Norges miljø- og biovitenskapelige universitet

Master`s Thesis 2023 60 stp
Faculty of Biosciences

Cryopreservation of strawberry and two aphid-transmitted strawberry viruses at different durations of PVS2 treatment

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Foreword

This thesis work has been done under the framework of the Kappaberry project NOBERRYVIRUSCZ (TO01000295), which is granted from Iceland, Liechtenstein and Norway through the EEA Grants and the Technology Agency of the Czech Republic. I am very appreciative to be a part of this intriguing project.

Before the start of the experiment, I had a four-day course at Sagaplant in Telemark. Sagaplant produces healthy and disease-free plant material and is specialized in keeping healthy stocks of vegetatively propagated plants, including cryopreservation. Due to this, Sagaplant was an excellent place to get training in the technique of cryopreservation and tissue culture and I am very grateful for their help.

I want to thank my supervisors Dr. Dag-Ragnar Blystad and Dr. Zhibo Hamborg for all their time, great help and support in this thesis work. I have learned a lot, which will be very valuable for me further in my career. I also want to thank Sissel Haugslien and Carl- Henrik Lensjø Alvin for their help and answers to many of my questions.

My husband, Clayton, has been a great support in stressful times.

Abstract

Cryopreservation is considered to be a valuable method for long-term preservation of plant germplasm and have in recent studies also shown to be a reliable method for preserving certain plant viruses. The presented thesis studies the effect of different PVS2 durations used in cryopreservation on: (1) regeneration rate of cryopreserved strawberry cv. Korona shoot tips, and (2) survival efficiency of paid-transmitted strawberry mild yellow edge virus (SMYEV) and strawberry vein banding virus (SVBV) following cryopreservation. Excised shoot tips of cv. Korona were cryopreserved with six different durations of PVS2 varying from 10 to 60 minutes, whereas virus-infected shoot tips were cryopreserved using either 10, 40 or 60 minutes of PVS2. The results showed that 40-60 minutes of PVS2 treatment seemed to be more efficient for preserving strawberry germplasm than lower duration times (10-30 min).

The results showed that SVBV was present in all of the regenerated shoots following cryopreservation in all the three durations of PVS2 examined. SMYEV, however, was more efficiently preserved in shoot tips exposed to 40 minutes (90%) of PVS2, in relation to 60 minutes (33%). Concentrations of the cryopreserved viruses were quantitatively analyzed by RT-qPCR.

This thesis demonstrates that SMYEV and SVBV can be successfully cryopreserved in living cells of *Fragaria ssp.* by droplet vitrification. The results indicates that cryopreservation has great potential in long-time preservation of both strawberry germplasm and aphid-transmitted strawberry-infecting viruses.

Abbreviations

AD	Apical dome
ANOVA	Analysis of variance
ASGV	Apple stem grooving virus
BAP	6-Benzylaminopurine, benzyl adenine (synthetic cytokinin)
CChMVd	Chrysanthemum chlorotic mottle viroid
CSVd	Chrysanthemum stunt viroid
DNA	Deoxyribonucleic acid
IBA	Indole-3-butyric acid (Plant hormone in the auxin family)
ITC	Internal control
LP	Leaf primordia
LN	Liquid nitrogen
LS	Loading solution
MP	Movement protein
PCR	Polymerase chain reaction
PLRV	Potato leafroll virus
PSTVd	Potato spindle tuber virus
PVS	Potato virus S
PVS2	Plant vitrification solution 2
qPCR	Quantitative polymerase chain reaction (real time PCR)
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SAM	Shoot apical meristem
SMYEV	Strawberry mild yellow edge
SVBV	Strawberry vein banding virus
SCV	Strawberry crinkle virus
SMoV	Strawberry mottle virus
SMYEV	Strawberry mild yellow edge
SVBV	Strawberry mild yellow edge
TMV	Tobacco mosaic virus
ULS	Unloading solution

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1 Introduction

Strawberry (*Fragaria x ananassa*) is considered one of the most important berry crops worldwide (Niino et al. 2003, Höfer 2016). Conserving genetic diversity of vegetatively propagated plants, such as strawberry, is more demanding than for seed-producing crops. Clonal crops are usually maintained in the field or in *in vitro* gene banks, where they may encounter challenges such as recurrent manual maintenance and exposure to diseases or contamination and abiotic pressures (Höfer 2016). Cryopreservation, however, may avoid these challenges, and is a preferred option for conservation of germplasm (Niino et al. 2003).

Many economically important crops are vegetatively propagated and are therefore especially susceptible to infection by obligate pathogens that are transmitted from generation to generation through vegetative propagation (Hull 2002). Cryopreservation allows for long term conservation of plant material without modification, protection against contaminants and limited maintenance, and protocols are developed for a large range of vegetatively propagated species (Engelmann 2011). This method is useful for preservation of plants that either do not produce seeds, produce non germinating seeds, or propagate vegetatively (Jiroutova and Sedlak 2020). Submerging plant material in liquid nitrogen (-196°C) halts cellular division and metabolic processes, and as a consequence of this, unaltered plant material can be stored for a theoretically unlimited time (Engelmann 2011).

Plant viruses are economically important plant pathogens and are widespread throughout agriculturally grown crops, as well as in nature (Hull 2009). Viruses are obligate intracellular parasites that replicate only inside living cells of their host, using their hosts biochemical machinery (Hull 2002). Long-term preservation of plant pathogens has many applications including plant pathogen research, preparation of antigens for virus detection by immunology-based methods, and genetic transformation to produce resistant transgenic plants (Zhao et al. 2019). Maintenance of virus-infected plant material for use as positive controls is often required for virus diagnostics and pathogen management programs (Hull 2002). Preservation of some plant viruses have been achieved through drying and freeze-drying of infected plant material (Yordanova et al. 2000). However, virus preserved in this matter can only be mechanically transmitted to the plant host, which is a problem for vector dependent viruses that cannot be transmitted this way (Yordanova et al. 2000, Hull 2002).

Viruses are unevenly distributed within plants, and the virus titer increases as the distance increases from the apical dome, resulting in lower virus titer or a virus free area in the

uppermost part of the apical dome (Zhao et al. 2019). When shoot tips are cryopreserved in liquid nitrogen, only the cells at the top layer of the apical dome and the youngest leaf primordia survive, whereas all other cells are killed (Wang et al. 2018, Zhao et al. 2019). Thus, plants regenerating from cryopreserved shoot tips might be free from viruses. Based on this potential, the method of virus eradication through cryotherapy was established (Wang et al. 2009). However, some viruses and viroids are known to infect meristematic cells, and cryotherapy has not been shown to effectively eradicate these viruses (Hamborg et al. 2014). This knowledge leads to the proposal that cryopreservation of shoot tips can be used for long-term preservation of viruses that do infect cells in the meristem or the youngest leaf primordia.

This thesis has been done under the framework of the Kappaberry project NOBERRYVIRUSSCZ (TO01000295). The full name of the project is "Healthy berries in a changing climate: development of new biotechnological procedures for virus diagnostics, vector studies, elimination and safe preservation of strawberry and raspberry". The main goal of this project is to increase the production potential of healthy strawberry and raspberry in a changing and challenging climate by using high-quality virus tested mother plants, improving virus control and associated vector approaches as well as safeguarding valuable plant germplasm. The project is divided into four areas of investigation, called subgoals: (1) Diagnostics and gene characterization of strawberry viruses in Norway and raspberry viruses in the Czech Republic and Norway using HTS and PCR, (2) identification of potential virus vectors in the field, (3) Production of high-quality virus tested nuclear stock material and preservation of plant health and gene resources with the use of cryopreservation and (4) long term preservation of "living" viruses and their sequences using cryopreservation.

The experiments being conducted in this thesis investigate the effect of cryopreservation of healthy plant material of the strawberry cv. Korona, as well as cryopreservation of strawberry infected with either strawberry mild yellow edge virus, (SMYEV, species *strawberry mild yellow edge virus*, genus *Potexvirus*, family *Aplhaflexiviridae*) or strawberry vein banding virus (SVBV, species strawberry vain banding virus, genus *Caulimovirus,* family *Caulimoviridae*). Cryopreservation is a process that consists of several steps that all could theoretically affect the outcome of the treatment. The variable investigated in this thesis is time of immergence in PVS2 (plant vitrification solution 2).

The aim of this thesis is to investigate the effect of cryopreservation of both healthy and virus infected strawberry shoot tips and examine if the two strawberry viruses survive the treatment

as well as the plant tissue itself. The goal is to explore which PVS2 time is most effective for cryopreservation of strawberry, and to work out if cryopreservation is a reliable method for long-term preservation of aphid transmitted strawberry viruses.

2 Literature

2.1 Strawberry

The genus of *Fragaria* belongs to the family Rosaceae (Husaini and Neri 2016). Within this genus we find the cultivated *Fragaria x ananassa* Duch., *Fragaria virginia* Mill. (wild strawberry native to North-America) and *Fragaria vesca* L. (wild strawberry native to Europe), amongst others (Husaini and Neri 2016). Botanically, the fruit of the strawberry plant is an enlarged flower receptacle and is embedded with achenes, popularly called seeds, and is thus not considered a true berry (Pritts 2017). Strawberry is a hybrid that can naturally propagate through seeds as well as vegetatively through runners (Husaini and Neri 2016). Propagation by seed is primarily used for breeding purposes to produce new genotypes with genetic improvements (Davis et al. 2007). Strawberry plants used in production are propagated through runners. Runners grow from auxiliary buds near the crown of the mother-plants and produce new self-rooted plants (Qiu et al. 2019).

Disease in strawberry production is predominantly caused by fungi, thus viral infections can often be overlooked due to few visible symptoms induced in commercial strawberry varieties (Babini et al. 2004). Strawberry is propagated vegetatively and are therefore subject to virus infection during propagation with the mother plant as the infection source, and may also get infected in later developmental stages through virus bearing vectors (Martin and Tzanetakis 2006).

2.2 Plant viruses

Control of plant pathogens is an important measure to ensure high yielding crops. Plant viruses, as agents of disease, can cause considerable losses in many important crops (Wilson 2014). As well as reducing yield, plant viruses may also affect the quality of the harvested product (source). The extent of damage and loss, however, can vary greatly.

Agrios (2005) defines a virus as a nucleoprotein that multiplies only in living cells and has the ability to cause disease. A virus consists of a type of nucleic acids, either RNA or DNA, protected by a protein coat (Blystad et al. 2020). Within a suitable host cell, the virus utilizes the hosts protein-synthesizing machinery for replication (Fletcher and Hickey 2013). The genome of plant viruses may be single or double-stranded DNA or RNA with positive sense, negative sense or ambisense (Wilson 2014), as precented in Figure 2.1. The majority of plant viruses possess single stranded, positive sense RNA genome (Wilson 2014).

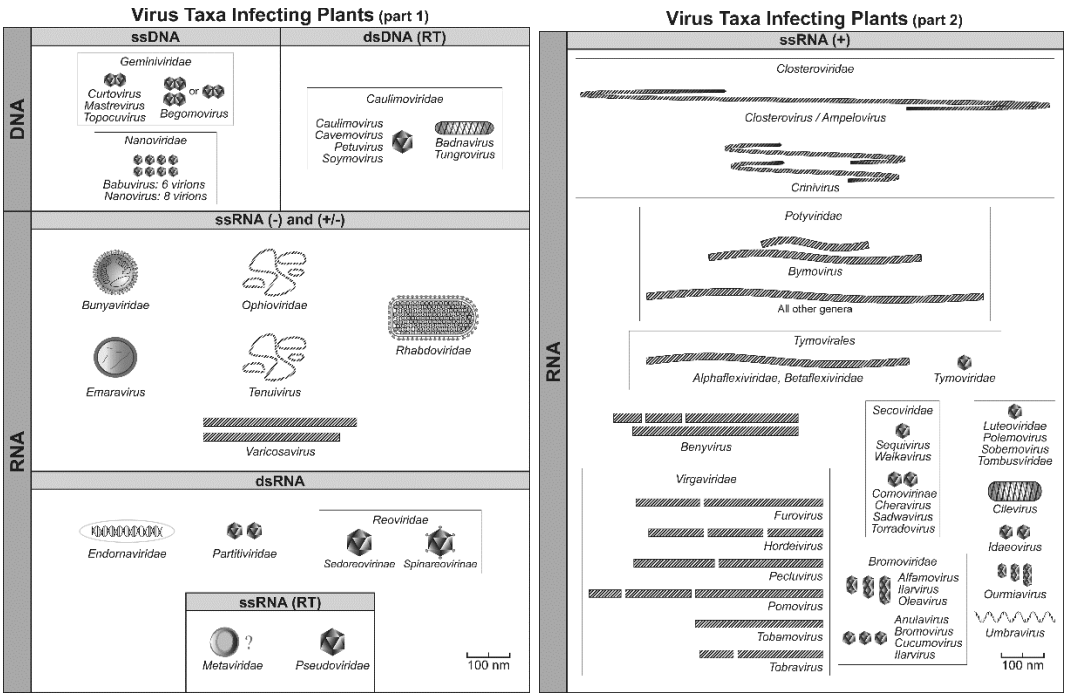

Figure 2.1: Schematic description of virus taxa infecting plants. Source: ICTV 9th report (King et al. 2011)

Whether a virus is considered alive or not has been a source of debate. A living organism is generally defined as a cellular structure that has its own metabolism and is able to reproduce and adapt to the environment by internal changes (Hull 2009). Viruses are able to reproduce and adapt but are not cellular and do not metabolize (Hull 2009). In other words, they do not fulfill all the requirements to be defined as a living organism.

In contrast to many necrotic fungal and bacteria pathogens, viruses rarely cause death of the plant or plant organ that they infect (Wilson 2014). However, their ability to systematically infect, propensity to spread, general lack of management tools and inability to eradicate viruses from infected plants make plant viruses significant plant pathogens (Wilson 2014).

For the virus to be able to systematically infect the plant, the virus must transfer their genome together with viral proteins from the site of replication to the plasmodesmata which facilitates movement to adjacent cells and furthermore enables movement to the phloem to spread systematically (Lucas 2006, Heinlein 2015). Virus encoded movement proteins (MP) help the virus move locally, from cell to cell, and longer distances systematically within the host plant (Gillespie et al. 2002). The two main MP facilitated intracellular and intercellular modes of movement can be broadly classified in two groups (Rui et al. 2022): (1) MPs that can bind directly to viral RNA to form a virus specific ribonucleoprotein complex (like TMV), and (2) MPs that can facilitate movement of whole virions by assembling hollow tubes, within the plasmodesmata, between cells (Gillespie et al. 2002, Sambade et al. 2008).

Viruses are obligate cellular parasites, meaning they must enter the plant host cell to utilize the cell's protein synthesis machinery, cellular components and sources of energy for their own reproduction (Wilson 2014). To be able to enter the plant cells cytoplasm, the virus needs to either find a way to penetrate the plant cells cuticula and cell wall, or avoid this problem by seed transmission or vegetative propagation (Hull 2002). To enter the cytoplasm of host cell, the virus needs to overcome cellular barriers, such as the cuticular layer, and a rigid cell wall primarily constructed of pectin, cellulose and hemicellulose (Wilson 2014). Plant viruses may be able to overcome these barriers through mechanical damage of the cell wall or vector transmission (Hull 2002, Wilson 2014).

There are several groups of biological virus vectors: invertebrates (such as nematodes, insects and mites), fungi and protozoa (Wilson 2014). The most common viral vector is aphids which vector over 60% of the invertebrate transmitted viruses (Wilson 2014). Aphids have characteristics that make them excellent as viral vectors: they feed by piercing through the cell barrier to enter live plant cells, they are able to generate large populations rapidly, they are highly mobile therefore enabling virus to spread, and many species are polyphagous encouraging movement between different host species (Wilson 2014).

Plant viruses are usually transmitted by insect vectors in non-persistent, semi-persistent, circulative persistent or propagative persistent ways (Wilson 2014, Shi et al. 2021). The non-persistent transmission is the most common mode of transmission found between plant viruses and their aphid vectors, and this plays a big role for many economically important plant viruses (Hull 2002). Plant viruses spread in this manner are retained in the stylet of their aphid vector, and do not enter and circulate within the vector, the virus is required rapidly, and the retention time is only a few minutes (Hull 2002, Shi et al. 2021). The semi-persistent manner of transmission is a somewhat intermediate between non-persistent and persistent modes with the acquisition and retention time being in-between (Hull 2002). Semi-persistent viruses are retained mainly in the foregut, or the stylet (Hogenhout et al. 2008). Non- and semi-persistent viruses does not require a latency period (the period from when the virus is acquired by the vector to when it is transmitted), are not replicated within the vector and are not retained following molting (Hull 2002, Hogenhout et al. 2008).

Persistent transmitted viruses are internalized within the aphid vector and are divided into two subgroups: propagative persistent where the virus replicates within the host, and circulative persistent where the virus does not replicate within the host (Wilson 2014). Persistently propagated viruses require a latency period and are retained after molting, the retention time

in the vector is days to weeks for the circulative viruses and for the propagative viruses the retention time is the lifespan of the vector (Wilson 2014).

2.2.1 *Strawberry viruses*

Many strawberry viruses were initially described based upon symptoms in *F. vesca* and *F. virginiana* plants after graft inoculation with different viruses (Martin and Tzanetakis 2006). However significant progress has been made in molecular diagnostics and characterization of viruses in recent times. Table 2.1 shows a list of viruses capable of infecting strawberries.

Table 2.1 Strawberry viruses, names, acronyms, mode of transmission, genus, and laboratory detection methods. Source: (Martin and Tzanetakis 2006)

Virus name	Acronym	Mode of transmission	Genus	Laboratory detection[b]
Apple mosaic	ApMV	Pollen, seed	*Ilarvirus*	ELISA, RT-PCR
Arabis mosaic	ArMV	Nematode, seed	*Nepovirus*	ELISA, RT-PCR
Beet pseudo-yellows	BPYV	Whitefly	*Crinivirus*	RT-PCR
Fragaria chiloensis cryptic	FClCV	Unknown	Unknown	RT-PCR
Fragaria chiloensis latent	FClLV	Pollen, seed	*Ilarvirus*	ELISA, RT-PCR
Raspberry ringspot	RpRSV	Nematode, seed	*Nepovirus*	ELISA, RT-PCR
Strawberry chlorotic fleck	StCFV	Aphid	*Closterovirus*	RT-PCR
Strawberry crinkle	SCV	Aphid	*Cytorhabdovirus*	RT-PCR
Strawberry feather leaf	NA	Unknown	Unknown	NA
Strawberry latent	StLV	Unknown	*Cripavirus*	RT-PCR
Strawberry latent C	SLCV	Aphid	*Nucleorhabdovirus*	NA
Strawberry latent ringspot	SLRSV	Nematode, seed	*Sadwavirus*	ELISA, RT-PCR
Strawberry mild yellow edge	SMYEV	Aphid	*Potexvirus*	ELISA, RT-PCR
Strawberry mottle	SMoV	Aphid	*Sadwavirus*	RT-PCR
Strawberry necrotic shock	SNSV	Thrips, pollen, seed	*Ilarvirus*	ELISA, RT-PCR
Strawberry pallidosis associated	SPaV	Whitefly	*Crinivirus*	RT-PCR
Strawberry pseudo mild yellow edge	SPMYEV	Aphid	*Carlavirus*	ELISA
Strawberry vein banding	SVBV	Aphid	*Caulimovirus*	PCR
Tobacco necrosis	TNV	Oomycete	*Necrovirus*	ELISA, RT-PCR
Tomato black ring	TBRV	Nematode, seed	*Nepovirus*	ELISA, RT-PCR
Tomato ringspot	ToRSV	Nematode, seed	*Nepovirus*	ELISA, RT-PCR

[a] NA = not available, indicates the virus disease has been described in the literature but that the authors are unaware of a known isolate of the virus currently maintained in a collection.
[b] Detection methods listed do not include sap inoculation, graft transmission, or vector transmission to indicator plants.

Strawberry mild yellow edge (SMYEV), strawberry crinkle virus (SCV), strawberry vein banding virus (SVBV) and strawberry mottle virus (SMoV) have been considered to be the four most economically important viruses in cultivated strawberry (Martin and Tzanetakis 2006) and are regarded as quarantine pests in Norway (Blystad and Spetz 2019). These four viruses are aphid transmitted, and the most important vector is *Chaetosiphon fragaefolii* (Martin and Tzanetakis 2006). *C. fragaefolii* has not yet been detected in Norway (Trandem et al. 2019), and chances of its vectored viruses establishing in Norway are therefore low. The aphid may, however, establish in Norway in the future, either due to it migrating further north, or through import of plant material. The use of certified planting materials and insecticides are

means that could be used to control the spread of aphid-borne strawberry viruses (Babini et al. 2004).

A study on occurrence and identification of the four most important aphid-borne strawberry viruses in five European countries (Italy, The Czech republic, Poland, Lithuania and Germany) was conducted by Babini et al. (2004). Approximately 4% of the tested strawberry plants were positive for at least one of the viruses. Plants positive for SMoV, SMYEV and SCV were found, whereas SVBV was not detected in any of the samples. The study discusses that the relatively low incidence of these viruses might indicate that the vector activity is quite low and/or that there are few sources of inoculum in the studied countries.

2.2.1.1 *Strawberry mild yellow edge*

The virus strawberry mild yellow edge virus, SMYEV (species *strawberry mild yellow edge virus,* genus *Potexvirus*, family *Aplhaflexiviridae*) has a single stranded positive-sense RNA (Martin and Tzanetakis 2006). The virus is vectored in a persistent manner (Martin and Tzanetakis 2006). SMYEV was first described in California in 1922 (Converse et al. 1987) and is today a widespread virus within cultivated *Fragaria* ssp. (EPPO 2017). Many of the cultivars grown today are tolerant to infection of this virus (EPPO 2017), and most strawberry cultivars infected are symptomless carriers (Converse et al. 1987). Symptoms in sensitive cultivars may be dwarfing, marginal chlorosis, reduced vigor (EPPO 2017). Cho et al. (2011) reports findings of mosaic symptoms on newly developed leaves of strawberry cv. Seolhyang and black colored edges on leaves of cv. Eyeberry. Babini et al. (2004) found that SMYEV induced reddish and necrotic spots on older leaves of the indicator plants UC4 and UC5. Further symptoms were epinasty of young leaves followed by death a month later.

SMYEV is usually not a significant disease agent alone, but complexes with other viruses like SMoV, SVBV or SCV can cause severe loss of plant vigor and decline in fruit quality and yield (Converse et al. 1987, EPPO 2017). Reports of the economic impacts of SMYEV are conflicting; a study conducted by Barrit & Loo (1973) showed no significant yield reduction, whereas a newer study by Torrico et al., (2017) found that SMYEV can cause notable losses, also when it acts alone. Martin & Tzenetakis (2016) notes that reported yield losses vary from 0% to 30%. The conflicting results may be due to different strains of the virus, different cultivars, synergetic effect with other viruses or different environmental conditions for the studies.

A study conducted by Cho et al. (2011) observed SMYEV particles in mesophyll cells and epidermal cells in leaves of strawberry and found that SMYEV could replicate well in cells of strawberry cv. Seolhyang. The study was conducted using RT-PCR and an electron microscope. The mass of virus particles was mainly an inclusion body induced by the virus in the epidermal cells, whereas in the parenchyma cells, virus particles were heavily scattered. Viral inclusion cells are structures generated by viral proteins together with cellular proteins and are formed as a platform for viral replication (Zhang et al. 2017).

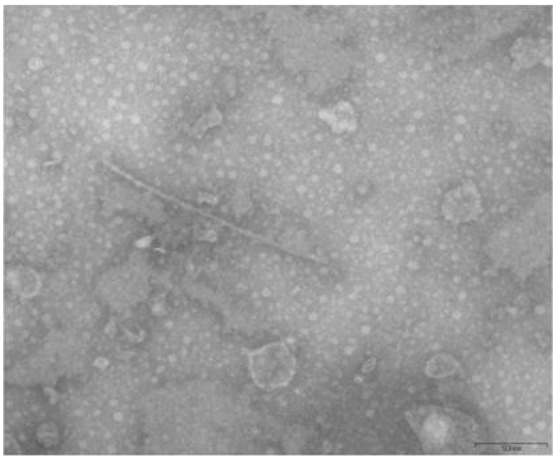

Figure 2.2: Virus particle of the Potexvirus SMYEV with the length of 550-600 nm. Observed by Quick dip electron microscopy of a leaf of strawberry. Source: (Cho et al. 2011)

A survey investigating viruses in imported strawberry plants was conducted by the Norwegian Food Safety Authority together with NIBIO in 2017 and 2018 (Blystad and Spetz 2019). In 2017 they tested 150 samples from different growers, and found one SMYEV infected strawberry plant, this plant was also infected with SCV. In 2018 they found one SMYEV infected strawberry plant amongst 156 tested samples. None of the SMYEV infected plants showed symptoms.

2.2.1.2 *Strawberry vein banding virus*

Strawberry vein banding virus, SVBV (Species *Strawberry vain banding virus*) belongs to the genus *Caulimovirus* of the *Caulimoviridae* family (Martin, 2006, EPPO, 2001). Viruses in the genus *Caulimovirus* have circular dsDNA (ICTV 2011), and their replication consists of two phases: transcription of RNA template from the virion DNA and reverse transcription of the template RNA to make dsDNA (Hull 2014).

Babini et al. (2004) found that SCV induced chlorotic spotting, crinkling of leaves and epinasty on indicator plants approximately 20 days after grafting. SVBV usually does not induce strong symptoms alone, but mixed infections with SCV or *Strawberry latent virus C*

can lead to more severe losses (Martin and Tzanetakis 2006). SVBV is known to cause symptoms on indicator clones of *F.vesca* and *F.virginia*, with UC-6 and UC-12 being the most sensitive (Martin and Tzanetakis 2006).

Ratti et al. (2009) found in 2007 and 2008 SVBV infected plants in a field in Northern Italy. The symptoms displayed in the field were poor growth, leaf chlorosis and reduction in fruit production. The younger leaves were reduced in size and had a yellow edge. The infected samples were grafted on indicator plants, and symptoms of chlorotic streaks on both sides of the main leaf veins appeared on UC-5, UC-10 and C-11. Kitajima et al. (1973) examined SVBV infected leaf material and found the presence of circular profiled cytoplasmic inclusions most commonly in cells of the vascular parenchyma and mesophyll, and less common in the epidermis.

Figure 2.3: Symptoms of SVBV on Fragaria ssp. grown in the greenhouse at Kirkejordet, SKP, NMBU. The plant has symptoms of vein banding, and chlorosis.

Table 2.2: An overview of aphid transmitted strawberry viruses. UC-4, UC-5, UC-6 is F.vesca hybrids and UC-10, UC-11, UC-12 is F.virginiana hybrids

Virus name	**Family**	**Genus**	**Genome**	**Indicator plants**	**Main Symptoms**	**Manner of transmission**	**Location in plant**	**Source**
SMYEV	*Aphaflexiviridae*	*Potexvirus*	+ssRNA	UC-3, UC-5	dwarfing, marginal chlorosis, reduced vigor	Persistent	Mesophyll and epidermal cells	(Babini et al. 2004, Martin and Tzanetakis 2006, Cho et al. 2011, EPPO 2017)
SVBV	*Caulimoviridae*	*Caulimovirus*	dsDNA	UC-6, UC-12 (UC-5, -10,-11)	Vein banding, leaf curl, necrosis	Semipersistent	Vascular parenchyma, mesophyll (epidermis)	(EPPO, 2001) (Martin and Tzanetakis 2006) (Kitajima et al. 1973)
SMoV	*Secoviridae*	*Sadwavirus*	+ssRNA	UC-5, *F. vesca* cv. Alpine	Mottling and chlorotic spots on leaves. Stunting	Semipersistent		(Martin and Tzanetakis 2006, Sanfaçon et al. 2020)
SCV	*Rhabdoviridae*	*Cytorhabdovirus*	-ssRNA	UC-4, UC-5 *F. vesca* cv. Alpine. Nicotiana ssp., Physalis ssp (mechanically)	Reduced yield and fruit size. Deformed and crinkled leaves, with chlorotic spots. Necrotic lesions on petals, petioles and runners	Propagative persistent	Vascular bundle and leaf parenchyma (necrotic cells)	(Martin and Tzanetakis 2006, Walker et al. 2018, CABI 2022)

2.2.1.3 *Strawberry mottle virus*

Strawberry mottle virus, SMoV (species *Strawberry mottle virus*), has recently been set to the newly composed subgenus *Stramovirus* in the genus *Sadwavirus* within the family *Secoviridae* (Sanfaçon et al. 2020). SMoV is considered the most common strawberry virus to infect *Fragaria* ssp. (Martin and Tzanetakis 2006). The virus is transmitted in a semi-persistent manner through the aphid vectors *Chaetosiphon ssp*. and *Aphis gossypii* (Martin and Tzanetakis 2006, Fan et al. 2022). SMoV causes symptoms ranging from mild mottling to severe stunting and even plant death on indicator plants (Fan et al. 2022). Babini et al. (2004) found that SMoV induced mild to severe mottling and chlorotic spots on younger leaves on indicator plants approximately 20 days after grafting.

Severe strains of SMoV can cause yield loss up to 30%, and can be even more detrimental in coinfection with other strawberry viruses (Martin and Tzanetakis 2006). Fan et al. (2022) found that plants infected with both SMoV and SVBV produced 25.5% lower fruit yield than the virus free control. The reason for the decline in yield were that the infection of these viruses caused cell death in leaves due to induction of ROS (reactive oxygen species), decrease in photosynthesis due to reduction of chlorophyll content, stomata aperture and anthocyanin content.

2.2.1.4 *Strawberry crinkle virus*

All species of *Fragaria* are susceptible to strawberry crinkle virus, SCV (species *Cytorhabdovirus fragariarugosus*, family *Rhabdoviridae*)(Walker et al. 2018). This virus is considered one of the most damaging viruses on strawberry (Martin and Tzanetakis 2006). Severe strains of SCV can cause symptoms on infected cultivars and reduce yield, however, great losses result from mixed infection with other aphid borne viruses (Martin and Tzanetakis 2006). This virus can also be transmitted from infected strawberry to solanaceous plants in the genera *Nicotiana* and *Physalis*, by first using injected green and pink potato aphids which work as surrogate vectors, and then by sap inoculation to new host plants (Richardson and Sylvester 1988). SCV particles are hard to find within infected plants, but have been detected in necrotic cells surrounding vascular bundles, and necrotic cells of mesophyll parenchyma of leaves and petioles (Jelkmann et al. 1988).

Strawberry plants infected with SCV are usually also infected with other aphid borne viruses, which makes their symptoms additive (CABI 2022). No strawberry cultivars have been shown

to be immune to single infection by SCV, but some cultivars may be tolerant and remain symptomless with infection (CABI 2022). Cultivars that show symptoms may have chlorotic spots, leaf deformation and crinkling as well as reduction in size of leaves and petioles. The severity of symptoms varies greatly with the strain of SCV. A comparison of healthy strawberry plant with SCV infected plants of the same cultivar showed a decrease of markable fruit yield by 26%, whereas plants coinfected with both SCV and SMoV had a reduction of 64% in the cultivar Hood (Barrit and Loo 1973). The cultivar Northwest, however, showed no significant difference in yield between healthy plants and virus infected plants.

Figure 2.4: SCV infected Fragaria ssp. grown in the greenhouse at Kirkejordet, SKP, NMBU. The plant has symptoms like crinkling and deformation of leaves.

2.2.2 *Diagnostics of plant viruses*

Accurate and precise diagnosis is important to find the most efficient control program and to prevent a major disease outbreak (Blystad et al. 2020). Identification of plant viruses can be done either by investigating symptoms on the virus host plant, symptoms on test plants, vector transmission, electron microscopy, serological methods or nucleic acid based methods (Blystad et al. 2020).

Virus symptoms are often associated with metabolic and physiological changes in the host plant (Wilson 2014). Specific symptoms can be characteristic for the specific virus, however, co-infection of several viruses within a plant could exacerbate the symptoms and other biotic or abiotic factors may cause virus like symptoms (Wilson 2014). In addition to this, viral

symptoms may vary with different strains of the virus, different cultivars and growth stages of the host plant, and different environmental conditions such as temperature (Wilson 2014). Even though some viruses may cause symptoms on their host plants that are so distinctive that they can be used for identification, investigating symptoms alone is usually not sufficient for a diagnosis, but may give an indication of a virus infection (Blystad et al. 2020). In these instances, the diagnostic work is moved to the laboratory.

2.2.2.1 *Biological indexing*

Biological indexing is based on indicator plant's ability to express characteristic symptoms when inoculated with a virus using manly mechanical inoculation or grafting (Legrand 2015). When testing for a possible infection of a known virus, a single test plant species could provide answers, whereas when identifying an unknown virus different test plants showing different responses are needed (Blystad et al. 2020). A test plant is characterized as a plant that gives familiar and characteristic responses to infection of a known virus (Blystad et al. 2020). Sap can be extracted from a virus-containing plant, mixed with a suitable buffer to maintain virion integrity, and rubbed on leaves on test plants (Wilson 2014). Following the mechanical inoculation, the test plants are observed for several weeks to months for expression of characteristic symptoms that may appear either on the inoculated leaves or newly emerging leaves, indicating local infection or systemic infection, respectively (Wilson 2014).

For viruses that are not sap transmissible and for viruses that infect wood species, grafting of a possible infected plant sample to an indicator plant can be a useful method (Wilson 2014). Vector transmission can also be used to investigate the viruses mean of transmission and for biological indexing of viruses that cannot be transmitted mechanically (Wilson 2014).

2.2.2.2 *Serological methods*

Serological methods are relatively inexpensive, and provide good sensitivity and specificity once an antiserum is developed (Blystad et al. 2020). These methods are based on the specific reaction between an antigen, in this case a virus, and the antibody raised against the specific antigen (Wilson 2014). The most used serological detection method for virus is ELISA (enzyme-linked immunosorbent assay) (Blystad et al. 2020). Results from ELISA tests can be analyzed visually or by a spectrophotometer due to color change following a reaction catalyzed by a conjugation of an enzyme to a detection antibody which binds to the target virus or a second antibody specific to the virus (Wilson 2014).

2.2.2.3 *Nucleic acid based methods*

Nucleic acids are the major component of the virus particle, and the sequence information will be specific to the virus species and can, due to this, be used as a diagnostics tool (Wilson 2014). Polymerase chain reaction (PCR) multiplies large amounts of a specific DNA sequence and can be used directly for detection of DNA viruses (Wilson 2014). RNA viruses, however, need an extra step: RNA needs conversion to complementary DNA (cDNA) by reverse transcription prior to the PCR reaction. To produce cDNA, RNA is extracted from the virus infected plant and is incubated with the reverse transcriptase enzyme together with either oligo-dT-, random- or virus-specific primers (Wilson 2014). Reverse transcriptase (RT) is a DNA polymerase enzyme able to transcribe single stranded RNA into DNA (Andréola et al. 2013). The cDNA serves as a template for the exponential amplification in the PCR reaction.

The quality of the extracted RNA can be determined by the absorbance ratio 260/280 and 260/230. Nucleic acid absorbs light at 260 nm (Lucena-Aguilar et al. 2016). The ratio of absorbance at 260 and 280 is used to determine the purity of the RNA and should optimally be around 2.0, however, if the ratio is lower than two, it might indicate that the sample is contaminated with either proteins, phenols or other contaminants that absorb at 280nm (Matlock 2015). The 260/230 rate should be around two as well, a lower rate may indicate presence of contaminants absorbing at 230, like salts, carbohydrates, or other organic contaminants (Lucena-Aguilar et al. 2016).

The amplified nucleic acid can be visualized by electrophoresis or quantified by the real-time qPCR machine (Blystad et al. 2020). Agarose gel electrophoresis is a traditional method to detect PCR-amplified DNA. The presence of a DNA band of the specific size will determine virus presence in the sample (Cassedy et al. 2021).

Real time PCR measures amount of a specific DNA molecules present in a sample, where the amount DNA molecules present after every cycle are measured using probes labeled with fluorescent tags that only florescent when annealed to their target sequence (Fletcher and Hickey 2013). The use of different fluorescent tags makes it possible to quantify more than one DNA species in each sample, and allows for inclusion of controls of known content and concentration (Fletcher and Hickey 2013).

Each cycle in the PCR reaction has three stages carried out in different temperatures: (1) denaturation – the reaction is heated to over 90°C to separate the strands in the DNA molecule into single strands, (2) annealing – the reaction is cooled to 40-60 °C to allow binding of the

primers to the single stranded template, (3) extension – the reaction is heated to 72°C, the temperature where DNA polymerase is most active, and allows for copying of the target sequence (Fletcher and Hickey 2013).

Efficiency of the RNA extraction and possible presence of inhibitors can be assessed by adding a known quantity of purified template DNA as a positive control standard (Loftis and Reeves 2012). A well characterized and highly expressed regulatory gene in the host plant should be included as an internal positive control and this should be detected to ensure that both the reverse transcription and the PCR worked (Loftis and Reeves 2012).

Establishment of methods that facilitate sequencing of high amount of nucleic acids, such as small RNA (sRNA) and all dsDNA in a plant, can be used to search for nucleic acids of viruses in short time (Blystad et al. 2020). These approaches, called next generation sequencing (NGS), do not require any presumptions of the virus present in the sample, making them very useful (Blystad et al. 2020).

2.2.2.4 *Electron microscopy*

Viruses are, due to their small size, hard to see in a normal light microscope, hence they require an electron microscope where the resolution is so good that the virus` morphology can be studied (Blystad et al. 2020). The method called negative staining is an important diagnostic tool, and is performed by grinding a small piece of infected plant material in a buffer to make a solution where the viruses can adhere to a grid (Blystad et al. 2020). This grid can then be investigated under the electron microscope, and the virus particles and other particles from the plant will appear bright against a darker background (Blystad et al. 2020). When the virus is observed in the sample, it gives a clear evidence for virus presence in the sample, and further investigation of the morphology of the virions can be used as further clues on the identification of the specific virus (Wilson 2014).

Other electron microscopy methods used in virus identification is TEM, which attempts to detect virion particles within a cell to provide additional information about the virions location within the cell and cytopathological changes induced by the virus, and ISEM (immunosorbent electron microscopy) that combines the sensitivity and specificity of serological methods with direct visualization of TEM (Wilson 2014).

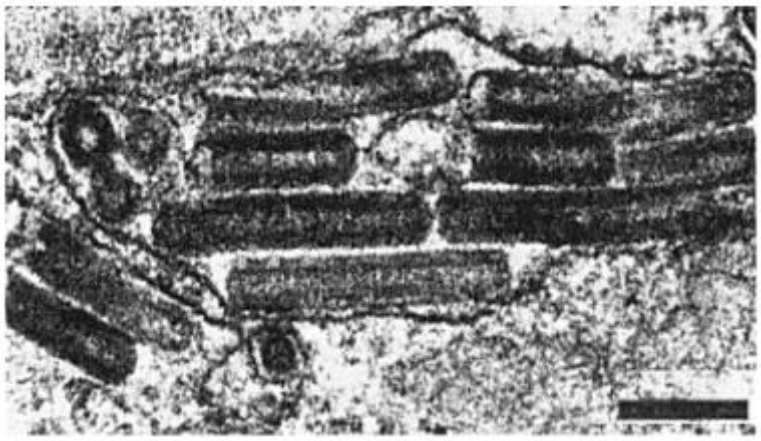

Figure 2.5: Electron micrograph of SCV. The virions are bacilliform, 163-383 nm in length. Bar= 200nm. Source: (Posthuma et al. 2000)

2.2.3 *Preservation of plant viruses*

To aid in diagnosis of viruses and for virus studies, it is often useful to store virus inoculum. This can be done by maintaining stock cultures in plants grown in greenhouses, however this method is dependent on space and work and can risk cross contamination or changes in the virus isolate (Hull 2002). Obligate plant pathogens, such as virus, viroid and phytoplasma can only replicate and colonize within a living cell of a host (Hull 2002). As a result of this, preservation of these pathogens can be difficult. Several strains of tobacco mosaic virus (TMV) can be stored in air-dried leaf for a long period of time, however, inoculum of most viruses loses infectivity rapidly unless certain requirements are met, and storage of these viruses for more than a few days can be a challenge (Hull 2002). Many viruses can be preserved in dried or lyophilized plant tissue, this however, is usually restricted to sap-transmissible viruses (Rochow et al. 1976). Following preservation, these viruses can only be transmitted by mechanical inoculation to the new host (Mckinney 1965, Yordanova et al. 2000), this causes a problem for viruses that cannot be transmitted in this matter, making long time-storage of vector dependent viruses are more challenging due to difficulties in recovering these viruses (Rochow et al. 1976). Due to these difficulties, cryopreservation has been researched as a method for long-term preservation of plant viruses.

2.3 Cryopreservation

2.3.1 *Definition*

Cryopreservation refers to the storage of living cells, tissue and organs at ultra-low temperatures, usually that of liquid nitrogen (-196°C) (Engelmann and Dussert 2012). At this temperature, all metabolic processes are stopped, and thus the material can theoretically be stored for an unlimited time without alternation or modification, while maintaining their genetic stability and avoiding contamination (Benson 2008, Engelmann and Dussert 2012).

Cryopreservation is considered to be the most valuable method for long-term preservation of plant germplasm (Jiroutova and Sedlak 2020). Several cryobanks have been established in different countries for various species like cassava, potato, banana, pear, apple, coffee, garlic and mulberry (Benelli 2021).

2.3.2 *Background*

The first work on cryopreservation was published over 60 years ago by Sakai (1956). This publication reported successful survival of mulberry twigs after exposure to liquid nitrogen. A challenge in the method of cryopreservation was to freeze fully hydrated plant tissues where there is a risk of formation of lethal intracellular ice-crystals, and due to this, slow freezing protocols were developed (Jiroutova and Sedlak 2020). This proved to be an efficient method for cryopreservation of non-organized tissues like callus, but remained problematic for organized tissues like meristems (Panis 2019). This led to the development of fast freezing protocols such as droplet-vitrification and encapsulation-dehydration. These processes are based on induction of explant "vitrification" (see 2.3.3) during a fast decrease in temperature. To this date several methods and techniques for cryopreservation have been reported. The initial motive for studies regarding cryopreservation was preservation of genetic resources (Sakai 1956). Further research has shown that cryopreservation also has other appliances, such as aiding in plant genetic engineering (Wang et al. 2014), preserving endangered species (Engelmann 2011), eradicating plant viruses (Wang et al. 2009) and preserving plant viruses (Zhao et al. 2019).

2.3.3 *Different cryopreservation methods*

Some plant materials can be cryopreserved without pretreatment, such as orthodox seeds or dormant buds due to their natural dehydration processes (Engelmann 2011). However, much of the material used for cryopreservation purposes contains high amounts of cellular water, such as cell suspensions, shoot tips, embryos and calluses. The high water content and the fact

that most are not freezing tolerant makes them very sensitive to freezing injury (Engelmann 2011). Thus, these kinds of cells must be dehydrated artificially to protect them from damages caused from crystallization of intracellular water (Mazur 1984).

Several methods of cryopreservation are developed, including both classical and modern methods (Figure 2.6).

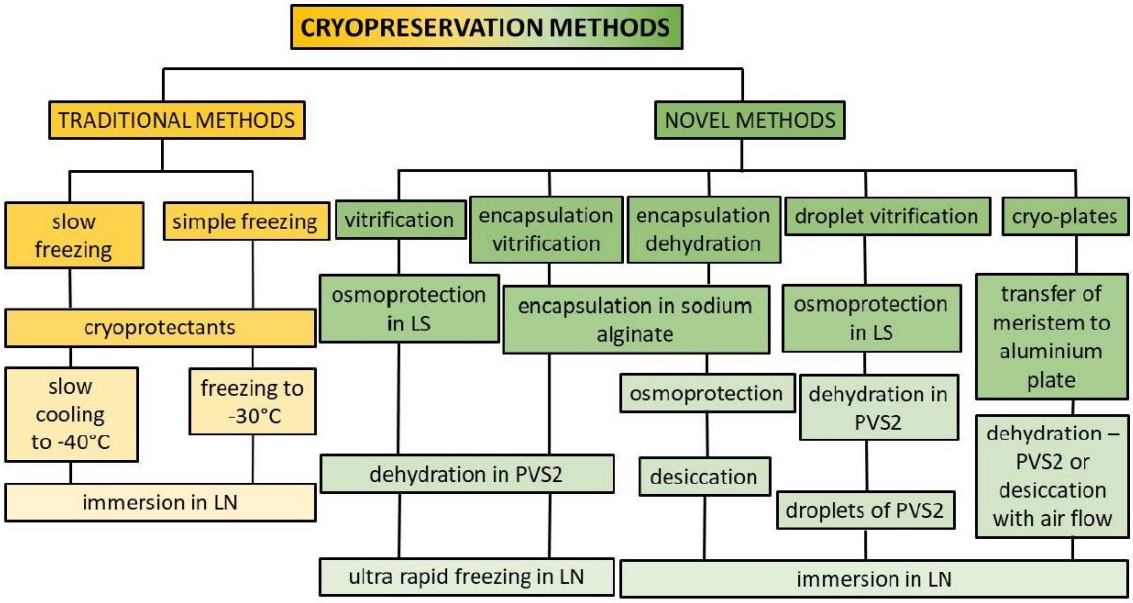

Figure 2.6: Schematic diagram of major cryopreservation methods of shoot tips. Source: (Jiroutova and Sedlak 2020)

The traditional methods were developed over 40 years ago and refer to the two techniques: (1) slow freezing (also known as two step-freezing) and (2) simple one step freezing (Kalaiselvi et al. 2017, Jiroutova and Sedlak 2020). These techniques consist of pretreatment of samples with cryoprotectants then slowly cooled to -40°C and then rapidly emerged in LN (1), or cooled to -30°C for dehydration following immergence in LN (2) (Jiroutova and Sedlak 2020).

Vitrification-based approaches are the most widely applied methods of cryopreservation due to high recovery rates, relatively easy to execute methods, and no requirement of special equipment (Jiroutova and Sedlak 2020). The term vitrification refers to the physical process of cells and tissues avoiding intracellular ice-crystallization during ultra-freezing, by the transition of the aqueous solution in the cytosol into an amorphous glass like state (Benelli 2021). When plant material is treated with a high concentrated vitrification solution, most of the freezable water is removed from the cells, and the material is ready for rapid freezing in LN (Jiroutova and Sedlak 2020). The combination of dehydration and rapid freezing of cells

causes residual water to solidify and thus prevents crystallization that could be harmful for living cells (Benson 2008). Plant vitrification solution 2 (PVS2) (Sakai et al. 1990, Nishizawa et al. 1993) and plant vitrification solution 3 (PVS3) (Nishizawa et al. 1993) are the most used cryoprotective solutions (Wang et al. 2014).

Shoot tips are often precultured on high sucrose media to increase the tolerance of the shoot tip to dehydration and freezing in LN (Wang et al. 2005). Accumulation of sugar in the plant can maintain the integrity of the plasma membrane by being a substitute for water in the surface of the plasma membrane and stabilizing proteins during dry freezing (Crowe et al. 1987). Osmoprotection prior to emergence in PVS2 is important to prevent harmful osmotic stress and chemical toxicity caused by PVS2, and this osmoprotection is achieved through preculture and loading solution (LS) (Wang et al. 2005). The reason for the cryoprotective effect of LS is not fully known (Wang et al. 2005), but treatment with this solution, containing glycerol and sucrose, promotes plasmolysis which might alleviate the mechanical stress during dehydration helping the shoot tips survive freezing (Hirai and Sakai 2003).

2.3.4 *Preservation of genetic resources*

2.3.4.1 *Conventional conservation*

Production of new plant varieties with improved agricultural characteristics and enhanced resistance to biotic and abiotic stresses are of utmost importance for success in the agricultural industry (Lynch et al. 2007). When breeding for new and improved plant varieties, it is important for the breeder to have access to a plethora of plant genetic resources such as wild relatives, land races, old varieties and genotypes from plant breeding programs (Lynch et al. 2007). Gene banks play a key role in preserving plant genetic resources for future plant improvement.

Crops producing orthodox seeds are now commonly stored in *ex-situ* gene banks under low relative humidity and low temperature conditions (Westengen et al. 2013). However, there are several plant species where conservation of seeds is not applicable, such as vegetatively propagated plants that do not produce “true to type” seeds (Lynch et al. 2007). Plants that are not suitable for seed banking because they are heterozygous or have recalcitrant seeds are often conserved in field collections (Jiroutova and Sedlak 2020). This type of collection is beneficial considering it gives direct access to the material, but on the downside, plants in field are exposed to several threats such as pests and diseases and it is labor intensive and expensive (Jiroutova and Sedlak 2020).

An alternative to preservation of plant resources in seed banks or in field is *in vitro* gene banks where plants are vegetatively propagated and grown on medium under sterile conditions (Jiroutova and Sedlak 2020). *In vitro* techniques are useful for conserving plant biodiversity such as genetic resources of vegetatively propagated plants, and elite genotypes and material for further breeding purposes, and ensure rapid multiplication of disease free material (Engelmann 2011). However, considering that this method requires periodically sub cultivation on new medium, this method is more suitable for short-term storage and propagation than long-term storage (Jiroutova and Sedlak 2020). In vitro conservation is also labor intensive and poses a risk of contamination which may lead to the loss of the germplasm (Wang et al. 2014), and repeated subcultures may lead to genetic variation (Reed et al. 2004). The method of cryopreservation has developed to be considered the most valuable method for long time preservation of plant germplasm (Jiroutova and Sedlak 2020).

2.3.4.2 *Cryopreservation of strawberry*

There are different approaches for conserving germplasm resources of *Fragaria x ananassa* cultivars and their wild relatives. Since strawberry is a vegetatively propagated plant, most common methods of conservation are in field gene banks, greenhouse or orchards as live plants (Gupta 2022). Strawberry resources can also be stored *in vitro*. With the use of this method, however, strawberry cultures can only be stored at for a maximum of 24 months (in 4°C) before they require repropagation (Reed 2002), and some DNA-methylation patterns have been found in slow growing strawberry cultures (Pinker et al. 2009). There have been several experiments conducted on cryopreservation of strawberry shoot tips, and several protocols has been published (Yamamoto et al. 2012) (Niino et al. 2003) (Halmagyi and C 2006) (Reed and Hummer 1995).

2.3.5 *Cryotherapy*

A plant that has succumbed to a plant virus will generally remain diseased, and there are few incidences where virus-infected plants recover from the infection (Wilson 2014). Management of virus disease therefore relies primarily on prevention of infection. Removing inoculum through planting virus-free planting material is a highly effective virus management strategy. Transmission of virus through seeds is generally inefficient, due to most viruses failing to pass to seeds of their host plants, leading to seed transmission being seldom, although few seed-borne viruses can occur (Wilson 2014). For many vegetatively propagated plants like *Fragaria ssp*., propagation often transfers incipient diseases that could reduce the quality and yield, and is especially important for viruses that move through the plant

systematically (Quiroz et al. 2017). Freeing a plant from a systemic virus infection may be achievable by using meristem tip culture, often combined with chemotherapy, thermotherapy or cryotherapy (Wilson 2014).

Shoot tip cryotherapy has been used as a procedure to eradicate plant obligate pathogens, as well as the contrasting preservation plant obligate pathogens. Due to this, cryopreservation has been referred to "a double edged sword" by Zhao et al. (2019). Virus particles are distributed unevenly within the plant, with low virus titer or even virus-free area in the upper parts of the apical dome, particularly for viruses that are phloem-limited (Zhao et al. 2019). Most viruses generally do not invade the meristem tissue primarily due to an RNA-dependent RNA polymerase facilitating antiviral RNA silencing as a consequence of virus infection, leading to protection of the meristem cells in the new growing points (Wilson 2014). This virus free region of the plant has frequently been used to obtain virus free plant meristem tip culture (Mochizuki and Ohki 2015).

Cryo-treatment usually kills the cells in the lower part of the apical dome and older leaf primordia (Wang et al. 2018). Less differentiated cells, however, that has a higher nucleus-cytoplasmic ratio and that contain lower levels of free water, such as meristem cells in the uppermost layer of the apical dome, survive (Zhao et al. 2019). As a result of this, cryo-treatment may kill the differentiated virus infected cells and allow for the pathogen free meristematic cells to survive and regenerate into new healthy plants**.**

2.3.6 *Development of cryopreservation for preserving obligate plant pathogens*

Several methods have been developed for preservation of plant viruses, including freezing (Fukumoto and Tochihara 1998), freeze-drying (Fukumoto and Tochihara 1998, Yordanova et al. 2000), dehydration by physical drying and chemicals (Mckinney 1965, Grivell et al. 1971) and in vitro culture (Chen et al. 2003). The freeze-drying method became the most widely used method amongst the conventional procedures. In this method, purified virions were freeze-dried and stored in ultra-low temperatures, and when thawed and mechanically inoculated into host plants (Wang et al. 2022). However, with this method, infection efficiency decreases as preservation time increases (Yordanova et al. 2000), and this method is not suitable for viruses that are not mechanically transmissible (Wang et al. 2018). A downside with in vitro culture for virus storage is that it requires labor and carries the risk of contamination, thus leading to total loss of the stored viruses (Wang et al. 2018). Viruses

recovered from cryopreserved shoot tips, however, can be transmitted to other hosts via mechanical transmission or grafting (Li et al. 2018), and are safe from contaminants during preservation in liquid nitrogen.

Cryotherapy is a method developed to eradicate obligate plant pathogens, and has shown to be an effective tool for eradicating several viruses from different plant species (Zhao et al. 2019). However, a certain proportion of plants recovered following cryotherapy are still pathogen-infected, depending on the pathogen-host relationship and location of the virus within the plant (Zhao et al. 2019). This is especially relevant when it comes to viruses that infect the meristematic cells in the shoot tips and viroids (Hamborg et al. 2014, Wang et al. 2018). This knowledge gives rise to the presumption that cryopreservation techniques developed for conservation of plant shoot tips could be used to preserve certain plant viruses.

Wang et al. (2018) reported successful cryopreservation of apple stem grooving virus (ASGV) in living shoot tips, a virus that infects the meristematic cells located in the upper area of the apical dome. Following cryopreservation by either droplet-vitrification or encapsulation-dehydration, about 62-67% of the shoot tips survived and regenerated, and ASGV was preserved in 100% of the recovered shoots. Shoot tip cryopreservation was also applied to long term preservation of potato leafroll virus (PLRV), potato virus S (PVS) and potato spindle tuber viroid (PSTVd) by Li et al. (2018). In this study, 100% of the recovered shoot tips were PVS and PSTVd preserved, whereas 0% to 35 % of the shoot tips were PLRV preserved depending on shoot size. The report notes that cell survival patterns and virus localization studies provided explanation for the varying results of the PLRV preservation in different shoot tip sizes. A study conducted by Li et al. (2019) on cryopreservation of chrysanthemum chlorotic mottle viroid (CChMVd) and chrysanthemum stunt viroid (CSVd) resulted in 100% of the recovered shoot tips maintaining their viroid-infected status.

2.3.7 *Genetic and morphological stability*

A successful cryopreservation procedure does not only imply high survival and regeneration percentage after immersion in liquid nitrogen, but also assures genetic stability in the plant material after regeneration (Martín et al. 2015). In vitro cultures of shoot tips are generally considered to be more genetically stable than callus or other forms of cultures (Martín et al. 2015).

Genetic stability is a major concern when it comes to cryopreservation of both germplasm and virus. When plant material is frozen in LN, cell division and all metabolic processes are

stopped, theoretically resulting in samples being able to be stored for an unlimited period of time with no changes in genetic integrity (Harding 2004). However, during the cryopreservation the samples are exposed to several more steps than immergence in LN, such as preculture on high sucrose medium and dehydration by chemical solutions. These factors may result in genetic variations in samples following cryopreservation, this is especially relevant in viruses which only consists of a nucleic acid surrounded by coat protein (Wang et al. 2018). However, studies indicate that cryopreservation can maintain the genetic integrity of cryopreserved viruses. Wang et al. (2018) sequenced three gene fragments in cryopreserved ASGV and found that the cryopreserved virus contained 99.89% nucleotide identity with viruses preserved in shoot tips.

Martín et al. (2015) found that the material recovered after droplet-vitrification, which is used in this thesis, showed higher genetic stability compared to recovered samples from encapsulation-dehydration. Wang et al. (2014) investigated the cryo-injury caused by three different cryopreservation procedures on potato shoot tips. The results showed that high sucrose preculture did not cause cell damage to the shoot tips, but dehydration and freezing in LN caused major damage to the cells.

Hao et al. (2002) found that DNA methylation status changed in plants that had recovered from cryopreservation in relation to non-cryopreserved plants. Cryopreservation induced stress and cryoprotectant toxicity may lead to DNA alterations and epigenetic mutations (Khosravizadeh et al. 2020).

Preculture with high sucrose medium can affect the morphological stability. Pinker et al. (2009) found that different concentrations of sucrose in the preculture medium affected the field performance, morphological features and could cause abnormalities. The plants which arose from preculture with the lowest sucrose content (0.1 M) showed the highest survival rate and fewest abnormalities in comparison to higher sucrose concentrations.

3 Materials and methods

3.1 Material

3.1.1 *Plant material*

In vitro grown and virus free cultivated strawberry (*Fragaria* x *ananassa*) plants of cv. Korona were used for cryopreservation in Experiment 1. These tissue cultures were supplied by Sagaplant AS, Akkerhaugen Telemark.

Virus infected strawberry plants used for cryopreservation in experiment 2, were acquired from NIBIO`s own positive control plants grown in the greenhouse at Kirkejordet, SKP, Ås. The strawberry plants were infected with either SMYEV or SVBV. The cultivars for these plants are unfortunately unknown.

3.1.2 Tissue culture medium

The *in vitro* cultures were maintained on basic medium composed of Murashige and Skoog (1962) medium (MS) supplemented with 3% (30g /L) sucrose, 6 g/ L agar (BRAND), 0,5 mg/L 6-benzylaminopurine (BAP) and 0,2 mg/L indole-3-butyric acid (IBA). The pH was adjusted to 5.8, and the medium was autoclaved at 121 °C for 20 min.

3.2 Methods

3.2.1 *Experimental setup*

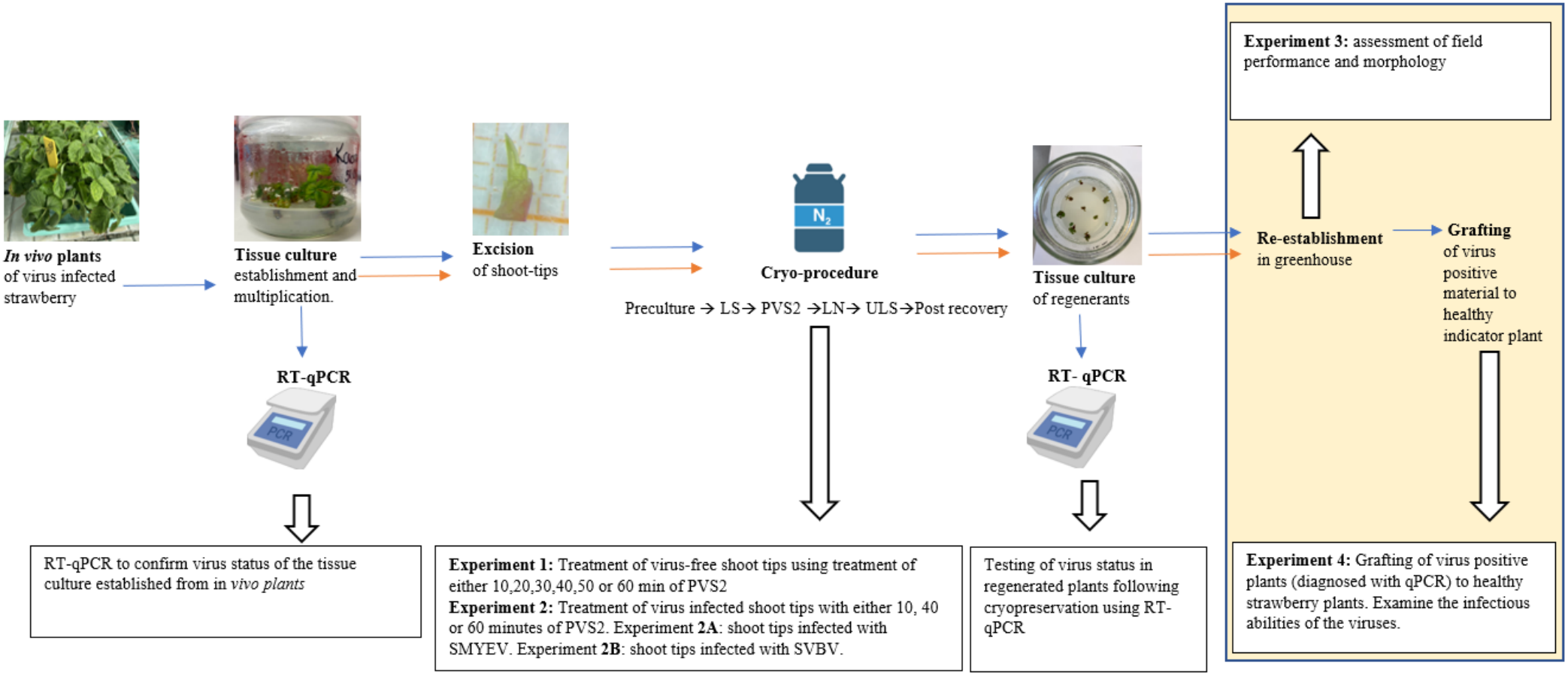

Figure 3.1: Flow-chart of the experimental set-up. Blue arrows indicate the plan for the virus- infected material, whereas orange arrows indicate the plan for the virus-free material. Black arrows indicate the main experiments conducted. The steps in the yellow square were planned experiment, but they were not conducted due to the time limit for the thesis. Shoots from runners of virus infected (SVBV or SMYEV) plants were established in tissue-culture, and qPCR confirmed the positive virus status. The virus-free plant material used was already established in tissue-culture (acquired from Sagaplant, Telemark). Shoot tips were excised from the established tissue culture. The cryo-procedure consisted of preculture on high sucrose medium, emergence in LS (loading solution), PVS2 (plant vitrification solution 2), LN (liquid nitrogen), ULS (unloading solution) and then post recovery medium. qPCR was conducted to test the survival of the viruses. Tissue culture of the regenerants were supposed to be established on soil in the greenhouse, and the morphology of regenerated plants from experiment 1 was going to be compared with plants that had not undergone cryopreservation. Virus positive regenerants from experiment 2 was supposed to be grafted to indicator plants.

3.3 Tissue culture establishment

One cm long runner segments, each containing an axillary bud were gathered from the explants and disinfected. The disinfecting method consisted of:

a. Washing with distilled water for 20 minutes
b. Immersion in 75% ethanol for 15 seconds
c. Immersion in 0,5% hypochlorite for 10 minutes
d. Rinsing with sterilized water three times

After disinfecting, the plant materials were placed under a stereo microscope in a flow hood bench. Leaf primordia were removed, leaving an approximately 5 mm shoot tip. The shoot tips were placed on basic medium explained above in in dark conditions at 22 °C. After 4-5 days the shoot tips were moved slightly to the side on the same medium, to prevent growth inhibition due to phenolic compounds and placed under indirect light (18 µmol m^{-2} s^{-1}). After 3 weeks they were placed under 50±5 µmol m^{-2} s^{-1} at 20±2 °C with a photoperiod of 16 hours light and 8 hours dark. Subculturing was conducted every 3 weeks. Each plant was divided into 2-4 new plants and cultured on new medium.

3.4 Cryopreservation

Before the start of the planned experiments, the cryopreservation protocol had been conducted three times. This was done to ensure fewer possible sources of error due to mistakes being made. Shoot tips are fragile, and during excision and handling it is important to have a steady hand to prevent damage on the shoot tips, which could affect the results.

3.4.1 *Shoot tips*

Shoot tips were excised from 4-6 weeks old subcultures, using two knives under a stereo microscope in sterile conditions. The shoot tips were placed on preculture MS media.

3.4.2 *Preculture*

Shoot tips were precultured on three different mediums with increasing concentration of sucrose prior to cryopreservation. They were precultured for one day for each medium, and then transferred to the next, making the preculture period a total of three days. The sucrose concentrations were 0,25 M, 0,5 M, and 0,75 M, respectively. During the three preculture days, the shoot tips were placed under 18 µmol m^{-2} s^{-1} light at 18 °C.

3.4.3 *Loading*

Pre-cultured shoot tips were transferred to 5 cm petri dishes containing 10 ml loading solution (LS) and treated for 20 minutes while placed on an orbital shaker (50 rpm). The loading solution contained 0.4M sucrose and 2M glycerol in MS medium and were room temperature.

3.4.4 *Vitrification*

Following treatment with loading solution, the shoot tips were soaked in 10 ml PVS2 consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycerol, 15 (w/v) dimethyl sulfoxide (DMSO) and 0.4 M sucrose in MS medium (Sakai et al. 1990), at room temperature. The petri dishes were still placed on an orbital shaker.

In Experiment 1, virus free shoot tips (Strawberry cv. Korona) were exposed to PVS2 for different periods of 10, 20, 30, 40, 50 or 60 minutes to find the most effective PVS2 exposure time. The experiment was repeated three times. There were 10 shoot tips exposed to each treatment in each replication. This resulted in 30 shoot tips receiving each of the six treatments, and 180 shoot tips cryopreserved in total in this experiment.

Table 3.1: Experimental set-up for experiment 1. Virus free strawberry plants were cryopreserved, with different period of PVS2 treatments. The experiment was replicated three times.

Plant material	**Cryopreservation**		**Replications**
	Number of shoot tips	Duration of PVS2 (min)	
Strawberry cv. Korona	10	10	X3
	10	20	
	10	30	
	10	40	
	10	50	
	10	60	

In Experiment 2 virus infected strawberry plants went through cryopreservation and thawing. Shoot tips from plants infected with either SMYEV or SVBV were collected from the greenhouse and used to establish tissue culture. To ensure that the plants were still virus positive after been moved to tissue culture, qPCR was performed prior to cryopreservation. Three rounds of cryopreservation were conducted for each of the two viruses. Each round included 10 shoot tips exposed to one of three different treatments of PVS2: 10, 40 or 60 minutes.

The three different PVS2 times (10, 40 and 60 minutes) in experiment 2 were chosen partly due to; the best results from experiment 1 (40 min), and partly to investigate the effect of the two most extreme times on virus preservation (10 and 60 minutes).

Table 3.2: Experimental set-up for experiment 2. SMYEV or SVBV infected plant material was diagnosed with the use of qPCR to confirm infection. Cryopreservation was conducted with different exposing times to PVS2. The experiment had three replications.

Plant material	**Diagnosis**	**Cryopreservation**		**Replications**
		Number of shoot tips	Duration of PVS2 (min)	
SMYEV infected strawberry culture	RT-qPCR	10	10	X3
		10	40	
		10	60	
SVBV infected strawberry culture	RT-qPCR	10	10	X3
		10	40	
		10	60	

3.4.5 *Freezing in liquid nitrogen (LN)*

The dehydrated shoot tips were transferred to sterilized aluminum foil strips in two mounds of five shoot tips per foil strip, followed by immersion in liquid nitrogen for 5 minutes. At this point, the foil strips containing the frozen shoot tips could have been placed in a cryotank for long time preservation. Due to this being an experiment, the shoot tips were only immerged in LN for five minutes, due to practical and time-saving reasons.

3.4.6 *Thawing in unloading solution*

The frozen foil strips were then rapidly transferred to 10 ml unloading solution (ULS) composed of MS medium enriched with 1.2 M sucrose for 20 minutes, placed in 5 cm petri dishes on an orbital shaker. The ULS was at room temperature.

Figure 3.2: Transfer of aluminum strips with frozen shoot tips from liquid nitrogen to unloading solution.

3.4.7 *Post culture*

After immersion in ULS, the thawed cryopreserved shoot tips were cultured on MS medium supplemented with 0,75 M sucrose for 3 days under indirect light (18 µmol m^{-2} s^{-1}) and after that transferred to basic medium (as explained in 2.1) for recovery.

The shoot tips stayed under indirect light (18 µmol m^{-2} s^{-1}) at 20 °C for 3 weeks, and thereafter placed under 50 ±5 m^{-2} s^{-1} at 20 °C. Six weeks following cryopreservation, the shoot tips were transferred to new basic medium. The tissue culture established from the virus infected cryopreserved shoot tips was usually moved to new medium every 3 weeks, due to slow growth. However, for one period of eight weeks the tissue culture remained on the same medium due to absence of the master student because of illness.

3.4.8 *Survival and regeneration percentage*

After cryopreservation, the plants were examined every week for survival and regeneration, and the final results were noted after 6 weeks. Survived shoot tips were defined as shoot tips that had visible cell growth, either callus or shoot formation (Figure 3.3) after 6 weeks. The survival percentage was calculated using the following formula:

$$Survival\ \% = \frac{Number\ of\ survived\ shoot\ tips}{Number\ of\ cryopreserved\ shoot\ tips} \times 100$$

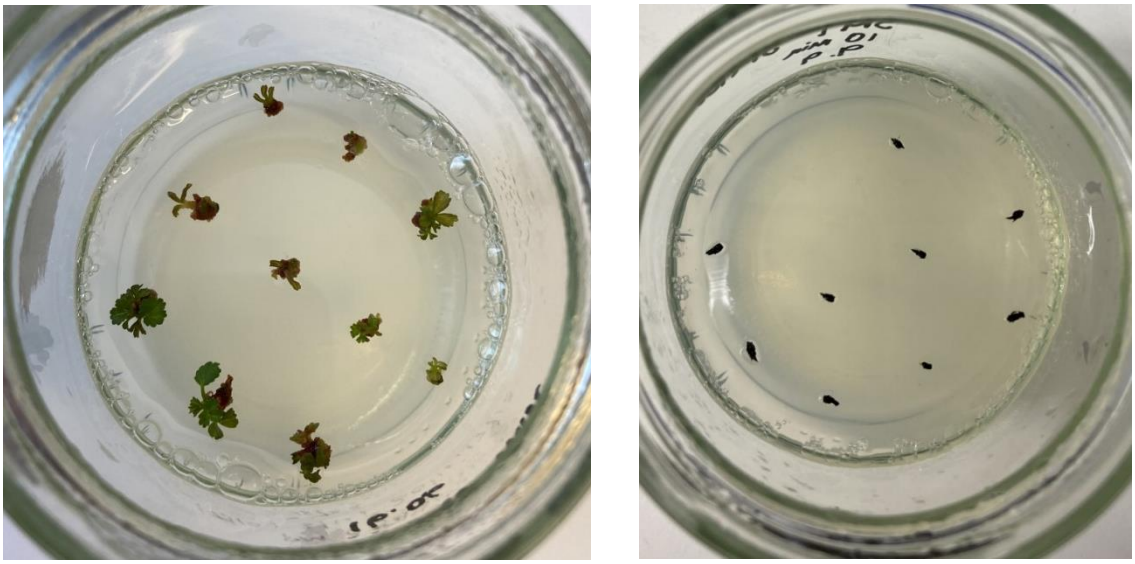

Figure 3.3: Indication of survived shoot tips (left) and not survived shoot tips (right), registered 6 weeks after cryotreatment.

Regeneration was defined as number of shoot tips that survived and that also developed visible shoots above 3 mm after 6 weeks.

Mean numbers of regenerated shoot tips were calculated using the following formula:

$$Mean = \frac{Regenerated\ shoot\ tips\ (repetition\ 1 + repetiton\ 2 + repetition\ 3)}{Number\ of\ repetitions\ (3)}$$

The mean regeneration percentage was calculated using the following formula:

$$Mean\ regeneration\ percentage\ = \frac{Mean\ number\ of\ regenerated\ shoot\ tips}{Number\ of\ shoot\ tips\ in\ each\ repetition\ (10)} \times 100$$

Standard deviation (SD) was calculated using the following formula, where $\bar{x}$ is the sample mean average, x is the i^{th} point in the sample and n is the sample size:

$$SD = \sqrt{\frac{\Sigma(x-\bar{x})^2}{n-1}}$$

Standard error (SE) was calculated using following formula, where n is the sample size:

$$SE = \frac{SD}{\sqrt{n}}$$

Mean numbers, standard deviation and standard error were calculated in Microsoft Excel.

The cryopreserved shoot tips of cv. Korona, that had both shoot induction and callus formation, were cleaned from their callus, and only the shoots were put on new medium. This was conducted after approximately 15-20 weeks.

3.5 Virus detection using RT-qPCR

To make sure the in vitro cultures were still virus infected after establishment of tissue culture, RT-qPCR was conducted to test for SMYEV and SVBV. RNA was extracted from seven cultures originating from shoot-tips from the SMYEV infected explant, and from three cultures of originating from the SVBV infected explant.

The cryopreserved virus infected shoot tips were tested again after cryopreservation using RT-qPCR to investigate if the virus survived the cryopreservation.

3.5.1 *Collection of material for testing*

Material for testing with RT-PCR was collected from plants in tissue culture. Primarily leaf material, but also some stems and roots were excised from the plantlets and packed in aluminum foil. The packages were numbered and put in the freezer (-20°C) and stored until RNA extraction.

Following cryopreservation, the regenerates of the SVBV infected plants were small and had a very dense morphology. Due to this, it was hard to excise primarily leaf material and the whole plantlet was used in the sample (Figure 3.5).

Figure 3.4: Regenerated plants from SMYEV infected cryopreserved shoot tip packed in an aluminum foil and stored for RNA extraction.

Figure 3.5: Regenerated plants from SVBV infected cryopreserved shoot tip, packed in an aluminum foil and stored for RNA extraction.

3.5.2 *RNA extraction*

Plant material from leaf and stem was wrapped in aluminum foil and kept in the freezer (-20°C) until RNA extraction. The plant material was grinded to fine powder using a mortar and pestle. To prevent RNA degradation, the plant material was made sure to always be frozen using liquid nitrogen. The material used was cooled with LN prior to use.

Figure 3.6: Plant material grinded to powder for use in RNA extraction.

Approximately 100 mg of the tissue powder was put in a pre-chilled 2 ml microcentrifuge tube (Eppendorf) and kept in LN. The remaining powder was kept in a backup tube. Extraction of the RNA was mainly done following the protocol of Spectrum Plant Total RNA Kit (Sigma-Aldrich), but for the last 40 samples, Norgen plant/fungi total RNA kit (Norgen biotek corp.) was used (due to shortage of elements in the Sigma- Aldrich kit).

Following RNA extraction, the concentration and quality of the RNA was determined by spectrophotometric analysis and analyzed through the program Nanodrop 2000 (Thermo Scientific). The program gives the RNA concentration in ng/µl. The purified RNA was stored at -20°C.

3.5.3 *cDNA synthesis (reverse transcription)*

The cDNA synthesis was performed using the Invitrogen **SuperScript IV First-Strand cDNA synthesis Reaction** (Thermo Fisher Scientific). The procedure was carried out according to the manufacturer's instructions, with some modifications. The first step was annealing primer to template RNA: 1µl random hexamer (50µM), 1 µL dNTP mix (10mM) was mixed with 1000-2000 ng of the template as well as nuclease free water. The manufacturer's instructions call for up to 5000 ng of RNA template, but due to strawberry tissue being renitent to extraction of pure and high-quality RNA (Chang et al. 2007, Hazman

et al. 2020), a lower amount was chosen. The solution was briefly centrifuged, thereafter heated to 65°C for 5 min and then cooled on ice for 1 min.

The second step was preparing the reverse transcription reaction mix: 4µL 5xSSIV buffer, 1µL DTT (100mM), 1µ SuperScript IV reverse transcriptase (200 U/µL) and 1µL nuclease free water was mixed, centrifuged. 7µL of the mix was added to each of the samples. The incubation reaction was done using the Bio-Rad T100 Thermal cycler with following protocol:

1. 23°C for 10 minutes
2. 55 °C for 10 minutes
3. 80°C for 10 minutes
3. 12°C for ∞

3.5.4 *qPCR*

StrawVir 1kit was used in the qPCR set-up. The result from this kit is unpublished, and the primers are confidential. The PCR was conducted in Bio Rad C1000 touch Thermal Cycler and converted to a real time system by Bio Rad CFX96 Real-time system. The internal control (ITC) used in the setup of this experiment was a mitochondrial NAD transcript (NAD5). The dye labeling used for the SMYEV isolates and internal positive control (IPC) were the FAM channel, whereas the ROX channel were used for the SVBV isolates. Sequence-specific synthesized DNA fragments were used as positive controls for both SMYEV and SVBV and included in the qPCR set up. The threshold level was set to 107, to avoid noise. Virus was regarded present in the sample when the Ct value was under 40.

Protocol used for PCR profile:

1: 94,0°C for 5:00
2: 94,0°C for 0:20
3: 58,0°C for 0:20
Plate Read
4: 72,0°C for 0:20
5: GOTO 2, 39 more times

3.6 Relative quantification calculations

Relative quantification analyzes the results from real-time PCR by relating the PCR signal of the target transcript in a treatment group to another sample such as an untreated control (Livak and Schmittgen 2001). In these calculations the untreated control is the Ct values of the first qPCR of the samples prior to cryopreservation. Internal control gene (NAD) was used to normalize the PCR for the amount of RNA added to the reaction. The $2^{-\Delta\Delta Ct}$ value is the fold change for the gene of interest, here the viral cDNA, in the test conditions, relative to the control condition, which has been normalized to the internal control gene (NAD).

ΔCt was calculated using following formula, where the gene of interest indicates SMYEV or SVBV:

$$\Delta Ct = Ct\ (gene\ of\ interest) - Ct\ (Internal\ control\ gene\ (NAD))$$

ΔΔCt was calculated using following formula, where the control group indicates the untreated samples diagnosed prior to cryopreservation:

$$\Delta\Delta Ct = Sample\ \Delta Ct - \ avrage\ \Delta Ct\ for\ the\ control\ group$$

$2^{-\Delta\Delta Ct}$ was calculated using following formula, where the value 2 is 1 plus a PCR efficiency of 1 (100%):

$$2^{-\Delta\Delta Ct}$$

Further, average $2^{-\Delta\Delta Ct}$ was calculated for each treatment.

3.7 Statistics

The graphs and calculations were made using Microsoft Excel (version 2211). Statistical analysis was conducted in Jamovi (The jamovi project (2021). *jamovi*. (Version 1.6) [Computer Software]. Retrieved from https://www.jamovi.org.

The significance level was set to 0.05 (if nothing else is noted), with a H_0 hypothesis of equal medians between treatments. Thus, if the P value is lower than 0.05 the H_0 hypothesis is rejected and the differences between medians are assumed statistically significant. The alternative hypothesis, H_1 is that the population medians are not equal. A small p-value, ($p<0.05$) means that there is stronger evidence in favor for the alternative hypothesis.

Table 3.3: Statistical test applied for specific applications source:(Sullivan et al. 2016)

Outcome variable	**Number of experimental groups**	**Group structure**	**Assumptions**	**Assumptions met (parametric)**	**Assumptions not met**
Continuous	2	Independent	Normality + homogeneity of variances	Unpaired t-test	Non-parametric (Mann-Whitney U)
Continuous	>2	Independent	Normality	ANOVA (Fisher)	Non-parametric (Kruskal-Wallis)
			Homogeneity of variances		ANOVA (Welch)
Binary/categorial	2	Independent	Independence of observations, expected count >5	Chi-square test	Fisher`s exact test

4 Results

The development of the regenerants from virus infected strawberry shoots went slower than expected. The waiting period following cryopreservation as the plants grew large enough to collect materials for RNA extraction was around 17-20 weeks, somewhat longer time than anticipated. This delay led to the demise of Experiment 3 and 4 (Figure 3.1). Several tissue cultures of regenerates of cryopreserved cv. Korona were lost due to contamination of fungi. This happened after 13-16 weeks following cryopreservation and lead to the dismissal of Experiment 3.

4.1 Tissue culture of SMYEV and SVBV infected plants

In vitro cultures of SMYEV and SVBV infected shoot tips were successfully established 8-10 weeks after the shoot tips were excised from the explants and cultured on the basic culture medium. After 8-10 weeks the shoot tips had developed into clusters of new shoots and were able to be divided into three new plants. The SMYEV- infected plants seemed to grow better and faster than SVBV- infected plants in tissue culture, however the differences were not significant, and could be due to cultivar differences.

Strawberry plants infected with SCV were originally intended to be a part of this thesis, however these plants did not establish well in tissue culture and propagated very slowly and ended up being excluded from further experiments.

4.2 Initial experiment to determine size of shoot tips for cryopreservation

In the practice period in the beginning of the experiment, the shoot tips were cut to either 2 mm with 1-2 leaf primordia (Figure 4.1 A), or 3-4 mm with 3-4 leaf primordia (Figure 4.1 B). Results from the initial experiment presumed higher survival rate in the bigger shoot tips (100%) than the smaller (52%) following cryopreservation (Table 4.1).

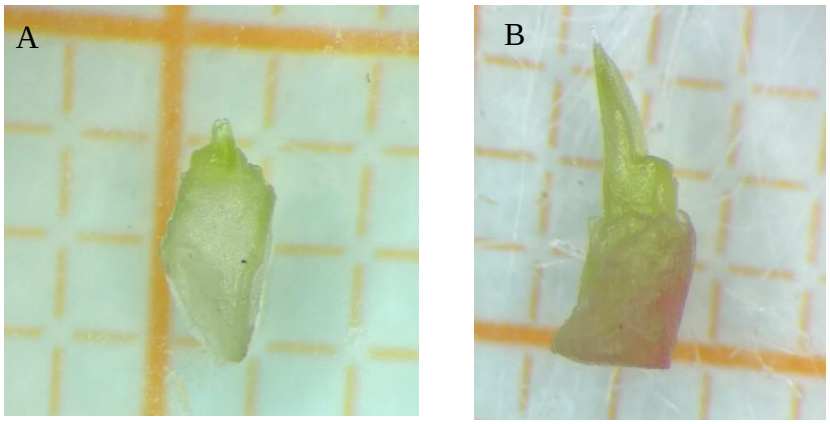

Figure 4.1: Sizes of shoot tips excised in the initial experiment.
A: 2 mm shoot tip. B: 3-4 mm shoot tip

Table 4.1: Effect of shoot tips size on survival following cryopreservation

Size of shoot tip	Number of survived shoot tips/ number of shoot tips cryopreserved	Survival percentage
2mm (1-2 leaf primordia)	31/60	51.7%
3-4 mm (3-4 leaf primordia)	31/31	100%

The results from the practice period were only used as an indicator for how the shoot tips should be excised further in the experiment. In Experiment 1 and Experiment 2 the shoot tips used for cryopreservation were 3-4 mm with 3-4 leaf primordia (Figure 4.1 B).

4.3 Experiment 1: Cryopreservation of virus-free strawberry cv. Korona

4.3.1 *Survival and regeneration of cryopreserved shoot tips following cryopreservation*

The cryopreserved shoot tips excised from virus free tissue culture showed high survival rate (Table 4.2). All the shoot tips cryopreserved in Experiment 1 survived, except form two; one shoot tip exposed to 20 minutes of PVS2 and one exposed to 50 minutes of PVS2.

Even though 178 of the180 shoot tips cryopreserved had cells that survived, not all developed shoots. All off the 178 shoot tips produced callus, whereas only 123 produced new shoots. It was difficult to distinguish if the new shoots originated from the meristem or from callus, due to all the survived shoot tips produced callus quite fast after post-culturing.

Table 4.2: survival of cryopreserved strawberry shoot tips cv. Korona. The shoot tips were exposed to either 10,20,30,40,50 or 60 minutes of PVS2. 30 shoot tips per PVS2 treatment were cryopreserved.

	PVS2 treatment (min)					
	10	20	30	40	50	60
Survived shoot tips	30/30	29/30	30/30	30/30	29/30	30/30
Survival rate	100%	97%	100%	100%	97%	100%

Due to the fact that several of the shoot tips that survived only produced callus, and therefore did not develop into a new plant, the number of survived shoot tips that also produced new shoots was recorded (see regenerated shoot tips, 3.4.8: Survival and regeneration percentage). The mean regeneration percentages for the treatments are presented in Figure 4.2.

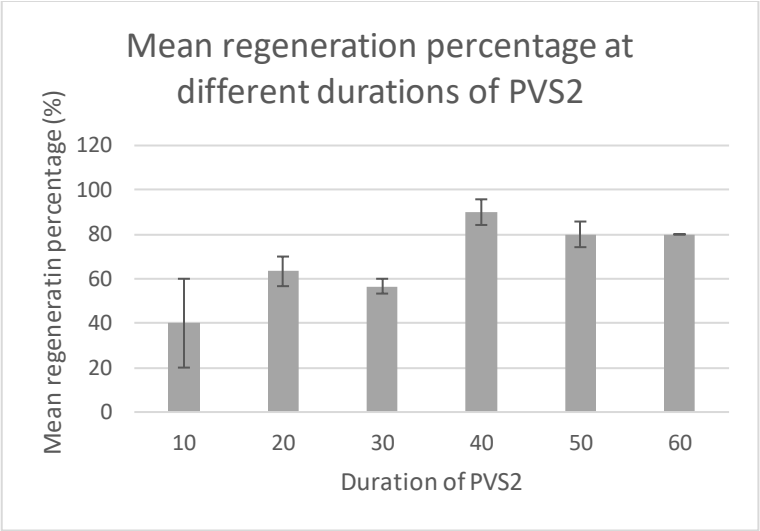

Figure 4.2: Mean regeneration percentage of cryopreserved shoot tips (strawberry cv. Korona) at treatment of different times of PVS2, with standard error bars.

PVS2 treatment of 40-60 minutes seemed to yield higher regeneration percentages than treatments of 10-30 minutes. Non-parametric ANOVA indicates differences (p=0.017), however, the DSCF pairwise comparison could not determine specific differences between treatment means. Tukey`s post hoc test, on the other hand, finds statistically significant differences between 10- and 40-minutes duration groups (p=0.024). There were no differences between replications within each treatment (p=0.42).

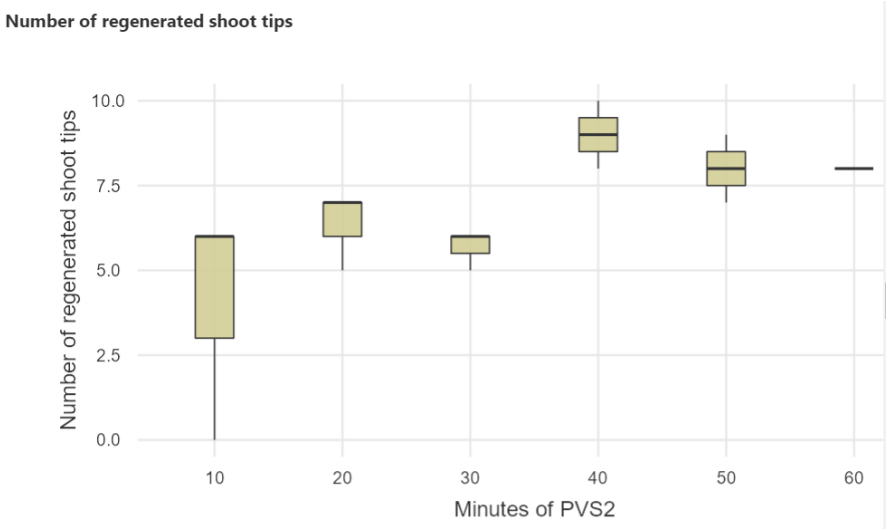

Figure 4.3: Box plot showing distribution of number regenerated cv. Korona shoot tips following cryopreservation, at different durations of PVS2. The thick line in the middle of the box is the median, whereas the whiskers go out to the extreme data points.

Figure 4.3 shows a great variation between the datapoints from the group within the 10 minutes treatment of PVS2, whereas the datapoints in the 60 minutes group are the most even.

4.3.2 *Morphology of regenerated plants following cryopreservation*

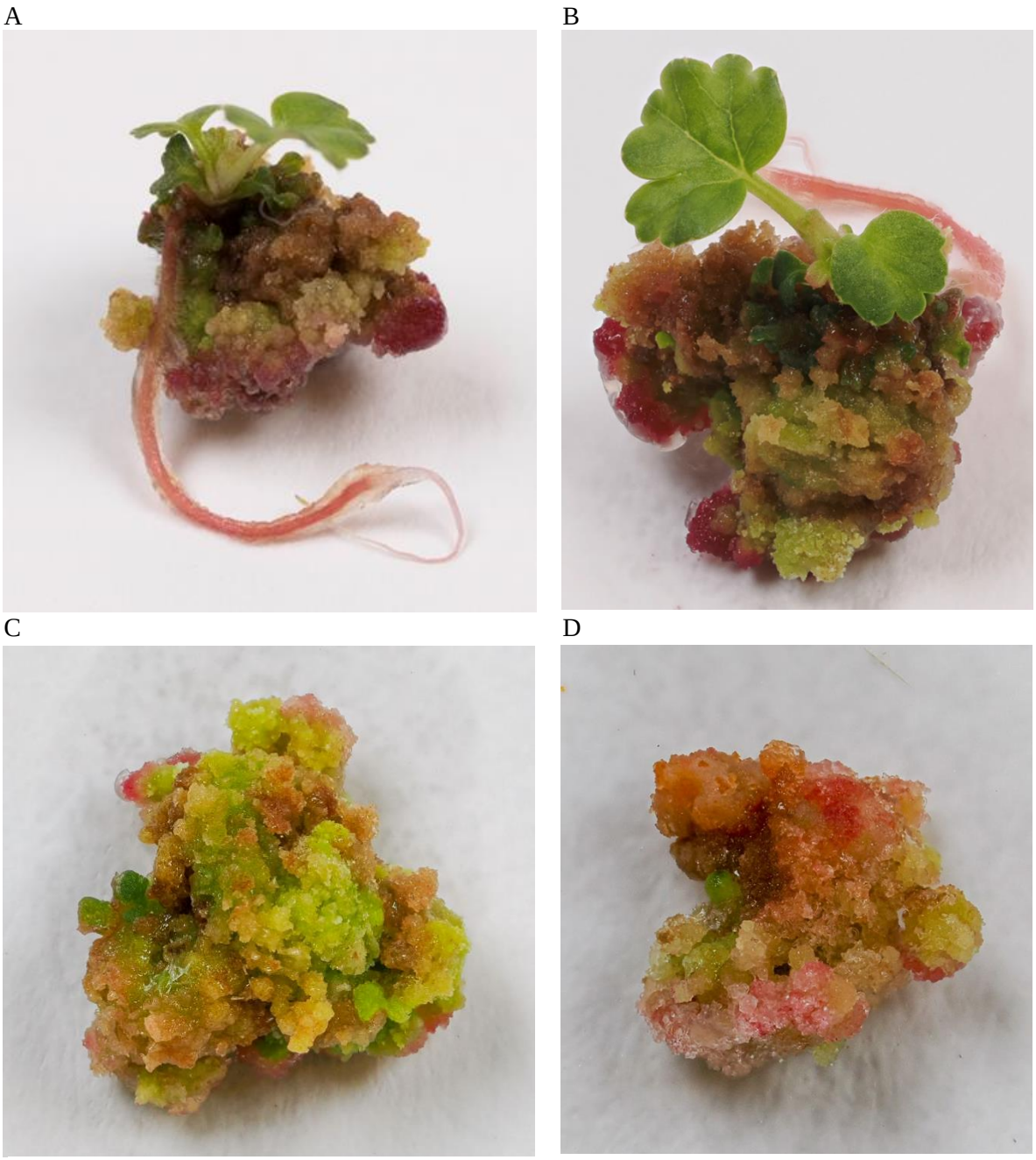

Figure 4.4: Different morphologies as callus, shoot and root formation originating from cryopreserved shoot tip of virus free strawberry culture. The pictures are taken 11 weeks after cryopreservation. A and B shows callus, root and shoot formation, whereas C and D shows only callus formation

Cryopreserved shoot tips of virus-free strawberry cv. Korona has shown different morphologies 11 week after cryopreservation treatment (Figure 4.4). Out of 180 cryopreserved shoot tips, 123 shoot tips produced callus, with shoot and root formation

appearing along with the callus (Figure 4.4 A and B), whereas 57 shoot tips produced only callus after cryopreservation treatment (Figure 4.4 C and D).

4.4 Experiment 2: Cryopreservation of plant viruses: SMYEV and SVBV

4.4.1 *Detection of virus infected tissue cultures prior to cryopreservation with RT-qPCR*

Analysis of the RNA extract from the samples showed a 260/230 absorbance ratio varying from 1.60 to 2, whereas the optimal value would be around or above 2. The slightly low 260/230 values did, however, not seem to affect the PCR results.

Table 4.3: Results of the RT-qPCR of the samples prior to cryopreservation. Sample 1-7 is RNA extract from regenerants before cryopreservation of cryopreserved SMYEV infected shoot tips. NAD is the internal control.

	Ct value	
Sample	**NAD**	**SMYEV**
1	20.51	17.14
2	21.39	17.28
3	20.68	17.75
4	20.21	17.02
5	19.96	16.32
6	20.17	17.68
7	19.62	17.23
Positive control SMYEV	N/A	32.73
Negative control	N/A	N/A

Table 4.4: Results of the RT-qPCR of the samples prior to cryopreservation. Sample 1-3 is RNA extract from plant material from regenerants after cryopreserved SVBV infected shoot tips. NAD is the internal control.

	Ct value	
Sample	**NAD**	**SVBV**
1	21.33	17.64
2	19.96	16.01
3	21.10	16.65
Positive control SVBV	N/A	32.43
Negative control	N/A	N/A

The Ct value is the PCR cycle number at which the sample reaction curve intersects the threshold line (Loftis and Reeves 2012). This means that the lower the Ct value, the higher the amount of the initial target sequence. The Ct values for the SMYEV samples are between 16.32 and 17.75 (Table 4.3) and the Ct value for the SVBV samples are between 16.01 and 17.64 (Table 4.4). The SMYEV and SVBV positive controls had a somewhat higher Ct value of 32.43 and 32.73, respectively. The negative controls had a Ct value of zero, meaning no DNA was detected, which is satisfactory. The internal control showed presence of nad5

mitochondrial gene in all the plant samples. The positive controls for SMYEV and SVBV gave somewhat high Ct values, but still within the accepted range.

Results of the RT-qPCR of the assumed virus infected tissue culture plants prior to cryopreservation showed that all samples contained either SMYEV or SVBV (Table 4.3, Table 4.4), as expected.

4.4.2 *Experiment 2A: Cryopreservation of SMYEV-infected strawberry*

4.4.2.1 *Regeneration following cryopreservation*

In contrast to the cryopreserved shoot tips from experiment 1, all of the shoot tips that survived also regenerated in experiment 2A. Due to this, only regeneration and regeneration percentage are presented.

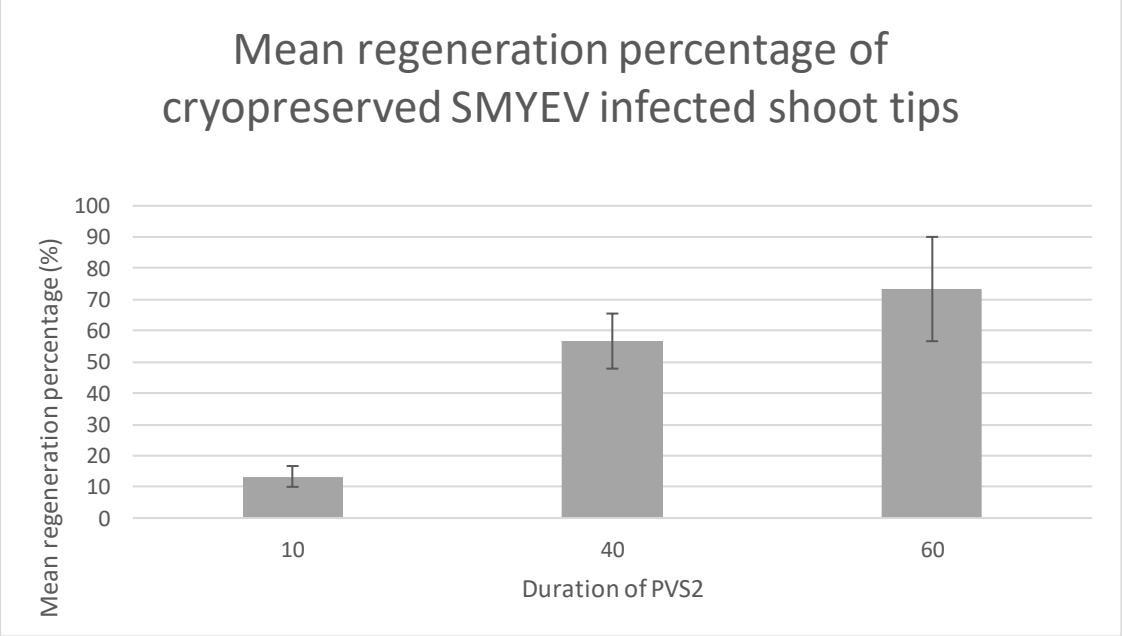

Figure 4.5: Mean regeneration percentage of cryopreserved SMYEV infected shoot tips at different treatment times of PVS2, with standard error bars.

After different treatment duration of PVS2, SMYEV-infected strawberry shoot tips showed that 40- and 60-minute duration times gave higher (57% and 73%) regeneration percentage than that of 10 minutes treatment (13%). Fisher`s ANOVA showed that there were statistically significant differences between treatments ($p=0.021$), and Tukey post-hoc test showed that 60 minutes treatment had significantly higher regeneration percentage than that of 10 minutes duration.

The mean regeneration percentage for 60 minutes seems to be higher than for 40 minutes, but the difference is not statistically significant (Tukey p=0.567). There was no significant difference between replications within each treatment (Fisher`s ANOVA p=0.537).

4.4.2.2 *Morphology of plants regenerated from cryopreserved SMYEV infected shoot tips*

Figure 4.6: Shoot formation of a cryopreserved SMYEV infected shoot tip 6 weeks following cryopreservation. Treated with 40 minutes PVS2.

Figure 4.6 shows a representative example of a regenerated plantlet of a cryopreserved SMYEV infected shoot tip. The plant shows no sign of callus formation.

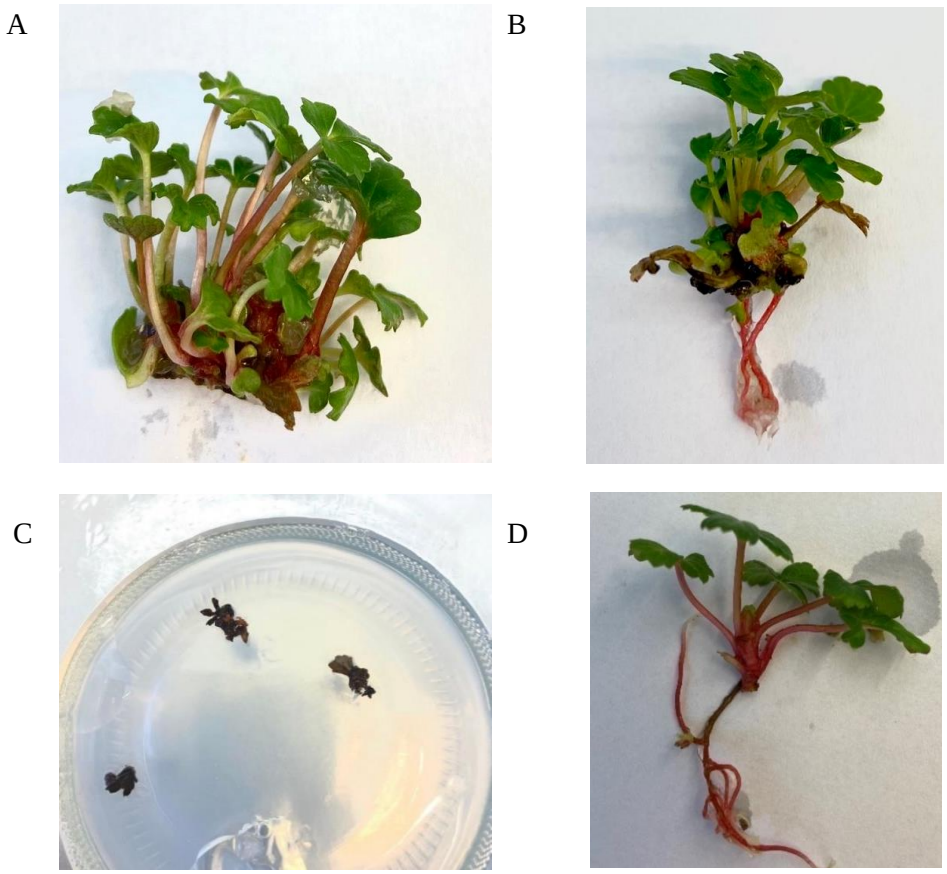

Figure 4.7: A, B and C shows Tissue culture of regenerants of cryopreserved SMYEV infected shot tips, 20 weeks following cryopreservation. A: The plant has several new shoots but no roots. B: the plantlet has both new shoot s and roots. C: Poorly developed and dead plants. The tissue culture had not been moved to new medium in eight weeks (see 3.4.7). D: Plantlet of SMYEV infected tissue culture prior to cryopreservation.

Figure 4.7 A, B and C shows the morphology of regenerants after cryopreserved SMYEV infected shoot tips, whereas D shows a plantlet from the tissue culture prior to cryopreservation. The morphology of regenerants after cryopreserves SMYEV infected strawberry shoot tips were not too different from the initial tissue culture. However, development of the regenerants was somewhat slower, and some regenerated plantlets stopped developing, and died as seen in Figure 4.7 C.

Out of the 90 cryopreserved SMYEV infected strawberry shoot tips, 43 survived and were considered regenerated after 6 weeks (shoots above 3 mm (paragraph 3.4.8: Survival and regeneration percentage)). 20 weeks following cryopreservation, 19 of these had developed enough to collect materials to perform RNA extraction. The remaining shoot tips that originally survived either developed too slowly or died after a period of time in tissue culture

(Figure 4.7 C), and due to this did not develop enough materials for RNA extraction. Five of the regenerated plantlets had developed roots after 20 weeks in tissue culture (Figure 4.7 B).

Table 4.5: Number of SMYEV infected shoot tips cryopreserved, number of shoot tips that survived cryopreservation, and number of samples that were tested with RT-qPCR.

Minutes PVS2	Cryopreserved	Regenerated	Number of plants for virus testing
10	30	4	0
40	30	17	10
60	30	22	9
SUM	90	43	19

Table 4.5 gives an overview over the number of samples that were tested with RT-qPCR in relation to how many shoot tips that were originally cryopreserved and how many of these that survived and regenerated.

4.4.2.3 *SMYEV survival in regenerated shoot tips*

Nineteen regenerated shoot tips from different PVS2 treatments were tested for SMYEV using RT-qPCR (Table 4.5)

Table 4.6: Number of cryopreserved SMYEV infected shoot tip regenerants analyzed, number of virus positive samples and percentage of virus presence within the samples. Relative RNA expression portrayed with Ct value. Ct data expressed as mean Ct value ± SE. Relative quantification expressed as fold change of SMYEV expression in relation to the control, portrayed with mean $2^{-\Delta\Delta Ct}$ value ± SE. Different letters (a and b) indicate a significant difference $P < 0.05$.

PVS2 treatment (min)	Samples analyzed	Virus presence	Virus presence (%)	Mean Ct value	Mean $2^{-\Delta\Delta Ct}$ value
10	0	-	-	-	-
40	10	9	90% *a*	18.76 ± 2.08	2.8 ± 0.52
60	9	3	33.33% *b*	26.21 ± 5.74 *	1.4 ± 1.15*

* *Calculated within the three virus positive samples*

Table 4.6 shows that 40 minutes of PVS2 treatment gave a statistically significant higher SMYEV survival than 60 minutes ($p=0.011$ X^2 test).

The virus titer was also higher in the shoot tips exposed to 40 minutes compared with those exposed to 60 minutes. The $2^{-\Delta\Delta Ct}$ values indicate higher gene expression in samples treated with 40 minutes than samples treated with 60 minutes. However, there was no statistically significant differences in $2^{-\Delta\Delta Ct}$ between treatments ($p=0.233$ Fisher one-way ANOVA) (Figure 4.8) as well as no statistical difference between Ct values ($p=0.125$).

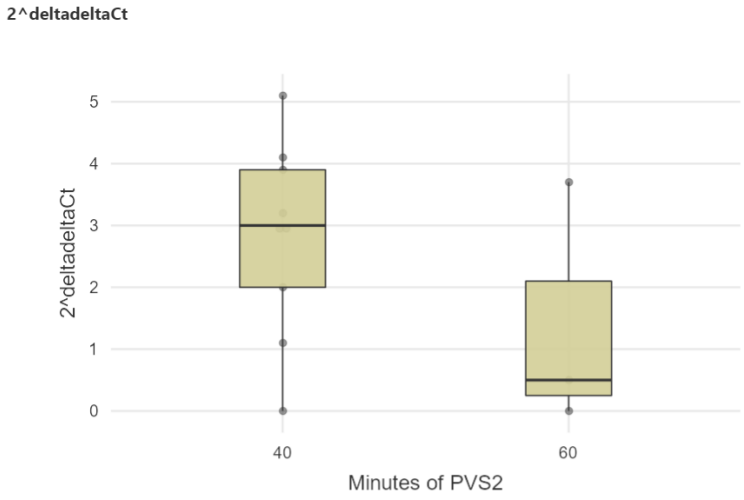

Figure 4.8: Box plot showing distribution of $2^{-\Delta\Delta Ct}$ values in the 40- and 60-minutes duration groups of PVS2.

There is a wide distribution of $2^{-\Delta\Delta Ct}$ values.

4.4.3 ***Experiment 2B*: Cryopreservation of SVBV-infected strawberry**

4.4.3.1 *Regenerated shoot tips following cryopreservation*

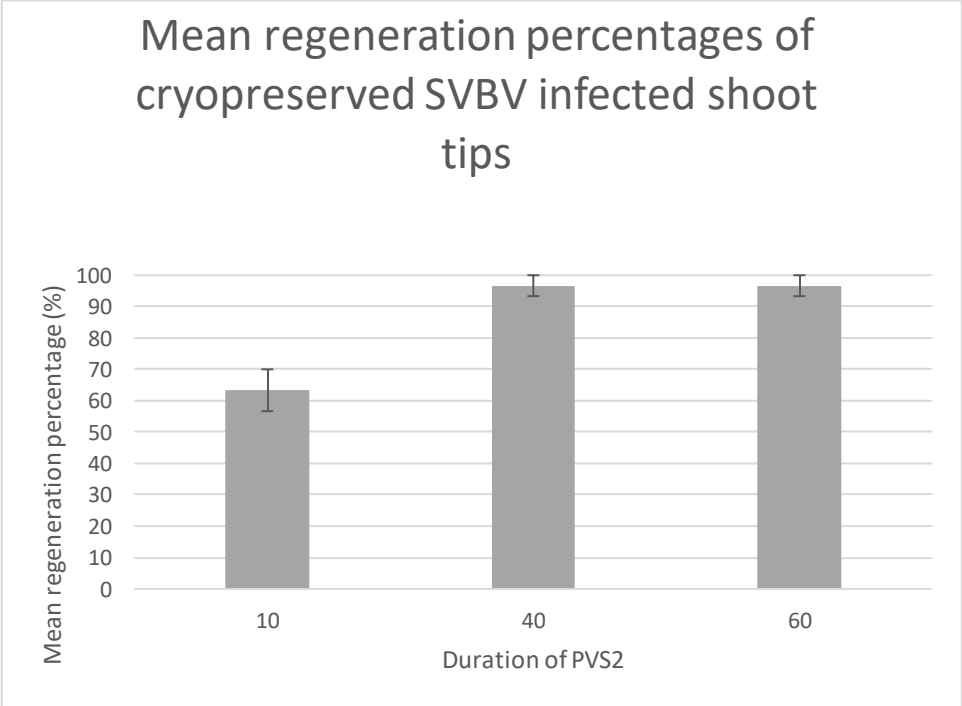

Figure 4.9: Mean regeneration percentage of cryopreserved SVBV infected shoot tips at different treatment times of PVS2. With standard error bars

Figure 4.9 shows that 40 and 60 minutes gave the best results for regeneration of cryopreserved SVBV infected shoot tips. 40 and 60 minutes of treatment were equally

effective. The mean regeneration percentage for 40 (96.7%) and 60 minutes (96.7%) is statistically greater (p=0.006 Tukey) than the mean regeneration percentage for 10 minutes (63.3%).

4.4.3.2 *Morphology of regenerants of cryopreserved shoot tips*

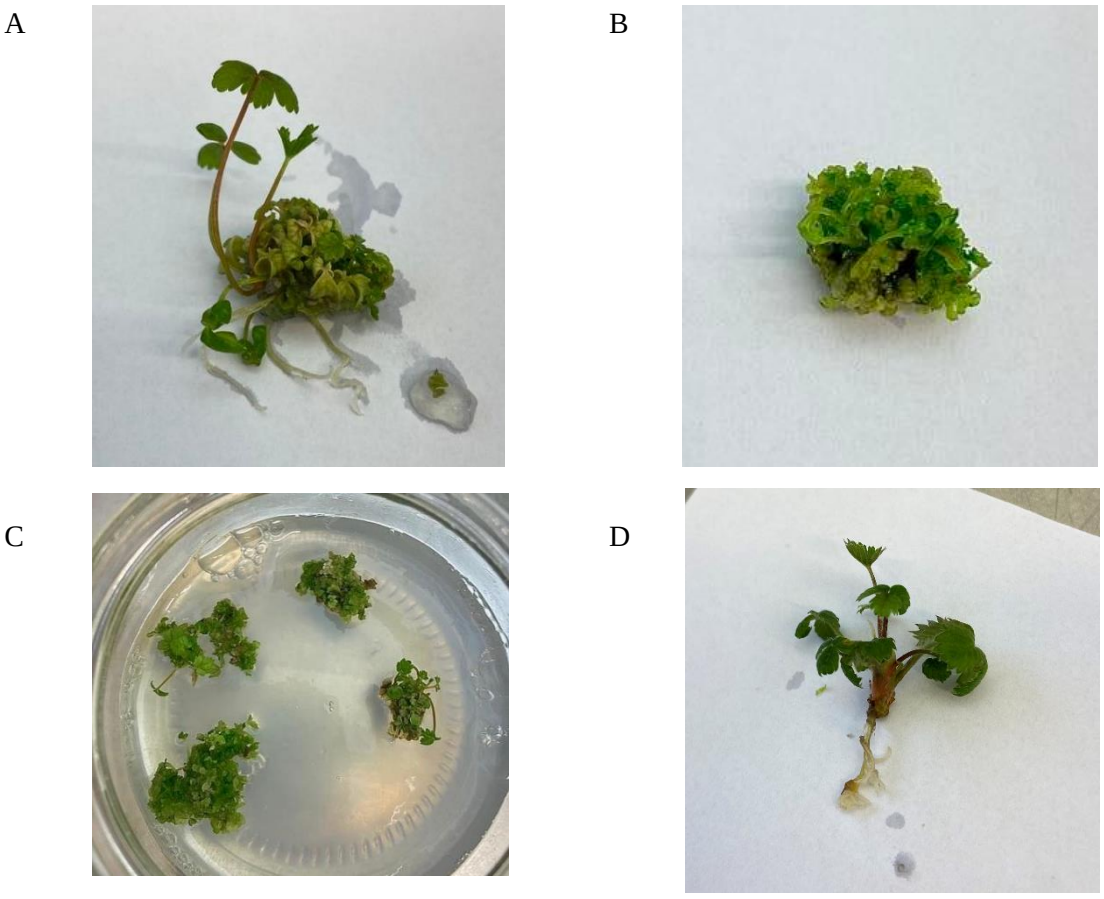

Figure 4.10: A, B, C: Tissue culture plant 17 weeks following cryopreservation. The plant is a regenerate from a SVBV infected shoot tip cryopreserved and treated with 40 minutes of PVS2. A: The plant has many small shoots, one big shoot and roots. B: The plant has many very small new shoots, but no roots. C: Four regenerated plants on medium. D: Plantlet of SVBV infected tissue culture prior to cryopreservation.

Figure 4.10 A, B and C shows the morphology of regenerants after cryopreserved SVBV infected shoot tips, whereas D shows a plantlet from the tissue culture prior to cryopreservation.

77 out of 90 SVBV positive cryopreserved shoot tips survived. After 17 weeks, 57 of these were developed enough to collect material for RNA extraction. The cryopreserved SVBV positive shoot tips that survived developed into small plants with a dense morphology and with a high number of small shoots. 3 of 77 plants had developed roots 17 weeks following

cryopreservation (Figure 4.10 A), whereas the rest only developed a large number of small shoots and no roots (Figure 4.10 B). Two of the plants that had developed roots were regenerates of shoot tips submerged in PVS2 for 60 minutes and one of the plants was regenerated from shoot tip submerged in PVS2 for 40 minutes.

Table 4.7: Number of SVBV infected shoot tips cryopreserved, number of shoot tips that survived cryopreservation, and number of samples that were tested with RT-qPCR.

Minutes of PVS2	Cryopreserved	Regenerated	Number of plants for virus testing
10	30	19	16
40	30	29	22
60	30	29	19
SUM	90	77	57

Table 4.7 gives an overview over the number of samples that were tested with RT-qPCR in relation to how many shoot tips that were originally cryopreserved and regenerated.

4.4.3.3 *SVBV survival in regenerated plants following cryopreservation*

Table 4.8: Number of cryopreserved SVBV infected shoot tip regenerants analyzed, number of virus positive samples and percentage of virus presence within the samples. Relative RNA expression portrayed with Ct value. Ct data expressed as mean Ct value ± SE. Relative quantification expressed as fold change of SMYEV expression in relation to the control, portrayed with mean $2^{-\Delta\Delta Ct}$ value ± SE

PVS2 treatment (min)	Samples analyzed	Virus presence	Virus presence (%)	Mean Ct virus	Mean $2^{-\Delta\Delta Ct}$
10	16	16	100%	23.50±1.31	0.8 ± 0.42
40	22	22	100%	22.62±1.12	1.4 ± 0.47
60	19	19	100%	22.11±1.37	2.1 ± 0.70

SVBV virions were preserved in all shoot tips cryopreserved in all three treatments. The $2^{-\Delta\Delta Ct}$ values indicate some increase in upregulation of gene expression with increasing time exposure to PVS2. The differences in $2^{-\Delta\Delta Ct}$ between treatments, is however, not significant ($p=0.312$). There is also no statistical difference in Ct value between treatments.

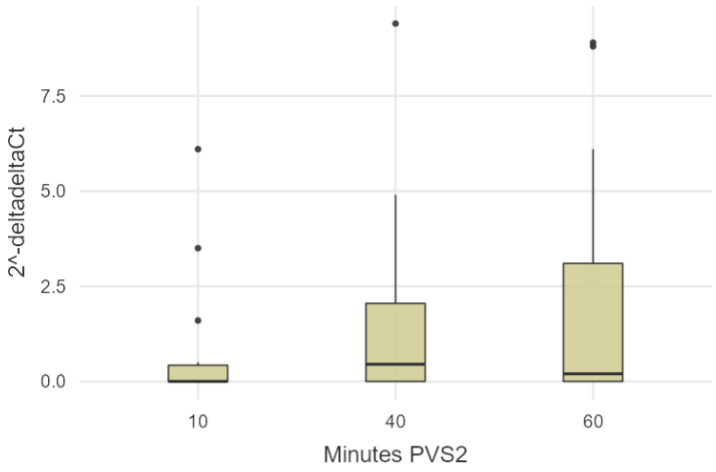

Figure 4.11: Box plot showing distribution of $2^{-\Delta\Delta Ct}$ values in the 10-, 40- and 60-minutes duration groups. Differences between treatments are not statistically different p=0.312.

Figure 4.11 shows a wide distribution of values of $2^{-\Delta\Delta Ct}$. The datapoints far from the center may create misleading results if only relying on the mean.

4.5 Comparison of regeneration percentage and virus preservation

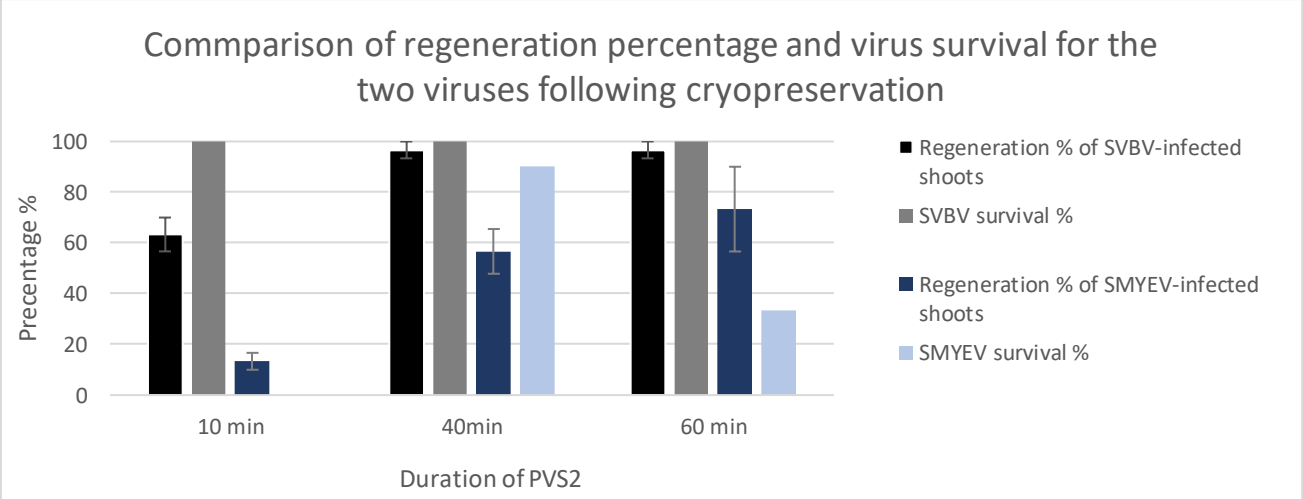

Figure 4.12: Comparison of regeneration percentage and virus survival for the two viruses following cryopreservation .

Figure 4.12 shows that cryopreserved SVBV infected shoot tips have both a higher regeneration percentage and virus survival percentage than cryopreserved SMYEV infected shoot tips. There does not seem to be a clear connection between regeneration percentage and virus survival.

5 Discussion

5.1 Tissue culture

Several of the parts of this project were delayed due to slower development of the tissue cultures than expected. This also led to experiments 3 and 4 being eliminated. The tissue culture took 8-10 weeks to establish and develop clusters of new shoots and were then divided into two to three new plants per cluster.

The conditions in the growth rooms used in this study were not necessarily adapted to the optimal conditions for strawberry propagation, due to several species of plant being cultivated in the same room. The temperature in the growth room used for the strawberry plants in this thesis was 20 ±2°C, whereas looking at other studies the temperature varies between 22°C (Gruchała et al. 2004) and 25°C (Bhatt and Dhar 2000, Nhut et al. 2003, Gruchała et al. 2004, Ashrafuzzaman et al. 2013, Dhukate et al. 2021, Sarıdaş et al. 2021). Temperature affects the properties of wide range of molecules and influences various physiological processes and metabolic functions in the plant (Willey 2018). The optimal temperature for a plant varies between different species and genotypes (Willey 2018). Increasing the temperature in the growth room used in this thesis might have accelerated the plants development.

The type of hormones and the concentration affects the shoot proliferation and root development of plants in tissue culture (Adak et al. 2016). The plant hormones cytokinin and auxin play a big role in plant regeneration in tissue culture, with both being important for cell division and meristem establishment, but different ratios between the two hormones may favor either shoot or root formation (Hill and Schaller 2013). Cytokinin plays an important role for shoot development and quality whereas auxin is widely used for the rooting stage (Hill and Schaller 2013).

Ashrafuzzaman et al. (2013) cultured runner segments of strawberry on MS medium enriched with 5 different BAP concentrations varying from 0 mg/L (control) to 2 mg/L. The concentration that gave the most shoots per culture and longest shoots was 0.5 mg/L, which is the same concentration used for the media in this thesis. The same results were also found by Sajjad et al. (1994) and Moradi et al. (2011). After five weeks, the cultures in the study from Ashrafuzzaman et al. (2013) were placed on a rooting medium containing IBA concentrations varying from 0 mg/L to 2 mg/L. The concentrations that gave the fastest root induction were 0.5 mg/L and 1 mg/L, which are higher than the concentration used in this thesis (0.2 mg/L). Moradi et al. (2011) however, found that the best IBA concentration was 0.2 mg/L. Bhatt and

Dhar (2000) found that better rooting was obtain with NAA (α-naphthalene acetic acid) than with IBA.

Sarıdaş et al. (2021) investigated the effect of varying concentrations of the cytokinin BAP and Thidiazuron (TDZ), and found that the media containing TDZ were more effective when it came to shoot proliferation than medium with just BAP. The results from this study also showed that the optimal growth medium varies between different cultivars of *Fragaria*. Gruchała et al. (2004) found that a medium with 0.4 mg/L IBA and 1.8 mg/L BAP gave the best results, but that the hormonal balance and ratio between auxin and cytokinin is a key factor in a successful growth medium. Adding gibberellin (GA_3) to the medium has also showed to be effective for shoot development (Biswas et al. 2007).

Looking into the literature regarding the effect of different media composition on strawberry growth, it seems that the best media and hormone composition vary between studies and different cultivars. Different medium composition could perhaps have affected the growth rate, propagation efficiency and shoot and root production for the tissue culture in this thesis. Other environmental factors such as light may have affected the growth rate. The growth rate of the virus infected tissue culture might also have been affected by the virus infection.

5.2 Cryopreservation of *Fragaria ssp*.

Strawberry shoot tips have been successfully cryopreserved using a variety of different approaches including vitrification (Niino et al. 2003), encapsulation vitrification (Hirai et al. 1998), droplet vitrification (Pinker et al. 2009, Bae et al. 2021, Chen et al. 2021) and cryo plate vitrification (Yamamoto et al. 2012).

Cold acclimatation is often applied to the strawberry tissue cultures prior to cryopreservation, but was not conducted in the protocol used in this thesis work. Hirai et al. (1998) observed an increase in shoot formation following cryopreservation in cold hardened explants (80-88%) in relation to non-hardened shoot tips (62%). Niino et al. (2003) cold hardened strawberry shoot tips at 5°C for 0 to 40 days prior to cryopreservation, and the results showed that this treatment significantly improved the survival rate. More than 10 days of cold hardening was necessary to obtain high survival rates.

Some cryopreservation approaches keeps the post culture under lower light intensity (Pinker et al. 2009) (5µmol $m^{-2}s^{-1}$) or in dark conditions (Chen et al. 2021), whereas in this thesis the post cultures were placed under 18 µmol $m^{-2}s^{-1}$. Adding active charcoal (Pinker et al. 2009) or polyvinylpyrrolidone (PVP) (Niino et al. 2003, Chen et al. 2021) in recovery medium has

shown to increase the survival rate in some studies. These substances may reduce problems with oxidation of shoot tips and phenolic exudation thus decreasing toxic metabolites (Thomas 2008).

Pinker et al. (2009) found that the sucrose concentration in the preculture strongly affected the percentage of regrowing shoot tips for the strawberry cv. Korona. The results of this study showed that 0.25M sucrose was the most effective sucrose concentration (66%), whereas 0.1 and 0.5 M gave lower regrowing shoot tips (16.6%) and 0.75 and 1 M gave the lowest (3.3%). The sucrose concentration used in the preculture medium in thesis exceeded the most effective sucrose concentration, from the study conducted by Pinker et al. (2009), for the cv. Korona, which might have affected the regeneration percentage and perhaps also the observed callus formation for this variety.

Many of the procedures in former studies on cryopreservation of *Fragaria ssp*., differ from the procedure used in this thesis when it comes to the use of cold acclimation, preculture methods and recovery medium, in addition to PVS2 treatment. The content and sucrose concentrations vary in the preculture medium, ULS and recovery medium between the procedures, as well as temperature of the solutions. The duration times of preculture, immergence in LN and ULS and post culture days also differ.

Looking at the literature on different cryopreservation procedures developed for strawberry, the time of exposure to PVS2 (at room temperature) varies some but seems to be within close proximity of the times found to be most successful in this thesis (40-60 min), with the exception of Pinker et al. (2009) that uses somewhat shorter time (20 min) than other droplet vitrification procedures. Niino et al. (2003) found that the highest survival percentage (70%) of cryopreserved strawberry shoots was obtained using treatment with 50 minutes of PVS2 (25°C) using the vitrification method. The exposure time also depends on the temperature of the PVS2 solution, with lower temperature increasing the time needed for exposure. Lower temperatures reduce the toxicity, thus extending the incubation time (Matsumoto et al. 1995). The optimal exposure time to PVS2 varies between plant species and also depends on the temperature of the solution (Wang et al. 2005).

With the virus infection aspect set aside, the successfulness of the cryopreservation protocol used in this thesis seemed to differ between the three different *Fragaria* species/cultivars examined. The cultivar effect is also found in other studies; the survival rate for the vitrification procedure developed by Niino et al. (2003) varied between cultivars of *Fragaria*,

ranging from 50-85%. The protocol used in this thesis did not seem optimal for the cv. Korona, due to callus formation, and some adjustment should be made to the protocol for further enhancement of the cryopreservation procedure for this cultivar. The other two unknown *Fragaria* species/cultivars seemed to benefit more from the cryopreservation procedure used, due to no callus being developed following cryopreservation. The regeneration percentage was quite high for all the three *Fragaria* species/cultivars for what seemed to be the best PVS2 treatments (40-60 minutes), with regeneration percentages varying from 56% (the SMYEV infected *Fragaria ssp.* treated with 40 min PVS2) to 96% (the SVBV infected *Fragaria ssp*. treated with 40 or 60 min PVS2).

The regenerants after the cryopreserved SVBV infected *Fragaria ssp*. experienced the biggest difference in morphology in relation to their initial tissue culture (Figure 4.10). The regenerated plantlets were dense, with a large number of shoots, with mostly short shoots with little elongation. The leaves were glassy, thick, translucent and brittle, thus experiencing symptoms of hyperhydricity. Hyperhydricity (vitrification, glassines) is a common physiological disorder in plant tissue culture and can be considered an adaptive response to stress factors such as osmotic shock, wounding and high humidity (Polivanova and Bedarev 2022). PVS2 can cause major stress to the cells of the shoot tips (Wang et al. 2014) through chemical toxicity and excessive osmotic stress (Rall 1987). The stress associated with cryopreservation and perhaps the tissue culture conditions, might be the reason for the observed morphological changes.

The development of regenerants after cryopreserved virus infected shoot tips in Experiment 2 took longer time than expected. This affected the time scheme for the following experiments and resulted in the termination of Experiment 3 and Experiment 4. The time from cryopreservation to RNA extraction of material from regenerants was around 17-20 weeks. The prolonged developmental time was probably affected by a period with lack of subculturing and new medium (eight weeks). Looking at the literature on cryopreservation indicates that the optimal cryopreservation procedure can accelerate the regeneration time. Hirai et al. (1998) cryopreserved *Fragaria ssp*. meristems that produced shoots within a week following cryopreservation with no intermediate callus formation.

Many steps in the cryopreservation procedure, like preculturing on high sucrose medium, dehydration and freezing in LN, cause stress to the plant cells (Wang et al. 2014). PVS2 has been shown to cause major stress to cells of the shoot tip (Wang et al. 2014). The samples

needed some time to recover their growth ability following cryopreservation, probably due to the stress applied to the cells (Figure 4.10, Figure 4.7).

Following cryopreservation, strawberry cv. Korona shoot tips developed callus. Once the callus was removed from the shoots, the regenerants developed a similar morphology as the initial tissue culture, but with somewhat decreased developmental rate. There was no significant difference in callus formation between different durations of PVS2. Unfortunately, further investigation of the growth of Korona regenerants was ceased due to fungal infection in the tissue cultures.

Regeneration of new shoots from callus increases the chances for genetic instability and somaclonal variation (Chang and Reed 1998, Castillo et al. 2010), and is therefore undesirable. A successful cryopreservation procedure does not only ensure high survival of cells after thawing, but does also ensure fully viable plant structures such as shoot tips that are able to form shoots without callus formation (Martín et al. 2015). Having this in mind, the results from Experiment 1 regarding formation of callus, might indicate that the cryopreservation procedure used is not optimal.

Maintenance of organization within the meristem and presence of viable meristematic cells following cryopreservation is important to ensure that the new shoots regenerate from the meristem and not callus (Martín et al. 2015). Whether the shoot formation registered originated directly from survived meristem cells or from callus was hard to distinguish, which might have affected the integrity of the results. These results might also question the viability of the meristematic cells following cryopreservation.

A study on regrowth of *Rubus* following cryopreservation conducted by Chang and Reed (1998), found that callus proliferation rather than shoot formation was induced when IBA (auxin) was included in the recovery medium following cryopreservation. After exposure to LN, the *Rubus* shoot tips were partially damaged causing a stimulation of callus formation. When IBA was added to the recovery medium, additional callus formation was induced. Supporting findings were noticed in a study developing a method for cryopreserving of cassava, conducted by Escobar et al. (2000). They found that reducing auxin and increasing cytokinin in the recovery medium favored shoot formation. They also found that reduction of NAA (auxin) and increasing of GA_3 (Gibberellin) in the recovery medium diminished growth of callus and stimulated shoot elongation. These results indicate that optimal recovery medium could prevent callus formation and be beneficial for recovery of cryopreserved shoot

tips. Reduction of IBA and changing the ratio between auxin and cytokinin, alternatively adding gibberellin, in the recovery medium used in this thesis could perhaps have been beneficial for optimal recovery.

Vollmer et al. (2017) regards cryopreservation of potato to be successful when the recovery rate is over 30%, whereas Chen et al. (2021) and Jenderek and Reed (2017) considers cryopreservation of strawberry shoot tips successful when at least 40% of the tested shoot tips are viable. The literature sited above indicates that, in relation to recovery, the cryopreservation protocol used in this thesis was successful.

5.3 Virus preservation

Plant viruses does usually not infect the shoot apical meristem (SAM) of the host plant (Mochizuki and Ohki 2015), with a few exceptions (Foster et al. 2002). The meristem portion in the shoot tips is usually 0.2-0.7 mm, depending on different species and cultivars, and smaller portions of tissue is considered to have a lower chance of virus presence (Panattoni et al. 2013). Figure 5.1 shows a representative diagram of a shoot tip, portrayed as a *Nicotiana tabacum* shoot. The picture shows that the SAM is a small part of the shoot tip, and under the SAM is stem tissue and vascular tissue. The virus distribution and movement in the plant affects whether an excised shoot tip will be virus free or not (Panattoni et al. 2013).

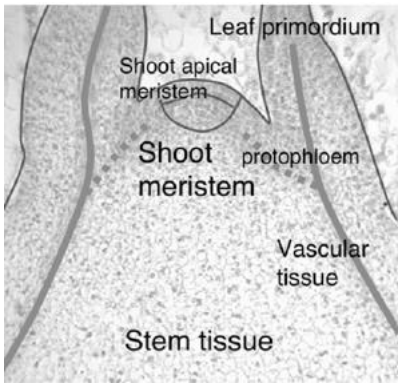

Figure 5.1: Longitudinal section of a shoot meristem of Nicotiana tabacum cv. Xanthi. Source: (Mochizuki and Ohki 2015)

The size of shoot tips used for cryopreservation might affect both survival rates and virus survival. Some of the existing studies on virus eradication through cryopreservation of shoot tips claims successful eradication despite shoot tip size (Zhao et al. 2019). Other studies, however, show that the virus free frequency was higher in smaller shoot tips than bigger shoot tips. Jeon et al. (2016) found higher eradication frequencies of Chrysanthemum stunt viroid (CSV) in cryopreserved shoot tips of chrysanthemum with 1 to 2 leaf primordia (LP), than shoot tips with 3 to 4 LP.

Li et al. (2018) successfully cryopreserved potato leafroll virus (PLRV), potato virus S (PVS) and potato spindle viroid (PSTVd) in potato shoot tips. They found that shoot tip size significantly affected regrowth levels in cryopreserved shoot tips, with the larger shoot tips (1.5 mm) producing significantly higher shoot regrowth levels than the smaller ones (0.5mm). This result matches the small initial experiment in this thesis (chapter 4.2). Shoot tip size also affected virus preservation efficiency of PLRV, with larger shoots resulting in 35% virus preservation whereas the smaller shoots produced no virus-preserved shoots. All PVS and PSTVd recovered shoots were virus-preserved, regardless of shoot size.

These studies indicate that the size of the shoot tip may influence the effect of virus eradication through cryopreservation and may therefore also influence the rate of virus preservation. According to this, one can suppose that the size of shoot tips excised in this thesis can have influenced the virus survival efficiency. Had the shoot tips been cut smaller, there might have been a different result. However, due to the fact that the viruses were able to be successfully cryopreserved, it can be assumed that the shoot tips size excised in this thesis were beneficial.

It is generally assumed that in shoot tip cryopreservation, only the cells located in the top of the apical dome (AD) and the youngest LPs survive following freezing in LN (Wang et al. 2009, Wang et al. 2014). The cells in the AD harbors less differentiated cells with high nucleocytoplasmic ratio and low levels of water (Wang et al. 2022). Li et al. (2018) found that the majority of cells in the AD and some in LP 1-3 in potato shoot tips survived cryopreservation, as well as some cells in LP 4 in 30% of the cryopreserved shoot tips.

Li et al. (2018) used histological virus localization to study the localization of PLRV in the shoot. PLRV was not detected in the AD or LP 1-3, but was found in LP 4 and older tissues, and reviews showed that this virus was phloem limited. PSTVd however is known to infect AD (Zhang et al. 2015). In this study, it is seen that differences in localization of the viruses in the plant effects the virus's ability to be successfully cryopreserved.

Little to no research is found on the localization of SMYEV and SVBV in the shoot tip. However, SMYEV and SVBV have been successfully eradicated from infected strawberry plants through meristem cutting (Miller and Belkengren 1963, Mullin et al. 1974, Quiroz et al. 2017). Mullin et al. (1974) found that meristem cutting (0.3-0.8 mm meristems including one LP) gave few SMYEV free plants, whereas meristem cutting in combination with heat treatment were more effective in virus eradication. Quiroz et al. (2017) found that 78%-100%

of plants regenerated from meristem cuttings of *F.chiloensis* were free of SMYEV, depending on different accessions. Miller and Belkengren (1963) found that SVBV could be eliminated from *Fragaria* through meristem culture of 0.5-1 mm shoot tips.

Due to the fact that SMYEV and SVBV can be eradicated through meristem cutting, one can suspect that these viruses do not infect the apical dome (AD) but are present in lower part of the shoot tip and/or LPs, but more research using techniques like immunohistochemical microscopy or in situ hybridization is needed to be able to conclude so. The shoot tips excised in this thesis were bigger than the size usually cut for virus eradication through meristem cutting.

Virus presence in the regenerants of cryopreserved shoot tips depends on the virus distribution within the shoot and the virus-infected cells ability to survive cryopreservation. In this thesis, the shoot tips are cut to include more cells than only the SAM. Hopefully this would include virus infected cells, assuming that the investigated viruses infect the cells in the older LPs and the basal part of the shoot tip. In this way, whether the virus survives the cryo-procedure will greatly depend on the ability of cells that located in the LPs or basal shoot tip area to survive cryopreservation.

A strategy, suggested by Bettoni et al. (2019), to improve virus eradication through cryopreservation was to use shorter or longer PVS2 duration time than what is optimal for survival rate for the shoot tips, which would lead to fewer cells surviving the procedure. This is supported by a study on eradication of *Potato Virus S, Potato Virus A* and *Potato Virus M* from potato using cryotherapy (Bettoni et al. 2022), where the results showed that survival of shoot tips decreased as duration of PVS2 treatment increased. A PVS2 duration over 135 min resulted in the lowest shoot tips survival and regrowth but was also the only effective PVS2 duration for virus eradication. In regard to this, it would be assumed that the optimal PVS2 duration time for survival rate would also be the best time for virus preservation. This, however, was not observed in this thesis. Figure 4.12 shows no clear connection between regeneration percentage and virus survival percentage across the two viruses.

The results from this thesis showed that 40- and 60-minute duration gave higher regeneration rate than 10-minute duration of PVS2. This indicates that higher PVS2 duration is needed for maintaining the viability of the meristem cells. SVBV was present in all the cryopreserved samples in the three PVS2 durations, whereas longer time (60 min) of exposure to PVS2 gave lower survival of SMYEV in relation to shorter time (40 min). This might indicate that longer

PVS2 duration killed more of the cells in the lower part of the shoot tip and LPs in relation to shorter time.

The type of plant vitrification solution used might also affect the outcome of virus preservation and virus eradication through cryopreservation. Jeon et al. (2016) fount that cryopreservation with PVS2 was able to obtain 13 % CSVd-free chrysanthemum plants, whereas cryopreservation with PV3 resulted in no viroid free plants.

The Ct results of the RT-qPCR showed that virus titers were somewhat lower in the regenerants of cryopreserved shoots in relation to the non-cryopreserved tissue culture. These results align with the RT-qPCR results of virus titer in cryopreservation of ASGV (Wang et al. 2018) and of PLRV, PVS and PSTVd (Li et al. 2018), where the virus titers were lower in cryopreserved shoots than in non-cryopreserved shoots. In these studies, the virus titer gradually increased as subculture duration increased, and reached a level of those not cryopreserved after 16-21 weeks. Cryopreservation reduces the number of live cells in the shoot tip below the meristem, thus reduces the number of virus infected cells, hence a reduction in virus concentration in the regenerants is reasonable. In this thesis, the final RT-qPCR was conducted 17-20 weeks following cryopreservation, however, it would be interesting to take another RT-qPCR after a longer period of time to see if the virus titer stabilizes at the initial level of the non-cryopreserved plants.

According to Wang et al. (2018) the most important factors determining virus preservation efficiencies are; frequencies of shoot regeneration of cryopreserved shoots tips, virus concentration preserved in the shoots as well as proliferation of regenerants of cryopreserved virus-infected shoots. The results from this thesis show a satisfactory regeneration percentage in shoot tips cryopreserved with a PVS2 duration of 40-60 minutes, as well as an adequate virus concentration in the regenerants, which would indicate efficient virus preservation. The propagation efficiency of the virus-infected regenerants, however, are uncertain.

Cryopreservation seemed to be a viable way to preserve SVBV due to the presence of this virus in all the sampled shoot tip following cryopreservation. Cryopreservation did also seem like a suitable way for preserving SMYEV, but more precautions should be taken regarding time of PVS2. SMYEV was more successfully cryopreserved in shoot tips treated with 40 minutes in relation to 60 minutes. Statistical analysis showed that the mean of virus preservation percentage of SVBV were statistically higher than the mean preservation percentage of SMYEV, when summarizing the results from all the treatments. The biggest

difference between the viruses were in the 60-minute duration group, whereas there was not statistical difference between the two viruses in the 40-minute treatment group.

The two viruses are located in two different genus and inhabit different characterizations, which may affect their ability to survive cryopreservation. It is also noteworthy that the virus infected material used in this thesis were two different species/cultivar of *Fragaria*, which might also have affected differences in results.

Due to the fact that SVBV is easier to preserve through cryopreservation, it could indicate that its capacity to infect shoots is stronger than that of SMYEV. To fully understand the difference in virus preservation frequencies between the two viruses, virus localization can be conducted. This, in addition to investigating cell survival patterns in cryopreserved shoot tips, could give more answers to both why the survival frequencies vary between the two viruses as well as between different durations of PVS2 (for SMYEV).

SVBV is a *Caulimovirus* and has dsDNA, whereas SMYEV is a *Potexvirus* with a ssRNA genome (ICTV 2011). Viruses with a DNA genome are considered more capable of surviving in the nucleus of their host cell due to the use of their hosts biochemical machinery for DNA replication. RNA viruses have a higher mutation rate because their RNA polymerase is incapable of repairing errors in the replication (Cross 2019). RNA is also more easily degraded than DNA, due to RNA containing ribose instead of deoxyribose and RNA containing a hydroxy group, making it more susceptible to hydrolysis than DNA (Fordyce et al. 2013). The different types of nucleic acid could possibly affect the ability to survive cryopreservation.

SVBV occurs in cytoplasmic inclusion bodies in the vascular parenchyma adjoining the xylem vessels (Adams and Antoniw 2005). These inclusion bodies are considered stable due to protection from degradation from host cell enzyme (Hull 2002), which might have affected SVBVs ability to survive cryopreservation.

5.4 Diagnosis procedure

The RT-qPCR procedure using the StrawVir kit worked well, with the exception of the positive controls. The positive controls gave results in the first qPCR procedure, but not in the one following cryopreservation. This is probably due to the controls being frozen and thawed several times, leading to degradation of the DNA.

The results from the first round of RNA extraction (prior to cryopreservation) gave somewhat low 260/230 rates, with the average being around 1.65, which might indicate presence of contaminants. The results from round two of extraction (following cryopreservation) gave a higher 260/230 rate, with most being above 2. There was no significant difference between the two extraction kits used.

Plant material from *Fragaria* ssp. contains a large amount of secondary metabolites such as polyphenols and polysaccharides, and can therefore be reluctant to extraction of virions and nucleic acids (Mercado et al. 1999). The challenges caused by the secondary metabolites in strawberry material can affect purification of nucleic acid and cause inconsistency in the detection of strawberry viruses. These problems have been reported for extraction of RNA from strawberry infected with SCV (Posthuma et al. 2001) and SVBV (Mráz et al. 1997) (Thompson et al. 2003). However, extraction of RNA from the strawberry material in this thesis did not cause any significant challenges. This might be due to the strawberry material originating from tissue culture.

Even though SVBV is DNA virus and can be detected by PCR directly (Mráz et al. 1997, Ren et al. 2022), its RNA intermediate can also be used as template for diagnosis through PCR (Chang et al. 2007). For viruses belonging to the genus *Caulimovirus*, RNA serves as a template in the replication of the virus genome and protein synthesis (Chang et al. 2007). SVBV belongs to a group of viruses called pararetroviruses, which are characterized by the existence of pre-genomic RNA (Vašková et al. 2004). The term pararetrovirus describes viruses with retroelements that do not integrate in the host genome and where the DNA phase of the replication is encapsulated rather than the RNA phase (ICTV 2011, Hull 2014).

5.5 Statistics

Each experiment had three replications, and there was no statistically significant difference between the results in the three replications for each treatment in either of the experiments, meaning that the results were not affected by difference in experimental conditions between the replications. Replication is a critical element in experimental set up, and provides additional information to quantify uncertainty in observed estimates (Sullivan et al. 2016).

The outcome of the different treatments in the experiments is presented as means for continuous variables (Regeneration percentage and Ct and $2^{-\Delta\Delta Ct}$ values), and proportions for binary variables, (virus presence or no virus presence), and are compared statistically with one-way ANOVA or chi-square test, respectively. The statistical tests assume specifical

characters for the data. Basic assumptions for ANOVA are independence, normally distributed outcomes and homogeneity of variances across the groups compared (Sullivan et al. 2016).

The experimental set-up had three replications with 10 samples for each of the treatments (10 x10 x 10 =30 samples for each treatment). However, many of the shoot tips did not survive cryopreservation, or did not develop sufficiently, leaving fewer samples to include in the statistical analysis of differences in virus preservation. The sample sizes tested were rather small, thus might not fully support formal statistical testing of the underlying hypothesis as well as preventing the findings from being extrapolated. Small sample sizes may increase vulnerability to assumption violations, like homogeneity of variance (Ogbonna et al. 2019). With small sample sizes, normality is not necessarily ensured, and the statistical test for normality is generally designed for larger sample sizes (Ghasemi and Zahediasl 2012).

The low number of samples in each treatment made it hard to conduct sufficient statistical analysis to be able to determine any significant differences in regeneration percentage and $2^{-\Delta\Delta Ct}$ value between different durations of PVS2, thus few conclusions can be drawn. However, there are some strong trends amongst the results, which can give indications. One of the trends, indicated by the results, was that longer treatment with PVS2 (40-60 minutes) gave better regeneration percentage than shorter treatment of PVS2 (10-30 minutes) for cv. Korona.

5.6 Potential sources of error and future perspectives

The time of freezing in liquid nitrogen varies in different studies on cryopreservation. If the samples are frozen for preservation purposes, they may be frozen for many years. However, in studies of cryopreservation, the samples are often exposed to LN for a much shorter time, often a few minutes to half an hour (Zhao et al. 2019). Both the viability of the cryopreserved shoot tip, and the preservation frequency of plant viruses may be affected. Jeon et al. (2016) found that eradication frequencies of CSVd in chrysanthemum were 13% when shoot tips were exposed to LN for 1 to 3 hours and decreased to around 7% when they were exposed to LN for 5 hours. Shoot tips of globe artichoke plants frozen in LN for 15 minutes showed lower virus eradication efficiency (20-30%) than shoot tips frozen for 30 minutes (Taglienti et al. 2013). (Kartha et al. 1980) found that after one weeks of storage in LN 95% of the cryopreserved shoot tips were viable, which decreased to 56% after 8 weeks. A study conducted by Caswell and Kartha (2009) found that the viability of strawberry meristems

after 28 years storage in LN was 59.3%. These results indicate that the time of storage in LN may affect both the viability of meristems as well as eradication frequencies of plant viruses.

The regenerants from cryopreserved virus-infected shoots had an extended period without subculturing, which assumably had a negative effect on the regeneration proliferation and possible the morphology of the plantlets. The light intensity and temperature during post culture varied some between thesis than in other studies which might have affected the results. Controls (shoot tips not immerged in LN) could have been added when the cryopreservation procedure was conducted, which would have given an indication on whether the negative effects of the cryopreservation process was caused by freezing in LN or by the other steps in the procedure such as PVS2.

The results from this thesis demonstrate that cryopreservation can maintain SMYEV and SVBV within living strawberry shoot tips. For further research it would also be interesting to examine whether the cryopreserved viruses are genetically stable and whether they can be transmitted through vegetatively propagation. It would also be interesting to examine the effect of cryopreservation on the two viruses infecting the same *Fragaria* cultivar, to eliminate possible cultivar effects on the results.

ROS-induced oxidative stress has been shown to cause somaclonal and epigenetic changes in regenerants after cryopreservation (Harding 2004, Kaczmarczyk et al. 2012), thus monitoring of the genetic and epigenetic integrity of the regenerated strawberry plants is important. Investigating field performance is useful for evaluating whether the cryopreserved plants are true-to-type, hence growing the strawberry regenerants in greenhouse conditions and examining the morphological features would be a beneficial continuation of this project.

Knowledge on the localization of the investigated virus within the shoot tip is important to optimize cryopreservation as a mean for virus preservation. Due to this it would be interesting to conduct histological studies on virus infected shoot tips, to find the virus` localization in the shoot. Micrografting of virus infected regenerants to pathogen-free plants would show whether the viruses can be efficiently transmitted and if they have maintained their infectious abilities following cryopreservation.

In this thesis, duration of PVS2 treatment was the variable investigated. In addition to this there are several other factors that affect the successfulness of a cryopreservation procedure, which would be interesting to investigate further. Looking at both the results from this thesis, and literature on cryopreservation of strawberry, it seems that different cultivars of *Fragaria*

ssp., require somewhat different cryopreservation procedures to achieve the best results, thus some adjustments can be made to the procedure to assure the best outcome of cryopreservation to the specific cultivar cryopreserved.

6 Conclusion

Frequencies of shoot regeneration following cryopreservation is an important factor determining the success of the cryopreservation protocol conducted. In this thesis, a PVS2 duration of 40-60 minutes seemed to give the best regeneration percentage, in comparison to a shorter duration (10-30 minutes). Within the 40–60-minute duration groups, the regeneration percentages from the two experiments varied from 56-96%, thus, the protocol used was considered successful. However, the cryopreservation protocol had some negative effects on the cryopreserved material of cv. Korona, which developed callus following cryopreservation, indicating that the protocol can be further improved for this cultivar.

In the presented thesis, SMYEV and SVBV was successfully cryopreserved in living cells of *Fragaria ssp.* by droplet vitrification. SVBV was present in all of the regenerated shoots following cryopreservation in all the three durations of PVS2 examined. SMYEV was more efficiently preserved in shoot tips exposed to 40 minutes (90%) of PVS2, in relation to 60 minutes (33%). The results presented in this thesis shows that cryopreservation has a great potential in long-time preservation of both strawberry germplasm and aphid-transmitted viruses in strawberry.

7 References

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Appendix

Appendix 1: Regenerated shoot tips after cryopreservation of strawberry cv. Korona for each of the three repetitions and the six treatments. Sum of the three repetitions calculated for each treatment, as well as mean and standard deviation.

Min. PVS2	Replication			SUM	Mean number	Mean regeneration percentage %	SD (for mean reg. rate %)
	1	2	3				
10	0	6	6	12/30	4	40	34.64
20	5	7	7	19/30	6.333	63.3	11.55
30	5	6	6	17/30	5.667	56.6	05.77
40	9	10	8	27/30	9	90	10
50	8	7	9	24/30	8	80	10
60	8	8	8	24/30	8	80	0

Appendix 2: Regenerated shoot tips after cryopreservation of SMYEV infected material for each of the three repetitions and the three treatments. Sum of the three repetitions calculated for each treatment, as well as mean number of regenerated plants, regeneration percentage and standard deviation for the regeneration percentage.

Min. PVS2	Replication			SUM	Regeneration percentage (%)	Mean number	SD (for mean reg. rate %)
	1	2	3				
10	1	1	2	4/30	13.3	1.333	5.77
40	6	4	7	17/30	56.7	5.667	15.28
60	9	4	9	22/30	73.3	7.333	28.87

Appendix 3: Regenerated shoot tips after cryopreservation of SVBV infected material for each of the three repetitions and the three treatments. Sum of the three repetitions calculated for each treatment, as well as mean and standard deviation.

Min. PVS2	Replication			SUM	Regeneration percentage (%)	Mean number	SD (for mean reg. rate %)
	1	2	3				
10	7	7	5	19/30	63.3	6.333	11.55
40	9	10	10	29/30	96.7	9.667	5.78
60	10	9	10	29/30	96.7	9.667	5.78

Appendix 4: Sample number, sample ID and Ct value following qPCR of the regenerants after cryopreserved SMYEV infected shoot tips. Both Ct value for SMYEV and the internal control NAD is included

Sample number	Sample ID			Ct	
	Virus	Replication number	PVS2 treatment (min)	SMYEV	NAD
2	SMYEV	1	40	N/A	21.72
3	SMYEV	1	40	16.25	21.75
4	SMYEV	1	40	17.28	22.02

5	SMYEV	1	40	17.31	22.02
6	SMYEV	1	40	18.84	22.08
7	SMYEV	1	40	16.05	20.89
8	SMYEV	2	40	15.11	20.29
9	SMYEV	2	40	15.3	20.43
11	SMYEV	3	40	17.64	21.77
12	SMYEV	3	40	35.08	21.01
13	SMYEV	1	60	16.73	21.77
14	SMYEV	1	60	31.43	22.28
15	SMYEV	1	60	N/A	21.40
16	SMYEV	1	60	N/A	22.05
17	SMYEV	2	60	N/A	22.15
18	SMYEV	2	60	N/A	22.48
19	SMYEV	3	60	30.47	22.60
20	SMYEV	3	60	N/A	22.87
21	SMYEV	3	60	N/A	23.31
	NTC			N/A	N/A
	ITC(SMYEV)			N/A	
	ITC (NAD)				21.66

Appendix 5:Sample number, sample ID and Ct value following qPCR of the regenerants after cryopreserved SVBV infected shoot tips. Both Ct value for SMYEV and the internal control NAD is included

Sample nr.	**Sample ID**			**Ct**	
	Virus	**Replication number**	**PVS2 treatment (min)**	**SVBV**	**NAD**
23	SVBV	1	10	16.69	23.32
24	SVBV	1	10	20.34	22.92
25	SVBV	1	10	23.63	22.82
26	SVBV	1	10	20.1	23.02
27	SVBV	1	10	16.91	22.73
28	SVBV	2	10	20.72	20.52
29	SVBV	2	10	27.69	20.95
30	SVBV	2	10	30.38	21.84
31	SVBV	2	10	30.39	21.99
32	SVBV	2	10	30.12	22.09
34	SVBV	2	10	23.69	22.40
35	SVBV	3	10	29.69	22.38
36	SVBV	3	10	17.68	22.43
37	SVBV	3	10	21.63	20.46
38	SVBV	3	10	17.57	19.82
39	SVBV	3	10	28.83	21.4
40	SVBV	1	40	28.91	23.26
41	SVBV	1	40	17.73	22.84
43	SVBV	1	40	29.14	23.38
44	SVBV	1	40	19.29	22.77
45	SVBV	1	40	29.52	22.53
46	SVBV	1	40	29.5	23.13
47	SVBV	1	40	18.67	23.43

48	SVBV	1	40	29.24	24.1
49	SVBV	2	40	24.07	24.11
50	SVBV	2	40	21.94	23.39
51	SVBV	2	40	17.3	22.29
52	SVBV	2	40	27.84	22.62
53	SVBV	2	40	18.24	22.17
54	SVBV	2	40	17.98	23.47
55	SVBV	2	40	17.64	23.96
56	SVBV	2	40	28.57	23.88
57	SVBV	3	40	19.46	24.06
58	SVBV	3	40	27.6	23.41
59	SVBV	3	40	18.35	23.77
60	SVBV	3	40	18.22	23,3
61	SVBV	3	40	24.68	23.4
62	SVBV	3	40	13.85	21.11
63	SVBV	1	60	19.74	21.20
64	SVBV	1	60	21.73	22.16
65	SVBV	1	60	29.15	22.68
66	SVBV	1	60	29.11	22.61
67	SVBV	1	60	28.61	22.01
69	SVBV	2	60	28.6	22.34
70	SVBV	2	60	16.13	22.78
71	SVBV	2	60	19.1	22.21
72	SVBV	2	60	16.29	23.47
73	SVBV	2	60	26.74	22.97
74	SVBV	2	60	18.11	22.32
75	SVBV	2	60	15.63	22.14
76	SVBV	2	60	16.31	22.12
78	SVBV	3	60	28.86	23.03
79	SVBV	3	60	17.3	22.82
80	SVBV	3	60	24.22	22.75
81	SVBV	3	60	19.74	22.72
82	SVBV	3	60	15.69	22.85
83	SVBV	3	60	29.08	22.69
	NTC			N/A	N/A
	Positive control (SVBV)			N/A	
	Positive control (NAD)				21.95

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