato *in vitro* bank if the percent survival shown is acceptable.

The trends seen from the mean height boxplots for month two and four shows that slow growth medium with 3% sucrose and 1 mg/L ABA would work the best. This medium has lower variance in mean length between varieties then the medium with 7% sucrose and 0.5 mg/L ABA, gave no early shoot cutting death, and had less abnormal growth. Switching to using 3% sucrose and 1 mg/L ABA medium in the potato *in vitro* bank, would involve little risk of loss of varieties, and should lessen the work needed to maintain it, as the varieties can stay on the same medium for longer.

in vitro cultures of the four virus infected varieties were made. Blåpotet were successfully eradicated of the viruses tested for with ELSIA and PVY infection was successfully removed from Kvit Rund Kvam-2. I was able to learn how to meristem culture, and was somewhat successful in eradicating the viruses.

The RPA test for PVY using the PVY1 primers works for the leaf samples, as they yield consistently high RNA quality and quantity samples. Using RPA on leaf samples is not much easier than running a PCR, so the original method of testing for PVY should be kept. Improving the gathering of the potato skin samples and altering the total RNA extraction from the tuber skins might improve the RNA samples quality and quantity. This could make the bands on the gel more readable and by that the test for PVY more accurate on potato tubers. There is still some work needed to improve the virus testing on tuber skins, before it can be considered accurate. I would urge NIBIO to try a kit specialized for RNA extraction from tubers, such as BIOREBA's Potato DNA/RNA rapid extraction set (*Potato DNA / RNA Rapid Extraction Set*, n.d.).

References

Atli Arnarson BSc, PhD & Alyssa Northrop, MPH, RD, LMT. (2019, March 7). *Potatoes 101: Nutrition Facts and Health Effects*. Healthline. https://www.healthline.com/nutrition/foods/potatoes

Bidlack, J. E., & Jansky, S. (2017). *Stern's Introductory Plant Biology* (14th edition). McGraw-Hill. https://lccn.loc.gov/2016044453

BIO RAD. (n.d.-a). *ChemiDoc and ChemiDoc MP Imaging Systems with Image Lab Touch Software User Guide* (3.0.1, p. 170). Bio-Rad Laboratories, Inc. https://www.bio-rad.com/en-no/category/chemidoc-imaging-systems?

BIO RAD. (n.d.-b). *Sub-cell GT Agarose Gel Electrophoresis System Instruction Manual* (1–170–4401 to 170–4406 and 170–4481 170–4486, pp. 1–14). Bio-Rad Laboratories, Inc. https://www.bio-rad.com/en-no/product/mini-sub-cell-gt-systems?

BIOREBA. (n.d.). *BIOREBA Universal Extraction Bags* [Lab supplies]. Retrieved April 13, 2024, from https://www.bioreba.ch/extraction-bags/Extraction-bags-Universal.aspx

Cassedy, A., Della Bartola, M., Parle-McDermott, A., Mullins, E., & O'Kennedy, R. (2022). A one-step reverse transcription recombinase polymerase amplification assay for lateral flow-based visual detection of PVY. *Analytical Biochemistry*, *642*, 114526. https://doi.org/10.1016/j.ab.2021.114526

Charoensub, R., & Phansiri, S. (2004). In vitro Conservation of Rose Coloured Leadwort: Effect of Mannitol on Growth of Plantlets. *Agriculture and Natural Resources*, *38*(5), 97–102.

Davis, L. A., & Addicott, F. T. (1972). Abscisic Acid: Correlations with Abscission and with Development in the Cotton Fruit. *Plant Physiology*, *49*(4), 644–648. https://doi.org/10.1104/pp.49.4.644

de Oliveira, Kívia Soares, & Magdi Ahmed Ibrahim Aloufa. (2022). Slow growth in vitro culture for conservation of Hancornia speciosa gomes. In *Floresta* (Vols. 007–016). https://www.sciencedirect.com/science/article/abs/pii/S2452219822001288

Department of Biosciences - University of Oslo. (2016). *Agaros gel elektroforese*.

https://www.mn.uio.no/ibv/om/skolelab/ressursarkivet/nedlasting-av-undervisningsmateriell.html

Dr. Qu Dongyu Director-general, FAO. (2022, May). *Role and Potential of Potato in Global Food Security*. https://www.fao.org/3/cc0330en/cc0330en.pdf

Evert, R. F., & Eichhorn, S. E. (2017). *Raven Biology of Plants* (8th ed.). W.H. Freeman & Company. https://www.akademika.no/realfag/biologi/raven-biology-plants/9781319156268r180

Ferrante, A., Vernieri, P., Serra, G., & Tognoni, F. (2004). Changes in Abscisic Acid During Leaf Yellowing of Cut Stock Flowers. *Plant Growth Regulation*, *43*(2), 127–134. https://doi.org/10.1023/B:GROW.0000040119.27627.b2

Food and Agriculture Organization. (1997). *Carbohydrates in human nutrition: Annex 2—Global trends in production and consumption of carbohydrate foods* (Viale delle Terme di Caracalla, Ed.). https://www.fao.org/3/W8079E/w8079e0g.htm

Fuentes, G., Talavera, C., Oropeza, C., Desjardins, Y., & Santamaria, J. M. (2005). Exogenous sucrose can decrease in vitro photosynthesis but improve field survival and growth of coconut (Cocos nucifera L.) in vitro plantlets. *In Vitro Cellular & Developmental Biology - Plant*, *41*(1), 69–76. https://doi.org/10.1079/IVP2004597

Gartner, C., & Winding, J. C. (1694). Horticultura, Det er: En kort Underviisning og Anledning, hvorledes en liden Lyst- Urte- Frugt- eller Kiøkken-Have i disse nordiske Lande, særligen her Nordenfields, best kand funderes, anlegges, indhægnes, afdeeles, Jorden dyrkes, plantes, saaes, vandes, formeeres og bevares. Udi nogle faa Capitler afdeelet. In *Schweigaardsamlingen* (1746th ed.). Trykt igjen paa nye 1746, efter eendeel Liebhaberes Begiæring, af Jens Christ. Winding. https://urn.nb.no/URN:NBN:no-nb_digibok_2020091428007

GSL Biotech LLC. (n.d.). *SnapGene* (7.1) [Computer software]. GSL Biotech LLC. https://www.snapgene.com/

Haider, M. W., Nafees, M., Ahmad, I., Ali, B., Maryam, Iqbal, R., Vodnar, D. C., Marc, R. A., Kamran, M., Saleem, M. H., Al-Ghamdi, A. A., Al-Hemaid, F. M., & Elshikh, M. S. (2022). Postharvest dormancy-related changes of endogenous hormones in relation to different

dormancy-breaking methods of potato (Solanum tuberosum L.) tubers. *Frontiers in Plant Science*, *13*. https://doi.org/10.3389/fpls.2022.945256

Jakobsone, G., Auzenbaha, D., & Megre, D. (2006). INFLUENCE OF ABA ON STORAGE POSSIBILITIES IN VITRO OF LILACS. *Acta Horticulturae*, *725*, 217–220. https://doi.org/10.17660/ActaHortic.2006.725.26

Joshi, C. J., Ke, W., Drangowska-Way, A., O'Rourke, E. J., & Lewis, N. E. (2022). What are housekeeping genes? *PLoS Computational Biology*, *18*(7), e1010295. https://doi.org/10.1371/journal.pcbi.1010295

Kamińska, M., Skrzypek, E., Wilmowicz, E., Tretyn, A., & Trejgell, A. (2016). Effect of light conditions and ABA on cold storage and post-storage propagation of Taraxacum pieninicum. *Plant Cell, Tissue and Organ Culture (PCTOC)*, *127*(1), 25–34. https://doi.org/10.1007/s11240-016-1026-1

Kekarainen, T., Merits, A., Oruetxebarria, I., Rajamäki, M.-L., & Valkonen, J. P. T. (1999). Comparison of the complete sequences of five different isolates of Potato virus A (PVA), genus Potyvirus. *Archives of Virology*, *144*(12), 2355–2366. https://doi.org/10.1007/s007050050649

Kumar, G. N. M., Iyer, S., & Knowles, N. R. (2007). Extraction of RNA from fresh, frozen, and lyophilized tuber and root tissues. *Journal of Agricultural and Food Chemistry*, *55*(5), 1674–1678. https://doi.org/10.1021/jf062941m

Landbruksdirektoratet. (2023). *Målpriser 2022-2023*. Landbruksdirektoratet. https://www.landbruksdirektoratet.no/nb/industri-og-handel/marked-og-pris/priser-og-prisuttak/frukt-og-gr%C3%B8nt/malpriser

Lobato, I. M., & O'Sullivan, C. K. (2018). Recombinase polymerase amplification: Basics, applications and recent advances. *Trends in Analytical Chemistry: TRAC*, *98*, 19–35. https://doi.org/10.1016/j.trac.2017.10.015

Lov data: Forskrift om settepoteter, Landbruks- og matdepartementet (1996). Authority: LOV-2003-12-19-124-§5, LOV-2003-12-19-124-§8, LOV-2003-12-19-124-§9, LOV-2003-12-19-124-§10, LOV-2003-12-19-124-§18, LOV-2003-12-19-124-§33, LOV-2003-12-19-124-§36, FOR-

2003-12-19-1790 https://lovdata.no/dokument/SF/forskrift/1996-07-02-1447/KAPITTEL_3#KAPITTEL_3

Merck. (n.d.). *Growth Regulators – Plant Tissue Culture Protocol*. Retrieved October 26, 2023, from https://www.sigmaaldrich.com/NO/en/technical-documents/technical-article/cell-culture-and-cell-culture-analysis/plant-tissue-culture/growth-regulators

Murashige, T., & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Physiologia Plantarum*, *15*(3), 473–497. https://doi.org/10.1111/j.1399-3054.1962.tb08052.x

National Library of Medicine. (n.d.). *Nucleotide BLAST: Search nucleotide databases using a nucleotide query*. Retrieved October 20, 2023, from https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&BLAST_SPEC=GeoBlast&PAGE_TYPE=BlastSearch

Norgen Biotek Corp. (n.d.). *Plant/Fungi Total RNA Purification Kit (Cat. 25800, 31350, 25850, 31900) | Norgen Biotek Corp.* Retrieved June 18, 2023, from https://norgenbiotek.com/product/plantfungi-total-rna-purification-kit

Pan, X. J., Zhang, W. E., & Li, X. (2013). In vitro conservation of native Chinese wild grape (Vitis heyneana Roem. & Schult) by slow growth culture. *Vitis*, *53*(4), 207–214. CABI.

Piepenburg, O., Williams, C. H., Armes, N. A., & Stemple, D. L. (2010). *Recombinase polymerase amplification* (United States Patent US7666598B2). https://patents.google.com/patent/US7666598B2/en

Posit. (2023). *R-studio* (2023.06.1 Build 524) [Computer software]. Posit Software, PBC formerly RStudio. https://www.posit.co/

Potato DNA / RNA rapid extraction set Nucleic acid extraction for potato samples User Guide (p. 4). (n.d.). BIOREBA AG. Retrieved May 10, 2024, from https://www.bioreba.ch/catalog/Kachel-Struktur-Potato-DNA-RNA-Extraction.aspx

Robin Harris, J. (1999). Negative Staining of Thinly Spread Biological Particulates. In M. A. Nasser Hajibagheri (Ed.), *Electron Microscopy Methods and Protocols* (pp. 13–30). Humana Press. https://doi.org/10.1385/1-59259-201-5:13

Rook, F., Hadingham, S. A., Li, Y., & Bevan, M. W. (2006). Sugar and ABA response pathways and the control of gene expression. *Plant, Cell & Environment*, *29*(3), 426–434. https://doi.org/10.1111/j.1365-3040.2005.01477.x

Sharp, R. E., Poroyko, V., Hejlek, L. G., Spollen, W. G., Springer, G. K., Bohnert, H. J., & Nguyen, H. T. (2004). Root growth maintenance during water deficits: Physiology to functional genomics*. *Journal of Experimental Botany*, *55*(407), 2343–2351. https://doi.org/10.1093/jxb/erh276

Slater, A., Fowler, M., & Scott, N. (2008). *Plant biotechnology: The genetic manipulation of plants* (2nd ed.). Oxford University Press.

Statistics Norway. (2023, March 6). *Potet- og grovfôravlingar*. SSB. https://www.ssb.no/jord-skog-jakt-og-fiskeri/jordbruk/statistikk/potet-og-grovforavlingar

Suttle, J. C., & Hultstrand, J. F. (1994). Role of Endogenous Abscisic Acid in Potato Microtuber Dormancy. *Plant Physiology*, *105*(3), 891–896. https://doi.org/10.1104/pp.105.3.891

Taiz, L., Zeiger, E., Møller, I. M., & Murphy, A. (2018). *Plant physiology and development* (International sixth edition.). Oxford University Press.

Thermo Fisher Scientific. (n.d.). *NanoDrop Spectrophotometer Resources—NO*. Retrieved May 6, 2023, from https://www.thermofisher.com/uk/en/home/industrial/spectroscopy-elemental-isotope-analysis/molecular-spectroscopy/uv-vis-spectrophotometry/instruments/nanodrop/resources.html

Thermo Fisher Scientific Inc. (n.d.). *SYBR Safe DNA Gel Stain—NO* [Lab supplies]. Retrieved April 25, 2024, from https://www.thermofisher.com/uk/en/home/life-science/dna-rna-purification-analysis/nucleic-acid-gel-electrophoresis/dna-stains/sybr-safe.html

Thermo Fisher Scientific Inc. (2022). *Product Sheet: M-MLV Reverse Transcriptase (200 U/uL)* (B.0, p. 4). Life Technologies Corporation. https://www.thermofisher.com/order/catalog/product/28025013

TwistDx limited. (n.d.). *TwistAmp® Basic* [Lab supplies]. Retrieved April 13, 2024, from https://www.twistdx.co.uk/product/twistamp-basic/

TwistDx limited. (2018). *Help designing your own isothermal RPA assays | TwistDx*.

https://www.twistdx.co.uk/support/rpa-assay-design/

TwistDx Limited. (2018). *TwistAmp Combined Instruction Manual* (p. 62). https://www.twistdx.co.uk/support/manuals-and-software/twistamp/

Veteläinen, M., & Bjor, T. (2001). *Potatis i Norden: En beskrivning av gamla nordiska potatissorter bevarade hos Nordiska genbanken* (Vol. 39). Nordiska genbanken.

Vishwakarma, K., Upadhyay, N., Kumar, N., Yadav, G., Singh, J., Mishra, R. K., Kumar, V., Verma, R., Upadhyay, R. G., Pandey, M., & Sharma, S. (2017). Abscisic Acid Signaling and Abiotic Stress Tolerance in Plants: A Review on Current Knowledge and Future Prospects. *Frontiers in Plant Science*, *8*. https://www.frontiersin.org/articles/10.3389/fpls.2017.00161

Vollmer, R., Villagaray, R., Castro, M., Cárdenas, J., Pineda, S., Espirilla, J., Anglin, N., Ellis, D., & Rennó Azevedo, V.C. (2022). The world's largest potato cryobank at the International Potato Center (CIP) – Status quo, protocol improvement through large-scale experiments and long-term viability monitoring. In *Frontiers in Plant Science.* https://cipotato.org/genebankcip/genebankcip/publications/the-worlds-largest-potato-cryobank-at-the-international-potato-center-cip-status-quo-protocol-improvement-through-large-scale-experiments-and-long-term-viability-monitoring/

Wang, M.-R., Chen, L., Zhang, Z., Blystad, D.-R., & Wang, Q.-C. (2018). Cryotherapy: A Novel Method for Virus Eradication in Economically Important Plant Species. In V. M. Loyola-Vargas & N. Ochoa-Alejo (Eds.), *Plant Cell Culture Protocols* (Vol. 1815, pp. 257–268). Springer New York. https://doi.org/10.1007/978-1-4939-8594-4_17

Wang, Y., Chen, R., Nie, X., Zhong, Z., Li, C., Li, K., Huang, W., Fu, X., Liu, J., & Nie, B. (2020). Rapid and sensitive detection of potato virus Y by isothermal reverse transcription-recombinase polymerase amplification assay in potato. *Molecular and Cellular Probes*, *50*, 101505. https://doi.org/10.1016/j.mcp.2019.101505

Wien, H. C., & Stützel, H. (2020). *The physiology of vegetable crops* (2nd ed.). CABI. https://www.cabidigitallibrary.org/doi/epdf/10.1079/9781786393777.0000

Wilson, C. R. (2014). *Applied Plant Virology*. CABI. https://www.cabidigitallibrary.org/doi/book/10.1079/9781780644257.0000

Yoon, J., Cho, L.-H., Tun, W., Jeon, J.-S., & An, G. (2021). Sucrose signaling in higher plants. *Plant Science*, *302*, 110703. https://doi.org/10.1016/j.plantsci.2020.110703

Yun-peng, D., Wen-yuan, L., Ming-fang, Z., Heng-bin, H., & Gui-xia, J. (2012). The establishment of a slow-growth conservation system in vitro for two wild lily species. *African Journal of Biotechnology*, *11*(8), Article 8. https://doi.org/10.5897/AJB11.2868

Attachments

Nr. 1 Table of mean heights after two months

Table showing the mean heights (cm) of the ten varieties after two months according to medium.

		N-94-30-22	Y-67-20-40	Beate	Bruse	Gullauge P2	Innovator P3	Kerrs Pink	Laila	Oleva	Peik
Sucrose [%]	**ABA [mg/L]**	**173**	**231**	**236**	**238**	**241**	**243**	**244**	**246**	**250**	**253**
3	**0**	9.5	10.9	7.9	11.4	11.3	9.5	11.9	10.5	11.3	9.1
7	**0**	8.2	8.8	6.8	10.0	8.7	9.4	11.1	5.8	10.1	7.5
15	**0**	4.5	2.3	1.1	1.4	2.4	2.7	5.7	1.4	1.2	0.8
3	**0.5**	4.1	7.1	2.0	4.7	7.2	1.8	9.1	2.7	3.0	1.6
7	**0.5**	0.8	5.1	0.8	2.4	1.3	1.7	7.5	0.5	3.6	0.4
15	**0.5**	0.3	0.3	0.3	0.5	0.1	0.3	0.3	0.2	0.2	0.1
3	**1**	1.4	4.5	0.7	1.3	3.7	1.3	2.6	0.8	2.5	1.1
7	**1**	0.7	2.3	0.5	0.6	2.2	0.2	1.2	0.3	1.1	0.2
15	**1**	0.2	0.1	0.1	0.5	0.1	0.1	0.1	0.2	0.3	0.0
3	**3**	0.2	1.0	0.6	0.5	0.6	0.6	0.8	0.4	0.9	0.6
7	**3**	0.5	0.3	0.3	0.5	0.2	0.3	0.5	0.3	0.4	0.3
15	**3**	0.3	0.2	0.1	0.3	0.2	0.0	0.1	0.3	0.1	0.0

Nr. 2 Table of Dead/Total Plants Ratio

Tables showing ratio of dead plants/total plants on 5/3-24 for the test experiment. The top table shows the ratio for the apical shoot cuttings. The lower table shows the ratio for the axillary shoot cuttings.

Variety number	ABA [mg/L]	234	238	241	377	393	449	458	508	562	636
	1	1	1	1	1	1	1	0	0	0.5	1
	3	0	0	0.5	0.5	0	0.5	0	0	1	0
	5	1	0.5	0	1	0.5	0	0.5	0	0	0
Variety number	**ABA [mg/L]**	**234**	**238**	**241**	**377**	**393**	**449**	**458**	**508**	**562**	**636**
	1	0.75	0	0.75	0.75	0.25	0	0.25	0.5	0.5	1
	3	0.75	0	0.25	0.25	0	0	0.25	0.25	0.5	0.25
	5	0.5	0.5	0	0	0.25	0	0	0.5	0	0.25

Nr. 3 A more detailed materials and methods

Slow Growth

Material

in vitro potato plants of ten varieties, test tubes with cork, 1 mg/mL ABA, tube racks, growth room, camera, measuring stick, Excel, R-studio (Posit, 2023), Murashige and Skoog basal medium with vitamins, distilled water, 1 L glass flask with cork, crystalline sucrose, magnet staff, magnet mixer, pH-meter, 1 M KOH, acetic acid, 7pH liquid, 4pH liquid, Bacto agar, autoclave, laminar flow bench, 75% ethanol spray, tissue paper, mason jars with plastic lids, scalpel, tweezers, glass bead oven, autoclaved paper, four 0.5 L flasks, 1N NaOH, autoclaved water, 2 mL tube, coloured stickers, water bath, precision dispenser, test tubes, coloured autoclavable tube racks, coloured tube caps, marker or pencil, white stickers, cold storage.

Test experiment

Ten potato varieties were put on nutrient medium with differing amounts of ABA added. The different ABA treatments were 0 mg/L for the control group, 0.5 mg/L, 0.1 mg/L, 3 mg/L and 5 mg/L. For each variety and ABA concentration there were made three tubes; one with two apical shoot cuttings from *in vitro* established potato, and two tubes with two axillary shoot cuttings with two nodes from *in vitro* potato in each. All the cuttings had at least one node over the medium for shoot development and one node under the medium for root development. The tubes were put together, three by ten, in racks that were four by ten tubes. This meant the middle row was subjected to lower light intensity. The growth room the plants stood in holds a constant temperature of 18°C and the light measured in between the racks of tubes where 40-45 $\mu mol/m^2/s$.

Every month the tubes were photographed in tube racks according to variety. The photos were used to measure the length of the potato plants and the numbers of opened leaves. A table for the data was made in Excel. The table was used to make boxplots, where every data point is the mean plant length of a variety on a specific medium, or every data point is the mean number of leaves on a plant of a variety on one of the media.

Calculation for numbers of observations needed

A test experiment was done, where *in vitro* potato plants were subjected to different concentrations of ABA. This made it possible to do an estimate of observations needed for every treatment. The height of the plants and number of nodes were recorded and used to find their standard deviation, for the plants who had the same treatment. It was apparent that the deviation became higher with growth of the plant. To be sure that the experiment could go on for longer than the test experiment, the mean standard deviation used for the power test came from the control plants and the plants subjected to the lowest amount of ABA, as they had given the highest plants.

The power tests were done in R-studio with the command power t-test. As the slow growth experiment had two parameters varying, an ANOVA power test would have been ideal for the estimate of "n", but as the test experiment only looked at the plant's response to ABA, the ANOVA power test was not possible.

Potato growth medium

The potato growth medium used in the potato bank and for the micropropagation, was made in 1L flasks. The flask was first half filled with distilled water, and 4.4 g Murashige and Skoog mix with vitamins was added. Next, 30 g of crystalline sucrose and a magnet staff was added. The flask was put on a magnet mixer until the liquid was homogenous. The pH should lie between 5.7 and 6. The pH was adjusted by either pipetting basic (1M KOH) or sour droplets (acetic acid) and using a pH-meter calibrated with a 7 pH and 4 pH liquid. 9 g of Baco agar was then added in the flask. The flask was filled to the 1L mark with more distilled water.

The medium was autoclaved at 121 °C for 20 minutes (the maximum pressure being 2 bar) with the cap of the flask screwed sideways, so the pressure did not build up inside the flask. After cooling down for around an hour, the medium was poured in sterile containers in a flow bench. The bench was wiped down with 75% ethanol before use and the flow of air must be continually on for the inside of the bench to be kept sterile. When used for micropropagation the medium was poured in mason jars. The medium has to be let cool until semi solid, before the containers are moved. This minimizes the condensation on the inside of the containers in cold storage.

Micropropagation

The *in vitro* potato plants for this thesis originate from the *in vitro* plants of the potato bank at NIBIO. Cuttings from these plants were micro-propagated in plastic boxes and glass mason jars on potato growth medium. The cuttings were made by picking off the leaf of a green node with tweezers and cutting a 1-2 cm segment with the node in the middle. The segments put on the new medium needed only one node to produce both roots and shoot. To combat the root growth pushing the cutting out of the medium, as much of the cutting as possible was put in the medium without submerging where the petiole had been attached, as this is where the growth points are. When the *in vitro* potato plants started to show patchy brown leaves and stems, axillary cuttings were taken for transfer to new medium.

Slow growth medium preparation

For the slow growth experiment, every medium treatment was made on 0.5 L flasks in batches of four according to sucrose content. A stock solution with 10 mL of 1 mg/mL ABA was made first with 1 mL 1N NaOH as solvent and 9 mL autoclaved water (Merck, n.d.). This was allocated and frozen in 2 mL tubes. The base nutrient medium was then made by adding 2.2 g Murashige and Skoog basal medium with vitamins to each 0.5 L flask, the sucrose amount was also added according to treatment (for 3% sucrose adding 15 g, 7% adding 35 g and 15% 75 g). Distilled water was added, filling the flasks to the 0.5 L mark.

The flasks should be marked with ABA concentration or color coded. The stock of ABA was pipetted so that one flask of medium ended up having 0.5mg/L ABA (0.25 mL of stock), another 1 mg/L ABA (0.5 mL of stock), a third with 3 mg/L (1.5 mL of stock) and the fourth with no ABA. The pH was then corrected so it lands around 5.8, mixing them with a magnet mixer. 4.5 g Bacto agar was added to the flasks, and the medium flasks were heated in a water bath.

Each flask was pipetted with a precision dispenser into 70 glass tubes with a diameter of 20 mm, in autoclavable tube racks. 5.7 mL of nutrient medium was dispensed in each. The racks had different colors, representing the difference in ABA concentration. Different colored caps were also used for the same effect. The tubes were capped and autoclaved for 25 minutes at 110°C. After cooling, the tubes were marked with white stickers and a pencil to show the

sucrose concentration. The tubes are put into metal racks after marking and left in cool storage, until cutting and transferal of axillary shoots.

Cutting and transfer of axillary shoot

For each variety, two stem segments with nodes were placed into six tubes of the same medium. There were twelve different mediums. This was done for all ten varieties in two repetitions. The stem segments were cut in a sterile bench. The surface was first wiped down with 75% ethanol. The scalpels were sterilized in a glass bead oven at 250°C and left to cool laying on a rack. Autoclaved printer paper was used as a cutting surface, cutting 1-2 cm long shoot segments with a node in the middle. The leaves on the stem were plucked off with the tweezers, holding the shoot down with the blunt back of the scalpel. Segments were pushed down in the medium with the tweezers, so the nodes were just barely above it. The segments had the same orientation on the medium as they had on the plant or were laid flat with the node facing up. The end result was 24 axillary shoots of each variety getting the same treatment. This means that 12 axillary shoots of one variety stood on the same medium for repetition 1.

Data gathering

After a repetition of the plants had been left to grow for two months, pictures were taken of the racks with a ruler on the side for scale. Three pictures were taken for each side of the rack: one straight on with the label showing, one with the stems of the plants in front and one taken with a perspective looking down on the medium. As some cuttings never grew, the picture of the medium showed this. The pictures were then used to measure the length of the plants, writing the results into Excel. Boxplots were made with every data point being a mean value length of a variety on a specific medium.

Virus eradication; meristem cutting

Material

Tubers of different varieties, sharpy, plastic tray, jam jar, paper towels, timer, 75% ethanol, wash basin, 1% sodium hypochlorite, (shake bench), autoclaved water, two 1 L autoclaved glass flasks with lids, distilled water, MS with vitamins, sucrose, casein hydrolysate, indole-3-butyric acid, adenine hemisulphate, gibberellic acid, agar, aluminum foil, hotplate, pot, pot bottom insert, test tubes with caps, 10 mL pipette or liquid dispenser, an autoclave, cool storage, laminar flow bench, sterile petri dish, sterile filter paper, scalpels, tweezers, glass bead oven, labels, potato plant growth medium, plastic potting containers, soil potting mix, see-through tray lid.

Sample preparation; Sprouting

Tubers from four varieties infected with potyviruses were given to me for virus eradication; Åkerøy (707), Early Rose from Arendal (714), Blåpotet from Snåsa (715), and Kvit Rund Kvam (763). The eyes of the potatoes had started sprouting as they sat in cold storage. This was before I received them for my experiment. The tubers were disinfected and put in a windowsill for a month, for the sprouts to grow longer. Some of the sprouts started to tuberize. The tuberized shoots were, together with the normal stems, used for meristem culturing.

For variety Kvit Rund Kvam stem cuttings were taken off a plant growing in one of NIBIO's greenhouse rooms. Cuttings were taken 20-th of June and disinfected 21. of June in the same way as the tuber shoots. Shoot-tips were then excised and put on medium to establish an *in vitro* culture for this variety. In the middle of October, meristems were cut from this *in vitro* culture to try and clean Kvit Rund Kvam of the viruses. This was done around the same time as the meristem culturing of the other three varieties.

Disinfection of sprouts

The sprouts were disinfected in mason jars with up to six shoots in each. To start the shoots were bent off the tuber and segmented into two-centimeter chunks, leaving some space from the cut to the next growth point. The jars were marked with the variety number and the shoots

were put in. 75% ethanol was added so that the solution at least covered all the shoots. The glass jar was swirled around to cover the whole inside of the jar. A shaker bench was used for 30 seconds, so the shoots were constantly moved around in the ethanol. The jar got decanted. 1% sodium hypochlorite was added so it covered the shoots, and the glass was swirled. The jar was shaken on a shaker bench for 10 minutes, and then decanted. Autoclaved water was added so it covered the shoots. The water was swirled and decanted. Autoclaved water was added a second time, and the jar was left on the shaker for a minute. Most of the water was decanted, leaving the shoots uncovered.

Potato meristem medium

For 1L of meristem medium a 1L flask was half filled with distilled water. The following was then added to the flask: 4.4 g Murashige and Skoog basal mix with vitamins, 20 g sucrose, 1 g Casein hydrolysate, 0.1 g Indole-3-butyric acid (100 µL of 1 mg/mL stock solution), 40 mg adenine hemisulphate, and 1 mg of gibberellic acid. The pH was then adjusted to between 5.7 and 6 in pH. Agar was then added to make hard-medium tubes, or skipped for the liquid-medium tubes. Aluminum foil was put over the top of the flasks and the flasks were warmed in a water bath for 20 minutes. The mediums were then pipetted with a precision dispenser, dispensing 5.7 mL in each tube. The tubes were capped and autoclaved for 25 minutes at 110°C. After cooling down and letting the agar harden, the tubes were put in a cold storage room.

Meristem cutting

A sterile petri dish was put under a binocular loop in a sterile laminar flow bench. Sterile filter paper was put in the dish. A little autoclaved distilled water was added on the paper, so the shoots would dry out slower. Scalpels and tweezers were sterilized in a glass bead oven at 250°C. All the visible leaves were bent off with the scalpel while holding the stem lightly with the tweezers as near to the shoot tip as possible, without directly holding it. The scalpel was swapped before reaching the innermost leaves or any leaf primordia. The last leaf primordia might be left on or slightly bent away, to avoid damaging the meristem. Cutting a "V" shape down into the shoot tip, the meristem was freed. The meristem needs to be put on medium as fast as possible to not dry out.

Some shoot-tips were also cut to make *in vitro* cultures of the four varieties, in case the meristems did not survive and regenerate plants. The shoot-tips were cut in the same way as meristems, with the exception of leaving leaf primordia around the meristem. This is an easier cut and protects the meristem, giving a higher rate of survival.

When the meristems and growth points had grown visibly on the meristem medium, they were transferred to potato plant growth medium. This was done to increase growth and lessen the chance of vitrification, especially the meristems/growth points that lay on liquid meristem medium.

Transferring to soil

When the surviving meristems had produced plants with roots and shoots, one clone from every meristem was planted in separate containers and put in a shared watertight container with a transparent lid. The plant with the visually biggest leaf area and longest roots were chosen for transfer to soil. Trying not to damage the root, the medium was squished off of the roots using paper towels, taking a new towel for each clone. This was done to minimize the risk of cross contamination with virus from one plant to the next, as we cannot know at this stage if the virus eradication worked. Soil mix was put into a pot and a hole for the roots was made. The plant was put in, with some stem under the soil. Soil was compacted around the stem. ELISA was done after the potato plants had grown on soil for a month.

Using RPA to test for PVY

Materials

Primer design tool (snapGene (GSL Biotech LLC, n.d.)), nucleotide database BLASTN tool (National Library of Medicine, n.d.), phosphate-buffered saline, Tween 20, polyvinylpyrrolydon, bovine serum albumin, 500 mL glass flask with cork, distilled water, label, marker, a peeler, milligram weight, extraction bags (BIOREBA, n.d.), ball bearing crushing machine, auto pipette, 1,5 mL PCR-certified centrifugation tubes, -20°C freezer, Norgen Plant/Fungi Total RNA Purification kit (Norgen Biotek Corp., n.d.), 96% ethanol, beta-mercaptoethanol, water bath, centrifuge tube holder, centrifuge, Nanodrop machine (Thermo Fisher Scientific, n.d.), 50-250 ng/mL random primers, 10 mM dNTP mix, nuclease free water, vortexer, timer, ice, 5x First strand buffer, incubator, 0,1 M DTT, M-MLV

Reverse Transcriptase, TwistAmp Basic kit (TwistDx limited, n.d.), primer pairs, TBE, buffer, 200 mL Erlenmeyer flask, agarose, cling film, Sub Cell GT System (BIO RAD, n.d.-b), microwave, heat protecting gloves, sink, SYBR-safe (Thermo Fisher Scientific Inc., n.d.), well combe, 6x DNA loading dye, 100 bp DNA ladder, imaging machine (ChemiDoc (BIO RAD, n.d.-a)), Blot/UV/Stain-Free Tray or blue light tray.

PVY primer design

A forward and reverse primer from two different papers seemed to have the most promising features. They had no secondary structures with high confidence and spanned a region which seemed mostly conserved. All three primer pairs for PVY amplified a part of the sequence coding for coat protein.

174bp, 43% GC, BLASTN at National Library of Medicine gave no other similarities:

ccaactgtgatgaatgggcttatggtttggtgcattgaaaatggaacctcgccaaatgtcaacggagtttgggttatgatggatgggaatg
aacaagttgagtacccgttgaaaccaatcgttgagaatgcaaaaccaacccttaggcaaatcatggcacatttctcagatgtt

Forward primer, 32bases, 47% GC: CCAACTGTGATGAATGGGCTTATGGTTTGGTG (Y. Wang et al., 2020)

Revers primer, 32bases, 38% GC: AACATCTGAGAAATGTGCCATGATTTGCCTAA (Cassedy et al., 2022)

Making of test samples

200 mL of buffer was made in preparation. This was done by adding 20 mL 10x phosphate-buffered saline (BSA), 10 mL Tween 20, 1 g polyvinylpyrrolydon, and 0.4 g bovine serum albumin to a 500 mL flask with lid. Distilled water was added to the 200 mL mark. The lid was fastened and the flask shook until the contents were homogeneous. The flask was labeled with the contents. Because of the BSA added to the buffer, it's pron to go bad from infection of bacteria or fungi, and has a short shelf life.

To make a test sample four tubers were skinned near their bud end. The bud end had been cut off for use in an ELISA test. The skins were weighed together and amounted to between 0,5-1

mg. 4,5 mL of the readymade buffer was added to an extraction bag with a separating mesh in the middle. The skins got put in behind the lattice of the pocket. A crushing machine was used on the pocket until there were no visible fragments left. 100 µL of liquid was pipetted from the front of the mesh into a 1,5 mL centrifugation tube. Another 1,5 mL centrifugation tube was filled to work as a backup tube.

Extracting total RNA

For the extraction of total RNA from the test samples, a Norgen Plant/Fungi Total RNA purification kit was used (Norgen Biotek Corp., n.d.). To prepare for extraction 42 mL of 96% ethanol was added to the provided "wash solution A" (18 mL). The box on the solution bottle was then marked to indicate the addition. 500 µL of lysis buffer C was mixed with 5 µL of beta-mercaptoethanol per test sample in a centrifuge tube. The water bath was turned on at 55°C. The different tubes used were put in a holder and numbered to keep track of the different samples treated. For each sample two RNAse-free 2 mL centrifuge tubes, a filter column in a collection tube, a spin column in a collection tube and an elution tube was needed.

The samples were taken out of the freezer and thawed on ice. 100 µL of the sample was transferred to a 2 mL centrifuge tube. 500 µL of the treated lysis buffer C was added to this centrifuge tube. The tube was put in the water bath for 5 minutes mixing occasionally by flipping the tube upside down. The liquid was pipetted into the filter column and the column was spun at 14 000 RPM for 2 min. The supernatant was transferred to a new RNAse-free centrifuge tube, by avoiding the debris that had collected at the bottom (pipetting from the side that faced inward under centrifugation).

96% ethanol was then added to the tube so that there was equal parts ethanol and supernatant. 600 µL of the supernatant and ethanol mix was pipetted into the spin column. This was then spun in the centrifuge for 1 minute at 6 000 RPM. The liquid that had flowed through was discarded. If there was any liquid left in the column after the centrifugation then it was spun again for 1 minute at 14 000 RPM. If there was any supernatant and ethanol mix left in the previous RNAse-free centrifuge tube, then this was added and spun as previously described.

400 µL of Wash solution A was added to the spin column, and this was centrifuged at 14 000 RPM for 1 minute (spinning an extra minute if not all solution has passed through). The flowthrough was discarded and the washing got repeated two more times. The column was

centrifuged for another 2 minutes at 14 000 RPM, to dry the column and the collection tube was thrown away.

The spin column was put in a 1,7 mL Elution tube and 30 µL Elution solution A was added in the column. Spinning the column at 2 000 RPM for 2 minutes the elution solution worked on the RNA and released it from the column. When spun at 14 000 RPM for 2 minutes the RNA and solution collected in the elution tube. If not all the solution had passed through the column, then it was spun for an additional minute at 14 000 RPM. The elution step was repeated by pipetting the solution in the elution tube on top of the spin column again, and repeating the centrifugation. The resulting RNA samples were stored at -20°C after testing concentration and quality.

Testing RNA concentration and quality

A Nanodrop machine was used to check the concentration and quality of the extracted total RNA. The pedestal and lid of the machine was wiped down with lens paper before use and between every measurement. Elution buffer was used as the reference blank pipetting 1,5 µL, sentering the drop on the lens of the machine. The lid was put down on the pedestal carefully. After calibrating with a blank, 1,5 µL of the RNA samples were pipetted on the lens to make an absorption spectrum read.

Reverse transcription into cDNA

Before the procedure the 5x First-strand buffer, 0,1 M DTT, and total RNA sample was thawed (Thermo Fisher Scientific Inc., 2022). All buffers were agitated, as some salts may have settled at the bottom. The water bath was turned on and set to 65 °C. All the reagents were held on ice after being defrosted. A master mix was made in a tube. This consists of 1 µL 50-250 ng random primers, 1 µL 10 mM dNTP mix and 2 µL nuclease free water for every sample plus one extra (in case of spills). The master mix was lightly vortexed. For each sample 8 µL of RNA sample was added to a tube. 4 µL of the master mix was pipetted into the sample tubes. The sample tubes were put in the water bath for 5 minutes, and were then put on ice to chill.

A second master mix was made in a new tube. The master mix was made up of 4 µL 5x First strand buffer, 2 µL of 0,1 M DTT and 1 µL nuclease free water per test sample plus an extra.

The mix was vortexed down. 7 µL of the second master mix was pipetted into every test sample tube and the tubes were gently centrifuged down. The tubes were then incubated at 37 ℃ for 2 minutes.

1 µL of M-MLV Reverse Transcriptase (Thermo Fisher Scientific Inc., 2022) was added to each sample tube and pipetted gently up and down to mix. As random primers were used, the sample tubes were first incubated at 25 ℃ for 10 minutes. The sample tubes were then incubated at 37 ℃ for 50 minutes. To inactivate the reverse transcriptase, the sample tubes were held at 70 ℃ for 15 minutes.

Running RPA reaction

A TwistAmp® Basic kit (TwistDx limited, n.d.) was used to do the RPA reaction. An incubator was set to 39 ℃. Rehydration solution was first prepared. A master mix was made in a 1.5 mL PCR certified tube adding 2,4 µL forward primer (10 µM), 2,4 µL reverse primer (10 µM), 29,5 µL Primer Free Rehydration buffer and 11,2 µL of nuclease free water per test sample plus an extra (in case of spills). The master mix was pipetted up and down.

45,5 µL of the master mix was pipetted into the new PCR certified tubes. 2 µL of cDNA sample was added for each sample. These rehydration solutions were vortexed and centrifuged briefly.

The 47,5 µL of rehydration solutions per sample, were pipetted onto their reaction pellets. These were pipetted up and down until the pellets were mixed in and the solution was clear.

2,5 µL of 280 mM magnesium acetate (MgOAc) were pipetted into the lids of every pellet tube, closing all the lids at approximately the same time. The tubes were then handshaken up and down three times and spun down. The sample tubes were transferred as soon as possible to the incubator (39 ℃) for 20 minutes. After 4 minutes of incubation the tubes were vortexed and spun down briefly. The tubes were then put in a -20℃ freezer.

Gel electrophoresis

TBE 10x was premade, and contains 108 g Tris base, 55 g boric acid, 40 mL of 0.5 M EDTA (pH 8.0) and are finally filled to an end volume of 1 L. This 10x stock is dissolved in 600 mL distilled water for use in gel electrophoresis.

A gel form was prepared. To make the gel for a medium tray 1,5 g agarose was mixed with 100 mL of TBE buffer solution in a 250 mL Erlenmeyer flask (BIO RAD, n.d.-b). For small gel trays only 0.75 g of agarose was added to 50 mL of TBE buffer.

Cling film was put over the opening of the Erlenmeyer flask, and a small hole was made in it. The gel mix was then warmed in a microwave on a low to medium setting. The flask was taken out and stirred every 30 seconds. After all the agarose had dissolved the gel was cooled down to around 60 °C by cooling the Erlenmeyer flask under running water.

SYBR-safe (Thermo Fisher Scientific Inc., n.d.) was added (1 µL to 10 mL of gel) to the gel mix and swirled in. The gel was poured into a form to harden, and a comb was added to make the wells near the cathode (black) end. The gel was left to harden for 20-40 minutes. The comb was then taken out of the gel, and the tray was unfastened.

The tray with gel was transferred to a horizontal electrophoresis chamber. There was then added a 6 mm layer of TBE buffer over the gel (BIO RAD, n.d.-b). 2 µL of 6x DNA loading dye, 5 µL of cDNA sample and 5 µL of water were mixed and then pipetted into the wells. The left most well was filled with 3 µL of 100 bp DNA ladder, 7 µL of Milli-Q water and 2 µL of 6x DNA loading dye. Loading dye indicates successful loading and marks how far the electrophoresis has dragged the DNA.

The lid was carefully put on so as to not disturb the wells contents. The cathode marked with black was matched with the black ring on the lid of the cell, and the same for the anode marked with red. The voltage was set to 100 V and the DNA was left to migrate until the loading dye had reached ⅔ of the way through the gel (around 40-60 minutes).

The gel was then pictured with a ChemiDoc imaging machine (BIO RAD, n.d.-a). The gel was put in the center of the imaging stage, checking if it was centered by zooming with the camera. The machine was closed and the Blot/UV/Stain-Free Tray was chosen on the display. SYBR-safe also works with the blue tray. Single Channel was chosen on the display and the image size was chosen (manually by tapping image size and pinching two fingers on the display). Under “application” nucleic acid gels were chosen, auto optimal was accepted. A region of interest showing the bands and 100 bp ruler was selected with “preview” and a picture was taken. The pictures were copied onto an USB stick. The machine was finally wiped down with kleenex, inside and out, and the gel was safely disposed of.

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