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IN VITRO VIRUS ELIMINATION FROM TARO (*Colocasia esculenta* (L.) Schott) FOR CONSERVATION AND SAFE INTERNATIONAL EXCHANGE

by

Amit Chand Sukal

A thesis submitted in fulfilment of the
requirements for the degree of
Master of Science

School of Biological and Chemical Sciences
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May, 2014

DECLARATION

Statement by Author

I, Amit Chand Sukal, declare that this thesis is my own work and that, to the best of my knowledge, it contains no material previously published, or substantially overlapping with material submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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Statement by Supervisor

The research in this thesis was performed under my supervision and to my knowledge is the sole work of Mr. Amit Chand Sukal.

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ABSTRACT

Taro (*Colocasia esculenta* (L.) Schott) is a very important Pacific Island crop for food and nutritional security, being ranked in the top five of food crops. It is also very important culturally and for a few countries, makes a significant contribution to its economic growth. However pests and diseases as well as the increasing challenge of climate change are affecting taro production. Failure to address these challenges could lead to serious consequences on food security and trade. Crop improvement either through selection or breeding offers a solution to these challenges, but availability of and access to taro diversity is essential for any worthwhile and effective selection and breeding programme to be implemented. The Centre for Pacific Crops and Trees (CePaCT) of the Secretariat of the Pacific Community (SPC) has a unique repository of taro diversity and is mandated to facilitate the conservation and safe exchange of this diversity. CePaCT, SPC is able to achieve this exchange through *in vitro* cultures but the challenge of virus infection in taro cultures has prevented the exchange of some of its valuable diversity. The aim of this research was to investigate methods for the elimination of different viruses in taro. Using *Dasheen mosaic virus* (DsMV) as a model virus, the research evaluates different elimination methodologies (meristem extraction, chemotherapy, electrotherapy and thermotherapy), and proposes possible techniques for the routine elimination of viruses from taro. All methods evaluated were successful in the elimination of DsMV from taro. However, with electrotherapy, the highest regeneration was obtained (*avg.* 91.66%), as well as effective elimination of DsMV (*avg.* 76.57%) compared to the other three methods. Importantly with electrotherapy plantlets recovered faster than the other therapies (at least 1½ month faster than the other therapies). Of the four techniques evaluated, electrotherapy and thermotherapy had the highest therapy efficiency rates, 71.66% and 43.3%, respectively. Either of these methods can be used routinely to eliminate DsMV and optimized for the elimination of other viruses infecting taro.

A prerequisite for virus diagnostics is the availability of reliable detection methodologies. Both serology and molecular based diagnostics are available for the detection of DsMV. For germplasm exchange it is necessary to ensure that the most reliable and sensitive of the methods is used for the purpose of virus testing. Commercially available DAS-ELISA kits (Agida® Inc, France) and RT-PCR developed by Maino (2003) were compared to see which test was more effective in detecting DsMV from naturally infected samples. Comparisons between the two methods found that RT-PCR was 10 times more sensitive than the DAS-ELISA. RT-PCR was able to detect DsMV from 10^{-3} diluted samples while the DAS-ELISA had a detection limit of 10^{-2}. ELISA however, is much cheaper per sample than RT-PCR (15 times cheaper), requires less technical expertise, and does not require purpose build infrastructure. ELISA can be incorporated into national surveys and testing schemes so that production can be maintained at optimum level by reducing DsMV infections in the field with annual testing and rouging. RT-PCR should be used at gene bank level as testing at the highest level of sensitivity and accuracy is required.

Availability of reliable testing method such as RT-PCR and the means (electrotherapy and thermotherapy) to eliminate DsMV will allow the dissemination of valuable taro diversity conserved at CePaCT, SPC. This research also provides foundation for future work on the elimination of other viruses from taro.

Key words: Taro, *Colocasia esculenta*, virus elimination, electrotherapy, meristem, chemotherapy, thermotherapy, ribavirin, adefovir, regeneration, *in vitro*, RT-PCR, *Dasheen mosaic virus* (DsMV), CTAB, ELISA

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LIST OF ABBREVIATIONS

µm	micron or micrometre
°C	degrees Celsius
µg	microgram(s) = 10-^{6} gram
µl	microlitre(s) = 10^{-6}litre
µl	microlitre
µM	micromolar(s) = 10^{-6} Molar
µM	micromolar
A	adenine
Abs	absorbance
ACLSV	*Apple chlorotic leaf spot virus*
AFLP	amplified fragment length polymorphism
AusAID	Australian Agency for International Development
BAP	6-benzyl-aminopurine
BBTV	*Banana bunchy top virus*
BMV	*Banana mosaic virus*
BP	before present
bp	base pair(s)
BSV	*Banana streak virus*
C	cytosine
Cat#	catalogue number
CBDV	*Colocasia bobone virus*
CePaCT	Centre for Pacific Crops and Trees
cm	centimetre(s)
CTAB	cetyltrimethylammonium bromide
DAS-ELISA	double antibody sandwich-ELISA
DC	direct current
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid

dNTPs	deoxynucleotide triphosphate
DsMV	*Dasheen mosaic virus*
ECL buffer	enhanced chemiluminescence buffer
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FAO	Food and Agriculture Organization of the United Nations
g	gram (s)
G	guanine
g/l	grams per litre
GBNV	*groundnut bud necrosis virus*
GE buffer	general extraction buffer
GR	genetic resource
GTP	guanosine triphosphate
HBV	Hepatitis B virus
HCl	hydrochloric acid
hr(s)	hour(s)
IBPGR	International Board for Plant Genetic Resources
IFS	International Foundation of Science
IMPDH	inosine monophosphate dehydrogenase
ITPGRFA	International Treaty on Plant Genetic Resources for Food and Agriculture
KRS	Koronivia Research Station
M^2s^{-1}	metres squared per second
mA	milliampere
mg/l	milligram per litre
mg/ml	milligram per mililitre
min	minutes
ml	millilitre
mM	millimolar
mm	millimetre

mRNA	Messenger Ribonucleic acid
MS	Murashige and Skoog
MS30	Murashige and Skoog medium with 30g/l sucrose
MTP	microtitre plate
NA	Neuraminidase
NaCl	sodium chloride
NaOH	sodium hydroxide
NCDs	non-communicable diseases
nm	nanometre
NtRTI	nucleotide analog reverse transcriptase inhibitor
PBST	phosphate buffered saline with tween
PCD	photon flux density
PCR	polymerase chain reaction
PEQ	post entry quarantine
PGR	plant genetic resource
PLRV	*Potato leaf roll virus*
PNP	44-Nitrophenol
PPV	*Plum pox virus*
psi	pounds per square inch
PVA	*Potato virus A*
PVP-40	polyvinylpyrrolidone
PVS	*Potato virus S*
PVX	*Potato virus X*
PVY	*Potato virus Y*
RDI	recommended daily intake
rev/s	revolutions per second
RNA	ribonucleic acid
RPM	revolutions per minute
rRNA	ribosomal ribonucleic acid
RT-PCR	reverse transcription -polymerase chain reaction

SAH	S-adenosylhomocysteine hydrolase
sec	seconds
sp.	species (singular)
SPC	Secretariat of the Pacific Community
spp.	species (plural)
SSR	simple sequence repeats
T.E.	therapy efficiency
t/ha	tonnes per hectare
TaBV	*Taro badna virus*
TaroGen	Taro Genetic Resources Conservation and Utilization
TaRV	Taro reovirus
TaVCV	*Taro vein chlorosis virus*
TBD	Tris-borate-EDTA
TDZ	Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea)
TLB	Taro leaf blight
Tris-HCl	tris(hydroxymethyl)aminomethane hydrochloride
U	units
USDA	United States Department of Agriculture
USP	University of the South Pacific
V	volt
V/h	volts per hour
w/v	weight/volume
XMP	xanthosine 5'-monophosphate
β-ME	β-mercaptoethanol
ρmol	picomoles

CHAPTER ONE

INTRODUCTION

1.1 Introduction

Taro, *Colocasia esculenta* (L.) Schott, is an important edible crop grown throughout the Pacific region, Asia and Africa. Taro is ranked 14th amongst staple food crops worldwide by production[1]. However, for most Pacific Island countries it is ranked in the top five (McGregor *et al.*, 2011). Taro has evolved from a subsistence crop to an export commodity, with significant economical contributions towards the growth of some of the Pacific Island countries, yet remains a very important subsistence crop in other Pacific Island countries.

1.1.1 Description of scientific problem under investigation

A growth in export demands has resulted in a significant increase in taro production in many Pacific Island countries; however, farmers face constant challenges due to pests and diseases (Harding, 2008; Revill *et al.*, 2005a). The future is likely to be more challenging as climate change brings changes in pest and disease dynamics, and extreme climate variability (IPCC, 2013). Now more than ever new diversity is needed for evaluation and inclusion in breeding programmes to develop varieties with tolerance and resistance to pests and diseases and also climatic conditions such as salinity, drought, water-logging, and high temperatures.

Taro leaf blight (TLB) has been a major constraint and challenge in the production of taro for countries like Samoa but it is also a pending disaster for other producers of taro. Taro exporting countries such as Fiji, which do not have TLB, are at risk as the climate changes. Studies carried out by Trujillo (1965) and Putter (1976)[2] directly link

[1] Food & Agriculture Organization of the United Nations. (2013). FAO Statistical Yearbook 2012. Retrieved January, 2013

[2] Putter (1976), working in PNG, found that temperature and relative humidity together explained 72.57 per cent of the variation in sporulation, with all these associations being highly significant

temperature and humidity with variation in the oomycete sporulation, and observations of weather patterns at the time of outbreaks, particularly in Samoa, do point to the influence of minimum (night) temperature on TLB outbreaks. A small increase in temperature linked with high humidity would increase the pressure from TLB inoculum. Future climate projections could be favourable for TLB outbreaks and therefore, devastating for taro production. Acquisition of germplasm and breeding activities after the TLB disaster epidemic in Samoa has led to the availability of many TLB resistant lines. Countries which do not have TLB are encouraged to access these germplasm so that they can build resistance in their existing taro gene pool.

Exchange of diversity is integral to ensure taro production remains viable in the future. Sharing genetic diversity however must exclude pests and diseases. National and international quarantine regulations require that plant genetic resources (PGR) are exchanged safely without introduction of pests and diseases. The Centre of Pacific Crops and Tress (CePaCT), of the Secretariat of the Pacific Community (SPC) is a custom build laboratory, specifically for the purpose to conserve and facilitate the safe exchange of PGR. A globally unique and important diversity of taro gene pool is conserved at the CePaCT, SPC. However, virus infections have hindered the distribution of some of these important taro genetic resources. Breeding lines that have been evaluated and found to be TLB resistant/tolerant cannot be exchanged as they are virus infected. These are important accessions especially for those countries currently free of TLB, but could be at risk due to projected climate conditions favouring the spread of TLB. Therefore they need to be distributed to help these countries manage TLB, should an outbreak occur. However, virus infection, especially *Dasheen mosaic virus* (DsMV) and *Taro badna virus* (TaBV), has been a constraint to these disseminations.

Protocols for cleaning of viruses are available but limited studies have been carried out to investigate the elimination of viruses from taro (*Colocasia esculenta*). The current

(P<01).Trujillo (1965) showed that a minimum night-time temperature of 21°C and a relative humidity of 100 per cent provided optimum conditions for the TLB. With RH less than 90%, no sporulation occurs and zoospores rapidly lost their viability.

research reviews the information available and evaluates therapies for the elimination of virus, namely DsMV, from taro. DsMV is used as a model virus to determine the optimum parameters of the different therapies so that they can be effectively used for DsMV elimination and in the future employed for the elimination of other viruses. This research would enable the important taro genetic diversity conserved at the CePaCT, SPC to be shared more effectively.

1.1.2 Implications of research

This research will strengthen Pacific Island's food security as well as build on knowledge in the field of virus diagnostic and elimination. The global staples, such as rice and wheat will be impacted more by climate change than the Pacific staples such as taro. Therefore it is important for Pacific food security that production and processing of taro is strengthened to reduce the reliance of many Pacific Island countries on rice and wheat-based products (Taylor 2013, pers. comm., April 25)

Elimination of viruses from taro would make more breeding lines, hybrids as well as wild material, available for exchange. The farmers of the Pacific as well as globally stand to benefit directly by being able to access the virus-free materials for direct utilization. Researchers would also benefit from the numerous cultivars being available for breeding.

1.1.3 Overall objectives of the research

The main aim of the research is to evaluate the different protocols available for plant virus elimination for usability in elimination of *Dasheen mosaic virus* (DsMV) in *C. esculenta.*

Specific Objectives:

1. Compare serological and molecular based methods for the detection of DsMV.
2. Evaluate the regeneration rates of taro accessions BL/SM-97, BL/SM-129 and BL/SM-145 exposed to meristem extraction, chemotherapy, electrotherapy, and thermotherapy.

3. Evaluate the DsMV elimination rates of meristem extraction, chemotherapy, electrotherapy, and thermotherapy from the three taro accessions.
4. Calculate the DsMV elimination efficiencies of meristem extraction, chemotherapy, electrotherapy, and thermotherapy
5. Propose a protocol that can be routinely used for DsMV elimination as well as elimination of other taro viruses.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction to taro (*Colocasia esculenta* (L.) Schott)

Taro, *Colocasia esculenta* (L.) Schott, is an ancient and important staple food crop cultivated throughout many Pacific Island countries. Close to half a billion people include taro in their diets worldwide (Bown, 2000). In addition to contributing towards food security in the Pacific, it has become a major export commodity for Fiji, with other countries like Samoa and Tonga also building up their export capacities (Deo, 2008; Revill *et al.*, 2005a; Plucknett *et al.*, 1970). Taro is grown under intensive cultivation as a carbohydrate source. It fits well amongst tree crops and agro forestry systems, and some varieties are well suited to unfavourable land and soil conditions such as those with poor drainage systems (Deo, 2008; Jianchu *et al.*, 2001).

2.1.1 Taxonomy and morphology of taro

Taro is an herbaceous, monocotyledonous crop. The main stem is a starch-rich underground structure called corm. The leaves grow from the apical bud at the top of the corm and roots grow from the lower portion. Cormels, daughter corms and runners grow laterally. The corms are cylindrical about 30cm in length and 15cm in diameter with short internodes and a superficial and fibrous root system, restricted to the top one metre of the soil. The whole plant grows to a height of 1-2m (Strauss, 1983; Purseglove, 1979). The corm is the nutrient storage organ of taro and shares some of the characteristics of the food storage organs of carrot, sweet potato and cassava.

Taro is a monocotyledonous angiosperm which belongs to the family *Araceae*, and genus *Colocasia* (Ivancic, 1992; Onwueme, 1978). Cultivated taro is classified as *Colocasia esculenta*, but the species is highly polymorphic (Ivancic & Lebot, 2000; Purseglove, 1979). One of the main reasons for the different botanical classifications is genetic variability. Some authors describe *C. esculenta* as one polymorphic species with

two or more botanical varieties, while others consider it to be two different species; *C. esculenta* (L.) Schott (referred to as dasheen) and *C. antiquorum* Schott (referred to as eddoe) (Purseglove, 1979).

The major difference between *esculenta* and *antiquorum* is in the shape and size of the main corm and the cormels (Figure 2.1 and Figure 2.2). The *esculenta* form is characterised by a large central or main corm and smaller 'side' cormels or suckers whilst *antiquorum* has relatively smaller central corm with well developed side cormels (Ivancic & Lebot, 2009; Deo, 2008; Lebot & Aradhya, 1991; Purseglove, 1979).

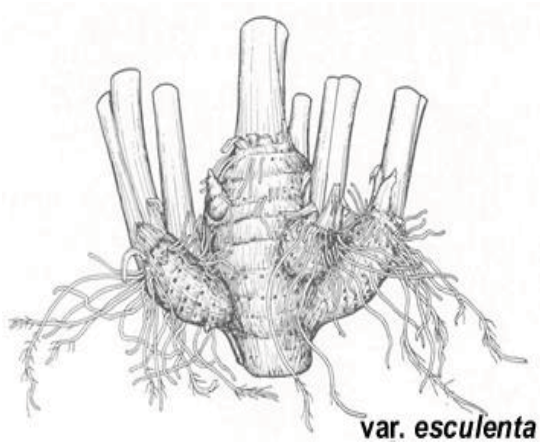


Figure 2.1: Diagram depicting *C. esculenta*

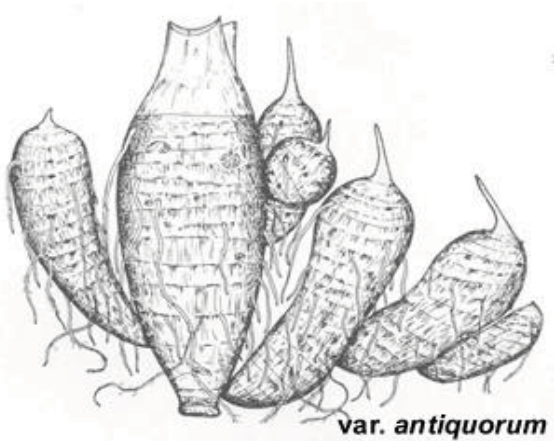


Figure 2.2: Diagram depicting *C. antiquorum*

In practice, two morphological groups exist; dasheen (corm is consumed) and eddoe (side corms are consumed) which do not match taxonomic and cytological groups. This grouping is not differentiated by molecular markers. From an agronomic consideration eddoe forms can be considered genetically less improved and having plant architecture resembling the wild taro forms with their non-edible corm competing for photosynthates with cormels and having lower yields when compared with the dasheen-type cultivars (Lebot, 2009).

2.1.2 Origin and distribution of taro

Taro is most likely the oldest crop on earth being cultivated in tropical Asia for more than 10,000 years. Taro is suggested to have originated from north-east India and South-east Asia since four species related to taro (*C. fallax, C. affinis, C. indica* and *C. gigantea*) are all confined within this area but evidence to support this theory does not exist. Wild *C. esculenta* can be found throughout India, South-east Asia, South China, Melanesia and Australia. This is much larger than the range of the related species thus can be argued that they were derived locally from *C. esculenta.* The problems associated with the origin, domestication and spread of taro has been studied by different groups all of which have concluded that a single centre of origin for taro is too simplistic a viewpoint (Lebot, 2009; 1992; Lebot & Aradhya, 1991; Matthews, 1990; Plucknett, 1984; Yen & Wheeler, 1968; Spier, 1951).

Domestication of taro was most likely at different locations at different times, spreading from South China, Melanesia and northern Australia. Wild populations isolated for a long period of time and the effect of insular isolation, combined with human and natural selection pressures, induced genetic diversity within a single, highly polymorphic species, *C. esculenta* (Lebot, 2009). The Asian origin of taro has been well documented (Matthews, 1990). Analysis of prehistoric use of stone tools for starch processing shows evidence that taro processing was active by at least 10,000 before present (BP) from the highlands of Papua New Guinea (Lebot, 2009), while *Alocasia* and *Colocasia* starch residues were found on stone tools from Buka, Solomon Islands dating back some 28,000 years (Manner & Taylor, 2010; Loy *et al.*, 1992). Prehistoric residues and starch granule analyses also indicate that taro was processed during the early and mid-Holocene. Although not possible to differentiate between the processing of wild or cultivated forms, from 6950 to 6440 BP, the fact that taro was processed suggests that it was domesticated and integrated into the cultivation practices (Lebot, 2009; Fullagar *et al.*, 2006).

Early settlers cultivated taro in the Pacific Islands from around 3050-2500 BP as elucidated by starch dating from Brourewa, south-west Viti Levu, Fiji (Horrocks &

Nunn, 2007). Austronesian sailors who settled the islands of Melanesia probably introduced taro to Bismarck Archipelago in Eastern New Guinea and Vanuatu, from 3500 to 3000 BP and later to Fiji. Indigenous people of New Guinea may already have been cultivating taro and the Austronesians distributed cultivars into the Pacific Islands, along with other crops (Yen, 1991; 1982).

Isozyme variation has been studied in more than 1400 cultivars and wild forms of taro collected from Asia and Oceania which have revealed a pattern of greater variation in Asia than Oceania with Indonesia having the greatest variation (Lebot, 2009; Lebot & Aradhya, 1991) . The existence of greater variability in Indonesia may indicate lack of crop improvement. Morphotypes often exhibit several 'wild' characters, including frequent flowering and stolon production. Oceania cultivars have originated from a common, narrow genetic base, which was probably domesticated in Melanesia. The genetic (isozyme) variation is nil in Hawaii and in most Polynesian Islands, suggesting an extremely narrow genetic base. Polynesian fields often have variegated forms of taro which are due to a long period of vegetative propagation and accumulation of somatic mutations and the cultivars existing in Polynesia are probably clones of a common Melanesian source (Lebot, 2009; Lebot & Aradhya, 1991). Studies with SSR (Simple Sequence Repeats) markers (Noyer *et al.*, 2004) and AFLP (Amplified Fragment Length Polymorphism) markers (Kreike *et al.*, 2004) confirm the existence of two distinct gene pools which suggest two independent domestication events of taro, one in South-east Asia and the other in the Pacific; therefore, it can now be considered as a native plant of the Pacific (Lebot, 2009). The AusAID-funded Taro Genetic Resources Conservation and Utilization (TaroGen) project revealed a pattern of decreasing diversity going from west to east with the greatest diversity in Papua New Guinea. Significant diversity is also present in the Solomon Islands and Vanuatu (Manner & Taylor, 2010). Taro is now found throughout the Pacific islands and worldwide. It is grown in more than 65 countries worldwide (USDA, 2001).

2.1.3 Nutrition

Taro is a staple food crop for Pacific Island countries and parts of Asia (Opara, 2003; Lebot & Aradhya, 1991). About half a billion people include taro in their diet (Lebot, 2009). The highest contribution to the diet occurs in the Pacific Islands (Ivancic, 1992), for example in Tonga where tuber crops represent approximately half the nation's calorie intake, 40% of this is contributed by taro. Similarly in the Solomon Islands about 10% of the people's dietary calories are sourced from taro and 30% from other tubers. Prior to the devastation of taro in Samoa, caused by taro leaf blight (TLB), virtually the entire population's dietary intake of calories from tubers (20% of the overall diet) came from taro (Deo *et al.*, 2009a ; Deo, 2008)

Taro is one of the crops where both the top (leaf and petiole) and underground part (corms) is important as a food source. Taro corms are an excellent source of carbohydrate and potassium but low in calories. It contains various forms of carbohydrates with starch forming the highest portion, 17-28% being amylase and the rest being amylopectin (Manner & Taylor, 2010; Deo, 2008; Oke, 1990). The digestibility of taro starch has been estimated at 98.8% and the grain size is estimated at 1/10 that of potato. Ease of assimilation makes it suitable for people with digestive problems. Since starch is hypoallergenic it is useful for people allergic to cereals and can be used as food for children sensitive to milk. Taro flour has been reported to have been used in infant food formulae and canned baby foods in the United States of America (Darkwa & Darkwa, 2013). Taro corm has low fat and protein content but is higher compared to that of other root crops such as yams, cassava or sweet potato. The protein is rich in some essential amino acids but is low in isoleucine, tryptophan and methionine (Onwueme, 1978).

Taro leaves contain higher levels of protein than the corm and are also an excellent source of carotene, potassium, calcium, phosphorous, iron, riboflavin, thiamine, niacin, vitamin A, vitamin C and dietary fibre (Opara, 2003; Bradbury & Holloway, 1988; Hanson & Imamuddin, 1983; Lambert, 1982; Onwueme, 1978). Half a cup of cooked leaves provide 97% and 39% of the U.S. RDI of vitamins A and C, respectively

(Yokoyama *et al.*, 1989). The leaves also contain greater amounts of B-complex than whole milk (Darkwa & Darkwa, 2013). Yellow fleshed taro contains higher level of β-carotene than the corms of white flesh. High levels of β-carotene promote the production of vitamin A which is important for a healthy immune system. It has been shown that foods with higher level of caroteniods protect against chronic disease, including certain cancers, cardiovascular disease, and diabetes (Englberger *et al.*, 2003). Having a low glycemic index makes taro an excellent food for diabetics, who require glucose to be released into the bloodstream slowly (Manner & Taylor, 2010). All parts of most cultivars contain calcium oxalate, which is destroyed by cooking but the time needed for cooking to effectively break down all the calcium oxalate varies with varieties. Tradition *palusami*, a dish that has taro leaves cooked with coconut milk, utilize the youngest leaves possible. The younger leaves have lower levels of calcium oxalate and require less cooking time than older leaves. Moisture levels are also high in taro leaves. Fresh leaf lamina and petiole contain 80% and 94% moisture respectively (Deo, 2008).

The Asia/Pacific region produces large quantities of taro. The corms are boiled, baked or fried and consumed with fish and coconut preparations. The leaves and petioles are often boiled and served as a kind of spinach. A dish known as *basese* is prepared in Fiji where the petioles are boiled and served with coconut cream (Manner & Taylor, 2010). A favourite and peculiarly Pacific way to prepare taro corm is to roast it on hot stones in dug-out earth ovens which is commonly used in feasts and ceremonies. Taro is also processed in storable forms such as taro chips and Poi. Taro chips are prepared by peeling the corm, washing, slicing into thin pieces and blanching; the pieces are then fried in oil, allowed to cool, then drained and packed. Poi is a sour paste made from boiled taro and its production and utilisation is quite limited- mainly to the Hawaiian Islands (Deo *et al.*, 2009b; 2009c).

2.1.4 Cultural and economical importance of taro

The evolution of taro has been in parallel with the culture of the people of Asia and Pacific region, where it has acquired considerable socio-cultural importance. Taro is considered a prestige crop and the crop of choice for royalty, gift-giving, traditional

feasting and the fulfilment of social obligations. Folklore and oral traditions of many cultures in Oceania and South-east Asia feature taro very prominently. Taro is also depicted on the currency of Samoa and Tonga (Onwueme, 1999). Murals, posters, original arts and other visuals of Hawaii have prominent depictions of taro and taro farmers throughout the islands; it's symbolic importance reflects its continuing role as a common food and common element in the agricultural landscape (Matthews, 1998). The socio-cultural attachment to taro has meant that taro itself has become a symbol of cultural identification, such that the people of Pacific Island origins continue to consume taro wherever they may live in the world. As such taro has acquired considerable economic importance (Hanson & Imamuddin, 1983). While much of the taro is produced and consumed on a subsistence basis, a considerable amount is produced as a cash crop. Surpluses from the subsistence production are found in the market, thereby playing a role in poverty alleviation. Taro has also developed into a lucrative export commodity for some Pacific Island countries, not only providing cash for farmers but also valuable foreign exchange for the countries. Export markets have become niche oriented towards populations of expatriate Pacific Islanders living in developed nations, such as Australia, New Zealand and USA. Taro exports to Australia, New Zealand and western North America amount to millions of dollars every year (Deo *et al.*, 2009a; Onwueme, 1999). Between 2005 and 2009, Pacific Island countries exported between 10,000-12,000 tonnes of taro annually (see Table 2.1) (McGregor *et al.*, 2011). Over this period (2005-2009) Fiji has had the highest export volume followed by Tonga while Samoa and Vanuatu have also contributed to exports though on a far lesser scale.

Table 2.1: Pacific Island taro exports 2005-2009 (tonnes) (McGregor *et al.*, 2011)

	2005	**2006**	**2007**	**2008**	**2009**
Fiji	9,959	11,434	11,894	10,794	9,482
Samoa	253	86	75	97	85
Tonga	220	208	626	249	323
Vanuatu	101	69	69	65	-
Total	**10,533**	**11,797**	**12,664**	**11,205**	**9,890**

2.2 Taro pests and diseases

2.2.1 Fungal and bacterial diseases

Despite its agronomic, cultural and economic importance, taro production in many countries is being replaced by sweet potato and cassava mainly due to pest and disease problems (Ivancic, 1992). Further both crops are more tolerant of variable climate conditions.

Taro Leaf Blight (TLB) is a major disease of taro caused by Oomycete water mould (*Phytophthora colocasiae*). TLB has characteristic symptoms of circular, water soaked, necrotic spots, on the leaves followed by the collapse of the petiole and plant death. It has been detected in Papua New Guinea, Federated States of Micronesia, Northern Mariana Islands, Palau and Solomon Islands for over 50 years. An outbreak of TLB in Solomon Islands after World War II devastated taro production and led to a permanent shift in some parts of the country away from taro to sweet potato and cassava production (Vinning, 2003). Similarly, TLB disease struck American Samoa and Western Samoa in 1993-1994 totally destroying production as all cultivars were susceptible to this pathogen. Resistant cultivars were not introduced to Samoa until 1996 but by then taro export from Samoa was dramatically reduced resulting in losses of millions of dollars (Iosefa *et al.*, 2003). In 2002, TLB reduced taro production in Morobe Province, PNG to such levels that food aid was requested (Vinning, 2003). Other Pacific Island countries, namely Fiji, Tonga, Cook Islands and Vanuatu are considered susceptible to an outbreak for a number of reasons. The AusAID-funded Taro Genetic Resources Conservation and Utilization (TaroGen) project demonstrated using molecular markers that the gene pools within these countries were very similar and narrow, especially for Fiji, Cook Islands and Tonga (Taylor 20013, pers. comm., 15 April). Further, these countries have similar climate and as discussed in section 1.1.1, the projected temperature increases due to climate change would increase the susceptibility of these countries to TLB. Taking all these factors into consideration TLB constitutes a significant threat to food security and economy of those countries which do not have TLB resistant varieties and where taro is a major staple diet and a viable export commodity. Even in those countries where taro

cultivation is possible because varieties are tolerant/resistant or because the inoculum pressure is low, changing climate conditions could result in increased inoculum pressure.

Taro soft rot is caused by several species of *Pythium*, which is soil borne and attacks roots and corms. Diseased plants display wilting and chlorosis of the leaves as well as proliferation of roots at the base of the shoot. The corms become soft and putrid and the plant often dies. Sclerotium corm rot is caused by *Sclerotium rolfsii*, which causes stunting of the plant, rotting of the corm and formation of numerous spherical sclerotia in the corm. Cladosporium leafspot is caused by *Cladosporium colocasiae* where brown spots appear on the older leaves (Deo, 2008).

2.2.2 Taro pests

Amongst taro pests, taro beetle belonging to the genus *Papuana* is of great concern. The adult beetles which are black, shiny and 15-20mm in length fly from the breeding sites to the taro field and tunnel into the soil just at the base of the taro corm. They feed on the growing corm leaving large holes that reduce the eventual market quality. The wounds created by the beetles while feeding cause secondary infections by rot-causing organisms. The feeding activity can cause wilting and even death of the affected plant. Other insect pests include taro leafhopper (*Tarophagus proserpina*), which is a vector for viruses and may also cause wilting and death of the plant in heavy infestations. The taro hawkmoth (*Hippotion celerio*) is another occasional pest of taro. The larvae can cause defoliation of the plant. Armyworm or cluster caterpillars also do extensive damage to the leaves (Lebot, 2009).

2.2.3 Taro viruses

Viruses are one of the most important pathogens of taro, with some infections resulting in severe yield reductions and even plant mortality. Viral infections manifest in reduction of corm size and quality, with some studies reporting as much as 20% reduction in yield (Reyes *et al.*, 2006). Five viruses, from four taxonomic groups, have been reported to infect taro with varying distribution throughout the Pacific. *Dasheen mosaic virus* (DsMV) is very widespread; *Taro bacilliform virus* (TaBV) occurs

throughout the region, often without producing symptoms; two rhabdoviruses occur, with *Taro vein chlorosis virus* (TaVCV) in both south and north of the Pacific, and *Colocasia bobone disease virus* (CBDV) confined to Papua New Guinea and Solomon Islands, is associated with different diseases depending upon the variety; and *Taro reovirus* (TaRV), reported in mixed infections from PNG, Solomon Islands and Vanuatu (Revill *et al.*, 2005a, b; Yang *et al.*, 2003a, b; Devitt *et al.*, 2001; Pearson *et al.*, 1999; Brunt *et al.*, 1990).

DsMV is a potyvirus with flexuous rod shaped virions and is found wherever taro is grown. It infects both the edible and ornamental aroids (Revill *et al.*, 2005a, b; Zettler & Hartman, 1987; 1986; Jackson, 1980; Shaw *et al.*, 1979). DsMV infected plants show a variety of infected mosaic patterns: small, irregular, grey/green (sometimes white) patches along or between the major veins or brilliant white or yellow feather-like patterns along the veins, sometimes throughout the leaf blade. DsMV causes such diverse symptoms that there is sometimes confusion such that different pathogens are thought to be responsible, until diagnosis proves otherwise. The main effect of DsMV is a reduction in corm size and quality (Zettler & Hartman, 1987). DsMV has been well characterised but these studies were based on isolates originating from the USA and Taiwan (Li *et al.*, 2011; Revill *et al.*, 2005a; Pappu *et al.*, 1994a, b). More recent studies analysed the coat protein-coding sequences from 16 DsMV isolates from PNG, Samoa, Solomon Islands, French Polynesia, New Caledonia and Vietnam and developed both serological and PCR-based diagnostic tests (Revill *et al.*, 2005a; Maino, 2003).

TaBV was initially classified as a putative badnavirus based on electron microscopy and mealybug transmission studies (Gollifer *et al.*, 1977; James *et al.*, 1973) but later studies by Yang *et al.,* (2003a, b) subsequently cloned and sequenced a PNG isolate of TaBV and confirmed its classification as a badnavirus. Yang *et al.,* (2003b) also showed that TaBV was widespread in the South Pacific Islands. TaBV alone results in a range of mild symptoms including stunting, mosaic and down-curling of the leaf blades (Macanawai *et al.*, 2005; Jackson, 1978) but co-infections of TaBV and CBDV, a rhabdovirus, is thought to result in the lethal alomae disease (Shaw *et al.*, 1979; James

et al., 1973) which is considered to be the most destructive viral disease of taro in Solomon Islands and PNG (Revill *et al.*, 2005a; Rodoni *et al.*, 1994; Jackson & Gollifer, 1975; Gollifer & Brown, 1972). Alomae causes leaf chlorosis, stunting, thickened veins and leaf blades, and irregularly shaped galls on the petioles. Leaves of infected plants fail to unfurl and the plants die from a systematic necrosis (Revill *et al.*, 2005a; Rodoni *et al.*, 1994). CBDV was initially classified as a putative rhabdovirus with characteristic bacilliform-shaped virons in sap dips (Shaw *et al.*, 1979; James *et al.*, 1973) but partial characterization of the CBDV genome and sequencing confirmed that it is a rhabdovirus (Revill *et al.*, 2005a). Taro infection with CBDV alone causes bobone disease with initial symptoms similar to that of alomae, although bobone-infected plants are rarely chlorotic and eventually recover (Revill *et al.*, 2005a; Jackson, 1978).

TaVCV was also initially identified as a putative rhabdovirus based on morphological studies using electron microscopy studies of sap dips (Pearson *et al.*, 1999) but sequence information of monopartite RNA of a Fijian isolate confirmed that it is a definite rhabdovirus with most similarity to members of the genus Nucleorhabdovirus (Revill, *et al.,* 2005b). TaVCV-infected plants show a striking leaf-vein chlorosis symptom, particularly at the leaf margin, which often leads to necrosis of the affected tissue (Pearson *et al.*, 1999).

TaRV is another virus that infects taro but there is no information on its significance. It has been classified as a reovirus based on the isolation of 10 segments of dsRNA from infected taro plants (Plant Biotechnology Program and the Regional Germplasm Centre, 2004). As yet no symptoms have been associated with the infection of TaRV and it has been detected in combination with other viruses and its impact as a taro disease is unclear. Preliminary sequence data suggest that TaRV is similar to insect-infecting reoviruses (Devitt *et al.*, 2001) and although unconfirmed, it is highly possible that TaRV is not a serious pathogen of taro.

2.2.4 Importance of DsMV and TaBV in germplasm exchange

The exchange and movement of plant genetic resources is very important for the improvement and adaptation of crops, and farmers have been carrying out this practice since the birth of agriculture. Collection, conservation and utilization of plant genetic resources as well as international exchanges are essential for national and international crop improvement programmes (Cruz-Cruz *et al.*, 2013; Zettler, 1989).

However before germplasm can be moved internationally, countries have to be satisfied that the plants are not infected by viruses and other internally borne pests. As discussed in section 2.2.3, several taro viruses are present in the Asia/Pacific region, having different distributions. FAO/IBPGR guidelines, developed in 1989 for the safe movement of edible aroid germplasm, suggest that vegetative propagating material should be transferred as sterile cultures derived from meristem-tips, and tested for microbial contamination and viruses (Plant Biotechnology Program and the Regional Germplasm Centre, 2004; Zettler *et al.*, 1989).

TaBV and DsMV are the most common viral diseases of taro, having a global distribution. DsMV reduces the photosynthesis rate of infected plants and, although not lethal, DsMV has been linked to corm yield reduction. Some studies report yield losses of up to 60% (Lebot, 2009). Yield studies conducted on performance of DsMV-infected and DsMV-free *Xanthosoma sagittifolium* in Nigaragua, found that DsMV-free planting material yielded larger number of cormels that were heavier and longer than those of infected plants. This resulted in larger yields from the virus-free plants compared to the infected plants (18.2 t/ha and 13.6 t/ha, respectively) (Reyes *et al.*, 2006).

TaBV, on the other hand, seems to have very minimal effect on the growth of taro and many of the infected plants remain symptomless (Harding, 2008). However, mixed infections of TaBV and CBDV have been attributed to the lethal disease known as alomae which can result in 100% yield loss (Rodoni *et al.*, 1994).

Infections of DsMV and TaBV are a major problem encountered in taro germplasm collections. Almost 50% of all taro accessions that have undergone virus testing at the CePaCT, SPC, have been found to be infected with either TaBV or DsMV or both[3]. This imposes severe limitations to the exchange of this globally important germplasm collection. Regardless of the fact that DsMV and TaBV have a widespread distribution, the diversity of these two viruses has not been fully studied and understood. There may be differences in strains globally, for example DsMV infected plants in French Polynesia show greater pathogenicity when compared to infections in other countries (Nelson, 2008).

To address the problem of virus infected taro germplasm and its consequence on international exchange, the current research makes initial attempts to identify potential virus elimination protocols suited to cleaning of the taro germplasm. Being able to clean the germplasm of viruses would make important genetic diversity, which is crucial for current agricultural breeding needs, and is available for regional and international germplasm exchange. New lines available at CePaCT, SPC which have come through the taro breeding programme in Samoa have tested resistant/tolerant to TLB. As discussed in section 2.2.1, some countries are vulnerable to TLB because of their narrow taro genetic base and projected climate change conditions. Other important taro diversity such as yellow fleshed taros, high in carotenoids, also needs to be shared to support good nutrition and assist in improving health of Pacific communities. Importantly carotenoids can help in the fights against non-communicable diseases (NCDs), as discussed in section 2.1.3., but all farmers and communities are being denied these benefits because of the contamination of the germplasm by viruses.

The need to mobilise the taro germplasm requires that viruses are eliminated. Virus elimination is necessary to be able to safely exchange the taro genetic diversity. Due to the limited time and resources, the research takes DsMV as a model virus to evaluate the efficacy of different elimination therapies.

[3] Approximation based on CePaCT virus indexing from 2009 to 2012.

2.3 Virus elimination techniques

Tissue culture and *in vitro* technologies, although effective in the removal of external pathogen, fail to ensure that the material is free of systemic agents such as viruses which can infect the plant which remains symptomless, and be transmitted during *in vitro* culture (Cruz-Cruz *et al.*, 2013). Techniques such as meristem extraction, chemotherapy, cryotherapy, electrotherapy and thermotherapy offer opportunities to eliminate viral infections. Using tissue culture the virus-free material can be multiplied rapidly and exchanged. Different strategies for production of virus-free plant material have been studied on many crops and a brief is given herein.

2.3.1 Meristem Culture

Meristem culture remains the most widely used method of virus elimination with success being achieved in a wide variety of crops. Morel and Martin (1952) were the first to show that meristem dissection can be used for the production of virus-free material. Presence of different viruses in the apical dome tissue has been documented in various plant species but virus-free plants have been regenerated using meristem culture (Faccioli & Marani, 1998). The use of meristem extraction is based on the principal that virus concentration is not uniformly distributed throughout the infected plant (Panattoni *et al.*, 2013). The meristematic regions are far away from differentiated vascular tissue; the vascular tissues of the primordia leaves are still developing and are not yet in contact with the vascular system of the plant. Therefore, virus particles present in mature vascular tissue can only reach the top of the meristematic region by moving slowly from cell to cell. Viruses infecting non-vascular tissues use plasmodesmata for cell to cell dissemination and this represents a slow process which makes it relatively difficult for viruses to infect the meristem (Panattoni *et al.*, 2013; Faccioli, 2001).

To ensure successful virus elimination, the meristems extracted need to be as small as possible but still be regenerated *in vitro* (Wang *et al.*, 2009). However, meristem extraction and subsequent regeneration requires a high level of skill, time and patience (Cowell, 2013; Wang & Valkonen, 2008; Love *et al.*, 1989).

Production of virus-free taro via meristem culture is often reported (Nelson, 2008). However, the data available does not confirm that meristem culture for the production of virus-free taro as a routine method. Of the literature reviewed, none described clearly the percentage of virus-free taro plantlets obtained through meristem culture. Preliminary work carried out at CePaCT, SPC was successful in the production of DsMV-free *C. esculenta* via meristem culture (Prasad 2007, pers. comm., 15 June) but the rates were low.

2.3.2 Chemotherapy

Antiviral chemotherapy has made a valuable contribution in clinical medicine but has not been used as extensively with plant viruses as thermotherapy (Panattoni *et al.*, 2013). Fruit tree research into virus elimination has shown that antiviral chemicals effectiveness in treating animal virus infections can also be successfully used to eliminate viruses in plants (James *et al.*, 1997). Chemotherapy has been used in virus elimination from a number of woody and herbaceous plant species. The antiviral agents can be sprayed directly on crops or included in the plant culture medium. Upon uptake by the plant, they inhibit virus replication. Some antiviral agents upon exposure even confer resistance (Nascimento *et al.*, 2003). Research on the use of chemotherapants for plant virus elimination from 1991-2010 has been summarized by Panattoni *et al*., (2013).

Unlike thermotherapy, the mode of action for chemotherapy has been poorly investigated. Several groups of antiviral agents that have shown potential towards virus elimination in plants belonging to inosine monophosphate dehydrogenase (IMPDH) inhibitors, S-adenosylhomocysteine hydrolase (SAH) inhibitors, and neuraminidase (NA) inhibitors. Ribavirin belongs to the IMPDH inhibitors. It has been shown to be a very effective antiviral agent against animal viruses (Panattoni *et al.*, 2013). Ribavirin targets the IMPD that converts IMP to XMP (xanthosine 5'-monophosphate) a key step in the *de novo* biosynthesis of GTP (guanosine triphosphate) and dGTP (deoxyguanosine triphosphate) (de Clercq, 2004). Adefovir dipivoxil is another antiviral agent which has been evaluated under this research. It has been shown to be active against hepadnaviruses, retroviruses and herpesviruses (Naesens *et al.*, 1997). Adefovir disrupts

DNA polymerase and reverse transcriptase (de Clercq, 2004). It has been used very effectively in the elimination of *Banana streak virus* (BSV) from banana (Helliot *et al.*, 2003).

Even though antiviral chemicals have been used successfully against many viruses, no study has reported the use of antiviral agents for the elimination of DsMV from *C. esculenta.*

2.3.3 Thermotherapy

In vivo and *in vitro* thermotherapy has been used successfully to eliminate many virus species, belonging to different families, from different plant hosts. It is one of the earliest methods used to eliminate viruses from plants (Pennazio, 1997), with the first successful heat treatment of a virus reported in 1949 (Kassanis, 1949). The mechanism by which heat eliminates viruses from plants is not fully understood but it is believed to inhibit viral replication and synthesis of movement proteins mainly by blocking transcription (Panattoni *et al.*, 2013; Mink *et al.,* 1998). Thermotherapy is based on the premise that at optimum temperature, equilibrium exists between virus replication and degradation within a virus infected plant. But if temperature increases virus replication is inhibited but virus degradation proceeds (Cowell, 2013; Panattoni & Triolo, 2010; Razdan, 2003; Walkey, 1991; Kassanis, 1957; Harrison & Harpenden, 1956). Therefore, when plants are exposed to higher than optimum temperatures (30-40°C) the virus synthesis halts but the plant is able to survive and grow. The resulting new growth (meristematic regions) becomes virus-free while the older tissue still remains virus infected (Panattoni & Triolo, 2010; Razdan, 2003; Walkey, 1991). The meristems can be removed and grown into virus-free plants.

Thermotherapy has been employed in a number of crops including grapevine, apple, peach, pear, apricot, plum, raspberry, garlic, potato, artichoke, chrysanthemum, sugarcane, sweet potato, banana, cherry, citrus, strawberry, mandarin, olive, rose, begonia, blueberry, caper, carnation, cassava, hop, horseradish, lily, peanut, phlox, strawberry, taro, tomato, *Ullucus* sp. and yam (Panattoni *et al.*, 2013).

Only a single reference was found where the elimination of DsMV was successful from *C. esculenta* with some application of thermotherapy (Li *et al.*, 2002). The authors used thermotherapy *in vivo* followed by meristem culture, reporting a success rate of 5%.

2.3.4 Electrotherapy

Black *et al.*, (1971) and Blanchard (1974) laid down the foundation for the concept of electrotherapy. Black *et al*., (1971) demonstrated that when tomato plants were exposed to low electric current densities (3-15µA/plant) growth simulation and increased levels of ions were observed in the tomato tissues. Virus-free tissues were obtained after 2-3 days by Blanchard using 1-4A direct current (DC) pulsed to 6500V/hr. These studies and their results served as a basis for electric current mediated plant virus elimination. González *et al.,* (2006) observed that electrotherapy could be reduced to temperature controlled thermotherapy at the cellular level.

Quacquarelli *et al.,* (1980) applied electric current to purified preparations of Almond mosaic virus. The virus particles were degraded and infectivity was lost. The translation of this result to *in vivo* studies by applying 500V for 5-10min to almond cuttings showing intense mosaic symptoms produced 90% cleaned plants. The buds of treated plants when grafted on healthy seedlings failed to transmit virus symptoms and no virus was recovered from sap inoculation of herbaceous host. The first comprehensive study using electrotherapy as a utility for virus elimination was in potato. Potato stems infected with Potato virus X (PVX) were exposed to different current (5, 10 or 15mA) for 5 or 15mins. These were cultured *in vitro* and upon successful regeneration tested for PVX. This study found that 60-100% of the regenerated plants were free of PVX (Lozoya-Saldana *et al.*, 1996). Since then electrotherapy has been used successfully to eliminate other viruses from potato; namely *Potato virus Y* (PVY), *Potato virus A* (PVA) and *Potato virus S* (PVS) (AlMaarri *et al.*, 2012; Pazhouhande, 2001; Mozafari & Pazhouhande, 2000).

Electrotherapy has since been successful in the elimination of *Onion yellow dwarf virus* and *Leek yellow stripe virus* from Garlic, *Banana streak virus* from Banana, *Dioscorea alata potyvirus* from yam (*Dioscorea alata*), *Dasheen mosaic virus* from *Xanthosoma sagittifolium*, *Grapevine leafroll virus* from grapevine (*Vitis vinifera*), *Tomato yellow leaf curl virus* from tomato and *Bean mosaic virus* from common bean (*Phaseolus vulgaris*) (Hormozi-Nejad *et al.*, 2011; Guta *et al.*, 2010; Falah *et al.*, 2007; Hernández *et al.*, 2002, 1997; Castro *et al.*, 2001; Lebas, 2002)

There is no reported case of elimination of DsMV (or any other virus) from *C. esculenta* using electrotherapy but electrotherapy has proven to be very successful in elimination of DsMV from *Xanthosoma sagittifolium (*L.) Schott (Castro *et al.*, 2001).

2.3.5 Cryotherapy

Cryopreservation has been utilized for the long-term conservation of plant genetic resources. Recently however, cryotherapy has also been described as an efficient method for virus elimination (Bayati *et al.*, 2011; Wang & Valkonen, 2009, 2008; Wang *et al.*, 2009, 2008, 2007, 2003; Helliot *et al.*, 2002). Brison *et al*., (1997) first demonstrated that cryopreservation could be used for virus elimination when 50% virus-free plants were obtained from plum shoots originally infected with plum pox virus.

Cryotherapy involves the excision of shoot tips, frozen in liquid nitrogen, and regenerated to plants post thawing and culture. When cryopreservation is used for cryotherapy, conditions are selected to allow survival of only a limited number of the least differentiated cells and eliminate a large portion of virus-infected tissues. Therefore, cryotherapy can result in virus-free regenerants with much greater frequency than what is typically obtained with the conventional meristem tip culture (Wang *et al*., 2008; Wang & Valkonen, 2008). The shoot tips used for cryotherapy can be relatively large without compromising successful virus elimination which makes preparation of the materials more feasible (Wang *et al.*, 2008; Wang *et al.*, 2003; Brison *et al.*, 1997).

2.3.6 Summary

The current research evaluates meristem dissection, thermotherapy, chemotherapy and electrotherapy as candidates for the elimination of DsMV from taro. Each method/therapy comes with its own challenges and opportunities. The research attempts to highlight the opportunities and address the challenges. Cryotherapy was not evaluated as it is technically difficult, resource intensive and very time consuming, because it requires mastering cryopreservation, for which there was insufficient time available. There is also the need to obtain chemicals, including liquid nitrogen which is expensive and can be inconsistent in supply. The other most important factor is the regeneration rate. Initial attempts at cryotherapy showed successful regeneration but the plantlets were very slow in growth. The aim of the research is to find an effective and quick therapy for DsMV elimination for taro. Other methods need to be evaluated first before technically challenging techniques like cryotherapy are evaluated.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 Reagents, consumables and equipment

All reagents and consumables for virus diagnostic and virus elimination were purchased either locally or sourced from overseas using funds provided by International Foundation of Science (IFS) and University of the South Pacific (USP). Tissue culture reagents and consumables were funded by the SPC, Suva, Fiji. All equipment used was available from the CePaCT, SPC lab.

3.2 Plant material

3.2.1 *In vitro* material for virus elimination

In vitro accessions of taro (*Colocasia esculenta* var. *esculenta*) were obtained from CePaCT, SPC. The taro collection at CePaCT, SPC is unique with 1159[4] accessions. It is the largest *in vitro* collection of taro diversity conserved globally. The contribution of this collection towards efforts to conserve plant genetic resource has been recognized globally and CePaCT, SPC receives long term funding from the Global Crop Diversity Trust (www.croptrust.org) to ensure that the collection is conserved safely.

Accessions for this research were selected from the breeding lines generated by the Taro Improvement Programme (TIP), Samoa. These selected accessions had demonstrated tolerance/resistance to taro leaf blight (TLB) when evaluated in field trials. The accessions were screened for viruses (see section 3.4) to determine which were infected with *Dasheen mosaic virus* (DsMV). Of the five accessions identified as infected with DsMV, only three BL/SM-97, BL/SM-129 and BL/SM-145 were available in sufficient numbers for multiplication and therefore available for use in this research.

[4] Number of accession as of July, 2013.

3.2.2 Leaf samples for DsMV diagnosis

All leaf samples needed for DsMV diagnostic studies (see Chapter 4**)** were collected from the taro field collection maintained at the Koronivia Research Station, Fiji[5]. Leaf samples were collected and packed in individual labelled zip lock bags (24cm X 18cm) (Ziploc® brand, Morris Hedstrom, Fiji) and brought to CePaCT, SPC virus diagnostic lab where they were either used immediately or stored at 4°C and used the next day.

3.3 *In vitro* culture

3.3.1 *In vitro* culture media and condition

Murashige and Skoog (1962) (MS) was used as the basic medium composition to provide the inorganic and organic compounds required for plant culture. Stock solutions of macronutrients, micronutrients and vitamins were prepared and stored in the refrigerator (≈4°C) for subsequent culture medium preparations. The components of stock solution were obtained from Sigma Aldrich, Australia.

The non thermolabile medium components were dissolved in distilled water at the required concentration and the pH adjusted to 5.8 using either 1M NaOH or HCL. Agar was added at a concentration of 7.5g/l and the solution heated on a hotplate to dissolve the agar. Medium was then transferred to culture vessels with 10ml of medium dispensed in 28ml McCartney bottles (referred to as small glass bottles henceforth) and 20ml of medium in 128ml cospack jars (referred to as big glass bottles henceforth), and autoclaved at 121°C and 15psi for 15min. Thermolabile components were filter sterilized using sterile syringe filters, (RC-membrane, 0.20µm; EBOS, New Zealand) and added to the autoclaved medium after cooling to 40-50°C. The culture room was optimized to 25°C ± 2°C with a photoperiod of 16 hours. Illumination was provided by Phillips cool daylight fluorescent tubes with photon flux density (PFD) between 30-40µMol m^{-2} s^{-1}.

[5] Koronivia research station is one of the research divisions of Ministry of Agriculture.

3.3.2 Multiplication of plant material

The selected accessions BL/SM-97, BL/SM-129 and BL/SM-145 were multiplied following protocols used at CePaCT, SPC for the mass micropropagation of taro. The basic culture medium MS, as described in section 3.3.1., was supplemented with 30g/l sucrose to provide the carbohydrate source. If solid medium was needed the MS30 (MS +30g/l sucrose) was supplemented with 7.5mg/l agar while no agar was added if liquid MS was required. The components of the media are included in the appendix (Table 3.3 and Table 3.4)

The culturing cycle used to produce material for experiments, were modified protocols of Tuia (1997). The cycle is as follows: plants were cultured on the MS30 as previously described supplemented with 0.5mg/l of Thidiazuron (N-phenyl-N'-1,2,3-thiadiazol-5-ylurea)(TDZ) for four weeks, then transferred to the MS30 supplemented with 0.8mg/l 6-Benzylaminopurine (BAP) for three weeks followed by another three weeks on the MS30 supplemented with 0.005mg/l BAP in 28ml small glass bottle containing 10ml volume of solid medium. Following the above cycle the explants were transferred to big glass bottles containing 20ml of liquid MS30 for 3 weeks to boost growth. The suckers were separated after this and the cycle repeated until enough material was available for the elimination experiments (≈200 plants for each accession). Once sufficient numbers were achieved, the accessions were cultured on solid MS30 for one month to minimize the carryover effects of the growth regulator treatments. All tissue culture activities were conducted under sterile conditions in a laminarflow cabinet, with air flow speed of $0.661 m^2 s^{-1}$.

3.4 Virus screening

Virus screening is a routine function of the CePaCT, SPC and the virus diagnostic work for this project was carried out at the CePaCT, SPC virus diagnostic lab. The protocols used for the virus diagnosis of taro are modified protocols of Revill *et al*., (2005a) and Plant Biotechnology Program and the Regional Germplasm Centre (2004). One DNA virus, *Taro badna virus* (TaBV) and four RNA viruses, *Dasheen mosaic virus* (DsMV), *Colocasia bobone disease virus* (CBDV) and *Taro vein Chlorosis* (TaVCV) have been

identified in taro as quarantine pathogens of concern. The selected accessions (section 3.2) were screened for these four viruses at the *in vitro* stage and accessions that tested positive for DsMV were grown in the screen house and retested at 3 and 6 months of growth to confirm that no other virus was present. Although only TaBV and DsMV are reported from Samoa (Davis & Ruabete, 2010) for the purpose of consistency CBDV and TaVCV were also tested at all stages. Once accessions positive for DsMV only had been identified, in this case BL/SM-97, BL/SM-129 and BL/SM-145 they were mass propagated *in vitro* for the different experiments.

3.4.1 Nucleic acid extraction

At initial stage of testing total RNA and DNA were extracted from samples using the DNeasy (Cat# 69106) and RNeasy kits (Cat# 74106) (Qiagen, Australia), respectively, according to the instructions provided by the manufacturers. Due to the need to screen large numbers and the high cost associated with kits, other methods were evaluated for use. The method described by Li *et al.*, (2008) was identified to be reliable and effective. In case of RNA extraction and stability Li *et al.,* (2008) protocol[6] was found to be better than the kit extractions (data not shown).

3.4.2 DNA virus PCR

TaBV was amplified using reaction mixture and cycling parameters described by Yang *et al*., (2003a). A 320bp fragment of the reverse transcriptase/ribonuclease H-coding region was amplified in a duplex reaction. The duplex reaction also amplified a 200bp portion of the chloroplast-encoded 23S rRNA gene from the plant chloroplast as an internal control to check the presence of amplifiable DNA. TaBV was amplified in a 25µl reaction containing 25ρmol of TaBV specific primers (TSBV-1 and TSBV-4), 1 ρmol of each internal control primer (Control 1F and Control 1R) (Table 3.1), 20mM Tris-HCl, pH8.4, 50mM KCl, 1.5mM $MgCl_2$, 200µM dNTPs (Cat #201912) (Qiagen, Australia) and 1.25U of HotStarTaq DNA Polymerase (Cat #203205) (Qiagen, Australia).

[6] See Section 4.2.4: Nucleic acid extraction

Table 3.1: Primer details for DNA virus and chloroplast DNA internal control with amplicon size and reference

Target	Primers	Sequence 5'-3'	Product Size	Reference
TaBV	TSBV-1	CKSTGYAARSAACATGGTCTTG	320bp	Yang *et al.*, 2003a
	TSBV-4	TAATCAAGYGGWGGGAGYTTCTC		
Chloroplast DNA Internal Control	Control 1F	GAGCCGTAGCGAAAGCGAGTC	200bp	Plant Biotechnology Program and the Regional Germplasm Centre, 2004
	Control 1R	GGCATTTCACCCCTAACCACAAC		

The mastercycler® gradient thermal cycler (Cat# 950000015) (Eppendorf, Australia) was used to carry out the PCR. The following cycling conditions were used: 94°C for 10min, 35 cycles of 94°C for 30sec, 57°C for 30sec, 72°C for 1min, and a final extension of 72°C for 10min).

3.4.3 RNA virus RT-PCR

CBDV, DsMV and TaVCV were all amplified using the Transcriptor One-Step RT-PCR system (Roche, New Zealand) according to the manufacturer's instructions, with each reaction containing 10ρmols of each virus-specific primer (Table 3.2). The assays for each virus differed only in their annealing temperature; these were 58°C for TaVCV, 55°C for CBDV and DsMV. Cycling conditions were as follows: 50°C for 35min followed by 1 cycle of 94°C for 2min, 35 cycles of 94°C for 30ses, 55°C (58°C for CBDV) for 30sec, 68°C for 1min followed by a final extension cycle at 68°C for 10min.

Table 3.2: Primer details for RNA virus and NAD5 gene with amplicon size and reference

Target	Primers	Sequence 5'-3'	Product Size	Reference
DsMV	DsMV 3F	AGTACAAACCTGARCAGCGTGAYA	540bp	Maino, 2003
	DsMV 3R	TTYGCAGTGTGCCTYTCAGGT		
CBDV	TaroP3F	ACTGCTGGAAACGGTTTCTTTGCC	150bp	M. Dowling, P. Revill, J. Dale and R. Harding, unpublished
	TaroP3R	ATTGCTGTGGAGTTCCCAGCAACA		
TaVCV	TVC1	ATAATCCAGCTTTACATTCACTGAC	220bp	Revill *et al.*, 2005b
	TVC2	TGCCTGGGCTTCCTGAGATGATCTC		
Plant *Nad5* mRNA	mtR1	ATCTCCAGTCACCAACATTGGCAT	185bp	Lee & Chang, 2006
	mtF2	GCTTCTTGGGGCTTCTTGTTCGATA		

3.4.4 Quality control

Appropriate controls were included in each PCR/RT-PCR reaction. Four controls were used to ensure accurate interpretation of the PCR results as there are occasions when non-specific bands, amplified from plant genomic DNA, may appear on the gel.

3.4.4.1 Positive and negative control

The first control used was a known infected sample (positive control). The inclusion of the positive control showed that the PCR was working and that the band produced was of the expected size. This was used as a comparison with other amplified bands. The second control was a known negative sample (healthy control). This gave the assurance that samples without viral infection were not amplifying PCR products. The third control was the water-only control (no template control), which ensures that the reagents being used were not contaminated.

3.4.4.2 Internal control

Internal controls were used for both RNA and DNA PCR to check that the extracted nucleic acid was of amplifiable quality. Primers for chloroplast DNA and Plant *Nad5* mRNA were used to amplify plant DNA and RNA respectively to ensure that nucleic acids were being extracted properly and that PCR was working as expected.

CHAPTER FOUR

DASHEEN MOSAIC VIRUS (DsMV) DETECTION IN TARO

4.1 Introduction

DsMV has been well characterised, based on isolates from USA, Taiwan and Korea (van der Want & Dijkstra, 2006; Kim *et al.*, 2004; Li *et al.*, 1998; Pappu *et al.*, 1994 a, b). The isolates from 16 countries including PNG, Samoa, Solomon Islands, French Polynesia, New Caledonia, and Vietnam were collected and the coat protein-coating sequence were sequenced and analyzed by Maino (2003) to develop both serological and PCR-based diagnostic tests.

Both serological and molecular methodologies are available for the detection of DsMV. Enzyme-linked immunosorbent assay (ELISA) is often the preferred method for the diagnosis of DsMV as it is thought to be less technical and resource demanding compared to RT-PCR, but recent work has shown that ELISA detection may not be as sensitive (Taylor *et al.*, 2012; Huang *et al.*, 2005). In this section we compare commercially available ELISA kits and RT-PCR developed by Maino (2003) for the detection of DsMV.

4.2 Materials and methods

4.2.1 Leaf samples

Fifty plants were randomly sampled from the Koronivia Research Station (KRS) taro germplasm collection, Fiji. The sampled plants were about 4 months old. The first three new leaves were collected from each plant and brought to the virus diagnostic lab of the CePaCT, SPC situated at Narere, Fiji. Leaves from each sampled plant was pooled together and used to extract RNA/total nucleic acid. In cases where the leaves could not

be processed on the same day they were wrapped in tissue paper and placed in the refrigerator at 4°C for processing the following day.

4.2.2 Symptomatology

During leaf collection any symptoms observed were recorded against the sample. This was tabulated alongside the results obtained from ELISA and RT-PCR.

4.2.3 DsMV ELISA

ELISA was performed using the DsMV double antibody sandwich (DAS)-ELISA kit (Cat# SRA48000) obtained from Agida® Inc, France. The DAS-ELISA was carried out as per the manufacture's instruction. The protocol is summarized below with buffer recipes append (see to Appendix):

Extract preparation

1. Leaf tissue from the first 3 new leaves was pooled and grinded at a ratio of 1:10 (tissue weight in gram: buffer volume in ml); 0.1g of leaf tissue in 1ml of general extraction (GE) buffer. The sample and buffer were placed in a 2ml microcentrifuge tube containing a 6mm steel bead (Qiagen, Australia) and grinded using the TissueLyser II (Cat# 85300, Qiagen, Australia) for 4min at 30rev/s. The TissueLyser rack was rotated after 2minutes to ensure uniform grinding. Appropriate positive (Cat# LPC 48000) and negative (Cat# LNC 48000) controls were included. All buffer recipes are listed in the appendix.
2. The extracts were centrifuged at 12,000 RPM for 1 min at room temperature using an eppendorf miniSpin centrifuge (Cat# 5452 000.018) (Eppendorf, Australia) and then stored on ice until used.

DAS-ELISA

3. Nunc microtitre plates (MTP) were labelled with sample locations.
4. The capture antibodies (Cat# CAB 48000) was diluted to 1:100 concentration using 1X carbonate coating buffer and each well of the MTP was loaded with

100µL of the diluted antibody. The plate was incubated for 2-4hrs at room temperature in a humid box.

5. Following the incubation the plate was emptied into a waste container. The plates were washed with 1X PBST by completely filling the wells and then quickly empting them. This was repeated twice. The plate was then turned upside down and tapped on a paper towel to remove all excess fluid.
6. The MTP plates were loaded with 100µL of the prepared extracts; duplicate wells per sample. The remaining extracts were stored at 4 ºC until completion of the test. The plate was incubated for 2hrs at room temperature in a humid box.
7. The enzyme conjugate (Cat# ECA 48000) was diluted to 1:100 with 1X ECL buffer 10 minutes before washing the plates.
8. After the completion of sample incubation the plate was emptied into a waste container. The plates were washed with 1X PBST by completely filling the wells and then quickly empting them. This was repeated seven times. The plate was then turned upside down and tapped on a paper towel to remove all excess fluid.
9. Then 100 µL of prepared enzyme conjugate was dispensed per well and the plate was incubated in the humid box for another 2 hours at room temperature.
10. Fifteen minutes before the completion of the conjugate incubation PNP tablet (Cat# ACC 00404) was dissolved in PNP solution, at a concentration of 1 mg/ml. The solution was kept away from light.
11. The plate was washed 8 times using the washing procedures described previously and tapped on paper towel to get rid of remaining fluid.
12. Then 100ml of PNP solution was dispensed per well of the plate and incubated for 60min in the humid box protected from light at room temperature.
13. After the incubation, colour change was observed visually and the absorbance measured on a Multiskan™ GO Microplate Spectrophotometer (Cat# 5111930) from Thermo Fisher Scientific, New Zealand.

The ELISA results were taken to be positive if their absorbance values were more than 2.5 times that of the negative control. ELISA test was carried out with three repetitions including positive and negative controls.

4.2.4 Nucleic acid extraction

4.2.1.1. Qiagen RNeasy kits

Total RNA was extracted using the RNeasy Plant Mini Kit as per the manufacturer's instructions. Lysis buffer was supplemented with 1% β-mercaptoethanol (β-ME) before use. 50mg of leaf sample was used for the extraction; tissues were taken from the first 3 new leaves and pooled together. TissueLyser II was used for the sample disruption and homogenisation. The samples were frozen in liquid nitrogen (BOC (Fiji) Ltd, Fiji) before disruption. 50µL of sterile water was used to elute the RNA into 1.5ml microcentrifuge tubes.

4.2.1.2. CTAB

CTAB extractions were carried out according to Li *et al.,* (2008) but with some modification. Briefly, 100mg of leaf tissue (sampled from the first 3 new leaves and pooled together) was placed in a 2ml microcentrifuge tube (Eppendorf, Australia) containing 6mm stainless steel bead and 1ml of CTAB buffer (2% CTAB, 2% PVP-40, 100 mM Tris–HCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, and 0.2% 2-mercaptoethanol). All extraction chemicals used, unless stated otherwise, was obtained from Sigma-Aldrich (Australia) and was molecular grade. The tubes were cooled to -20°C for 5-15mins until the contents of the tubes were partially frozen. After cooling, the samples were homogenized in the TissueLyser II for 4min at 30rev/s. The homogenate was incubated at 65°C for 15 min and centrifuged at 10,000g for 5 min. Then 650µl of the supernatant was transferred to a 1.5ml microcentrifuge tube and mixed with an equal volume of chloroform/isoamyl alcohol (24:1) before centrifugation at 14,000g for 10min. After centrifugation, 500µl of the supernatant was transferred to a 1.5ml microcentrifuge tube containing 350µl of isopropanol, and mixed by inverting. The mixture was centrifuged at 14,000g for 10 min. The pellet was washed with 70% ethanol by centrifugation at 14,000g for 5 min, air-dried and resuspended in 100µl of 20mM Tris–HCl, pH 8.0. The extracts were used for RT-PCR the same day or kept at -20°C until they were used.

4.2.5 Primers

DsMV RT-PCR was carried out using the primers developed by Maino (2003). The primers specific to plant mitochondrial NADH dehydrogenase (nad5) gene were used as internal control to check the integrity of the extracts and rule out false negatives (Lee & Chang, 2006). Working solutions of primers were kept at -20°C. Primers were obtained from Invitrogen (Australia).

Table 4.1: The targets, names, sequences and the expected product sizes of the primer pair for RT-PCR

Target	Primers	Sequence 5'-3'	Product Size	Reference
DsMV	DsMV 3F	AGTACAAACCTGARCAGCGTGAYA	540bp	Maino, 2003
	DsMV 3R	TTYGCAGTGTGCCTYTCAGGT		
Plant *Nad5* mRNA	mtR1	ATCTCCAGTCACCAACATTGGCAT	185bp	Lee & Chang, 2006
	mtF2	GCTTCTTGGGGCTTCTTGTTCGATA		

4.2.6 RT-PCR

RNA integrity check was performed prior to DsMV PCR. This was carried out by using a *NAD5* PCR. Both *NAD5* mRNA and DSMV were amplified in simplex reactions using the Transcriptor One-step RT-PCR kit (Cat# 04655885001) from Roche, New Zealand, according to the manufacturer's instructions with each reaction containing 10ρmols of specific primer.

PCR was carried out using each set of primers. The amplification was carried out using the MyCycler^{TM} Personal Thermal Cycler (BioRad, New Zealand). Cycling conditions for each reaction was as follows:

Table 4.2: The cycling parameters of each target under RT-PCR

Target	Cycling Parameters
DsMV	25min at 50°C, 2 min denaturation step at 94°C, followed by 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 68°C for 30 sec, and a final extension step at 68°C for 10 min
Plant *Nad5* mRNA	25min at 50°C, 5min denaturation step at 94°C, followed by 30 cycles of 94°C for 30 sec, 52 for 30 sec, 72 for 30 sec, and a final extension step at 72 for 10 min

Following PCR amplification the DNA was analyzed by electrophoresis in 2% agarose gels in Tris-Borate-EDTA (TBE) buffer (Qiagen, Australia) containing 1ppm of SYBR™ Safe Dye (Cat # S33102, Life Technologies, Australia). The PCR products were mixed with 5X nucleic acid sample loading buffer (50 mM Tris-HCl, pH 8.0, 25% Glycerol, 5 mM EDTA, 0.2% Bromophenol Blue, 0.2% Xylene Cyanole FF) (Cat # 161-0767, Bio-Rad Laboratories Pty Ltd, Australia) and loaded on the gel. DNA ladder (100bp) from BioRad (Cat #170-8202) was also included in the gel for size determination. DNA was subsequently electrophoresed at 70 volts for an hour and visualized and photographed using the Gel Doc™ System (Bio-Rad Laboratories Pty Ltd, Australia).

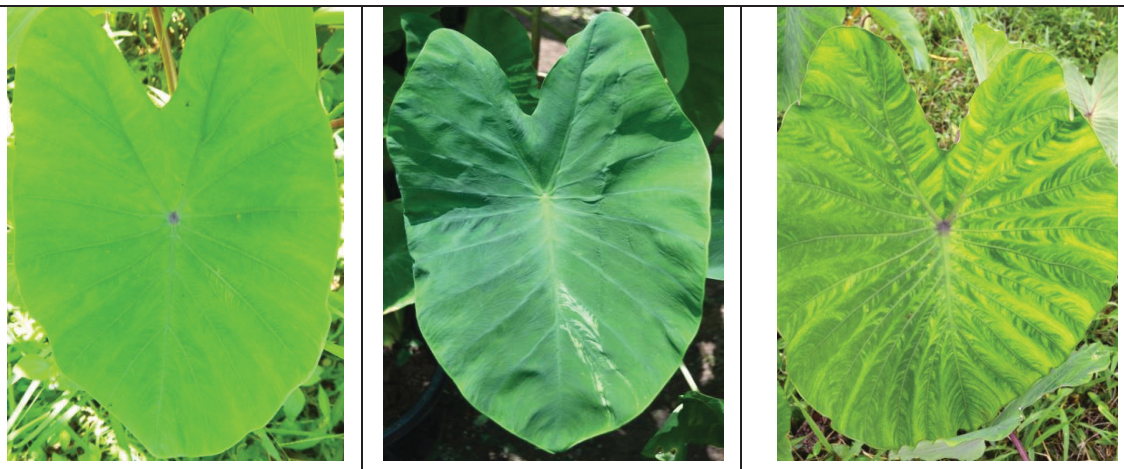
4.2.7 Statistical analysis

Comparison of DsMV infection rates as detected by ELISA and RT-PCR was carried out using chi-square contingency test (with Yate's correction) so as to determine an association between the two diagnostic tests, ELISA versus PCR and the frequency of positive versus negative diagnosis.

4.3 Results

4.3.1 Symptomatology

Virus symptoms were recorded when sampling the taro plants at KRS. Some of the symptoms observed during taro leaf collection are shown in Figure 4.1.



(A.) No symptoms	(B.) Mild Symptom	(C.) Severe symptoms

Figure 4.1: Leaf showing no symptom, mild symptom and severe DsMV symptoms

In DsMV infected plants where symptoms are present, a conspicuous feathery-like mosaic is seen. DsMV symptoms are seen above in B and C.

4.3.2 Detection of DsMV using ELISA

The sensitivity of ELISA was determined by using a tenfold serial dilution (down to 10^{-3}) of infected taro leaf extract. The absorbance values are represented in Figure 4.2.

As seen in the graph (Figure 4.2) the undiluted sample gave the highest absorbance with 1/10 also giving a significantly higher absorbance value than the positive threshold. The absorbance at 1/100 was very close to the positive threshold and was taken as a suspect result while the absorbance beyond this point was taken as unreliable.

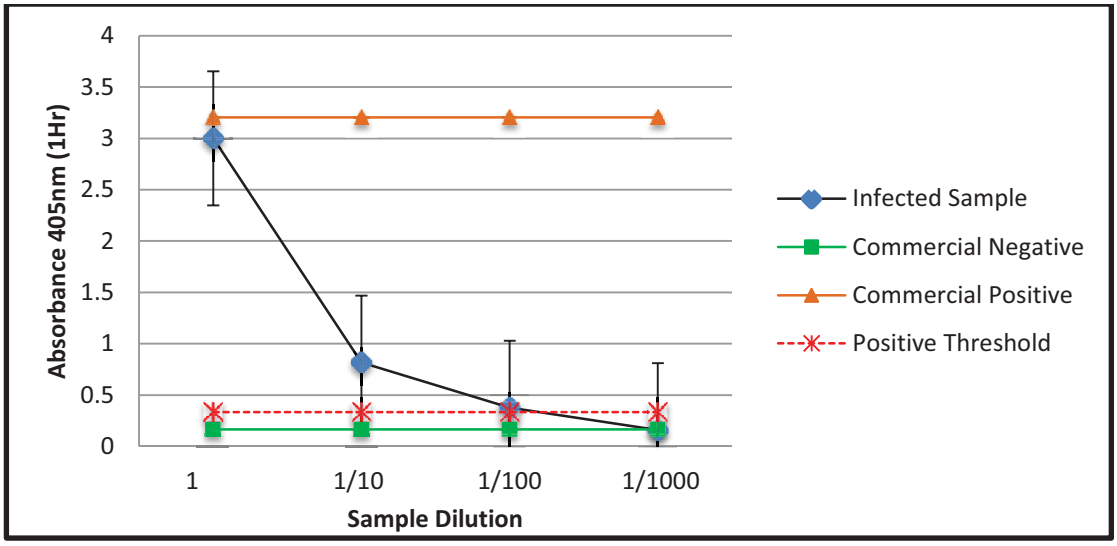


Figure 4.2: Graphical representation of absorbance values of taro leaf samples tested by ELISA.

A sample was taken to be positive if the average absorbance of the 3 replicates was more than 2.5 times the value of the average absorbance of the negative sample. Table 4.4 summarizes the results of DsMV testing of samples collected from KRS and the results of individual samples tested by ELISA testing.

4.3.3 Nucleic acid extraction and RT-PCR

Two extraction methods were compared to determine which one would be most efficient for use in this project. The RNeasy kit required small amount of plant material and produced low yields of total RNA. In addition, on several occasions, the nucleic acid failed to amplify the products in PCR when checked with the NAD5 gene primers. However the CTAB method was very effective. This was shown by the NAD5 gene amplification. The CTAB extraction failed only 1 out of 50 extracts while it was observed that the RNeasy extracts would fail 5 out of every 50 attempts. Further CTAB extraction did not require the use of liquid nitrogen. Liquid nitrogen is hazardous and also there is only one company, BOC Fiji Ltd, supplying it in Fiji and they do not have a dependable supply. CTAB extraction was much cheaper compared to the RNeasy kits.

The TissueLyser allows for the effective homogenization of 48 samples at one time and avoids the laborious task of sample grinding. It also avoids possible contamination that can be introduced when grinding samples in liquid nitrogen using mortar and pestle, and it is time efficient

RT-PCR amplification of nucleic extracted using the DsMV 3F and DsMV 3R primer pair produced a specific DNA band of about 540bp while the RT-PCR using the mt-R1 and mt-R2 primer pair amplified an 185bp sized band conforming to the presence of DsMV and *NAD5* gene RNA (Figure 4.3).

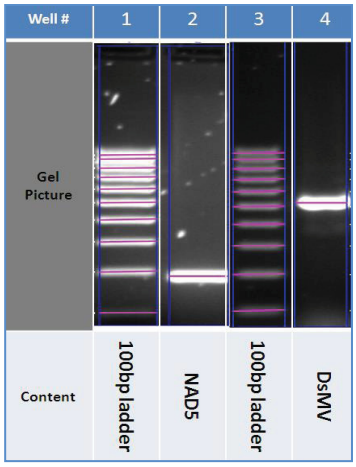


Figure 4.3: Gel picture showing the NAD5 and DsMV PCR products

4.3.4 Comparison of ELISA and RT-PCR results

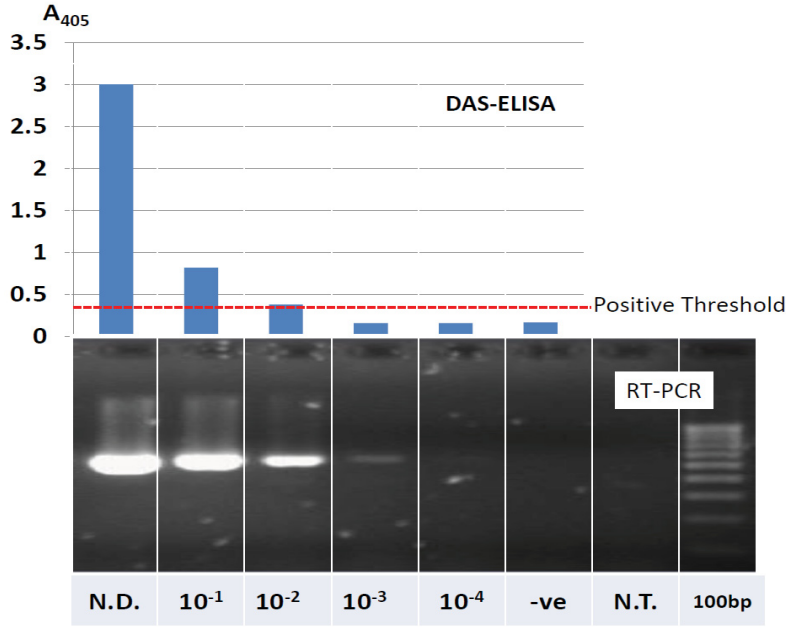
Eighteen (36%) out of 50 samples collected from KRS tested positive for DsMV by RT-PCR and 10 (20%) by ELISA (

Table 4.3). Forty two samples (84%) showed identical results in both assays (10 positive and 32 Negative), while 8 samples tested positive by RT-PCR but negative by ELISA. There was no statistically significant association between the diagnostic test (ELISA vs. PCR) and the frequency of positive versus negative results, χ^2 (d.f. =1) =2.43, p=0.119. Refer to figure 4.5, 4.6, 4.7 & 4.8

Table 4.3: Comparison of ELISA and RT-PCR assays for the detection of DsMV from field collected taro leaves.

	RT-PCR		ELISA	
	# of sample	% of sample	# of sample	% of sample
DsMV negative	32	64%	40	80%
DsMV positive	18	36%	10	20%
Total	**50**		**50**	

The sensitivity of ELISA and RT-PCR was compared using a tenfold serial dilution of the nucleic acid extracts. Samples containing DsMV were ELISA-positive in undiluted preparations or in preparations diluted not more than 100 times while PCR was able to ampilfy the viral RNA from 1/1000 dilution. The results show that RT-PCR is capable of detecting the virus titre up to 10^{-3} as depicted by the weak band (Figure 4.4). RT-PCR was more sensitive than ELISA for the detection of DsMV.



N.D. - undiluted sample, 10^{-1} to 10^{-4} tenfold serial dilution of DsMV infected, -ve –healthy *C. esculenta* sample, N.T. - No Template Control, 100bp - BioRad 100bp DNA ladder.

Figure 4.4: Sensitivity of DsMV detection by ELISA and RT-PCR.

4.4 Discussion

The diagnostic for DsMV has been developed using both serological and molecular techniques. The aim of this research was to compare the available serological and molecular techniques to check their sensitivity towards DsMV detection. An apparent uneven distribution of DsMV has been observed in taro and reliable results were only obtained using pooled tissues from different leaves rather than individual leaves (Hu *et al.*, 1995). This was also observed by Taylor *et al.,* (2012) whereby in a single plant, DsMV was not detected on the first and second new leaf but was only present on the third leaf. To overcome false negatives due to uneven distribution of virus particles, leaf tissue used for each protocol was taken randomly from the first 3 leaves and pooled together for RNA extraction.

Sensitivity assays for DsMV revealed that RT-PCR was more sensitive than ELISA, with detection thresholds of 10^{-3} and 10^{-2}, respectively. In summary detection sensitivity for DsMV by RT-PCR was 10 times higher than ELISA. These results were further confirmed by the testing of field samples. Eighteen samples out of 50 were detected as DsMV positive by RT-PCR but ELISA was positive for only 10 of these 18. None of the samples that tested negative by RT-PCR came out positive in ELSIA. Randles *et al.,* (1996) states that PCR-based detections are generally reported to be more sensitive than serological-based for the detection of plant pathogens such as viruses.

The results of field sample testing indicate that ELISA could detect DsMV from all symptomatic samples but failed to detect DsMV in some of the symptomless samples (Table 4.4), however RT-PCR was able to detect DsMV regardless of whether symptoms were present or not. Revill *et al.,* (2005) in their surveys reported detecting DsMV from taro showing typical feathery mosaic symptoms and also detecting DsMV in a large number of plants that appeared healthy. The sensitivity of RT-PCR makes it ideal for the detection of DsMV, especially since it is able to detect DsMV at lower concentrations.

To avoid "false negative" results, internal controls were used to check the integrity and quality of the RNA extracts before testing for DsMV. Different plant internal controls have been developed for verification of plant RNA quality and incorporated into virus detection systems. Some of these are 18S rRNA (ZhiYou *et al.*, 2006), chloroplast NADH dehydrogenase ND2 and subunit (ndhB) mRNA (Thompson *et al.*, 2003) and mitochondrial NADH dehydrogenase (nad5 and nad2) mRNAs (ZhiYou *et al.*, 2006; Menzel *et al.*, 2002). The primer pairs specific to plant mitochondrial nad5 mRNA was used as the internal control in this research. It amplified a 185-bp fragment of the plant mitochondrial nad5 mRNA (Figure 4.3). However if a sample failed to amplify the required fragment it was discarded and the extraction was redone.

In the current research ELISA was able to detect DsMV albeit not as effectively as RT-PCR. It can still be an important tool for routine screening for DsMV. ELISA is relatively cheap, simple to use and it can be used to test large number of samples simultaneously. ELISA testing does not require as much technical expertise as required for RT-PCR. Also when comparing costs of ELISA to RT-PCR, ELISA is 15 times cheaper[7]. ELISA can still serve an important purpose in national DsMV surveillance and quarantine screening. ELISA can be used as a tool to test planting material, to rogue out infected material to maintain clean seed stock. If yearly or two yearly testing's were done and DsMV infected material rouged out, then the inoculum level of the virus would be reduced and further spread would be suppressed.

However, for germplasm exchange the most sensitive of methods is needed to ensure that the material being exchanged is of the highest phytosanitary standards. To date RT-PCR detection of DsMV still remains the most sensitive test available and is used for testing of germplasm prior to distribution. RT-PCR and ELISA can play a complementary role in the production of DsMV-free *C. esculenta.* RT-PCR systems can be used by germplasm repositories such as CePaCT, SPC, to test and certify material DsMV-free. These materials then can be used by countries which can use ELISA in local systems to ensure that a local clean stock is maintained and that DsMV load in the

[7] Estimates based upon 2012 costs of extraction reagents, RT-PCR kits and ELISA kits

field is kept low by annual screening and rouging of infected plants. This would ensure that a uniform production is maintained and any yield losses due to DsMV infection negated. This is very appropriate for many South Pacific countries as they do not have infrastructure required for molecular diagnostics, yet at the same time need to maintain good production of taro to meet the local and export demands. As reported by Reyes *et al.,* (2006) DsMV is linked to yield losses.

CHAPTER FIVE

DASHEEN MOSAIC VIRUS (DsMV) ELIMINATION FROM TARO

5.1 Introduction

Movement of taro germplasm or for that matter any planting material must meet the phytosanitary standards requirements of the importing country. National quarantine regulations and international laws restrict movement of plant genetic resource (PGR) that do not adhere to these standards. Of the pathogens of concern, viruses are of particular importance. Viruses infect most crop species but are particularly common in vegetatively propagated crops like potato, sugarcane, cassava, sweet potato, beet, taro, cocoyam, yam, onion, banana, citrus, apple, strawberry and certain ornamental plants. These are generally propagated continuously over many years and this provides ample opportunities for infection with one or more virus (Sastry & Zitter, 2014). The virus infection can range from being insignificant (such as infections by latent viruses) to severe impacts on crop production (e.g. *Banana bunchy top virus* in banana, Alomae disease in taro). These present challenges in both production and germplasm exchange. A classic example is *Banana bunchy top virus* (BBTV) which is a debilitating virus disease of banana. It has been inadvertently spread both nationally, regionally and internationally via the exchange of infected plant materials, such as suckers and corms, before proper diagnostic procedures were available (Tsao, 2008; Wardlaw, 1961).

The symptomless nature of many viruses' means that infected planting material can go unnoticed and continue to be used in subsequent planting cycles. Accumulation of virus infection over years of planting can result in reduced productivity. Reduction in productivity has been recorded in many crop species. Reyes *et al.,* (2006) showed that yields from DsMV-infected *Xanthosoma sagittifolium* were lower than DsMV-free *X. sagittifolium*.

Utilisation of virus-free planting material is one solution to controlling viral diseases of vegetatively propagated crops (Cowell, 2013; El Far & Ashoub, 2009; Lozoya-Saldaña & Dawson, 1982a). The loss in field production due to virus infection can also be reduced by using virus-free planting material. Provision of virus-free planting material would require a repository, such as CePaCT, SPC, to be able to produce virus tested clean material that can be accessed by national country genebanks, which then would supply on to the farmers.

Virus-free planting material is necessary not only for sustaining production levels but also for introduction/exchange of new cultivars/varieties. Disease resistant taro varieties such as those that have been identified as taro leaf blight (TLB) resistant as well as taro varieties identified tolerant to climatic conditions such as salinity need to be exchanged between countries. For the safe exchange and to meet country quarantine regulations, taro plant materials need to be virus tested and where found to be infected with virus(es) these materials must be cleaned.

Protocols for the virus diagnostic of taro (see Chapters 2 & 3) are well defined and routinely used for testing of taro accessions conserved at CePaCT, SPC. However the elimination of viruses infecting taro have not been defined yet. Very few studies have looked at the elimination of viruses from taro. In fact only one study was found to have looked at the elimination of DsMV from taro using thermotherapy (Li *et al.,* 2002) while another looked at the elimination of DsMV from *Xanthosoma* sp. (Castro *et al.*, 2006). However this is limited work and does not provide a good foundation for a protocol to routinely be used for the cleaning of DsMV from taro.

The research in this study has focused on the elimination of DsMV only, reason being:

1. It is one of the most widespread virus infection in the CePaCT, SPC taro collection
2. It is of quarantine concern due to apparent differences in strains that exist. Strains found in French Polynesia appear to be more virulent (Nelson, 2008) compared to elsewhere.

3. It is attributed to yield loss (Reyes *et al.*, 2006).
4. Limited resource available meant that it would be beneficial to work on a model virus, i.e. DsMV, to evaluate the elimination potential of the suite of protocols available.

A review of plant virus elimination methodologies has been reported in section 2.3. As discussed, there are 5 main techniques used for plant virus elimination, meristem extraction, chemotherapy, thermotherapy, electrotherapy and cryotherapy, but only four therapies were evaluated under the current research.

In this chapter meristem extraction, chemotherapy, thermotherapy, and electrotherapy have been used to eliminate DsMV from taro. TLB resistant taro accessions (BL/SM-97, 129 and 145) were used for the elimination experiments. These accessions were chosen because they are important for exchange as they are resistant to TLB. In addition, these accessions had singular infection of DsMV as well as numbers conducive to building up stocks for elimination trial within the timeframe of the research. Since very little research has been aimed at the elimination of viruses from taro, preliminary experiments were conducted to determine suitable parameters (optimum dosages, duration etc) for chemotherapy, thermotherapy and electrotherapy. Based on the results, experiments were designed to evaluate the elimination potential of each technique.

5.2 Materials and methods

5.2.1 *In vitro* plant material

DsMV infected accessions BL/SM-97, BL/SM-129 and BL/SM-145 were multiplied *in vitro* as described in section 3.3.2. The plantlets after multiplication were maintained in 28ml McCartney bottles (referred to as small glass bottles henceforth) containing 10ml of solid MS30 for at least a month prior to carrying out any research work. This was done to minimize carryover effects of the growth regulators used during the multiplication process. These were considered as stock plants.

For each virus elimination technique, 20 replicates of DsMV-infected plantlets of each of the three accessions; BL/SM-97, BL/SM-129 and BL/SM-145, were used. The plants were placed randomly on the culture shelves with standard lighting conditions (as described in Chapter 3) before and after elimination treatments. For each treatment the plants were randomly selected from the stock plants.

Four techniques of virus cleaning/elimination were evaluated; these are meristem extraction, thermotherapy, chemotherapy and electrotherapy. Meristem extraction was used individually as well as in combination with the other techniques to determine the optimum elimination and recovery frequencies.

Differences in response to each treatment by the different accessions were not considered in the research and taken as negligible because the aim of this research was to consider the efficiency of DsMV elimination on taro (*Colocasia esculenta*) rather than on the different genotypes/varieties.

5.2.2 Plant growth

The time taken at each growth stage was recorded. Not all plants grew at the same rate so the time recorded was when 90% of the plants had reached the completion of the stage. After each treatment the following procedure was followed for meristem extraction, establishment and plant growth:

Meristem extraction and establishment (Stage 1) - Meristems (size dependent on treatment; ≈0.5mm for meristem extraction and 1-3mm for all other treatments) were excised in sterile conditions under the Olympus dissecting microscope (Biolab, New Zealand) using scalpels and forceps. Following meristem extraction the meristems were soaked in 5% bleach (Brand name: Whit King® containing 4.2% (w/v) sodium hypochlorite) for 5min and washed twice with sterile distilled water for 10mins. For meristem extraction, thermotherapy and electrotherapy experiments the meristems were cultured in small glass bottles containing solid MS30, however, for chemotherapy the meristems were cultured in liquid MS30 supplemented with the antiviral agent (section 5.2.6). The cultures were kept in the dark (placed in a box covered with aluminium foil) in the tissue culture room optimized for temperature of 25°C ± 2°C for 7 days, and then under standard illuminated conditions (16hr light/8hr dark regime at 25°C ± 2°C with illumination of photon flux density (PFD) between 30-40µMol m^{-2} s^{-1} provided by Phillips cool daylight fluorescent tubes). The meristems were kept under this condition until they were at least 1cm in length.

Plant growth (Stage 2) – When the plants reached a minimum length of 1cm (after meristem establishment stage) they were transferred to solid MS30 in small glass bottles and left at standard illuminated conditions until they reached heights of 3-4cm.

Final growth stage (*in vitro*) (Stage 3) – after stage 2 plants were transferred to 128ml cospak jars (referred to big glass bottles henceforth) containing 20ml of liquid MS30 and placed under standard illuminated conditions until they were above 6cm in height. This was taken to be the optimum plant size for transfer to the post entry quarantine screen house (PEQ).

Acclimatisation (*in vivo*) (Stage 4) – after the final growth stage the plants were transferred to the PEQ for acclimatisation. The *in vitro* plants were washed of all media without damaging the roots and planted in pots containing 1 part styrofoam balls (3mm)

to 3 parts of potting mix. The plants were covered with clear plastic bags to ensure that humidity was maintained. The plastic bags were gradually removed after one month.

5.2.3 DsMV testing

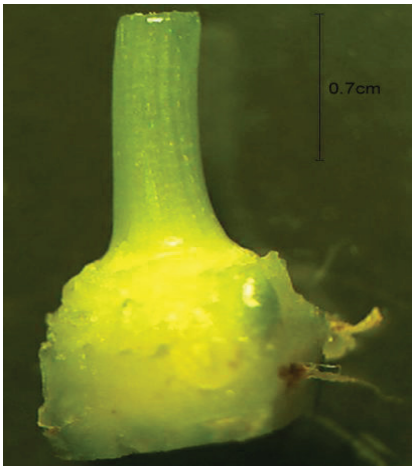
After three months of growth in the PEQ, the first three new leaves were sampled for 1st DsMV testing. They were grown for an additional six months and again tested for DsMV using the pooled samples of the first three new leaves. Each plant was tested twice (i.e. 3 and 6 months) for DsMV using RT-PCR as described in Chapters 3 & 4. If the first two DsMV test results were positive the plant was not tested any further but if it was still found to be DsMV-negative a third testing was done at seven months after planting. The rationale being that all the positive plants were detected at least in one of the testing and six months is taken to be a long enough period for all infections to be picked up. The third testing just confirms the results of the DsMV-free accessions.

5.2.4 Elimination via *in vitro* multiplication/control

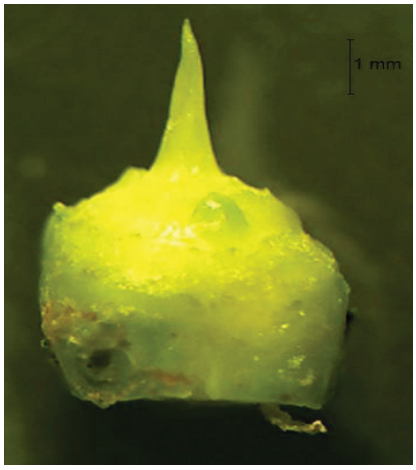
To find out if DsMV was being eliminated during the tissue culture and multiplication cycle, 20 plantlets from each accession were randomly selected and meristem of 1-3mm were extracted and cultured as described in section 5.2.2. The meristems were allowed to grow until they were ready for virus testing. The plants were tested for DsMV as described in sections 5.2.3 and chapters 3 & 4. This treatment was taken as the control treatment.

5.2.5 Meristem extraction

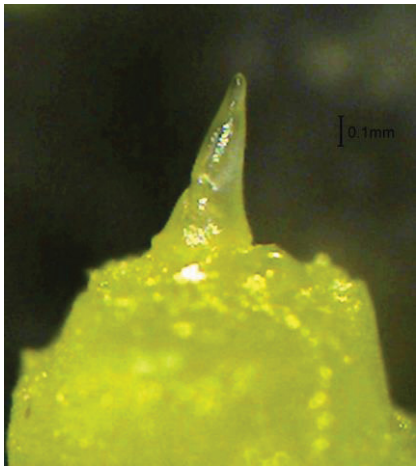
Meristems (apical domes with 1-2 leaf primodia or ≈0.5mm size) of each variety were stereoscopically excised under sterile conditions using the dissecting microscope (Figure 5.1). Meristems were then cultured as described in section 5.2.2 and tested for DsMV according to methods described in section 5.2.3 and Chapters 3 & 4.



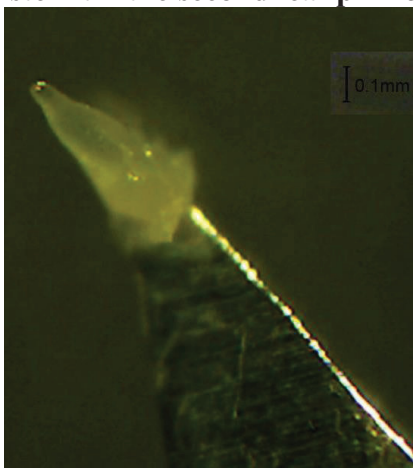
Trimmed shoot



Meristem till the second leaf primodia.



Last leaf primodia



Meristem on the tip of a scalpel

Figure 5.1: Photos depicting the meristem extraction process

5.2.4 Thermotherapy

5.2.5.1. Determination of thermotherapy parameters

Prior to thermotherapy the plantlets were transferred from small glass bottles (stock plants) to big glass bottles containing 20ml of liquid MS30 and grown for two weeks before any thermotherapy treatment.

Contherm Biosyn Series 6000 (EBOS, New Zealand) plant growth camber was used for all thermotherapy experiments. The plants were kept under standard lighting conditions

(16 hours photoperiod with illumination provided by Phillips cool daylight fluorescent tubes with photon flux density (PFD) between 30-40μMol m^{-2} s^{-1}) for 28 days.

To determine the thermotherapy treatment parameters that would be best suited for DsMV elimination two experiments were designed:

Experiment one: Constant temperature regime

Five replicates of plantlet from each of the three accessions were exposed to different temperature treatments, 25°C, 30°C, 34°C and 35°C[8] (Table 5.1) for 28days after which the meristem were extracted and cultured as described in section 5.2.2. and percentage surviving after 56 days was recorded as the survival rate.

Table 5.1: Constant temperature regime experiment design

Accession	Temperature (°C)				
	25°C	30°C	34°C	35°C	36°C
BL/SM 97	5	5	5	5	5
BL/SM 129	5	5	5	5	5
BL/SM 145	5	5	5	5	5
	15	15	15	15	15
Total Plants = 75					

Experiment two: Alternating temperature regime[9]

For each treatment five replicates of plantlet from each of the three accessions were exposed 16hrs (standard lighting conditions) at 30°C followed by 8hrs (darkness) at 25°C for 28days. This was repeated for 34°C, 35°C, 36°C, 37°C, 38°C and 40°C (Table 5.2). After the experiment meristem were extracted and cultured as described in section 5.2.2. The percentage surviving after 56 days was recorded as the survival rate.

[8] Temperatures beyond 35°C were not evaluated as the survival rate at 35°C was only 20%.

[9] Data not shown but 37°C day (16hrs) and 28°C night (8hrs) were also evaluated but the survival rates were below 40%.

Table 5.2: Alternating temperature regime experiment design

Accession	Temperature (°C)[10]							
	25°C	30°C	34°C	35°C	36°C	37°C	38°C	40°C
BL/SM 97	5	5	5	5	5	5	5	5
BL/SM 129	5	5	5	5	5	5	5	5
BL/SM 145	5	5	5	5	5	5	5	5
	15	15	15	15	15	15	15	15
	Total Plants = 120							

5.2.5.2. Thermotherapy treatment

Results from experiments conducted under section 5.2.5.1 were used to determine the parameters for thermotherapy experiment. It was found that under constant exposure to elevated temperatures (Experiment 1 of section 5.2.5.1) taro plantlets suffered stress such as wilting and leaf browning and meristem survival was lower than 50%. However, when exposed to alternating temperature regime the survival was higher at the same temperatures (results presented in Section 5.3.3). Virus elimination is more likely to happen at higher temperatures (35-40°C) (Panattoni, 2013) but survival of taro when exposed to constant temperatures above 35°C for 28 days was greatly reduced. Taking into consideration that the likelihood of virus elimination is greater at higher temperatures (Fløistad *et al*., 2011; Tan *et al*., 2010; El Far & Ashoub, 2009; Křižan & Ondrušiková, 2009; Paprstein *et al*., 2008; Sharma *et al*., 2008; Wang *et al*., 2006a; Arif *et al*., 2005; Verma *et al*., 2005; Robert *et al*., 1998; Keller, 1992; Lozoya-Saldaña & Dawson, 1982a, 1982b; Nyland, 1969) and that the taro plantlets are able to survive higher temperatures for shorter periods than a period of longer duration, therefore, an alternating temperature regime was developed as discussed in section 5.2.5.1. After thermotherapy meristem were treated as described in section 5.2.2.

[10] For each treatment the plant was exposed to 16hrs (normal lit condition) to the treatment temperature followed by 8hrs (dark) at 25°C. This alternating temperature regime for treatment was maintained for 28days.

5.2.6 Chemotherapy

5.2.6.1 *In vitro* phytotoxic assay of ribavirin and adefovir

Ribavirin (1-β-D-ribofurasonyl-1, 2, 4 triazone-3-carboxamide; RBV) and adefovir dipivoxil ({[2-(6-amino-9*H*-purin-9-yl)ethoxy]methyl}phosphonic acid) used in this study were obtained from Sigma-Aldrich (Australia). To determine the phytotoxic effect of ribavirin and adefovir dipivoxil on taro, meristems (1-3mm) were transferred to 10ml liquid MS30 amended individually with ribavirin and adefovir at the concentrations of 0, 10, 20, 30, 40 and 50mg/l in small glass bottles. Five replicates of each accession were exposed to each concentration. The phytotoxic effect was calculated on the basis of survival and mortality rates of the meristems after 28 days of growth on the antiviral amended MS30 followed by 56 days on solid MS30.

5.2.6.2 Chemotherapy treatment

The elimination treatments were the same for both ribavirin and adefovir. Meristems (about 1-3mm) were excised and cultured in 10ml liquid MS30 amended individually with filter sterilized antiviral agents at 50mg/l concentration for 28 days and then transferred to solid MS30 medium. After chemotherapy the meristems were treated as described in section 5.2.2.

5.2.7 Electrotherapy

5.2.7.1 Determination of electrotherapy voltage

Two methods of electrotherapy were trialled to determine the ideal one for electrotherapy experiments. First was a dry method (Figure 5.2). Plantlets were grown for a month in big glass bottles then trimmed to 3-4cm (2cm shoot and 1cm root end). The ends were attached to the negative and positive terminals of the power supply. Voltage of 5, 10, and 15V were passed through the plantlets for 5mins. The dry method was not very effective because after the first couple minutes the circuit would break and voltage would not pass through the plant. On careful examination it was found that the

ends of the plants had dried out when a voltage was applied, therefore, breaking the circuit.

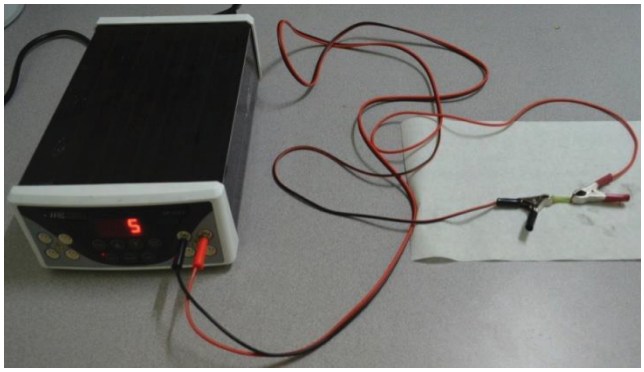


Figure 5.2: Photo depicting the dry electrotherapy method.

Following the failure of the dry method it was decided that a wet method would be used similar to the ones used by Castro *et al.,* (2001), Lebas (2002) and Hormozi-Nejad *et al.,* (2011). In the wet method the plants were trimmed to 3cm (2.5cm shoot and 0.5cm shoot) and immersed in 1M NaCl in an electrophoresis tank and held submerged by a gel comb (Figure 5.3). For each treatment 5 replicates of plantlet from each of the three accessions were exposed to the three different voltages; 5, 10 and 15V for 5 minutes[11] (Table 5.3). After the treatment meristems were extracted and cultured as per section 5.2.2. The percentage surviving after 56 days was taken as the survival rates for each of the voltage.

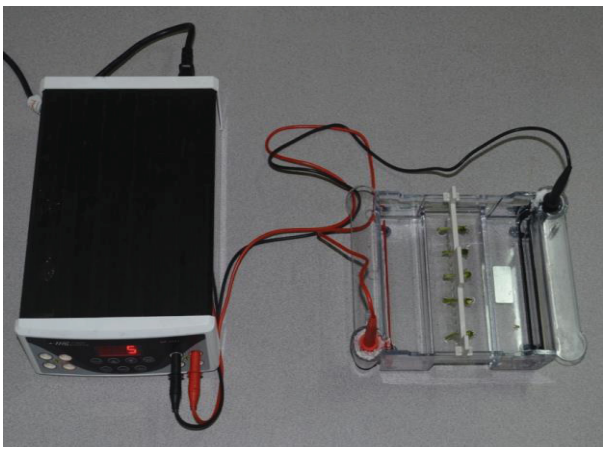


Figure 5.3: Photo depicting the wet electrotherapy method.

[11] The duration of five minutes was chosen from Castro *et al., (*2001) as it had had the highest regeneration and DsMV elimination from *Xanthosoma sagittifolium.* Longer durations had lower regeneration.

Table 5.3: Electrotherapy (wet method) experiment design

Accession	Treatment (duration 5min)		
	5V	10V	15V
BL/SM 97	5	5	5
BL/SM 129	5	5	5
BL/SM 145	5	5	5
	15	15	15
Total Plants = 45			

5.2.7.2 Electrotherapy treatment

Electrotherapy was carried out on 20 plants of each accession. Five plants were immersed in electrophoresis chamber containing 1M NaCl at one time and exposed to 5V for 5min on each occasion. The parameters for electrotherapy was determined from the survival rates of section 5.2.7.1 and also based on the work done by Castro *et al.,* (2001). Castro *et al.,* (2001) found that at 5V for 5min 75% of regeneration is achieved with DsMV elimination of 100% from *Xanthosoma sagittifolium* (L.) Schott. This was repeated for all the plants. After electrotherapy the meristems were treated as described in section 5.2.2.

5.2.8 Calculation and analysis

The following formula as described by Meybodi *et al., (*2011), Pazhouhandeh (2001) and Lozoya-Saldaña *et al.,* (1996), was used to determine the efficiency of therapy (T.E.) for all treatments:

$$\mathbf{T.E.} = \frac{\mathbf{\%\ plant\ regenerated}^{12} \times \mathbf{\%\ virus-free\ plants}}{\mathbf{100}}$$

[12] Regeneration was taken to be the number of plants surviving and transferred to PEQ for virus testing.

To determine the efficiency of *in vitro* DsMV elimination by meristem extraction and meristem extraction after thermotherapy, chemotherapy and electrotherapy the T.E. for each therapy was compared.

5.3 Results

5.3.1 Elimination via *in vitro* multiplication

All 60 plants (20 of each accession) tested positive for DsMV. Some plants tested negative at 3 months but all were positive when tested after 6 months in PEQ. DsMV was not eliminated during the tissue culture and multiplication cycles.

5.3.2 Meristem extraction

Meristem tips turned light green following one week of growth in the dark. They showed some growth during the early phase but then the outer leaves turned black. Some meristems recovered following removal of the outer leaves (Figure 5.1). Some of the meristems became contaminated with fungus but these were successfully recovered except for a single meristem of BL/SM-145[13] which was tested positive on the 2nd and 3rd testing. The regeneration rates for BL/SM-97, BL/SM-129 and BL/SM-145 individually were 40, 35 and 26%, respectively (Table 5.9, 5.10 and 5.11). The average regeneration rate for all accessions was 33%. All regenerated plants of BL/SM-129 and BL/SM-145 were free of DsMV but a single plant of BL/SM-97 was found to be infected with DsMV. The efficiency of elimination of DsMV using meristem extraction was 26-35% (Table 5.4).

[13] Not considered in the calculations when evaluating the efficiency of meristem extractions as DsMV-elimination technique.

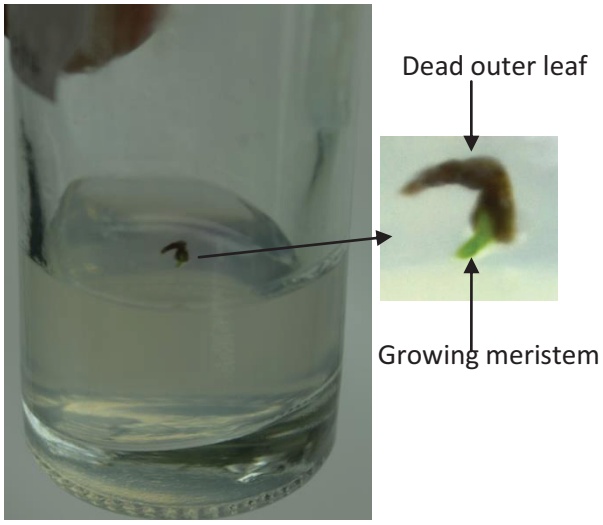


Figure 5.4: Photo of meristem showing the dead outer leaf that needs removal

Table 5.4: Regeneration efficiency and virus elimination of meristem rates calculated from regenerated meristem

Accession Code	Number of plants treated (a)	Regeneration		Elimination		Therapy efficiency
		Rate (b/a)	%	DsMV-free (c/b)	%	%
BL/SM-97	20	8/20	40	7/8	87.5	35
BL/SM-129	20	7/20	35	7/7	100	35
BL/SM-145	19*	5/19	26.31	5/5	100	26.31

[a] number of explants treated

[b] number of regenerated plantlets

[c] number of DsMV-free plants

*Only 19 explants of BL/SM-145 were considered in the calculations as one plant had died due to acute fungal contamination.

5.3.3 Thermotherapy

5.3.3.1 Determination of thermotherapy temperature

It was found that constant temperature regime had low meristem survival at temperatures of 35°C and 36°C, 4/15 (26.67%) and 2/15 (13.33%) respectively, (Figure 5.5). However, taro plants exposed to alternating temperature regimes had higher survival. All meristem (100%) survived 25, 30 and 35°C for 16hrs photoperiod and 25°C in the dark for 8hrs. Signs of heat damage, such as leaves wilting and browning, on to plants were observed at 35°C (Figure 5.6). In alternating temperature regimes where photoperiod temperatures were beyond 35°C mortality was observed. Increasing temperature above 35°C, reduced plant survival significantly (Figure 5.5). At 37°C photoperiod and 25°C in the dark for 8hrs was taken as the ideal temperature for the thermotherapy treatment. This parameter allowed for growth of accessions while in the heat chamber and survival rate for meristem were greater than 60%. At 38°C plant growth was greatly reduced and there was lot of browning of leaves and necrosis observed.

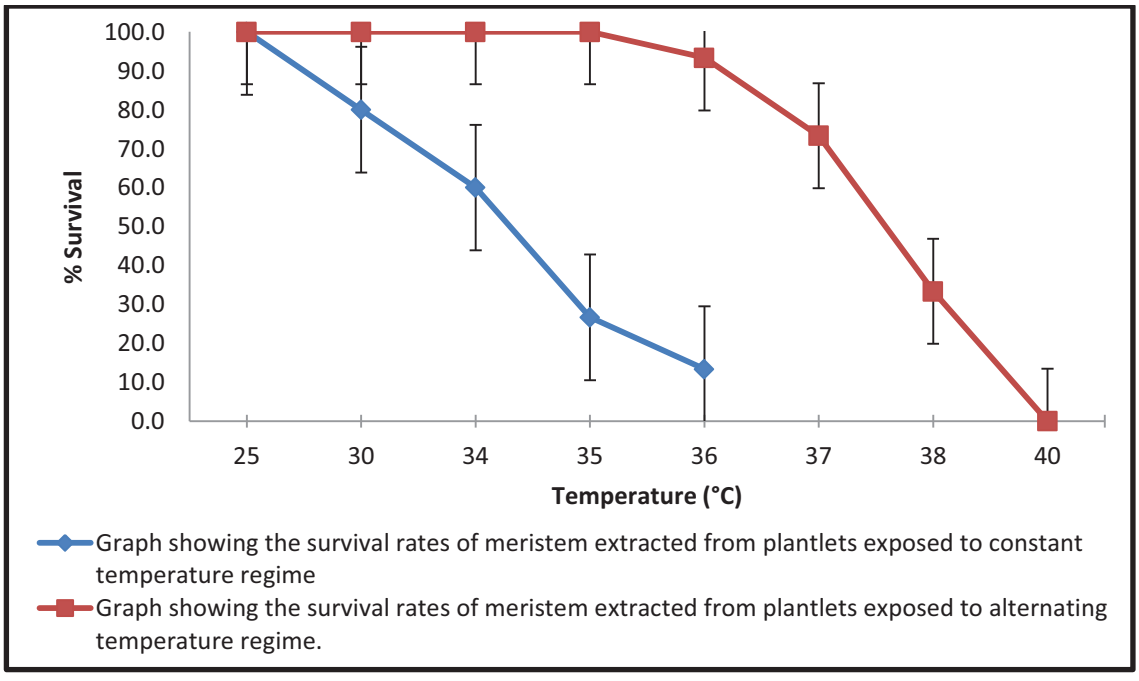


Figure 5.5: Graph showing the survival rates of meristem extracted exposed to constant and alternating temperature regimes

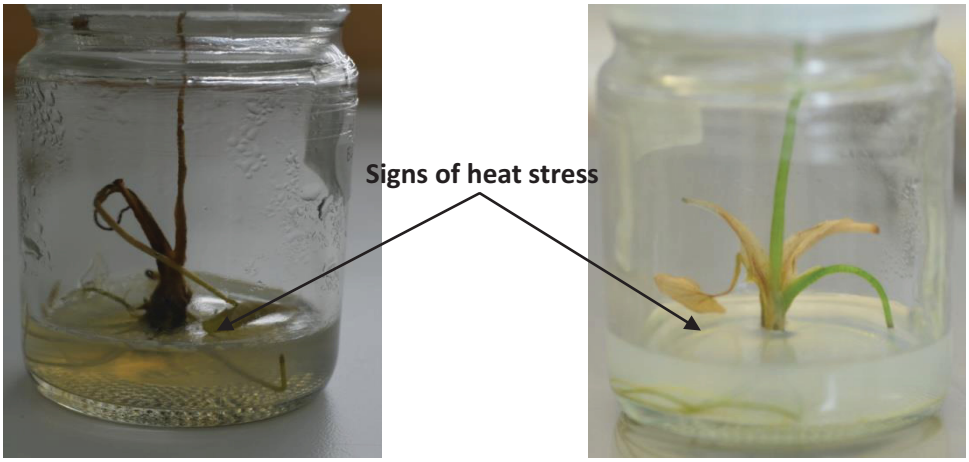


Figure 5.6: Signs of heat stress on *in vitro* plants after 28days in the thermotherapy chamber

5.3.3.2 Thermotherapy treatment

Of the accessions subjected to the selected thermotherapy treatment, 70% of meristem of accessions BL/SM-97 and BL/SM-145 survived while BL/SM-129 had 80% survival. Surviving eight meristems out of fourteen (57.14%) of BL/SM-97 and BL/SM-145 tested DsMV-free while surviving ten meristems out of sixteen meristems (62.5%) of BL/SM-129 tested DsMV free. Efficiencies of elimination for BL/SM-97 and BL/SM-145 were 40% while for BL/SM-129 was slightly higher, 50%. The average efficiency for elimination was 43.3% (Table 5.5).

Table 5.5: Effects of thermotherapy on the regeneration rate of *in vitro* plantlets and elimination rate of DsMV for the three taro accessions.

Accession Code	Number of plants treated (a)	Regeneration		Elimination		Therapy efficiency
		Rate (b/a)	%	DsMV-free (c/b)	%	%
BL/SM-97	20	14/20	70	8/14	57.14	40
BL/SM-129	20	16/20	80	10/16	62.5	50
BL/SM-145	20	14/20	70	8/14	57.14	40

[a] number of explants treated, [b] number of regenerated plantlets, [c] number of DsMV-free plants

5.3.4 Chemotherapy

5.3.4.1 *In vitro* phytotoxicity assay

Adefovir showed greater phytotoxicity effect than ribavirin (Figure 5.7). At 50mg/l concentration of antiviral agents the survival of meristem was 60% and 40% for ribavirin and adefovir, respectively. Despite the difference in survival rates, 50mg/l was used as the concentration for both the antiviral agents for chemotherapy so that final elimination rates and treatment efficiency could be compared for both the agents at the same concentration.

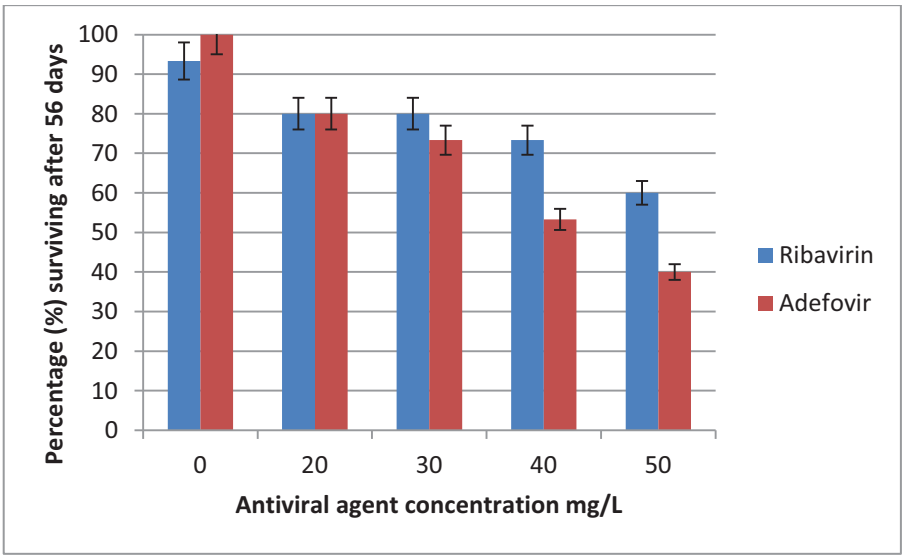


Figure 5.7: Survival rates of taro meristems 56 days post exposure to different concentrations of ribavirin and adefovir

5.3.4.2 Chemotherapy treatment

5.3.4.2.1 Ribavirin

Approximately 60% of meristems survived for BL/SM-97 (13/20), BL/SM-129 (12/20) and BL/SM-145 (10/20) that were cultured in ribavirin amended medium. Of these surviving plantlets 46% of BL/SM-97 tested DsMV-free while 67% of BL/SM-129 and 50% of BL/SM-145 tested DsMV-free. The elimination efficiency for the different accessions was 30, 40 and 25% for BL/SM-97, BL/SM-129 and BL/SM-145,

respectively. The DsMV elimination efficiency using ribavirin was calculated to be 31.66% (Table 5.6).

Table 5.6: Effects of ribavirin (50m/l) on the regeneration rate of *in vitro* plantlets and elimination rate of DsMV for the three taro accessions.

Accession Code	Number of plants treated (a)	Regeneration		Elimination		Therapy efficiency
		Rate (b/a)	%	DsMV-free (c/b)	%	%
BL/SM-97	20	13/20	65	6/13	46.15	30
BL/SM-129	20	12/20	60	8/12	66.67	40
BL/SM-145	20	10/20	50	5/10	50	25

[a] number of explants treated

[b] number of regenerated plantlets

[c] number of DsMV-free plants

5.3.4.2.2 Adefovir

Approximately 48% of meristems survived from each of the 20 different accessions of BL/SM-97, BL/SM-129 and BL/SM-145 exposed to adefovir. Of the surviving plantlets 8 were DsMV-free for BL/SM-97, 7 were DsMV-free for BL/SM-129 and 6 were DsMV-free for BL/SM-145. The efficiencies for elimination of DsMV from each of the accessions BL/SM-97, BL/SM-129 and BL/SM-145 were 40%, 35% and 30% respectively. The average efficiency of DsMV elimination using adefovir was 35% (Table 5.7). Two plants of BL/SM 145 generated from meristems treated with adefovir showed signs of morphological abnormalities (Figure 5.8).

Table 5.7: Effects of adefovir (50m/l) on the regeneration rate of *in vitro* plantlets and elimination rate of DsMV for the three taro accessions.

Accession Code	Number of plants treated (a)	Regeneration rate		Elimination rate		Therapy efficiency
		Rate (b/a)	%	DsMV-free (c/b)	%	%
BL/SM-97	20	10/20	50	8/10	80	40
BL/SM-129	20	11/20	55	7/11	63.63	35
BL/SM-145	20	8/20	40	6/8	75	30

[a] number of explants treated

[b] number of regenerated plantlets

[c] number of DsMV-free plants

Figure 5.8: Picture of plant showing signs of vein clearing after one month of growth in the PEQ[14].

[14] The plant was tested DsMV negative therefore the absence of chlorophyll could be due to treatment with adefovir. The holes are from 6mm biopsy punch, used to sample leaf for DsMV testing.

5.3.5 Electrotherapy

There were no differences observed in the survival of plantlets exposed to the different voltages. Survival of 90% and above was recorded for each of the voltages (5, 10, 15V) therefore 5V for 5min was taken as the appropriate treatment for electrotherapy as this was also the parameter that achieved greatest DsMV elimination from *Xanthosoma* sp. in studies done by Castro *et al.,* (2001).

For BL/SM-97 19/20 meristem survived whilst BL/SM-129 and BL/SM-145 both had 18/20 meristem surviving. Of the surviving meristems 14, 13 and 15 of BL/SM-97, BL/SM-129 and BL/SM-145 respectively tested DsMV-free. An average T.E. of 70% was observed (Table 5.8).

Table 5.8: Effects of electrotherapy (5V, 5min) on the regeneration rate of in vitro plantlets and elimination rate of DsMV for the three taro accessions.

Accession Code	Number of plants treated (a)	Regeneration rate		Elimination rate		Therapy efficiency
		Rate (b/a)	%	DsMV-free (c/b)	%	%
BL/SM-97	20	19/20	95	14/19	73.68	70
BL/SM-129	20	18/20	90	13/18	72.22	65
BL/SM-145	20	18/20	90	15/18	83.33	75

[a] number of explants treated

[b] number of regenerated plantlets

[c] number of DsMV-free plants

5.4 Discussion

Tissue culture can often produce virus-free plant material due to the inconsistent transmission of some viruses during micropropagation. This has been observed with *Banana bunchy top virus* (BBTV) infecting bananas. BBTV has inconsistent distribution within banana plantlets and continuous *in vitro* culture eliminates BBTV from some of the clones (Thomas *et al.*, 1995). This has also been observed with DsMV in taro (Taylor *et al.*, 2012). To test if DsMV was being eliminated during the multiplication process of the current research, 60 plantlets were randomly selected (20 plants of each accession) and meristems extracted (sized 1-3mm), regenerated and tested for DsMV. All 60 plants tested positive for DsMV. The difference between the procedure used in this research and that reported by Taylor *et al*., (2012), is the very rapid rate of multiplication using TDZ, which could have led to the elimination of DsMV. Multiplication using TDZ can produce plants very rapidly but it can also lead to morphological abnormalities (Shirani *et al.*, 2009) and somatic mutation, producing somaclonal variation (Bidabadi *et al.*, 2010). To ensure that collections are conserved effectively, clonal integrity must be maintained. Therefore in normal *in vitro* culture and maintenance DsMV is highly transferable to clones.

Meristem culture alone and meristem culture coupled with chemotherapy, thermotherapy and electrotherapy have been successfully used for virus elimination from many different plant species (see Section 2.3). The results of present research adds taro to the suite of crops in which these methods have been successfully used to eliminate viruses.

Plant regeneration is integral to the success of any virus elimination therapy. In this study regeneration differed between therapies with electrotherapy having the highest regeneration rate of 90-95%. This was followed by thermotherapy with 70-80% regeneration while chemotherapy treatments with adefovir and ribavirin had 40-55% and 50-65% regeneration, respectively. In contrast, meristem extraction (size ≈0.5mm) produced the lowest regeneration with only 26-40% of the meristems developing into plantlets.

Although plant regeneration is dependent on different factors; genotype, physiological state of the explant, culture medium, the cultivation conditions and the interactions between these factors (Hormozi-Nejad *et al.*, 2011; Svetleva *et al.*, 2003) these factors were not considered as the aim of the research was to find an effective virus elimination method for DsMV. Therefore a more generic protocol was the aim of the research.

Trends similar to plant regeneration were also observed with the duration of plant development for the different therapies (see Figure 5.9). Meristem dissection took the longest duration, followed by chemotherapy and thermotherapy. Electrotherapy treated plants showed the fastest growth and developed even faster than the control. Goldsworthy (1987) reported that electric pulses acted as stimulants of plant differentiation *in vitro*. Electrotherapy was also seen to have increased regeneration in potato plant tissues when they were treated with electric currents of 5 to 10mA for 5 to 10 min (Lozoya-Saldana *et al.*, 1996). Chemotherapy using adefovir showed slower growth than ribavirin. Adefovir seemed to have a greater phytotoxic effect than ribavirin (see Figure 5.7).

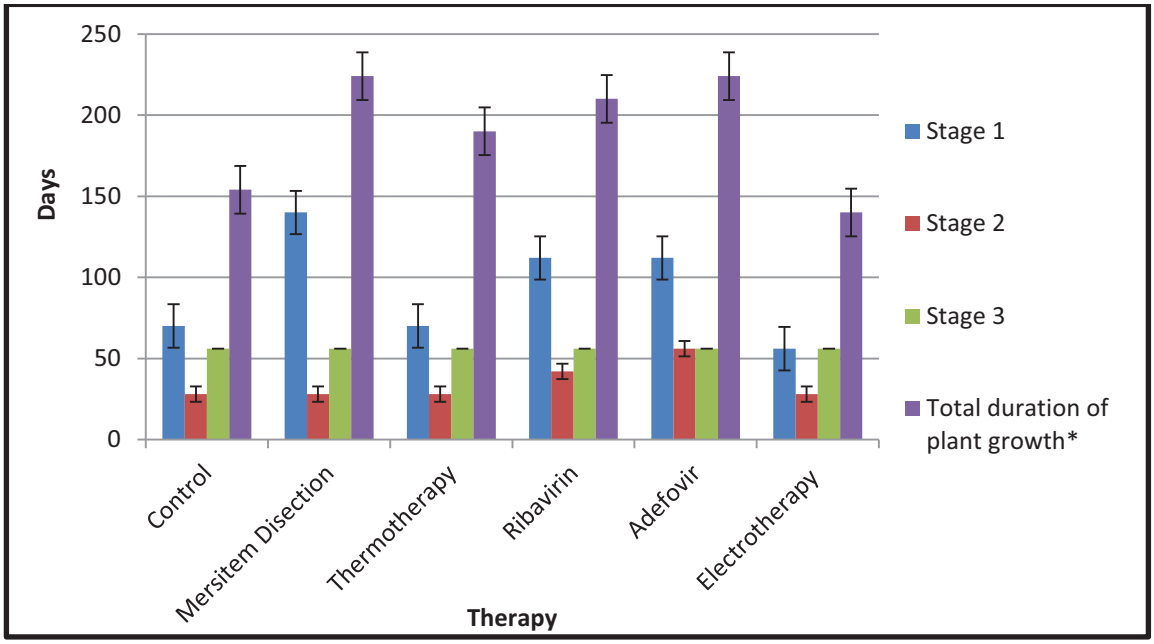


Figure 5.9: Days of growth at different stages *in vitro* until plants were ready for planting in PEQ

Therapy efficiency (T.E.) is dependent on the therapy. Thermotherapy is a very important tool for virus elimination from germplasm and has been utilized in many crops species (Panattoni *et al.*, 2013). Alconero (1972) attempted to eliminate DsMV from *Xanthosoma* sp. using thermotherapy but was unsuccessful since then only one publication highlights the successful use of thermotherapy for the elimination of DsMV. Li *et al., (*2002) established virus-free taro variety 'Fenghuayunaitou' by thermotherapy combined with meristem-tip culture. However thermotherapy by Li *et al.,* (2002) was done on *in vivo* plants before the meristems were removed and cultured *in vitro.* In preliminary trials carried out as part of this research to determine the appropriate temperatures to use for thermotherapy it was found that keeping plants exposed to steady temperatures (37°C & 38°C) was lethal. The fragile nature of *in vitro* plants meant that they were more heat sensitive than *in vivo* plants, as used by Li *et al.,* (2002), where the corms were exposed to 37°C for 30 days. Two experiments were carried out to determine thermotherapy parameters. One was with constant temperature while the other was an alternating temperature regime. It was found that taro has low tolerance to elevated temperatures therefore very low meristem survival was achieved from plants exposed to constant temperature of above 34°C. However, alternating temperature regimes had higher survival at the same temperatures as that of constant temperature regime (See Figure 5.5). Virus elimination is more probable at higher temperatures but plant tolerance is lower at higher temperatures (Fløistad *et al*., 2011; Tan *et al*., 2010; El Far & Ashoub, 2009; Ondrušiková, 2009; Paprstein *et al*., 2008; Sharma *et al*., 2008; Wang *et al*., 2006a; Arif *et al*., 2005; Verma *et al*., 2005; Robert *et al*., 1998; Keller, 1992; Křižan & Lozoya-Saldaña & Dawson, 1982a, 1982b; Nyland, 1969). Therefore, an alternating temperature regime was determined to be more appropriate for DsMV elimination from taro as the plants could be exposed to higher temperatures then what was possible with constant temperature regime. The current research was carried out on *in vitro* plants as they were easier to handle and required fewer sanitation steps because they had already been aseptically cultured.

In the current research thermotherapy was carried out using an alternating temperature regime of 37°C for 16hrs in standard lit conditions followed by 25°C for 8hrs in dark. Higher dark temperatures also had deleterious effect[15] therefore to minimize the effects of temperature to tolerable levels and to allow for plant regeneration 25°C was selected as an effective dark temperature. A T.E. of 40-50% was achieved by thermotherapy during this research. This effectively is the first reported study on *in vitro* elimination of DsMV from *C. esculenta* using thermotherapy.

The use of thermotherapy can be limited due to plant sensitivity towards heat and low percentage of regeneration (Cowell, 2013; Paunovic *et al.*, 2007; Deogratias *et al.*, 1989). Chemotherapy can be used as an alternative to thermotherapy. It has been used successfully in the elimination of viruses from many vegetatively propagated crops (Panattoni *et al.*, 2013) but there are no reports of it being used for the elimination of DsMV from taro. Chemicals used for chemotherapy can be phytotoxic (Špak *et al.*, 2010), therefore, the concentration that allow for sufficient plant regeneration must be determined by a phototoxic assay. Two chemical compounds, ribavirin and adefovir were used in this research. The phytotoxicity of both ribavirin and adefovir increased with increasing concentration. Ribavirin at 50mg/l had a mean survival of 58.33% (50-65%) while adefovir had a mean survival of 48.33% (40-55%). Therefore 50mg/l was taken to be an effective concentration for both compounds for the chemotherapy research. Ribavirin has been used at similar concentrations for virus elimination studies in other crop species. Hazaa *et al.,* (2006) found that 40mg/l of ribavirin had a 71% survival of meristems (3mm size) which is similar to that observed for taro (Figure 5.7). Haaza *et al.,* (2006) achieved 90% elimination of *Banana mosaic virus* (BMV) from banana using 40mg/l of ribavirin. Paunovic *et al.,* (2007) found that plum plants that underwent chemotherapy with 10 and 20mg/l of ribavirin remained infected with *Plum pox virus* (PPV). Similarly Parmessur and Saumtally (2002) found that chemotherapy with 10–30mg/l of ribavirin was ineffective in the elimination of *Sugarcane yellow leaf virus* and *Sugarcane bacilliform virus* from infected sugarcane. Similar results were

[15] Data not shown but 37°C day (16hrs) and 28°C night (8hrs) were also evaluated but the taro plants didn't survive the treatment.

reported by Deogratias *et al.,* (1989) where they found that low ribavirin concentrations (10-20mg/l) were ineffective in eliminating *Apple chlorotic leaf spot virus* (ACLSV) from sweet cherry (*Prunus* spp.). All these studies supported the use of ribavirin at higher concentration, such as the one used in the current research. Higher concentrations have been attributed to greater phytotoxic effect (Paunovic *et al.*, 2007). Phytotoxic effects of adefovir were studied by Helliot *et al.,* (2003) who found that at 50mg/l concentrations of adefovir 58% of banana meristem survived and was an effective concentration for the elimination of *Banana streak virus* (BSV). Higher concentrations of adefovir had greater phytotoxicity (Helliot *et al.*, 2003).

Ribavirin is an effective antiviral agent against various animal viruses (both RNA and DNA viruses) and has been commonly used for plant virus elimination research (de Clercq, 2004). It belongs to the class of chemicals known as purine or pyrimidine analogues (Pennazio, 1997). Ribavirn has been used in many crops and its usefulness in elimination of plant viruses is well documented (Cowell, 2013; Niedz *et al.*, 2013; Špak *et al.*, 2012; Budiarto, 2011; Budiarto *et al.*, 2011; Hauptmanová & Polák, 2011; Guta *et al.*, 2010; Danci *et al.*, 2009; Cieślińska, 2007; Panattoni *et al.*, 2007; Paunovic *et al.*, 2007; Sharma *et al.*, 2007; Awan *et al.*, 2005; Iwanami & Ieki, 2005; Verma *et al.*, 2005; Nascimento *et al.*, 2003; O'Herlihy *et al.*, 2003; Yi *et al.*, 2003; Faccioli & Colalongo, 2002; Fletcher *et al.*, 1998; James *et al.*, 1997; Porter & Kuehnle, 1997; Dewi & Slack, 1994; Toussaint *et al.*, 1993; Chen & Sherwood, 1991; de Fazio *et al.*, 1990; Griffiths *et al.*, 1990; Albouy *et al.*, 1988; Lerch, 1987; Hansen & Lane, 1985; Lozoya-Saldana *et al.*, 1984; Klein, 1983; Klein & Livingston, 1982). In this research ribavirin at a concentration of 50mg/l was able to eliminate DsMV from 57.23% of the surviving taro plants. This equated to a T.E. of 31.66%.

Adefovir on the other hand has been studied much less as an antiviral compound for plant virus elimination. Adefovir is an acyclic nucleotide analog reverse transcriptase inhibitor (NtRTI) and has been shown to be effective for the treatment of hepatitis B virus (HBV). Adefovir acts as chain terminator, following intracellular phosphorylation to the diphosphate form, and incorporation at the 3'-end of the viral DNA chain and is

effective for both DNA and RNA viruses (de Clercq, 2004). Helliot *et al., (*2003) used adefovir for the elimination of *Banana streak virus* (BSV) from banana. They used 10mg/l of adefovir and kept the plants in adefovir containing medium for 6 months. In the current research 50mg/l of adefovir was used and the plants were kept exposed to adefovir for 28 days similar to the procedure for ribavirin. Helliot *et al.*, (2003) obtained 88% elimination of BSV from banana after 6 months. In the current research the mean elimination rate of DsMV from taro was 72.88% with a mean T.E of 35% (33-40%). Morphological abnormalities were observed in some plants after been grown out in the PEQ (see Figure 5.8).

Thermotherapy is time consuming, it can take from several days to months to complete with low efficiencies in virus elimination (Lozoya-Saldana *et al.*, 1996) while chemotherapy requires expensive antiviral agents and is prone to be affected by phytotoxicity. However, electrotherapy is a simple method of virus elimination without the need to use any special or expensive equipment or chemicals (Sastry & Zitter, 2014). Blanchard (1974) established a basis for electric current mediated plant virus elimination. He obtained virus-free tissues after 2-3 days by applying 1-4A direct current pulsed to 6500V/h. Although the foundation for electric current mediated virus elimination was laid down in 1974 it has received much less attention than other techniques such as meristem extraction, chemo and thermo-therapy but recently it has been used more often for plant virus elimination (AlMaarri *et al.*, 2012; Bayati *et al.*, 2011; Hormozi-Nejad *et al.*, 2011; Meybodi *et al.*, 2011; Guta *et al.*, 2010; Mahmoud *et al.*, 2009; Dhital *et al.*, 2008; Falah *et al.*, 2007; Yi *et al.*, 2003; Hernández *et al.*, 2002; 1997; Lebas, 2002; Castro *et al.*, 2001; Pazhouhande, 2001; Lozoya-Saldana *et al.*, 1996). Castro *et al.,* (2001) studied the utility of electrotherapy for the elimination of DsMV from *Xanthosoma* sp. and achieved an elimination rate of 100%. However in the current research a mean elimination rate of 76.41% was achieved with a T.E. of 70%.

Meristem extraction without any additional treatments had a T.E. of 26-35%. Although meristem regeneration was not very high, higher levels of virus elimination were possible. Meristem size undoubtedly plays an important part in the regeneration and

virus elimination. The smaller the meristem the higher the chances of obtaining virus-free plants however meristem size and regeneration are inversely related. As such smaller meristem results in lower regeneration (Marcel *et al.*, 2012; Naz *et al.*, 2009; Wang *et al.*, 2006). Figure 5.10 shows the regeneration of each treatment. Meristem extraction had the lowest regeneration compared to other treatments. This correlates to size as well. Meristems for evaluation of meristem dissection as a method for virus elimination were kept at sizes below 1mm while the meristems extracted for the other therapies were between 1-3mm.

The advantage of *in vitro* culture is that even if one plant is found to be virus-free it can be multiplied to produce large numbers. A technique that gives a low regeneration rate but is effective at virus elimination can still be considered as useful because the resulting clean plant(s) can be used for clonal propagation. However if a plant is slow to recover from the therapy then the time needed for testing and declaration of virus status is longer which tends to make the method less viable.

Ideally any of the four techniques evaluated in this research can be used for the elimination of DsMV. Of all the different elimination techniques used, meristem had the highest mean elimination rate (95.83%; 87.5-100%) but lowest plant regeneration (Figure 5.10) and also took the longest to grow into plantlets suitable for acclimatising. Electrotherapy had the second highest mean elimination rate (76.41%; 72.22-83.33%) but had the highest regeneration rate when compared to other therapies. Plantlets treated with electrotherapy also developed faster than other treatment. This has also been reported by Castro *et al.,* (2001), whereby they observed accelerated growth of plant that underwent electrotherapy.

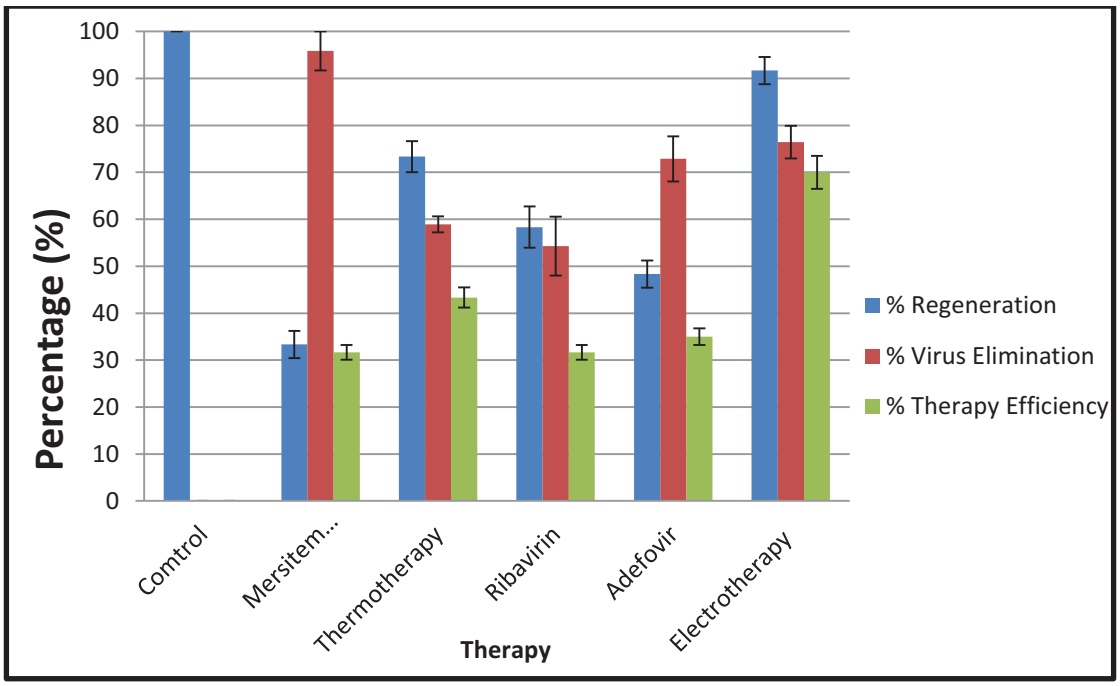


Figure 5.10: Mean values of plant regeneration, virus elimination rate and therapy efficiency for the different therapies and control.

The current research shows that electrotherapy has some distinct advantages. It has a high regeneration and also promotes plant growth; therefore, it shortens the time taken by plants from therapy to virus testing. It also has high DsMV elimination rates so there are higher chances of obtaining DsMV-free material. Electrotherapy is technically easy and the equipment used is inexpensive and can be used repeatedly. This is an added advantage over chemotherapy and thermotherapy which require purchase of expensive chemicals and equipments respectively. Electrotherapy unlike chemotherapy did not show any signs of morphological abnormalities. Electrotherapy is a sound technique that can be adopted as routine technique for DsMV elimination from taro (*C. esculenta*) and should be evaluated further for the elimination of other viruses such as TaBV from taro.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSIONS

The safe exchange of plant genetic resource (PGR) both nationally, regionally and internationally prerequisites pathogen and disease free material. Tissue culture or *in vitro* culture provides a means to eliminate most pathogens but viruses still remain a problem. Taro (*Colocasia esculenta*) is a very important crop for the Pacific Island countries. Besides having a very high cultural significance, taro has steadily become an economical crop. Taro exports from Fiji, Samoa, Tonga and Vanuatu have a value in millions of dollars with potential for export expansion, but no significant growth has been observed over recent years (McGregor *et al.,* 2011). Future production could see increased pressure from pests and diseases and climate change (such as increased soil salinity, drought, water logging etc). Research, evaluation, acquisition and breeding for new taro varieties is integral if the production levels are to be maintained or increased, all of which require availability of and access to taro diversity.

The CePaCT, SPC is the global repository for taro PGR. It is tasked with the conservation and safe exchange of taro PGR. The current research is aimed to supplement this globally important activity. Quarantine regulations restrict the movement of taro PGR without meeting regulated phytosanitary standards. CePaCT, SPC therefore ensures that the taro PGR that is being exchange confer to these standards.

The current research which was jointly funded by International Foundation for Science (IFS), Secretariat of the Pacific Community (SPC) and University of the South Pacific (USP), evaluated RT-PCR and ELISA as possible diagnostic methods for *Dasheen mosaic virus* (DsMV) and methods for elimination of DsMV from taro. DsMV being a quarantine virus with a widespread distribution and attributed to yield reduction was taken as virus to be investigated under the current research. The result of DsMV

diagnostic are presented in Chapter 4 and while Chapter 5 presents results and discuses possible methods for the elimination of DsMV.

Virus diagnostics is vital to ensure that taro germplasm is free of viruses meeting phytosanitary standards for importing countries. There are some viruses of taro that can lead up to 100% yield losses, such *Colocasia bobone diseases virus* (CBDV) which is restricted to PNG and Solomon Islands. Even DsMV which is very widespread can have serious impact depending on the strain as has been recorded from French Polynesia (Nelson, 2008). The virus diagnostic protocols for taro have been well defined and are routinely used at CePaCT, SPC to test for viruses in the taro germplasm collection. These protocols are based on polymerase chain reaction (PCR).

Chapter 4 compares the commercially available DAS-ELISA (Agida® Inc, France) with RT-PCR for the detection of DsMV. Results show that DsMV detection by RT-PCR is 10X more sensitive than ELISA in detecting DsMV from samples diluted to 10^{-3} while the later can only detect to 10^{-2} dilution. However when costs are compared ELISA is a much cheaper option; per sample it is 15 times cheaper. ELISA is not as reliable as RT-PCR and may not be suitable for use as a method for testing DsMV in germplasm collections. Gene banks need to use the most sensitive methods available, so that they can ensure that the virus status of the plant material is reliably diagnosed. The high cost of molecular diagnostic facilities and RT-PCR, and technical expertise needed for molecular based diagnostic prevents countries, especially in the Pacific, from using RT-PCR to ensure that their national collections and farmer fields remain at low levels of DsMV infections. But ELISA detection of DsMV can still be a very important tool at national level surveys and clean seed schemes as it is much cheaper and less technically oriented. For the movement of taro germplasm RT-PCR should be used as the method for detection of DsMV but when DsMV-free germplasm reaches the country gene banks and farmer's field, ELISA then can be used to ensure that the germplasm remains healthy. Regular testing and rouging of infected plants would keep DsMV loads in the field down and this would not affect the production.

Access to clean taro germplasm is essential so that new diversity can be introduced to broaden existing country taro gene pools. Broadening the existing gene pool would ensure that countries can respond to pressure from pests and diseases (such as TLB) and climate change (such as salinity). CePaCT, SPC is mandated to conserve and facilitate the distribution of taro genetic resource however the existence of virus infection in the collection is hindering the distribution of some very important taro germplasm. Chapter 5 looks at how to address the issue of virus infection in taro germplasm by using DsMV as a model virus. Virus elimination methods, namely meristem dissection, chemotherapy, electrotherapy and thermotherapy are compared to find out which method can be used for DsMV elimination routinely and in the future used on elimination of other viruses from taro.

All the methods were able to produce DsMV-free taro but electrotherapy showed the highest efficiency. It had the highest regeneration (*avg.* 91.66%), a good DsMV-elimination rate (*avg.* 76.57%) as well as the fastest plantlet recovery compared to other three methods. Studies on elimination of DsMV from *Xanthosoma* sp. have also given very high elimination rates (Castro *et al*., 2001). The second highest efficiency was that of thermotherapy, followed chemotherapy using adefovir, ribavirin and meristem dissection.

This forms part of the first comprehensive research on the elimination of a virus from taro. Using DsMV a suite of protocols has been evaluated pointing out their weakness and strengths. Electrotherapy and thermotherapy have shown reliable efficiency of DsMV elimination and can be possible methods to use for future work on the elimination of other viruses infecting taro. Meristem extraction has a high elimination rate but the regeneration rate is low and the perquisite for a small size to effect elimination is challenging. Chemotherapy on the other hand induced some morphological abnormalities in the plants. Haaza *et al.,* (2006) also report observing genetic variation of 4% after virus BBTV elimination from banana using ribavirin. Gene banks need to ensure that the clonal and genetic integrity of the germplasm is maintained

therefore chemotherapy which may induce genetic variation cannot be readily used unless there is a mechanism to test that the clonal integrity is maintained.

The current research will now be up-scaled for use at the CePaCT, SPC to start the virus elimination process from numerous accessions (≈500) conserved from 24 different countries from the Pacific and Asia region. This is a significant step in making available this taro diversity, both within the region and globally. It will also help CePaCT, SPC to fulfil its global commitment, which was undertaken by the signing of Article 15 of the ITPGRFA. Upon signing, CePaCT, SPC has agreed to ensure the safe conservation and sustainable use of taro genetic resources for food and agriculture, and to ensure the fair and equitable sharing of benefits arising out of their use. The outcomes of the research will ensure that CePaCT, SPC is able to provide Pacific Island countries, as well as globally, safe access to taro genetic diversity. This is crucial for building a food secure Pacific.

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APPENDIX

Media Preparation

Table 3.3: Recipe for MS stock solutions

Compound	Media concentration mg/l	Stock Concentration g/l
Stock A (x40)		
KNO_3	1900	76
NH_4NO_3	1650	66
$CaCl_2.2H_20$	440	17.6
KH_2PO_4	170	6.8
Stock B (x40)		
$MgSO_4.7H_2O$	370	14.8
$MnSO_4.H_2O$	22.3	0.54
Stock C (x200)		
$ZnSO_4.7H_2O$	8.6	1.72
H_3BO_3	6.2	1.24
KI	0.83	0.166
$CuSO_4.5H_2O$	0.025	0.005
$Na_2MoO_4.2H_2O$	0.25	0.05
$CoCl_2.6H_2O$	0.025	0.005
Stock D (x200)		
(EDTA) di-sodium salt	36.7	26.1
$FeSO_4.7H_2O$	27.8	24.9
Stock E (x200)		
Myo-inositol	100	10
Thiamine-HCl	0.4	0.04

Table 3.4: MS Components

Stock Solution	Volume in 1L (ml)
Stock A	25
Stock B	25
Stock C	5
Stock D	5

Note:

- For MS30 30g of Sucrose is added to 1L of the MS.
- After the addition of all components the pH is adjusted to 5.6-5.8 using NaOH or HCL
- For solid MS agar is added at a concentration of 7.8 to 8.0g/l

After the addition of agar the solution is heated until all components have dissolved and the solution as turned clear. The media is then autoclaved at 120°C (103KPa) for 15 minutes.

Standard ELISA Buffers

5×PBS (Phosphate buffered saline)

For 1 litre of solution:

NaCl	40.0g
KH_2PO_4	1.0g
Na_2HPO_4	5.75g (or 14.5g of $Na_2HPO_4.12H_2O$)
KCl	1.0g

Dissolve chemicals in about 900ml of dH_2O, then **adjust to pH 7.4 using 3M NaOH,** then make up to 1L with dH_2O. Store at 5°C.

1× PBS - Tween

Dilute 5×PBS stock solution to 1× PBS in dH_2O and add 0.05% (0.5ml/l) of Tween-20 and mix using a stirring bar. Store 1× PBS - Tween at 5°C.

1× PBS - Tween – PVP

Add 2g PVP (soluble polyvinyl pyrrolidone) to 100ml PBS-Tween.

Carbonate Coating Buffer, pH 9.6

For 1L of solution:

Na_2CO_3	1.59g
$NaHCO_3$	2.93g

Dissolve chemicals in about 900ml of dH_2O, and check pH is 9.6. If pH needs to be adjusted slightly, use HCl or NaOH. Then make up to 1L with dH_2O. Store at 5°C.

Diethanolamine Buffer, pH 9.8 (Substrate buffer)

Make a 9.7% solution of diethanolamine in dH_2O. For 1L of solution dissolve 97 ml of diethanolamine in about 850ml of dH_2O, adjust to pH 9.8 with about 2M HCl, then make up to 1L. Store at 5°C.

Addition of Mg^{2+}, a cofactor for alkaline phosphatase, as 0.01% $MgCl_2$ can greatly increase the rate of substrate conversion.

Substrate (for alkaline phosphatase conjugates)

1 mg pNPP (p-nitrophenylphosphate) / 1ml diethanolamine buffer

ELISA results for DsMV testing Koronivia samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank 0.123	S1 (R1) 0.140	S2 (R1) 1.584	S3 (R1) 0.140	S4 (R1) 0.190	S5 (R1) 0.135	S6 (R1) 0.513	S7 (R1) 0.144	S8 (R1) 0.154	S9 (R1) 0.137	S10 (R1) 0.135	S11 (R1) 0.123
B	Blank 0.126	S12 (R1) 1.719	S13 (R1) 0.129	S14 (R1) 0.143	S15 (R1) 0.141	S16 (R1) 1.395	S17 (R1) 0.137	S18 (R1) 0.135	S19 (R1) 0.130	S20 (R1) 0.927	S21 (R1) 0.141	S22 (R1) 0.147
C	Blank 0.123	S23 (R1) 0.146	S24 (R1) 0.148	S25 (R1) 0.148	S26 (R1) 0.129	S27 (R1) 0.138	S28 (R1) 0.137	S29 (R1) 0.223	S30 (R1) 0.208	S31 (R1) 0.140	S32 (R1) 0.138	S33 (R1) 0.136
D	Blank 0.134	S34 (R1) 0.153	S35 (R1) 0.145	S36 (R1) 0.855	S37 (R1) 0.192	S38 (R1) 0.139	S39 (R1) 0.143	S40 (R1) 0.141	S41 (R1) 0.135	S42 (R1) 0.685	S43 (R1) 0.941	S44 (R1) 0.145
E	Blank 0.145	S45 (R1) 2.841	S46 (R1) 0.155	S47 (R1) 0.150	S48 (R1) 0.145	S49 (R1) 0.146	S50 (R1) 0.966	DsMV 1X 2.727	DsMV 1/10 0.652	DsMV 1/100 0.220	DsMV 1/1000 0.148	-ve Cntrl 0.145
F	Blank 0.139	S1 (R2) 0.152	S2 (R2) 2.632	S3 (R2) 0.167	S4 (R2) 0.273	S5 (R2) 0.161	S6 (R2) 0.969	S7 (R2) 0.171	S8 (R2) 0.176	S9 (R2) 0.179	S10 (R2) 0.178	S11 (R2) 0.155
G	Blank 0.146	S12 (R2) 2.704	S13 (R2) 0.156	S14 (R2) 0.174	S15 (R2) 0.172	S16 (R2) 2.852	S17 (R2) 0.188	S18 (R2) 0.175	S19 (R2) 0.192	S20 (R2) 2.022	S21 (R2) 0.180	S22 (R2) 0.148
H	Blank 0.163	S23 (R2) 0.176	S24 (R2) 0.207	S25 (R2) 0.199	S26 (R2) 0.185	S27 (R2) 0.177	DsMV 1X 3.205	DsMV 1/10 0.915	DsMV 1/100 0.246	DsMV 1/1000 0.165	-ve Cntrl 0.172	Blank 0.145

Figure 4.5: DsMV DAS-ELISA plate one

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank 0.139	S1 (R3) 0.167	S2 (R3) 1.916	S3 (R3) 0.184	S4 (R3) 0.269	S5 (R3) 0.181	S6 (R3) 0.787	S7 (R3) 0.197	S8 (R3) 0.200	S9 (R3) 0.180	S10 (R3) 0.209	S11 (R3) 0.196
B	Blank 0.154	S12 (R3) 2.238	S13 (R3) 0.179	S14 (R3) 0.176	S15 (R3) 0.184	S16 (R3) 2.182	S17 (R3) 0.188	S18 (R3) 0.176	S19 (R3) 0.180	S20 (R3) 1.598	S21 (R3) 0.212	S22 (R3) 0.170
C	Blank 0.151	S23 (R3) 0.189	S24 (R3) 0.194	S25 (R3) 0.204	S26 (R3) 0.184	S27 (R3) 0.179	S28 (R3) 0.183	S29 (R3) 0.333	S30 (R3) 0.350	S31 (R3) 0.194	S32 (R3) 0.187	S33 (R3) 0.178
D	Blank 0.153	S34 (R3) 0.174	S35 (R3) 0.175	S36 (R3) 1.039	S37 (R3) 0.240	S38 (R3) 0.184	S39 (R3) 0.169	S40 (R3) 0.177	S41 (R3) 0.183	S42 (R3) 1.019	S43 (R3) 1.507	S44 (R3) 0.195
E	Blank 0.154	S45 (R3) 3.050	S46 (R3) 0.190	S47 (R3) 0.181	S48 (R3) 0.176	S49 (R3) 0.188	S50 (R3) 1.744	DsMV 1X 3.069	DsMV 1/10 0.887	DsMV 1/100 0.267	DsMV 1/1000 0.164	-ve Cntrl 0.186
F	Blank 0.160	S28 (R2) 0.199	S29 (R2) 0.403	S30 (R2) 0.387	S31 (R2) 0.221	S32 (R2) 0.204	S33 (R2) 0.206	S34 (R2) 0.200	S35 (R2) 0.203	S36 (R2) 1.532	S37 (R2) 0.267	S38 (R2) 0.199
G	Blank 0.170	S39 (R2) 0.177	S40 (R2) 0.184	S41 (R2) 0.198	S42 (R2) 1.140	S43 (R2) 1.860	S44 (R2) 0.214	S45 (R2) 3.416	S46 (R2) 0.219	S47 (R2) 0.198	S48 (R2) 0.169	Blank 0.161
H	Blank 0.138	DsMV 1X 2.636				Blank 0.170	Blank 0.176	Blank 0.179	Blank 0.191	Blank 0.169	Blank 0.171	Blank 0.167

Figure 4.6: DsMV DAS-ELISA plate two

Key:

S1	Sample number
(R1)	Replicate number
0.140	Absorbance

Note: Sample S49 & S42 were only done twice since the sample had spilled over during the loading of the second plate. The duplicate results complement each other therefore it was though unnecessary to repeat the whole test again.

Gel results of DsMV RT-PCR for Koronivia samples

Gel One - Results for KRS Sample DsMV RT-PCR

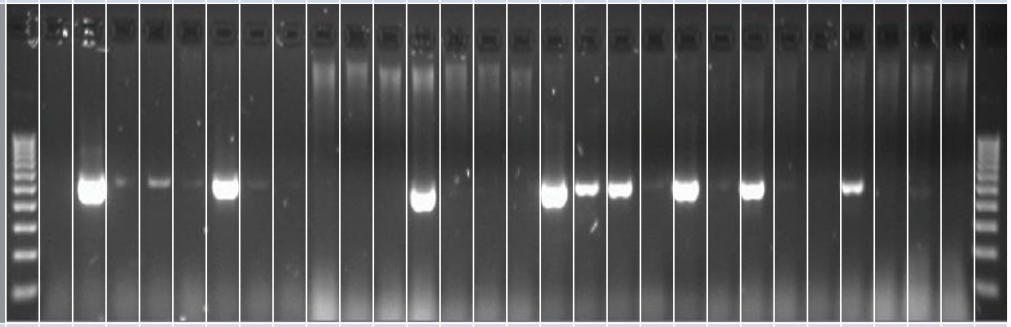
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
Content	100bp ladder	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Sample 7	Sample 8	Sample 9	Sample 10	Sample 11	Sample 12	Sample 13	Sample 14	Sample 15	Sample 16	Sample 17	Sample 18	Sample 19	Sample 20	Sample 21	Sample 22	Sample 23	Sample 24	Sample 25	Sample 26	Sample 27	Sample 28	100bp ladder
Result	OK	-	+	-	+	-	+	-	-	-	-	-	+	-	-	-	+	+	+	-	+	-	+	-	-	+	-	-	-	ok
Comments																														

Figure 4.7: Gel One- Results of KRS DsMV RT-PCR

Gel Two - Results for KRS Sample DsMV RT-PCR

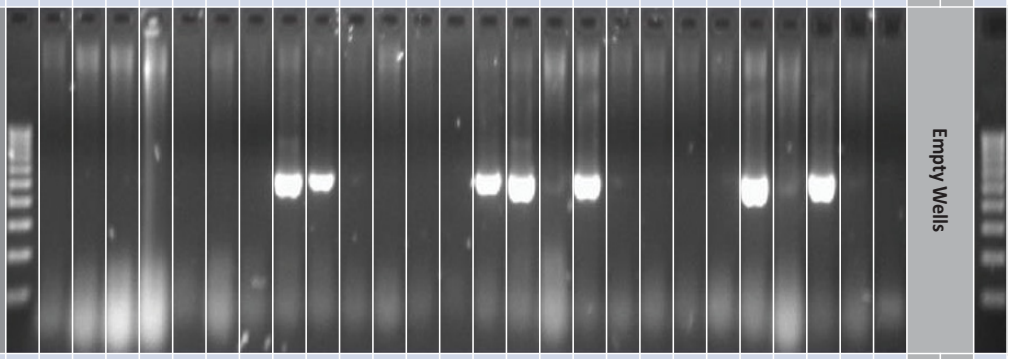
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																												Empty Wells		
Sample #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	-	-	-			-
PCR tube #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54			-
Content	100bp ladder	Sample 29	Sample 30	Sample 31	Sample 32	Sample 33	Sample 34	Sample 35	Sample 36	Sample 37	Sample 38	Sample 39	Sample 40	Sample 41	Sample 42	Sample 43	Sample 44	Sample 45	Sample 46	Sample 47	Sample 48	Sample 49	Sample 50	Dried sample*	Positive Control	Negative Control	No Template			100bp ladder
Result	OK	-	-	-	-	-	-	-	+	+	-	-	-	-	+	+	-	+	-	-	-	-	+	-	+	-	-			
Comments	One of CePaCT old samples included in the PCR to check if it was still of amplifiable quality.																													

Figure 4.8: Gel Two- Results of KRS DsMV RT-PCR

Table 4.4: Summary results of DsMV testing using DAS-ELISA and RT-PCR

Sample ID:	Nature of symptoms	DAS-ELISA	RT-PCR
Sample 1.	No Symptoms	- (0.1531)	-
Sample 2.	Feathering	+ (2.0438)	+
Sample 3.	No Symptoms	- (0.1635)	-
Sample 4.	No Symptoms	- (0.2441)	+
Sample 5.	No Symptoms	- (0.1590)	-
Sample 6.	Mild Feathering	+ (0.7563)	+
Sample 7.	No Symptoms	- (0.1706)	-
Sample 8.	No Symptoms	- (0.1762)	-
Sample 9.	No Symptoms	- (0.1653)	-
Sample 10.	No Symptoms	- (0.1737)	-
Sample 11.	No Symptoms	- (0.1581)	-
Sample 12.	No Symptoms	+ (2.2205)	+
Sample 13.	No Symptoms	- (0.1546)	-
Sample 14.	No Symptoms	- (0.1643)	-
Sample 15.	No Symptoms	- (0.1657)	-
Sample 16.	No Symptoms	+ (2.1428)	+
Sample 17.	No Symptoms	- (0.1709)	+
Sample 18.	No Symptoms	- (0.1622)	+
Sample 19.	No Symptoms	- (0.1676)	-
Sample 20.	No Symptoms	+ (1.5157)	+
Sample 21.	No Symptoms	- (0.1775)	-

Sample 22.	No Symptoms	- (0.1549)	+
Sample 23.	No Symptoms	- (0.1700)	-
Sample 24.	No Symptoms	- (0.1830)	-
Sample 25.	No Symptoms	- (0.1838)	+
Sample 26.	No Symptoms	- (0.1659)	-
Sample 27.	No Symptoms	- (0.1647)	-
Sample 28.	No Symptoms	- (0.1732)	-
Sample 29.	No Symptoms	- (0.3196)	-
Sample 30.	No Symptoms	- (0.3151)	-
Sample 31.	No Symptoms	- (0.1849)	-
Sample 32.	No Symptoms	- (0.1764)	-
Sample 33.	No Symptoms	- (0.1734)	-
Sample 34.	No Symptoms	- (0.1756)	-
Sample 35.	No Symptoms	- (0.1743)	-
Sample 36.	No Symptoms	+ (1.1419)	+
Sample 37.	No Symptoms	- (0.2331)	+
Sample 38.	No Symptoms	- (0.1739)	-
Sample 39.	No Symptoms	- (0.1627)	-
Sample 40.	No Symptoms	- (0.1672)	-
Sample 41.	No Symptoms	- (0.1718)	-
Sample 42.	No Symptoms	+ (1.0378)	+
Sample 43.	Feathering	+ (1.4357)	+

Sample 44.	No Symptoms	- (0.1846)	-
Sample 45.	Severe Feathering symptoms	+ (3.1025)	+
Sample 46.	No Symptoms	- (0.1881)	-
Sample 47.	No Symptoms	- (0.1765)	-
Sample 48.	No Symptoms	- (0.1630)	-
Sample 49.	No Symptoms	- (0.1671)	-
Sample 50.	Mild feathering symptoms	+ (1.3551)	+

Results of DsMV testing for the different therapies

Meristem

Summary of results for DsMV testing of the meristem extraction experiment

Table 5.9: DsMV testing results of BL/SM 97 accessions treated with meristem extraction

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 97/MD1‡	NT	+**	+
BL/SM 97/MD2‡	NT	NT	NT
BL/SM 97/MD3	NT	NT	NT
BL/SM 97/MD4	-	-	-
BL/SM 97/MD5	-	-	-
BL/SM 97/MD6	NT	NT	NT
BL/SM 97/MD7‡†	-	-	-
BL/SM 97/MD8	NT	NT	NT
BL/SM 97/MD9	NT	NT	NT
BL/SM 97/MD10	NT	NT	NT
BL/SM 97/MD11‡	-	-	-
BL/SM 97/MD12	NT	NT	NT
BL/SM 97/MD13	NT	NT	NT
BL/SM 97/MD14‡	-	-	-
BL/SM 97/MD15	NT	NT	NT
BL/SM 97/MD16	-	-	-
BL/SM 97/MD17	-	-	-
BL/SM 97/MD18	NT	NT	NT
BL/SM 97/MD19	NT	NT	NT
BL/SM 97/MD20	-	-	-

	#	%
Surviving	8	40
Virus-free	7	87.5
Therapy efficiency		35

Table 5.10: DsMV testing results of BL/SM 129 accessions treated with meristem extraction

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 129/MD1	NT	NT	NT
BL/SM 129/MD2	NT	NT	NT
BL/SM 129/MD3‡	-	-	-
BL/SM 129/MD4	NT	NT	NT
BL/SM 129/MD5	NT	NT	NT
BL/SM 129/MD6	NT	NT	NT
BL/SM 129/MD7	NT	NT	NT
BL/SM 129/MD8	NT	NT	NT
BL/SM 129/MD9	NT	NT	NT
BL/SM 129/MD10‡	NT	NT	NT
BL/SM 129/MD11‡	-	-	-
BL/SM 129/MD12‡†	-	-	-
BL/SM 129/MD13	-	-	-
BL/SM 129/MD14	NT	NT	NT
BL/SM 129/MD15	NT	NT	NT
BL/SM 129/MD16†	-	-	-
BL/SM 129/MD17†	-	-	-
BL/SM 129/MD18	NT	NT	NT
BL/SM 129/MD19	-	-	-
BL/SM 129/MD20	NT	NT	NT

	#	%
Surviving	7	35
Virus-free	7	100
Therapy efficiency		35

Table 5.11: DsMV testing results of BL/SM 145 accessions treated with meristem extraction

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 145/MD1	-	-	-
BL/SM 145/MD2‡	-	-	-
BL/SM 145/MD3	NT	NT	NT
BL/SM 145/MD4	NT	NT	NT
BL/SM 145/MD5	NT	NT	NT
BL/SM 145/MD6	NT	NT	NT
BL/SM 145/MD7‡	NT	NT	NT
BL/SM 145/MD8	NT	NT	NT
BL/SM 145/MD9	NT	NT	NT
BL/SM 145/MD10	NT	NT	NT
BL/SM 145/MD11†	-	-	-
BL/SM 145/MD12	NT	NT	NT
BL/SM 145/MD13	NT	NT	NT
BL/SM 145/MD14	NT	NT	NT
BL/SM 145/MD15*	NT	NT	NT
BL/SM 145/MD16‡	-	-	-
BL/SM 145/MD17	-	-	-
BL/SM 145/MD18	NT	NT	NT
BL/SM 145/MD19	NT	NT	NT
BL/SM 145/MD20	NT	NT	NT

	#	%
Surviving	5	26.31
Virus-free	5	100
Therapy efficiency		26.31

* replicate where fungal contamination proved lethal and meristem could not recovered. These accessions were not considered in the calculations.

**Doubtful result as there were many band was light but confirmed by third testing.

† Replicate that got contaminated with fungus but was recovered.

‡ Meristems where the outer leaves turned black during the early phase and had to be removed to all for meristem to grow.

Gel results for DsMV testing of the meristem extraction experiment

First (3months) Testing Results

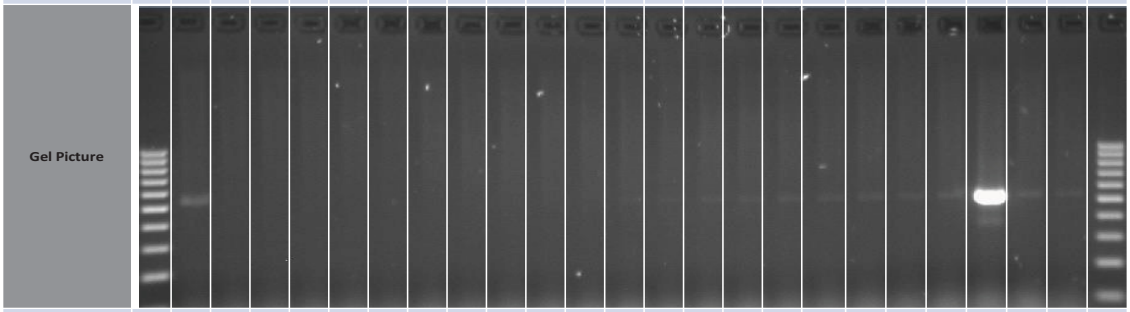
PCR Results of First Testing for DsMV for Meristem Experiment

Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Gel Picture																										
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	12	13	14	15	16	17	18	19	20	21	22	23	
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	12	13	14	15	16	17	18	19	20	21	22	23	
Content	100bp ladder	BL/SM 97/MD4	BL/SM 97/MD5	BL/SM 97/MD7	BL/SM 97/MD11	BL/SM 97/MD14	BL/SM 97/MD16	BL/SM 97/MD17	BL/SM 97/MD20	BL/SM 129/MD3	BL/SM 129/MD11	BL/SM 129/MD12	BL/SM 129/MD13	BL/SM 129/MD13*	BL/SM 129/MD16	BL/SM 129/MD17	BL/SM 129/MD19	BL/SM 145/MD1	BL/SM 145/MD2	BL/SM 145/MD11	BL/SM 145/MD16	BL/SM 145/MD17	DsMV Positive Control	Negative Control	No Template Control	100bp ladder
Result	Ok	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	Ok
Comment	**BL/SM 97/MD4** was listed in first testing but it has come out as negative in this PCR however the NAD5 test showed that the RNA was amplifiable. The first test had a few erroneous results therefore **BL/SM 97/MD4** coming out as positive may have been one of the errors. ***BL/SM 129/MD13** was loaded twice by mistake.																									

Figure 5.11: Gel 1 of first testing of DsMV for meristem extraction experiment

Second (6months) Testing Results

PCR Results of second Testing for DsMV for Meristem Experiment



Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Gel Picture																									
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	
Content	100bp ladder	BL/SM 97/MD4	BL/SM 97/MD5	BL/SM 97/MD7	BL/SM 97/MD11	BL/SM 97/MD14	BL/SM 97/MD16	BL/SM 97/MD17	BL/SM 97/MD20	BL/SM 129/MD3	BL/SM 129/MD11	BL/SM 129/MD12	BL/SM 129/MD13	BL/SM 129/MD16	BL/SM 129/MD17	BL/SM 129/MD19	BL/SM 145/MD1	BL/SM 145/MD2	BL/SM 145/MD11	BL/SM 145/MD16	BL/SM 145/MD17	DsMV Positive Control	Negative Control	No Template Control	100bp ladder
Result	Ok	+	-	-	-	-	-	-	-	-	-	-	-	-	-*	-*	-*	-*	-*	-*	-*	+	-*	-*	Ok
Comment	*Wells 14 – 23 are showing very light bands consistent with DsMV. However it is suspected that these are probably contamination. The second testing will confirm this. They are taken as negative but listed as suspect results. Since a slight amplification is also present in the negative and the no template control.																								

Figure 5.12: Gel of second testing of DsMV for meristem extraction experiment

Third (7months) Testing Results

PCR Results of third testing for DsMV for meristem experiment

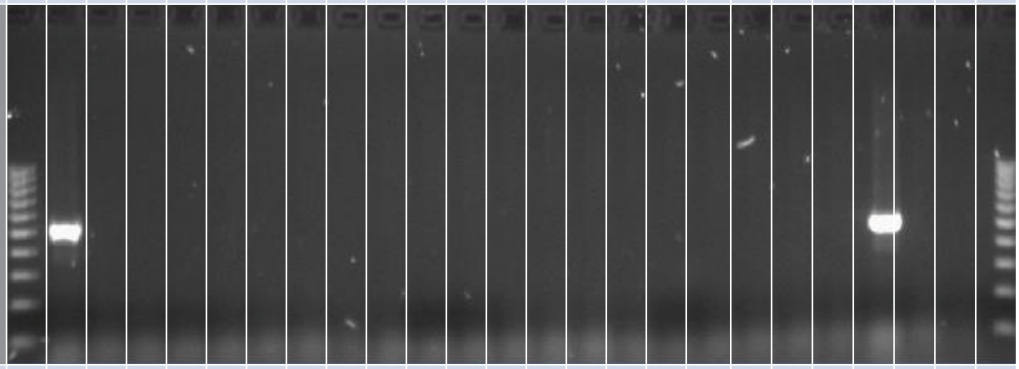
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Gel Picture																									
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	-	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	-
Content	100bp ladder	BL/SM 97/MD4	BL/SM 97/MD5	BL/SM 97/MD7	BL/SM 97/MD11	BL/SM 97/MD14	BL/SM 97/MD16	BL/SM 97/MD17	BL/SM 97/MD20	BL/SM 129/MD3	BL/SM 129/MD11	BL/SM 129/MD12	BL/SM 129/MD13	BL/SM 129/MD16	BL/SM 129/MD17	BL/SM 129/MD19	BL/SM 145/MD1	BL/SM 145/MD2	BL/SM 145/MD11	BL/SM 145/MD16	BL/SM 145/MD17	DsMV Positive Control	Negative Control	No Template Control	100bp ladder
Result	Ok	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	Ok

Figure 5.13: Gel of third testing of DsMV for meristem extraction experiment

Thermotherapy

Summary of results for DsMV testing of the thermotherapy experiment

Table 5.12: DsMV testing results of BL/SM 97 accessions treated with thermotherapy

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 97/TT1	-	-	-
BL/SM 97/TT2	NT	NT	NT
BL/SM 97/TT3	+	+	NT
BL/SM 97/TT4	NT	NT	NT
BL/SM 97/TT5	NT	NT	NT
BL/SM 97/TT6	NT	NT	NT
BL/SM 97/TT7	+	+	NT
BL/SM 97/TT8	-	-	-
BL/SM 97/TT9	-	-	-
BL/SM 97/TT10	-	-	-
BL/SM 97/TT11	-	-	-
BL/SM 97/TT12	+	+	NT
BL/SM 97/TT13	+	+	NT
BL/SM 97/TT14	-	+	+
BL/SM 97/TT15	-	+	+
BL/SM 97/TT16	-	-	-
BL/SM 97/TT17	NT	NT	NT
BL/SM 97/TT18	-	-	-
BL/SM 97/TT19	-	-	-
BL/SM 97/TT20	NT	NT	NT

	#	%
Surviving	14	70
Virus-free	8	57.14
Therapy efficiency		40

Table 5.13: DsMV testing results of BL/SM 129 accessions treated with thermotherapy

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 129/TT1	-	+	+
BL/SM 129/TT2	-	+	+
BL/SM 129/TT3	-	-	-
BL/SM 129/TT4	-	-	+
BL/SM 129/TT5	-	-	-
BL/SM 129/TT6	NT	NT	NT
BL/SM 129/TT7	-	-	-
BL/SM 129/TT8	-	-	-
BL/SM 129/TT9	-	-	-
BL/SM 129/TT10	NT	NT	NT
BL/SM 129/TT11	+	+	NT
BL/SM 129/TT12	-	-	-
BL/SM 129/TT13	-	-	-
BL/SM 129/TT14	-	-	-
BL/SM 129/TT15	-	-	-
BL/SM 129/TT16	NT	NT	NT
BL/SM 129/TT17	-	-	-
BL/SM 129/TT18	NT	NT	NT
BL/SM 129/TT19	-	+	+
BL/SM 129/TT20	+	+	NT

	#	%
Surviving	16	80
Virus-free	10	62.5
Therapy efficiency		50

Table 5.14: DsMV testing results of BL/SM 145 accessions treated with thermotherapy

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 145/TT1	NT	NT	NT
BL/SM 145/TT2	NT	NT	NT
BL/SM 145/TT3	-	-	-
BL/SM 145/TT4	-	-	-
BL/SM 145/TT5	NT	NT	NT
BL/SM 145/TT6	-	+	+
BL/SM 145/TT7	NT	NT	NT
BL/SM 145/TT8	-	-	-
BL/SM 145/TT9	-	+	+
BL/SM 145/TT10	NT	NT	NT
BL/SM 145/TT11	-	-	NT
BL/SM 145/TT12	-	-	-
BL/SM 145/TT13	+	+	NT
BL/SM 145/TT14	-	-	-
BL/SM 145/TT15	-	-	-
BL/SM 145/TT16	+	NT	NT
BL/SM 145/TT17	NT	NT	NT
BL/SM 145/TT18	-	-	-
BL/SM 145/TT19	-	-	-
BL/SM 145/TT20	-	+	+

	#	%
Surviving	14	70
Virus-free	8	57.1
Therapy efficiency		40

Gel results for DsMV testing of the thermotherapy experiment

First (3months) Testing Results

PCR Results of First Testing of DsMV for Thermotherapy Experiment

Gel 1

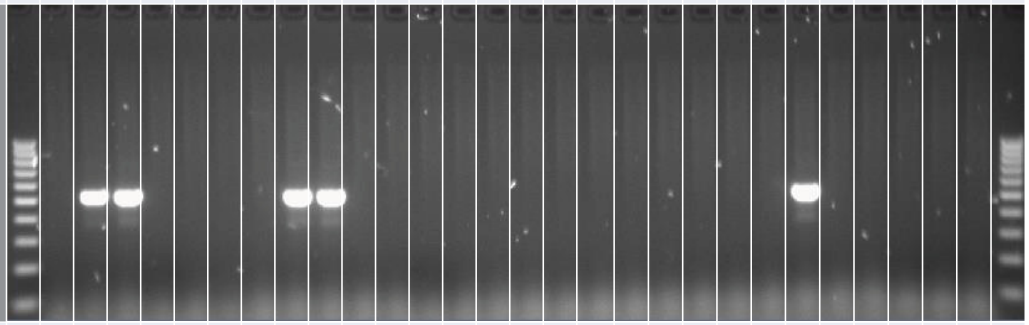
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
Content	100bp ladder	BL/SM 97/TT1	BL/SM 97/TT3	BL/SM 97/TT7	BL/SM 97/TT8	BL/SM 97/TT9	BL/SM 97/TT10	BL/SM 97/TT11	BL/SM 97/TT12	BL/SM 97/TT13	BL/SM 97/TT14	BL/SM 97/TT15	BL/SM 97/TT16	BL/SM 97/TT18	BL/SM 97/TT19	BL/SM 129/TT1	BL/SM 129/TT2	BL/SM 129/TT3	BL/SM 129/TT4	BL/SM 129/TT5	BL/SM 129/TT7	BL/SM 129/TT8	BL/SM 129/TT9	BL/SM 129/TT11	BL/SM 129/TT12	BL/SM 129/TT13	BL/SM 129/TT14	BL/SM 129/TT15	BL/SM 129/TT17	100bp ladder
Result	Ok	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	Ok

Figure 5.14: Gel 1 of first testing of DsMV for thermotherapy experiment

PCR Results of First Testing of DsMV for Thermotherapy Experiment

Gel 2

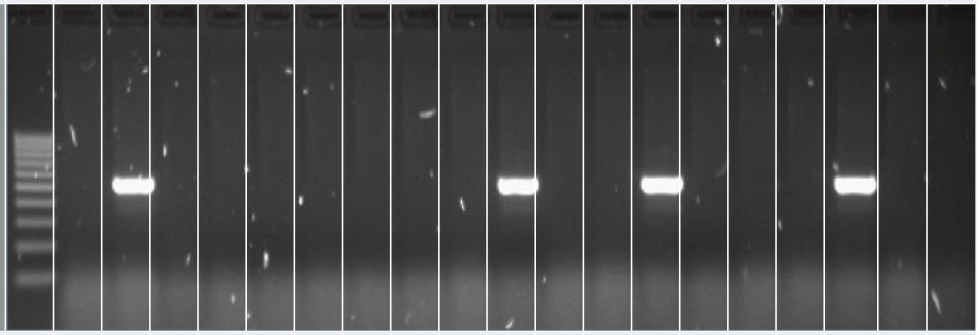
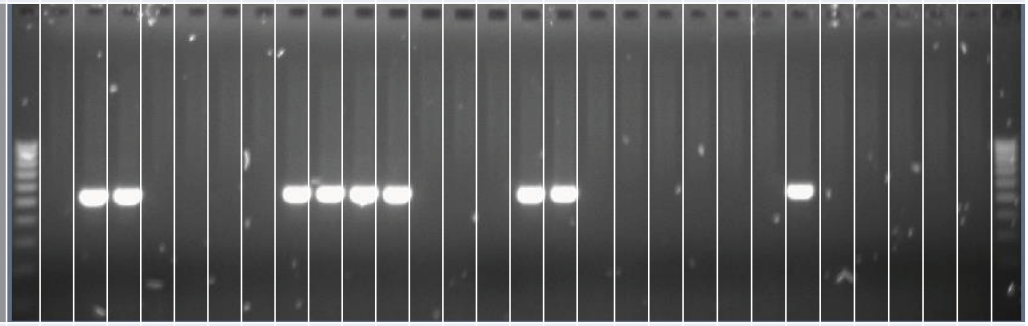
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gel Picture																				
Sample #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	-	-
PCR tube #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	48	-
Content	100bp ladder	BL/SM 129/TT19	BL/SM 129/TT20	BL/SM 145/TT3	BL/SM 145/TT4	BL/SM 145/TT6	BL/SM 145/TT8	BL/SM 145/TT9	BL/SM 145/TT11	BL/SM 145/TT12	BL/SM 145/TT13	BL/SM 145/TT14	BL/SM 145/TT15	BL/SM 145/TT16	BL/SM 145/TT18	BL/SM 145/TT19	BL/SM 145/TT20	DsMV Positive Control	Negative Control	No Template Control
Result	Ok	-	+	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	-	-

Figure 5.15: Gel 2 of first testing of DsMV for thermotherapy experiment

Second (6 months) Testing Results

PCR Results of Second Testing of DsMV for Thermotherapy Experiment

Gel 1

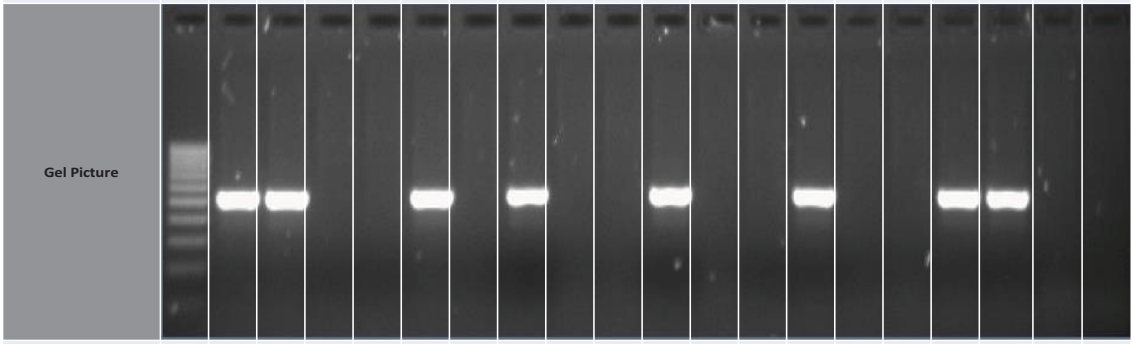


Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
Content	100bp ladder	BL/SM 97/TT1	BL/SM 97/TT3	BL/SM 97/TT7	BL/SM 97/TT8	BL/SM 97/TT9	BL/SM 97/TT10	BL/SM 97/TT11	BL/SM 97/TT12	BL/SM 97/TT13	BL/SM 97/TT14	BL/SM 97/TT15	BL/SM 97/TT16	BL/SM 97/TT18	BL/SM 97/TT19	BL/SM 129/TT1	BL/SM 129/TT2	BL/SM 129/TT3	BL/SM 129/TT4	BL/SM 129/TT5	BL/SM 129/TT7	BL/SM 129/TT8	BL/SM 129/TT9	BL/SM 129/TT11	BL/SM 129/TT12	BL/SM 129/TT13	BL/SM 129/TT14	BL/SM 129/TT15	BL/SM 129/TT17	100bp ladder
Result	Ok	-	+	+	-	-	-	-	+	+	+	+	-	-	-	+	+	-	+	-	-	-	-	-	+	-	-	-	-	Ok

Figure 5.16: Gel 1 of second testing of DsMV for thermotherapy experiment

PCR Results of Second Testing for DsMV for Thermotherapy Experiment

Gel 2



Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gel Picture																				
Sample #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	-	-
PCR tube #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	48	-
Content	100bp ladder	BL/SM 129/TT19	BL/SM 129/TT20	BL/SM 145/TT3	BL/SM 145/TT4	BL/SM 145/TT6	BL/SM 145/TT8	BL/SM 145/TT9	BL/SM 145/TT11	BL/SM 145/TT12	BL/SM 145/TT13	BL/SM 145/TT14	BL/SM 145/TT15	BL/SM 145/TT16	BL/SM 145/TT18	BL/SM 145/TT19	BL/SM 145/TT20	DsMV Positive Control	Negative Control	No Template Control
Result	Ok	+	+	-	-	+	-	+	-	-	+	-	-	+	-	-	+	+	-	-

Figure 5.17: Gel 2 of second testing of DsMV for thermotherapy experiment

Third (7 months) Testing Results

PCR Results of third Testing for DsMV for Thermotherapy Experiment

Gel 1

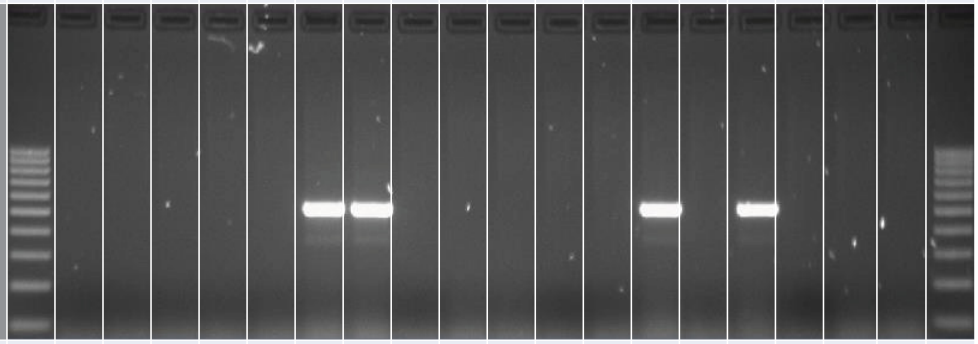
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gel Picture																				
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	-
Content	100bp ladder	BL/SM 97/TT1	BL/SM 97/TT8	BL/SM 97/TT9	BL/SM 97/TT10	BL/SM 97/TT11	BL/SM 97/TT14	BL/SM 97/TT15	BL/SM 97/TT16	BL/SM 97/TT18	BL/SM 97/TT19	BL/SM 145/TT3	BL/SM 145/TT4	BL/SM 145/TT6	BL/SM 145/TT8	BL/SM 145/TT9	BL/SM 145/TT12	BL/SM 145/TT14	BL/SM 145/TT15	100bp ladder
Result	Ok	-	-	-	-	-	+	+	-	-	-	-	-	+	-	+	-	-	-	Ok

Figure 5.18: Gel 1 of third testing of DsMV for thermotherapy experiment

PCR Results of third Testing for DsMV for Thermotherapy Experiment

Gel 2

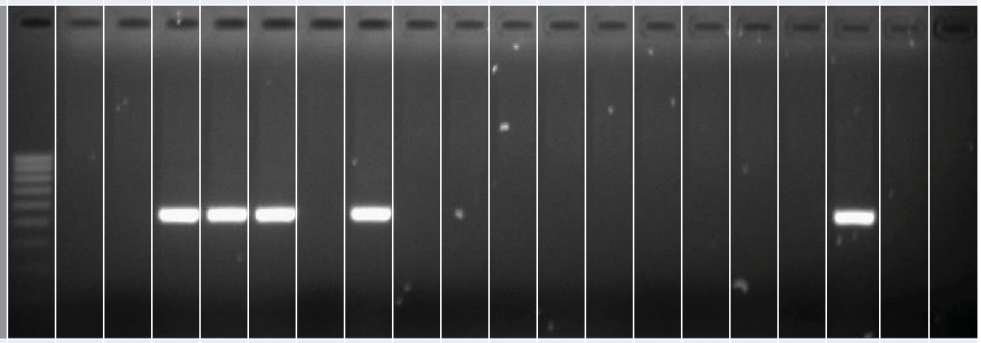
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gel Picture																				
Sample #	-	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	-
PCR tube #	-	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	-
Content	100bp ladder	BL/SM 145/TT18	BL/SM 145/TT19	BL/SM 145/TT20	BL/SM 129/TT1	BL/SM 129/TT2	BL/SM 129/TT3	BL/SM 129/TT4	BL/SM 129/TT5	BL/SM 129/TT7	BL/SM 129/TT8	BL/SM 129/TT9	BL/SM 129/TT12	BL/SM 129/TT13	BL/SM 129/TT14	BL/SM 129/TT15	BL/SM 129/TT17	DsMV Positive Control	Negative Control	No Template Control
Result	Ok	-	-	+	+	+	-	+	-	-	-	-	-	-	-	-	-	+	-	-
Comment	*BL/SM 129/TT19 was missed out during testing as it was mixed into other batch of plants. It was tested in another testing session and was positive for DsMV.																			

Figure 5.19: Gel 2 of third testing of DsMV for thermotherapy experiment

Chemotherapy

Summary of results for DsMV testing of the chemotherapy experiment

Ribavirin

Table 5.15: DsMV testing results of BL/SM 97 accessions treated with 50mg/l of ribavirin

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 97/RIB1	NT	NT	NT
BL/SM 97/RIB2	NT	NT	NT
BL/SM 97/RIB3	-	+	+
BL/SM 97/RIB4	+	NT	NT
BL/SM 97/RIB5	NT	NT	NT
BL/SM 97/RIB6	-	-	-
BL/SM 97/RIB7	NT	NT	NT
BL/SM 97/RIB8	+	+	NT
BL/SM 97/RIB9	NT	NT	NT
BL/SM 97/RIB10	-	-	-
BL/SM 97/RIB11	-	-	-
BL/SM 97/RIB12	-	-	-
BL/SM 97/RIB13	-	+	+
BL/SM 97/RIB14	+	+	NT
BL/SM 97/RIB15	+	+	NT
BL/SM 97/RIB16	-	-	-
BL/SM 97/RIB17	-	-	-
BL/SM 97/RIB18	+	+	NT
BL/SM 97/RIB19	NT	NT	NT
BL/SM 97/RIB20	NT	NT	NT

	#	%
Surviving	13	65
Virus-free	6	46
Therapy efficiency		30

Table 5.16: DsMV testing results of BL/SM 129 accessions treated with 50mg/l of ribavirin

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 129/RIB1	NT	NT	NT
BL/SM 129/RIB2	NT	NT	NT
BL/SM 129/RIB3	NT	NT	NT
BL/SM 129/RIB4	-	-	-
BL/SM 129/RIB5	NT	NT	NT
BL/SM 129/RIB6	+	-	-
BL/SM 129/RIB7	-	-	-
BL/SM 129/RIB8	-	-	-
BL/SM 129/RIB9	-	-	-
BL/SM 129/RIB10	-	-	-
BL/SM 129/RIB11	-	-	-
BL/SM 129/RIB12	-	+	+
BL/SM 129/RIB13	NT	NT	NT
BL/SM 129/RIB14	NT	NT	NT
BL/SM 129/RIB15	-	-	-
BL/SM 129/RIB16	NT	NT	NT
BL/SM 129/RIB17	-	+	+
BL/SM 129/RIB18	NT	NT	NT
BL/SM 129/RIB19	+	+	NT
BL/SM 129/RIB20	+	+	NT

	#	%
Surviving	12	60
Virus-free	8	67
Therapy efficiency		40

Table 5.17: DsMV testing results of BL/SM 145 accessions treated with 50mg/l of ribavirin

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 145/RIB1	-	+	+
BL/SM 145/RIB2	+	+	NT
BL/SM 145/RIB3	-	-	-
BL/SM 145/RIB4	NT	NT	NT
BL/SM 145/RIB5	NT	NT	NT
BL/SM 145/RIB6	NT	NT	NT
BL/SM 145/RIB7	-	+	+
BL/SM 145/RIB8	-	-	-
BL/SM 145/RIB9	NT	NT	NT
BL/SM 145/RIB10	-	+	+
BL/SM 145/RIB11	NT	NT	NT
BL/SM 145/RIB12	NT	NT	NT
BL/SM 145/RIB13	NT	NT	NT
BL/SM 145/RIB14	-	-	-
BL/SM 145/RIB15	-	-	-
BL/SM 145/RIB16	-	+	+
BL/SM 145/RIB17	NT	NT	NT
BL/SM 145/RIB18	NT	NT	NT
BL/SM 145/RIB19	+	+	NT
BL/SM 145/RIB20	-	-	-

	#	%
Surviving	10	50
Virus-free	5	50
Therapy efficiency		25

Adefovir

Table 5.18: DsMV testing results of BL/SM 97 accessions treated with 50mg/l of Adefovir

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 97/AD1	NT	NT	NT
BL/SM 97/AD2	-	-	-
BL/SM 97/AD3	-	-	-
BL/SM 97/AD4	-	-	-
BL/SM 97/AD5	-	-	-
BL/SM 97/AD6	NT	NT	NT
BL/SM 97/AD7	NT	NT	NT
BL/SM 97/AD8	-	-	-
BL/SM 97/AD9	NT	NT	NT
BL/SM 97/AD10	-	-	-
BL/SM 97/AD11	NT	NT	NT
BL/SM 97/AD12	NT	NT	NT
BL/SM 97/AD13	+	+	NT
BL/SM 97/AD14	+	+	NT
BL/SM 97/AD15	NT	NT	NT
BL/SM 97/AD16	-	-	-
BL/SM 97/AD17	-	-	-
BL/SM 97/AD18	NT	NT	NT
BL/SM 97/AD19	NT	NT	NT
BL/SM 97/AD20	NT	NT	NT

	#	%
Surviving	10	50
Virus-free	8	80
Therapy efficiency		40

Table 5.19: DsMV testing results of BL/SM 129 accessions treated with 50mg/l of Adefovir

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 129/AD1	+	+	NT
BL/SM 129/AD2	+	+	NT
BL/SM 129/AD3	-	-	-
BL/SM 129/AD4	NT	NT	NT
BL/SM 129/AD5	-	-	-
BL/SM 129/AD6	-	-	-
BL/SM 129/AD7	-	-	-
BL/SM 129/AD8	-	-	-
BL/SM 129/AD9	NT	NT	NT
BL/SM 129/AD10	-	-	-
BL/SM 129/AD11	-	-	-
BL/SM 129/AD12	NT	NT	NT
BL/SM 129/AD13	NT	NT	NT
BL/SM 129/AD14	-	+	+
BL/SM 129/AD15	NT	NT	NT
BL/SM 129/AD16	-	+	+
BL/SM 129/AD17	NT	NT	NT
BL/SM 129/AD18	NT	NT	NT
BL/SM 129/AD19	NT	NT	NT
BL/SM 129/AD20	-	+	+

	#	%
Surviving	11	55
Virus-free	7	64
Therapy efficiency		35

Table 5.20: DsMV testing results of BL/SM 145 accessions treated with 50mg/l of Adefovir

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 145/AD1	NT	NT	NT
BL/SM 145/AD2	NT	NT	NT
BL/SM 145/AD3	-	-	-
BL/SM 145/AD4	-	-	-
BL/SM 145/AD5	-	-	-
BL/SM 145/AD6	-	-	-
BL/SM 145/AD7	NT	NT	NT
BL/SM 145/AD8	NT	NT	NT
BL/SM 145/AD9	NT	NT	NT
BL/SM 145/AD10	NT	NT	NT
BL/SM 145/AD11	NT	NT	NT
BL/SM 145/AD12	-	+	+
BL/SM 145/AD13	NT	NT	NT
BL/SM 145/AD14	-	+*	+
BL/SM 145/AD15	NT	NT	NT
BL/SM 145/AD16	NT	NT	NT
BL/SM 145/AD17	NT	NT	NT
BL/SM 145/AD18	NT	NT	NT
BL/SM 145/AD19	-	-	-
BL/SM 145/AD20	-	-	-

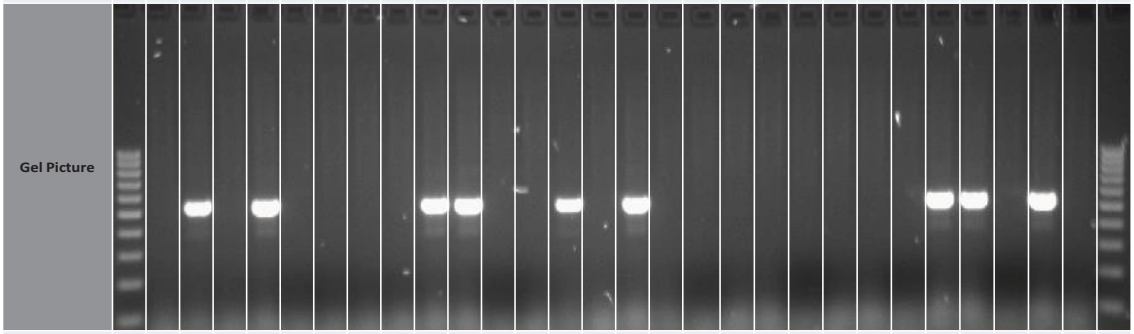
	#	%
Surviving	8	40
Virus-free	6	75
Therapy efficiency		30

Gel results for DsMV testing of the chemotherapy experiment

First (3 months) Testing Results

PCR Results of First Testing for DsMV for Chemotherapy Experiment

Gel 1

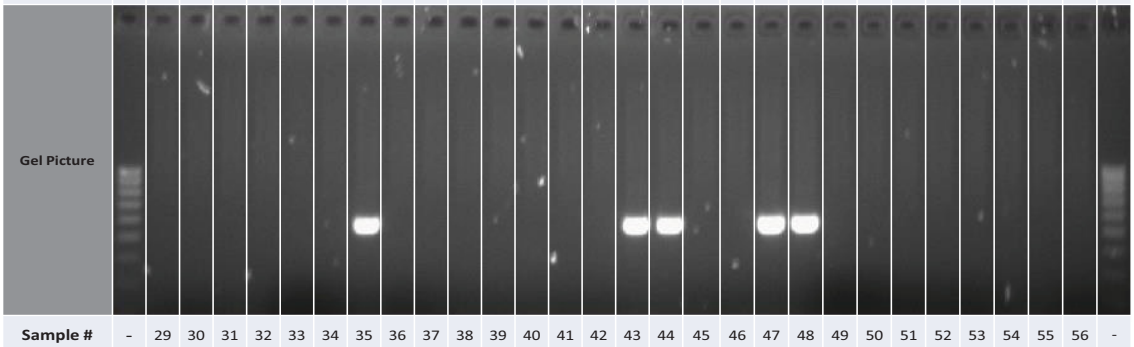


Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
Content	100bp ladder	BL/SM 97/RIB3	BL/SM 97/RIB4	BL/SM 97/RIB6	BL/SM 97/RIB8	BL/SM 97/RIB10	BL/SM 97/RIB11	BL/SM 97/RIB12	BL/SM 97/RIB13	BL/SM 97/RIB14	BL/SM 97/RIB15	BL/SM 97/RIB16	BL/SM 97/RIB17	BL/SM 97/RIB18	BL/SM 129/RIB4	BL/SM 129/RIB6	BL/SM 129/RIB7	BL/SM 129/RIB8	BL/SM 129/RIB9	BL/SM 129/RIB10	BL/SM 129/RIB11	BL/SM 129/RIB12	BL/SM 129/RIB15	BL/SM 129/RIB17	BL/SM 129/RIB19	BL/SM 129/RIB20	BL/SM 145/RIB1	BL/SM 145/RIB2	BL/SM 145/RIB3	100bp ladder
Result	Ok	-	+	-	+	-	-	-	-	+	+	-	-	+	-	+	-	-	-	-	-	-	-	-	+	+	-	+	-	Ok

Figure 5.20: Gel 1 of first testing of DsMV for chemotherapy experiment

PCR Results of First Testing for DsMV for Chemotherapy Experiment

Gel 2



Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	-
PCR tube #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	-
Content	100bp ladder	BL/SM 145/RIB7	BL/SM 145/RIB8	BL/SM 145/RIB10	BL/SM 145/RIB14	BL/SM 145/RIB15	BL/SM 145/RIB16	BL/SM 145/RIB19	BL/SM 145/RIB20	BL/SM 97/AD2	BL/SM 97/AD3	BL/SM 97/AD4	BL/SM 97/AD5	BL/SM 97/AD8	BL/SM 97/AD10	BL/SM 97/AD13	BL/SM 97/AD14	BL/SM 97/AD16	BL/SM 97/AD17	BL/SM 129/AD1	BL/SM 129/AD2	BL/SM 129/AD3	BL/SM 129/AD5	BL/SM 129/AD6	BL/SM 129/AD7	BL/SM 129/AD8	BL/SM 129/AD10	BL/SM 129/AD11	BL/SM 129/AD14	100bp ladder
Result	Ok	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	-	Ok

Figure 5.21: Gel 2 of first testing of DsMV for chemotherapy experiment

PCR Results of First Testing for DsMV for Chemotherapy Experiment

Gel 3

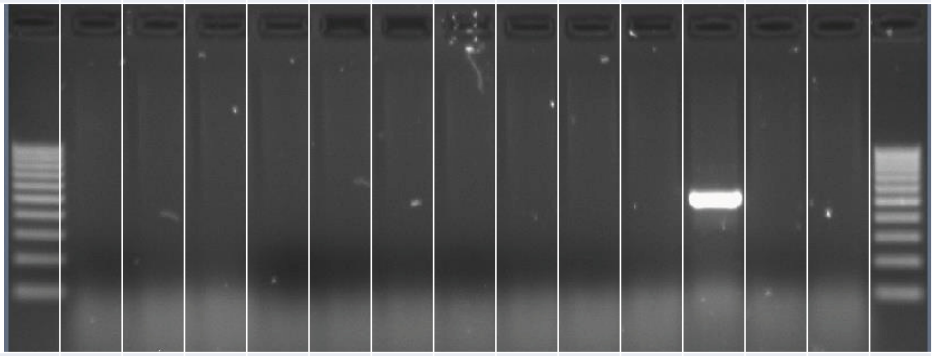
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Gel Picture															
Sample #	-	57	58	59	60	61	62	63	64	65	66	67	68	-	-
PCR tube #	-	57	58	59	60	61	62	63	64	65	66	67	68	69	-
Content	100bp ladder	BL/SM 129/AD16	BL/SM 129/AD20	BL/SM 145/AD3	BL/SM 145/AD4	BL/SM 145/AD5	BL/SM 145/AD6	BL/SM 145/AD12	BL/SM 145/AD14	BL/SM 145/AD19	BL/SM 145/AD20	DsMV Positive Control	Negative Control	No Template Control	100bp ladder
Result	Ok	-	-	-	-	-	-	-	-	-	-	+	-	-	Ok

Figure 5.22: Gel 3 of first testing of DsMV for chemotherapy experiment

Second (6 months) Testing Results

PCR Results of Second Testing for DsMV for Chemotherapy Experiment

Gel 1

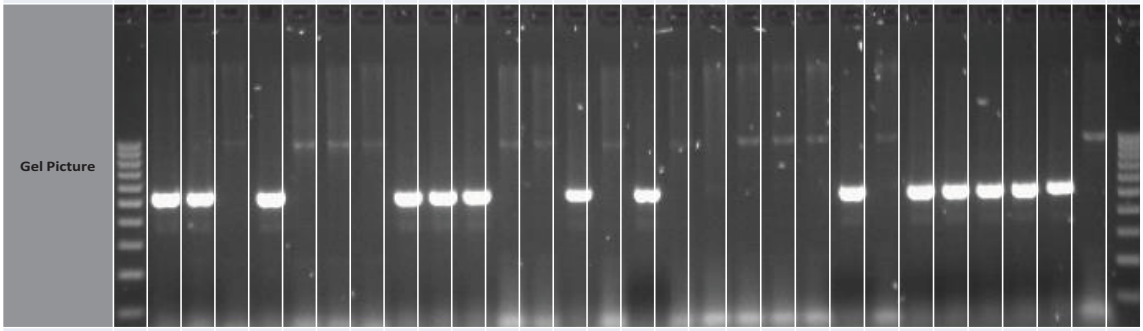
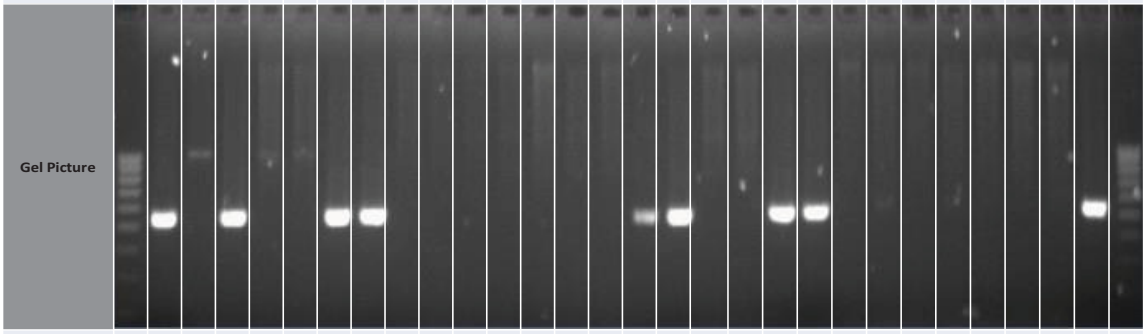
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
Content	100bp ladder	BL/SM 97/RIB3	BL/SM 97/RIB4	BL/SM 97/RIB6	BL/SM 97/RIB8	BL/SM 97/RIB10	BL/SM 97/RIB11	BL/SM 97/RIB12	BL/SM 97/RIB13	BL/SM 97/RIB14	BL/SM 97/RIB15	BL/SM 97/RIB16	BL/SM 97/RIB17	BL/SM 97/RIB18	BL/SM 129/RIB4	BL/SM 129/RIB6	BL/SM 129/RIB7	BL/SM 129/RIB8	BL/SM 129/RIB9	BL/SM 129/RIB10	BL/SM 129/RIB11	BL/SM 129/RIB12	BL/SM 129/RIB15	BL/SM 129/RIB17	BL/SM 129/RIB19	BL/SM 129/RIB20	BL/SM 145/RIB1	BL/SM 145/RIB2	BL/SM 145/RIB3	100bp ladder
Result	Ok	+	+	-	+	-	-	-	+	+	+	-	-	+	-	+	-	-	-	-	-	+	-	+	+	+	+	+	-	Ok

Figure 5.23: Gel 1 of second testing of DsMV for chemotherapy experiment

PCR Results of Second Testing for DsMV for Chemotherapy Experiment

Gel 2

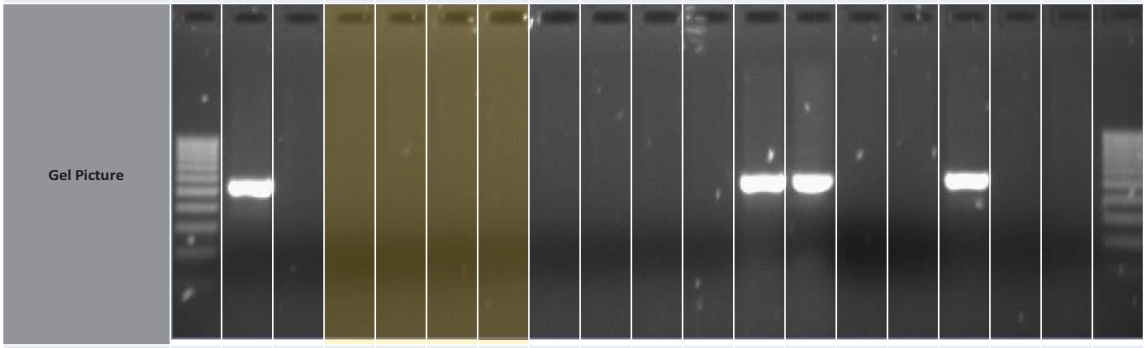


Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	-
PCR tube #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	-
Content	100bp ladder	BL/SM 145/RIB7	BL/SM 145/RIB8	BL/SM 145/RIB10	BL/SM 145/RIB14	BL/SM 145/RIB15	BL/SM 145/RIB16	BL/SM 145/RIB19	BL/SM 145/RIB20	BL/SM 97/AD2	BL/SM 97/AD3	BL/SM 97/AD4	BL/SM 97/AD5	BL/SM 97/AD8	BL/SM 97/AD10	BL/SM 97/AD13	BL/SM 97/AD14	BL/SM 97/AD16	BL/SM 97/AD17	BL/SM 129/AD1	BL/SM 129/AD2	BL/SM 129/AD3	BL/SM 129/AD5	BL/SM 129/AD6	BL/SM 129/AD7	BL/SM 129/AD8	BL/SM 129/AD10	BL/SM 129/AD11	BL/SM 129/AD14	100bp ladder
Result	Ok	+	-	+	-	-	+	+	-	-	-	-	-	-	-	+	+	-	-	+	+	-	-	-	-	-	-	-	+	Ok

Figure 5.24: Gel 2 of second testing of DsMV for chemotherapy experiment

PCR Results of Second Testing for DsMV for Chemotherapy Experiment

Gel 3



Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Gel Picture																			
Sample #	-	57	58	59	60	61	62	59	60	61	62	63	64	65	66	67	68	-	-
PCR tube #	-	57	58	59	60	61	62	59	60	61	62	63	64	65	66	67	68	69	-
Content	100bp ladder	BL/SM 129/AD16	BL/SM 129/AD20	BL/SM 145/AD3	BL/SM 145/AD4	BL/SM 145/AD5	BL/SM 145/AD6	BL/SM 145/AD3	BL/SM 145/AD4	BL/SM 145/AD5	BL/SM 145/AD6	BL/SM 145/AD12	BL/SM 145/AD14	BL/SM 145/AD19	BL/SM 145/AD20	DsMV Positive Control	Negative Control	No Template Control	100bp ladder
Result	Ok	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	Ok
Comment	*During gel loading it was thought that there was an error in wells 4-7 therefore these samples were reloaded in gels 8-11. None of these wells amplified any DsMV band.																		

Figure 5.25: Gel 3 of second testing of DsMV for chemotherapy experiment

Third (7 months) Testing Results

PCR Results of Third Testing for DsMV for Chemotherapy Experiment

Gel 1

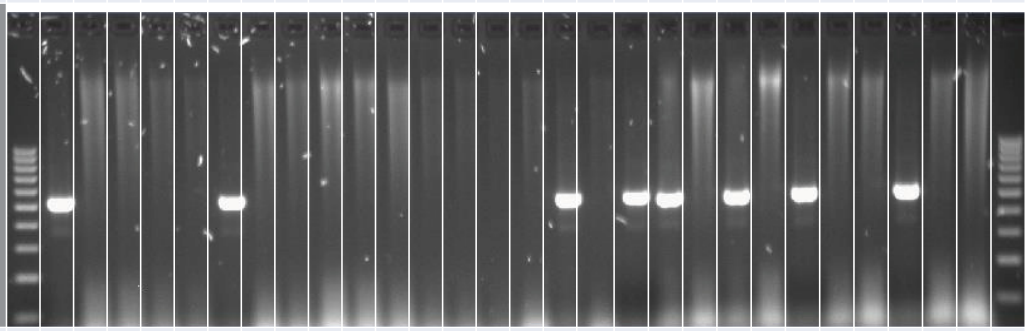
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
Content	100bp ladder	BL/SM 97/RIB3	BL/SM 97/RIB6	BL/SM 97/RIB10	BL/SM 97/RIB11	BL/SM 97/RIB12	BL/SM 97/RIB13	BL/SM 97/RIB16	BL/SM 97/RIB17	BL/SM 129/RIB4	BL/SM 129/RIB6	BL/SM 129/RIB7	BL/SM 129/RIB8	BL/SM 129/RIB9	BL/SM 129/RIB10	BL/SM 129/RIB11	BL/SM 129/RIB12	BL/SM 129/RIB15	BL/SM 129/RIB17	BL/SM 145/RIB1	BL/SM 145/RIB3	BL/SM 145/RIB7	BL/SM 145/RIB8	BL/SM 145/RIB10	BL/SM 145/RIB14	BL/SM 145/RIB15	BL/SM 145/RIB16	BL/SM 145/RIB20	BL/SM 97/AD2	100bp ladder
Result	Ok	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	+	-	+	-	+	-	-	+	-	-	Ok

Figure 5.26: Gel 1 of third testing of DsMV for chemotherapy experiment

PCR Results of Third Testing for DsMV for Chemotherapy Experiment

GEL 2

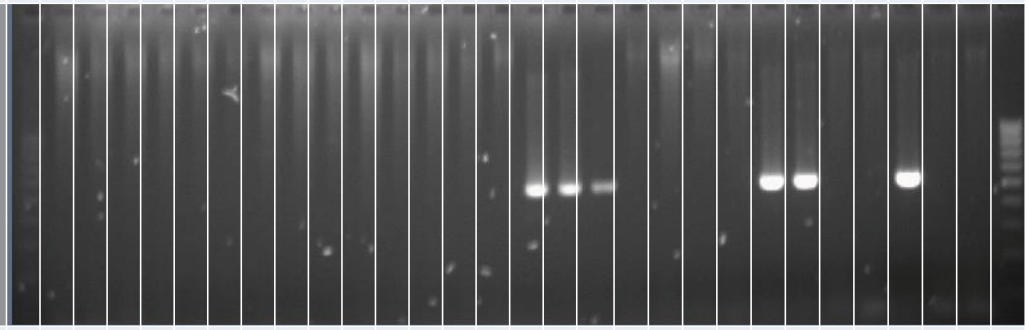
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	-	-
PCR tube #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	-
Content	100bp ladder	BL/SM 97/AD3	BL/SM 97/AD4	BL/SM 97/AD5	BL/SM 97/AD8	BL/SM 97/AD10	BL/SM 97/AD16	BL/SM 97/AD17	BL/SM 129/AD3	BL/SM 129/AD5	BL/SM 129/AD6	BL/SM 129/AD7	BL/SM 129/AD8	BL/SM 129/AD10	BL/SM 129/AD11	BL/SM 129/AD14	BL/SM 129/AD16	BL/SM 129/AD20	BL/SM 145/AD3	BL/SM 145/AD4	BL/SM 145/AD5	BL/SM 145/AD6	BL/SM 145/AD12	BL/SM 145/AD14	BL/SM 145/AD19	BL/SM 145/AD20	DsMV Positive Control	Negative Control	No Template Control	100bp ladder
Result	Ok	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+*	-	-	-	-	+	+	-	-	+	-	-	Ok
Comment	*This sample was negative in the first and second testing but positive in the third. May have been an error in PCR in secondtesting. It was tested a fourth time and the results were positive.																													

Figure 5.27: Gel 2 of third testing of DsMV for chemotherapy experiment

Electrotherapy

Summary of results for DsMV testing of the electrotherapy experiment

Table 5.21: DsMV testing results of BL/SM 97 accessions treated with electrotherapy

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 97/ET1	-	-	-
BL/SM 97/ET2	NT	NT	NT
BL/SM 97/ET3	-	-	-
BL/SM 97/ET4	-	-	-
BL/SM 97/ET5	-	+	+
BL/SM 97/ET6	-	-	-
BL/SM 97/ET7	-	-	-
BL/SM 97/ET8	-	-	-
BL/SM 97/ET9	+	+	NT
BL/SM 97/ET10	-	-	-
BL/SM 97/ET11	-	-	-
BL/SM 97/ET12	+	+	NT
BL/SM 97/ET13	-	-	-
BL/SM 97/ET14	-	-	-
BL/SM 97/ET15	-	+	+
BL/SM 97/ET16	-	-	-
BL/SM 97/ET17	-	-	-
BL/SM 97/ET18	-	-	-
BL/SM 97/ET19	-	-	-
BL/SM 97/ET20	-	+	+

	#	%
Surviving	19	95
Virus-free	14	4
Therapy efficiency		70

Table 5.22: DsMV testing results of BL/SM 129 accessions treated with electrotherapy

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 129/ET1	-	+	+
BL/SM 129/ET2	-	+	+
BL/SM 129/ET3	-	+*	+
BL/SM 129/ET4	-	-	-
BL/SM 129/ET5	-	-	-
BL/SM 129/ET6	-	-	-
BL/SM 129/ET7	+	+	NT
BL/SM 129/ET8	-	-	-
BL/SM 129/ET9			-
BL/SM 129/ET10	-	-	-
BL/SM 129/ET11	-	-	-
BL/SM 129/ET12	-	-	-
BL/SM 129/ET13	-	+	+
BL/SM 129/ET14	-	-	-
BL/SM 129/ET15	-	-	-
BL/SM 129/ET16	-	-	-
BL/SM 129/ET17	-	-	-
BL/SM 129/ET18	-	-	-
BL/SM 129/ET19	NT	NT	NT
BL/SM 129/ET20	NT	NT	NT

	#	%
Surviving	18	90
Virus-free	13	72
Therapy efficiency		65

Table 5.23: DsMV testing results of BL/SM 145 accessions treated with electrotherapy

Accession Code	1st testing	2nd Testing	3rd Testing
BL/SM 145/ET1	-	-	-
BL/SM 145/ET2	-	-	-
BL/SM 145/ET3	-	-	-
BL/SM 145/ET4	-	-	-
BL/SM 145/ET5	-	+	+
BL/SM 145/ET6	-	-	-
BL/SM 145/ET7	-	-	-
BL/SM 145/ET8	-	+	+
BL/SM 145/ET9	NT	NT	NT
BL/SM 145/ET10	-	-	-
BL/SM 145/ET11	-	-	-
BL/SM 145/ET12	-	-	-
BL/SM 145/ET13	-	-	-
BL/SM 145/ET14	-	-	-
BL/SM 145/ET15	-	-	-
BL/SM 145/ET16	-	-	-
BL/SM 145/ET17	NT	NT	NT
BL/SM 145/ET18	-	-	-
BL/SM 145/ET19	-	-	-
BL/SM 145/ET20	-	-	-

	#	%
Surviving	18	90
Virus-free	15	83
Therapy efficiency		75

Gel results for DsMV testing of the electrotherapy experiment

First (3 months) Testing Results

PCR Results of First Testing for DsMV for Electrotherapy Experiment

Gel 1

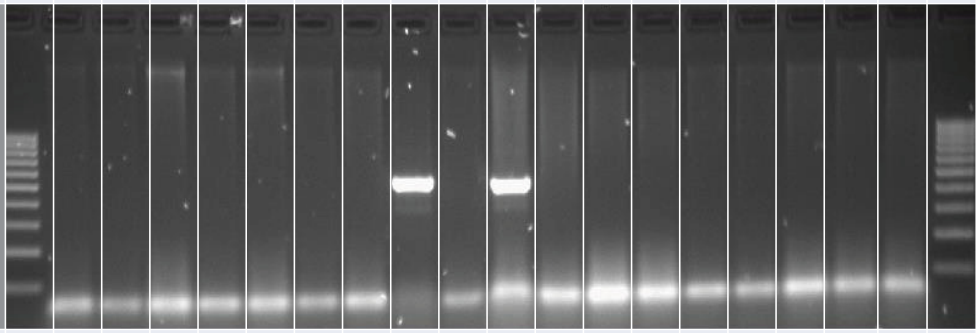
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gel Picture																				
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	-
Content	100bp ladder	BL/SM 97/ET1	BL/SM 97/ET3	BL/SM 97/ET4	BL/SM 97/ET5	BL/SM 97/ET6	BL/SM 97/ET7	BL/SM 97/ET8	BL/SM 97/ET9	BL/SM 97/ET10	BL/SM 97/ET11	BL/SM 97/ET12	BL/SM 97/ET13	BL/SM 97/ET14	BL/SM 97/ET15	BL/SM 97/ET16	BL/SM 97/ET17	BL/SM 97/ET18	BL/SM 97/ET19	100bp ladder
Result	Ok	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	Ok

Figure 5.28: Gel 1 of first testing of DsMV for electrotherapy experiment

PCR Results of First Testing for DsMV for Electrotherapy Experiment

Gel 2

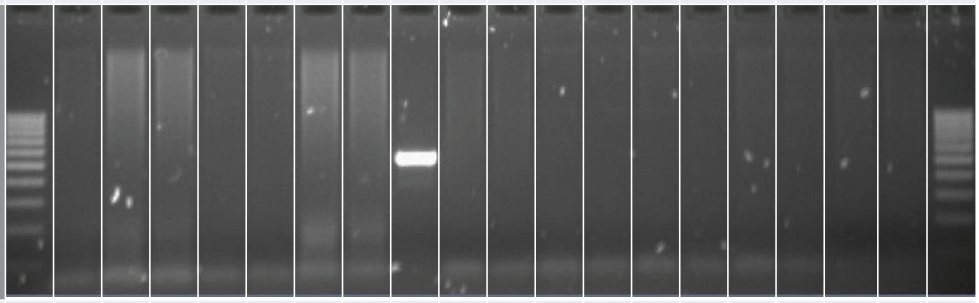
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gel Picture																				
Sample #	-	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	-
PCR tube #	-	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	-
Content	100bp ladder	BL/SM 97/ET20	BL/SM 129/ET1	BL/SM 129/ET2	BL/SM 129/ET3	BL/SM 129/ET4	BL/SM 129/ET5	BL/SM 129/ET6	BL/SM 129/ET7	BL/SM 129/ET8	BL/SM 129/ET9	BL/SM 129/ET10	BL/SM 129/ET11	BL/SM 129/ET12	BL/SM 129/ET13	BL/SM 129/ET14	BL/SM 129/ET15	BL/SM 129/ET16	BL/SM 129/ET17	100bp ladder
Result	Ok	-	-	-	-	-	-	-	+	-		-	-	-	-	-	-	-	-	Ok
Comment																				

Figure 5.29: Gel 2 of first testing of DsMV for electrotherapy experiment

PCR Results of First Testing for DsMV for Electrotherapy Experiment

Gel 3

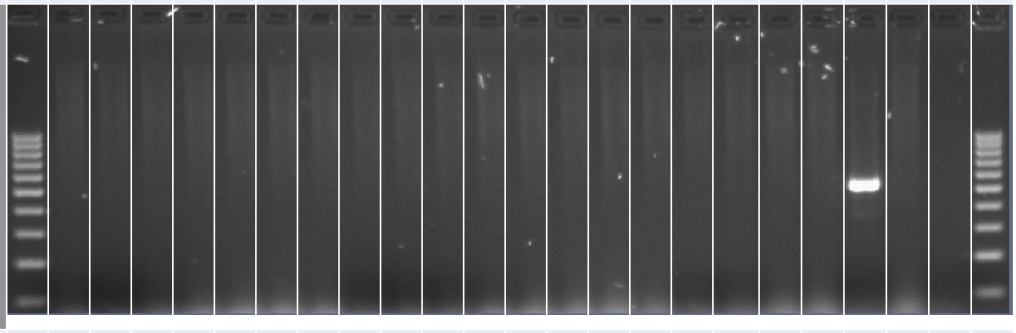
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Gel Picture																								
Sample #	-	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	-	-
PCR tube #	-	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	-
Content	100bp ladder	BL/SM 129/ET18	BL/SM 145/ET1	BL/SM 145/ET2	BL/SM 145/ET3	BL/SM 145/ET4	BL/SM 145/ET5	BL/SM 145/ET6	BL/SM 145/ET7	BL/SM 145/ET8	BL/SM 145/ET10	BL/SM 145/ET11	BL/SM 145/ET12	BL/SM 145/ET13	BL/SM 145/ET14	BL/SM 145/ET15	BL/SM 145/ET16	BL/SM 145/ET18	BL/SM 145/ET19	BL/SM 145/ET20	DsMV Positive Control	Negative Control	No Template Control	100bp ladder
Result	Ok	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	Ok

Figure 5. 30: Gel 3 of first testing of DsMV for electrotherapy experiment

Second (6 months) Testing Results

PCR Results of Second Testing for DsMV for Electrotherapy Experiment

Gel 1

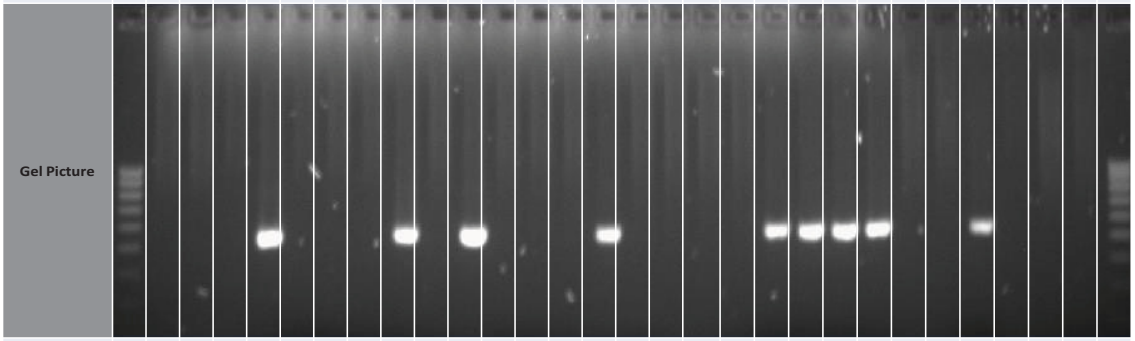
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
Content	100bp ladder	BL/SM 97/ET1	BL/SM 97/ET3	BL/SM 97/ET4	BL/SM 97/ET5	BL/SM 97/ET6	BL/SM 97/ET7	BL/SM 97/ET8	BL/SM 97/ET9	BL/SM 97/ET10	BL/SM 97/ET11	BL/SM 97/ET12	BL/SM 97/ET13	BL/SM 97/ET14	BL/SM 97/ET15	BL/SM 97/ET16	BL/SM 97/ET17	BL/SM 97/ET18	BL/SM 97/ET19	BL/SM 97/ET20	BL/SM 129/ET1	BL/SM 129/ET2	BL/SM 129/ET3	BL/SM 129/ET4	BL/SM 129/ET5	BL/SM 129/ET6	BL/SM 129/ET7	BL/SM 129/ET8	BL/SM 129/ET9	100bp ladder
Result	Ok	-	-	-	+	-	-	-	+	-	+	-	-	-	+	-	-	-	-	+	+	+	+	-	-	+	-	-	-	Ok

Figure 5.31: Gel 1 of second testing of DsMV for electrotherapy experiment

PCR Results of Second Testing for DsMV for Electrotherapy Experiment

Gel 2

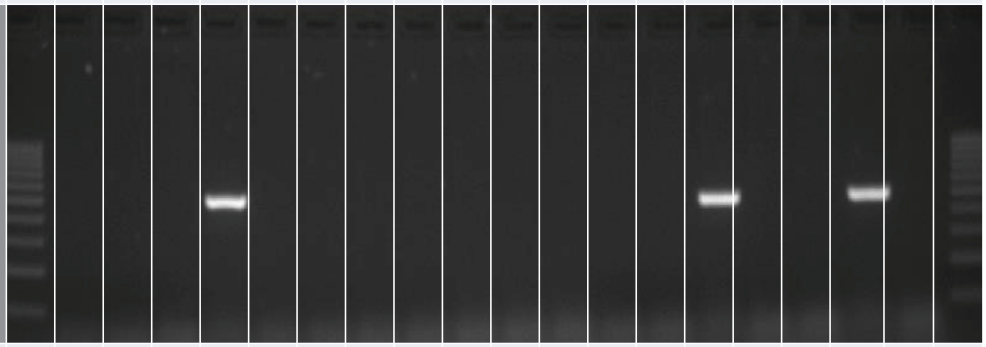
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Gel Picture																				
Sample #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	-
PCR tube #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	-
Content	100bp ladder	BL/SM 129/ET10	BL/SM 129/ET11	BL/SM 129/ET12	BL/SM 129/ET13	BL/SM 129/ET14	BL/SM 129/ET15	BL/SM 129/ET16	BL/SM 129/ET17	BL/SM 129/ET18	BL/SM 145/ET1	BL/SM 145/ET2	BL/SM 145/ET3	BL/SM 145/ET4	BL/SM 145/ET5	BL/SM 145/ET6	BL/SM 145/ET7	BL/SM 145/ET8	BL/SM 145/ET10	100bp ladder
Result	Ok	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	Ok

Figure 5.32: Gel 2 of second testing of DsMV for electrotherapy experiment

PCR Results of Second Testing for DsMV for Electrotherapy Experiment

Gel 3

Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Gel Picture														
Sample #	-	47	48	49	50	51	52	53	54	54	56	57	-	-
PCR tube #	-	47	48	49	50	51	52	53	54	54	56	57	58	-
Content	100bp ladder	BL/SM 145/ET11	BL/SM 145/ET12	BL/SM 145/ET13	BL/SM 145/ET14	BL/SM 145/ET15	BL/SM 145/ET16	BL/SM 145/ET18	BL/SM 145/ET19	BL/SM 145/ET20	DsMV Positive Control	Negative Control	No Template Control	100bp ladder
Result	Ok	-	-	-	-	-	-	-	-	-	+	-	-	Ok

Figure 5.33: Gel 3 of second testing of DsMV for electrotherapy experiment

Third (7 months) testing results

PCR Results of Third Testing for DsMV for Electrotherapy Experiment

Gel 1

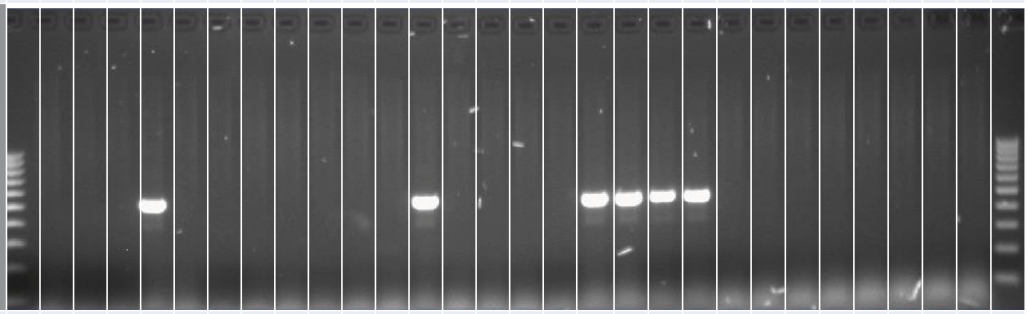
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																														
Sample #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
PCR tube #	-	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	-
Content	100bp ladder	BL/SM 97/ET1	BL/SM 97/ET3	BL/SM 97/ET4	BL/SM 97/ET5	BL/SM 97/ET6	BL/SM 97/ET7	BL/SM 97/ET8	BL/SM 97/ET10	BL/SM 97/ET11	BL/SM 97/ET13	BL/SM 97/ET14	BL/SM 97/ET15	BL/SM 97/ET16	BL/SM 97/ET17	BL/SM 97/ET18	BL/SM 97/ET19	BL/SM 97/ET20	BL/SM 129/ET1	BL/SM 129/ET2	BL/SM 129/ET3	BL/SM 129/ET4	BL/SM 129/ET5	BL/SM 129/ET6	BL/SM 129/ET8	BL/SM 129/ET9	BL/SM 129/ET10	BL/SM 129/ET11	BL/SM 129/ET12	100bp ladder
Result	Ok	-	-	-	+	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	Ok

Figure 5.34: Gel 1 of third testing of DsMV for electrotherapy experiment

PCR Results of Third Testing for DsMV for Electrotherapy Experiment

Gel 2

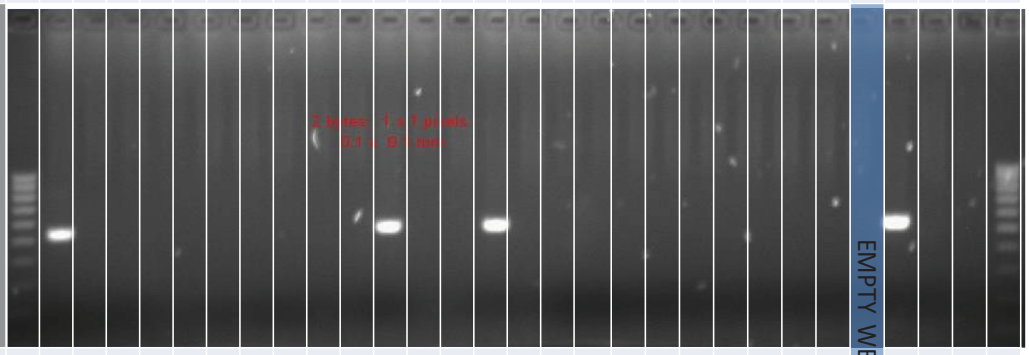
Well #	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
Gel Picture																										EMPTY WELL				
Sample #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52					-
PCR tube #	-	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52					-
Content	100bp ladder	BL/SM 129/ET13	BL/SM 129/ET14	BL/SM 129/ET15	BL/SM 129/ET16	BL/SM 129/ET17	BL/SM 129/ET18	BL/SM 145/ET1	BL/SM 145/ET2	BL/SM 145/ET3	BL/SM 145/ET4	BL/SM 145/ET5	BL/SM 145/ET6	BL/SM 145/ET7	BL/SM 145/ET8	BL/SM 145/ET10	BL/SM 145/ET11	BL/SM 145/ET12	BL/SM 145/ET13	BL/SM 145/ET14	BL/SM 145/ET15	BL/SM 145/ET16	BL/SM 145/ET18	BL/SM 145/ET19	BL/SM 145/ET20		DsMV Positive Control	Negative Control	No Template Control	100bp ladder
Result	Ok	+	-	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-		+	-	-	Ok

Figure 5.35: Gel 2 of third testing of DsMV for electrotherapy experiment