Development of improved methods for phytoplasma diagnostics

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Abstract

Phytoplasmas are plant pathogens that are small prokaryotic, wall-less bacteria causing significant diseases in hundreds of plant species globally. They have been named as distinct '*Candidatus* phytoplasma' species and belong to the Mollicutes. These microorganisms are non-cultivable plant pathogens (obligate), so their diagnoses are primarily achieved by molecular techniques. In infected plants, they inhabit phloem tissues (sieve cells) and they are transmitted between plants by insects (phloem feeders) such as those in the orders Cicadellidae, Psyllidae and Fulgoridae. Phytoplasmas have extremely small genomes and also have very low levels of the nucleotides cytosine and guanine.

Polymerase Chain Reaction (PCR) has been developed and employed for investigating phytoplasmas in their hosts, and such techniques have been advanced through the use of nested PCR and real-time PCR to improve the diagnosis of phytoplasmas with low titre in their different hosts, and also to be able to identify the different taxonomic groups of the pathogens. In addition, real-time PCR can be used to quantify phytoplasmas in the infected plants or insects.

The purpose of this project was to concentrate initially on 16S rRNA, leucyl tRNA synthetase and *secA* based PCR assays and test these on periwinkle plants containing phytoplasmas from six taxonomic groups, 16SrI, II, III, V, VI and X. Work also focussed on transmitting the six phytoplasma strains between periwinkle plants and other host plants

such as Napier grass, oil palm, and tomato plants through dodder shoots. Cuttings were taken from mother plants, that are all from the same periwinkle genetic background, to enable the propagation of new plants containing isolates from the six groups. These were treated with different potential control strategies, and conventional and real-time PCR and (Loop-mediated isothermal amplification) LAMP were used to test the levels of phytoplasma in these plants following treatments to determine the efficacy of the different control treatments. In more detail, the control strategies being tested included altered nitrogen fertilization regimes, treatment with salicylic acid, and UV-C pulse treatments.

As a consequence, phytoplasma titre of different phylogenetic groups were affected by the application of different nitrogen fertilizers and different amounts, and this showed the potential for use of various nitrogen sources as a control method for phytoplasma diseases. Furthermore, this research has shown that UV-C light can be applied to phytoplasma-infected plants to enhance the resistance characteristics of plants against phytoplasma and reduce phytoplasma titre within diseased plants.

Through applying different control approaches against phytoplasma infected plants, it has been shown that low concentrations of salicylic acid application had an optimal significant effect on phytoplasma quantity and reduced infection on diseased periwinkle plants. However, a high concentration of the same solution produced a negative effect and phytotoxicity on periwinkle plants.

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Abbreviations and acronyms

AN	Ammonium Nitrate
AP	Apple Proliferation Phytoplasma
AS	Ammonium Sulphate
AY-WB	Aster Yellows Witches' broom Phytoplasma
CN	Calcium Nitrate
Ca. P.	'*Candidatus* Phytoplasma'
C_T	Cycle Threshold
CTAB	Cetyltrimethylammonium bromide
COX	Cytochrome Oxidase
EY	Elm Yellows Phytoplasma
IRPCM	International Research Project for Comparative Mycoplasmology
LAMP	Loop-Mediated Isothermal Amplification
Leu	Leucyl tRNA synthetase gene
LNI	Plum leptonecrosis Phytoplasma
MEGA	Molecular Evolutionary Genetics Analysis
MLOs	Mycoplasma-like Organisms
NGS	Napier Grass Stunt Phytoplasma
OY-M	Onion Yellows Mild Strain Phytoplasma
PAa	Australian Grapevine Yellows Phytoplasma
PCR	Polymerase Chain Reaction
PMU	Potential Mobile Unit
PWB	Potato Witches' Broom Phytoplasma
PWX	Peach Western X Phytoplasma
qPCR	Real-time Quantitative PCR, or Real-Time PCR
RG	Rehmannia Glutinosa Phytoplasma
RIV	Ribes in Vinca Phytoplasma

SA	Salicylic Acid
SGP	Strawberry Green Petal Phytoplasma
SP	Soybean Phyllody Phytoplasma
SPLL	Sweet Potato Little Leaf Phytoplasma
T-RFLP	Terminal restriction fragment length polymorphism
UV	Ultraviolet Light
VCP	Vinca Coconut Phyllody Phytoplasma

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Chapter 1. Introduction

1.1 hytoplasmas

1.1.1 History of phytoplasma discovery

Phytoplasmas (previously named mycoplasma-like organisms or MLOs) were firstly discovered by Japanese scientists in 1967 as the casual agent of mulberry dwarf disease and were observed to be similar to mycoplasmas based on their morphology, fine structures and sensitivity to tetracycline. Because of these similarities, these micro-organisms were named as mycoplasma-like organisms that infect plants. All yellows diseases of plants were presumed to be caused by viruses before the discovery of phytoplasmas, even though in many cases viruses could not be visualized in diseased tissues or isolated from infected plants consistently (Lee et al., 2000).

The trivial name 'phytoplasma' has been approved as the common name for wall-less, non-helical prokaryotes that colonize plant phloem and insects (IRPCM, 2004). These kinds of plant pathogens are a group of bacteria. Phytoplasmas and mycoplasmas belong to the Mollicutes class, which are prokaryotes lacking cell walls. They are currently non-

culturable and have resisted all endeavours to culture *in vitro* in cell-free media (Bertaccini and Duduk, 2009). Phytoplasmas have the ability to multiply and survive in the isotonic environments provided by plant phloem and insect haemolymph (Bertaccini et al., 2014). Their life cycle is unique in that it involves replication in both plants and insects, and they can have both negative and positive impacts on their insect vectors by killing the vectors or by enhancing longevity, respectively. These microorganisms are the cause of many ornamental and fruit tree diseases (Kamińska and Korbin, 1999, Gasparich, 2010, Hodgetts et al., 2013).

Many researchers use periwinkle plants as an experimental host for maintaining collections of phytoplasmas due to the fact that it is easy to infect them with many different phytoplasmas. *C. roseus* have been cultivated as ornamental plants in tropical and subtropical countries. They are moreover, easy to grow and quick in responding to infections by phytoplasmas. Madagascar periwinkle or vinca is a shrubby, herbaceous perennial native to the Indian Ocean island of Madagascar, and it is widely cultivated as a garden ornamental and medicinal plant in tropical and subtropical areas around the world. It is possible to infect Madagascar periwinkle by many known phytoplasmas through graft inoculation, dodder or insect transmission (Marcone et al., 1999a, Nejat et al., 2013).

Based on the application of molecular technologies the phylogeny of these pathogens is being resolved and has resulted in the designation of a new taxon named '*Candidatus* Phytoplasma' (Duduk and Bertaccini, 2011). However, there can be difficulties in identifying the presence of phytoplasmas in plants as a result of their low concentration, particularly in woody plants, and their erratic distribution in the infected plants. There are various methods such as grafting on indicator plants, staining of the pathogen nucleic acid with DNA-specific dyes, and electron microscopy observations, which have been used to detect phytoplasmas in symptomatic plants. However, these often give unreliable results (Marzachi, 2006, Bertaccini and Duduk, 2009). Electron microscopy examinations have shown that the phytoplasma concentration varies greatly from one infected plant to another; for instance, American elms (*Ulmus americana*) infected with the elm yellows agent have a very low phytoplasma titre, and in flavescence dorée infected grapevines the concentration of phytoplasma is often below the detection level of microscopical methods (Berges et al., 2000).

Different phytoplasmas and different stages of infection can make the symptoms induced vary in diseased plants. Internally, phytoplasma infections have the ability to cause extensive phloem necrosis and, often, excess formation of phloem tissue, resulting in swollen veins (Lee et al., 2000). Mostly, phytoplasma infections cause symptoms that have a visible detrimental effect on the plants, but the plant's response can vary, with some showing a form of resistance or tolerance, while some

plants are symptomless or exhibit mild symptoms. In addition, the economic impact of phytoplasma infections on the plant vary, ranging from partial reduction in yield and quality to nearly total crop loss (Chen and Chen, 1998, Lee et al., 2000, Bertaccini and Duduk, 2009).

Phytoplasma diseases are very difficult to control. Disease-resistant host plants (when available) and pesticide applications to control insect vectors have been used to control them with limited success (Weintraub, 2007, Gasparich, 2010).

Applications of tetracycline into infected plants has been done in some crops but is too costly and environmentally damaging since resistance to antibiotics in other microbial populations may develop, not to mention potential entry into the human food chain. Genetically engineered host plants have also been developed to try and deter insect vector feeding or interfere with phytoplasma replication (Gasparich, 2010).

1.1.2 General characteristics of phytoplasmas

Phytoplasmas are very small with a diameter of 200 to 800 μm, which is almost the same size as a plant cell's chloroplast; they also have very small genomes (530 - 1350 kb). Their shapes are pleomorphic or filamentous and they are surrounded by a triple layered unit membrane, with their DNA free in the cytoplasm. In addition to that, they have

strands of nuclear material and ribosomes (Marcone et al., 1999b, Nejat and Vadamalai, 2010, Bertaccini et al., 2014).

According to Nejat and Vadamalai (2010), the genomes of phytoplasmas are significantly variable in size. The smallest one is believed to be around 530 kbp which belongs to the Bermudagrass white leaf phytoplasma and may represent the smallest chromosome known of any living organism to date. The largest ones amongst the phytoplasmas appear to be the aster yellows phytoplasma genomes, which are around 1350 kbp but are still much smaller than their closest relatives, a member of the genus *Acholeplasma* sp.

Whole genome sequences have already been completed for two strains of aster yellows ('*Candidatus* Phytoplasma asteris'), two strains of '*Candidatus* Phytoplasma australiense', and a strain of '*Candidatus* Phytoplasma mali'. This is making it possible to understand the molecular mechanisms underlying pathogen-host interactions and also virulence, due to conserved sequences can be used as a major breakthrough in detection, identification, and classification of phytoplasmas (Bertaccini et al., 2014). The season, tissue, host and pathogen species have an impact on the phytoplasmas colonization of the plant, which results in different symptoms due to complex interference with the host physiology (Marzachi, 2006).

In a natural ecosystem, phytoplasmas are transmitted from one plant to another plant by insect vectors and they are mainly dependent on insect

transmission for their spread and survival. The lifecycles of these pathogens are inside their hosts, so their lifecycles involve replication in insects and plants. They have to cross the gut and salivary gland cells inside the insect in order to reach the saliva for subsequent introduction into the phloem during insect feeding. When the insects are feeding on the plants, initially they absorb phytoplasmas during their feeding, and after the latent period (i.e. the time between acquisition of the phytoplasmas by insect and transmission back into the plants, which can vary between 7 and 80 days), the insect vectors have the ability to introduce phytoplasmas back into the plants (Hogenhout et al., 2008).

These pathogens are unique biologically, as they have the ability to survive and parasitise a diverse range of hosts, such as plants and insect vectors, and they can also reside endocellular within the plant phloem and feeding insects (Oshima et al., 2011). The phytoplasma life cycle between plants and insect vectors is shown in Figure 1.1.

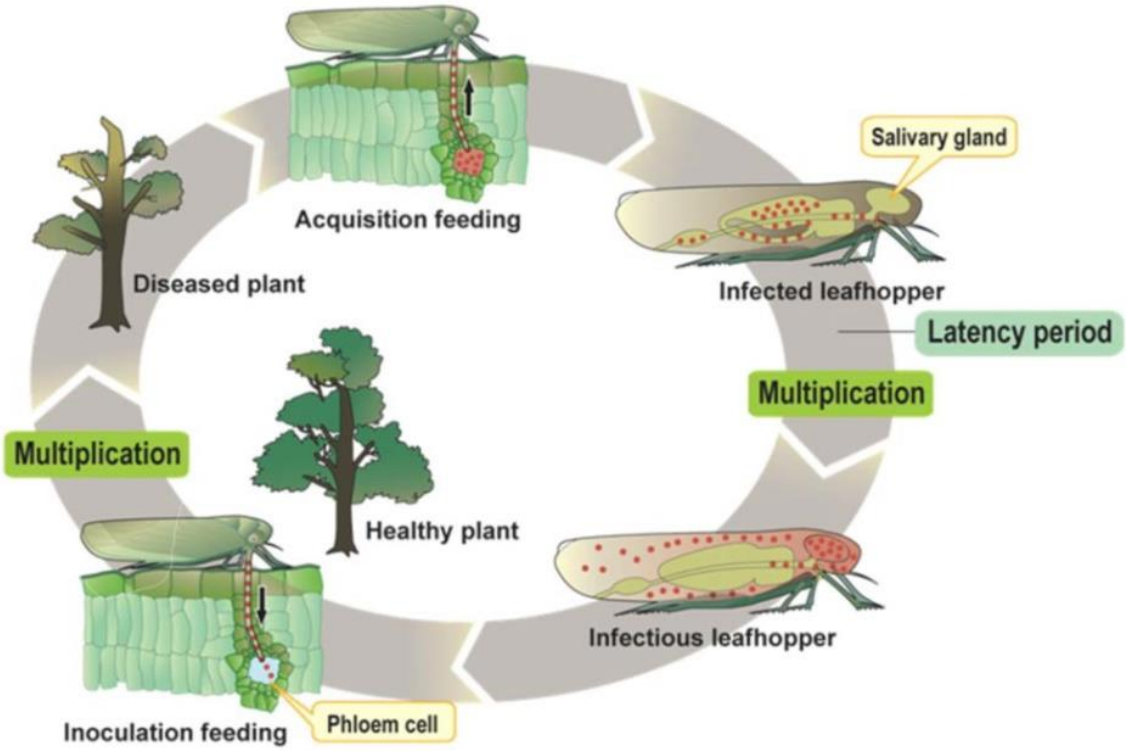


Figure 1.1 Lifecycle of phytoplasmas (Oshima et al., 2011). The phytoplasma is transmitted by infected leafhoppers from infected plants into healthy plants through insect vectors feeding. Each red dot represents a phytoplasma cell.

1.1.3 Classification of phytoplasma

Phytoplasmas have been assigned to the provisional genus '*Candidatus* Phytoplasma' under the family Acholeplasmataceae and belong to the class Mollicutes. Recent molecular data has provided considerable insights on phytoplasmas genetic interrelationships and diversity that can be used as a basis information to make some comprehensive studies on phylogeny and taxonomy of phytoplasma. Identification, classification and separation amongst the '*Ca*. P. species' can be done based on the 16S rRNA gene, with new '*Ca.* P. species' being defined as when the similarity of the 16S rRNA gene is less than 97.5%. Exceptionally, it is possible to create a novel '*Ca.* P. species' that has a different host range and/or insect vectors, even if the similarity of the 16S rRNA gene is greater than 97.5% (Bertaccini and Duduk, 2009, IRPCM, 2004, Hogenhout et al., 2008, Siewert et al., 2014). In addition, more definition between species and within species can be obtained using the internal transcribed spacer between the 16S and 23S rRNA, the 23S rRNA, ribosomal protein (rp), *secA, secY* and *tuf* genes (Gundersen et al., 1996, Lee et al., 2006, Duduk and Bertaccini, 2011).

The '*Candidatus* phytoplasma' species are determined by the sequence analysis based on 16S rDNA. Restriction fragment length polymorphism (RFLP) has been used as a basis for the first comprehensive phytoplasma classification scheme for analysis polymerase chain reaction (PCR)-amplified 16S rDNA (Duduk and Bertaccini, 2011), which is providing the

most comprehensive and widely accepted phytoplasma classification system for the differentiation of a broad array of phytoplasmas. According to a summary provided by (Bertaccini et al., 2014) phytoplasmas have been classified into 33 16S rRNA groups. All the currently recognised groups of phytoplasma are shown in Table 1.1.

Table 1.1 Classification of phytoplasmas based on 16S rDNA, adapted from Bertaccini et al. (2014).

Representative strain (acronym)	**Disease(s)**	**Place of isolation**	**16Sr Group**
Aster yellows witches' broom (AY-WB) '*Ca*. P. asteris' - Onion yellows mild strain (OY-M) '*Ca*. P. asteris' Clover phyllody Soybean purple stem (SPS) *Rehmania glutinosa* (RG) Chrysanthemum Yellows (ChY) Tomato big bud (BB) Ribes in Vinca (RIV)	Aster yellows	America, Europe, Asia, Africa	I
Peanut witches' broom (PnWB) Lime witches' broom (WBDL) '*Ca*. P. aurantifolia' Sweet potato little leaf (SPLL) Faba bean phyllody (FBP) Vinca Coconut Phyllody (VCP) Papaya mosaic (PpM) '*Ca*. P. australasia' Crotalaria Saltiana Phyllody (witches' broom) (CSP)	Peanut witches' broom	America, Africa, Europe, Asia, Australia	II
Peach X-disease '*Ca*. P. pruni'	X-disease	America, Europe, Asia	III

Clover yellow edge (CYE) Poinsettia Branching Factor (PBF) Potato purple top (MT117) Plum Leptonecrosis (PL)			
Coconut lethal yellowing Yucatan coconut lethal decline (LDY) Tanzanian coconut lethal decline (LDT)	Coconut lethal yellows	America, Africa	IV
Elm yellows (EY) '*Ca*. P. ulmi' *Flavescence dorée* Balanite witches' broom (BltWB) *'Ca*. P. balanitae' Rubus stunt '*Ca*. P. rubi"	Elm yellows	Europe, America, Asia, Africa	V
Clover proliferation '*Ca*. P. trifolii' Potato witches' broom (PWB) Catharanthus phyllody phytoplasma (CPS) Brinjal Little Leaf (BLL) Strawberry multiplier disease (MC)	Clover proliferation	Europe, America, Asia	VI
Ash yellows '*Ca*. P. fraxini' Erigeron witches' broom (ErWB) Argentinian alfalfa witches' broom (ArAWB)	Ash yellows	America, Europe	VII
Loofah witches' broom	Loofah witches' broom	Asia	VIII

Pigeon pea witches' broom Almond witches' broom '*Ca*. P. phoenicium' Naxos periwinkle virescence (NAXOS)	Pigeon pea witches' broom	Europe, Asia, America	IX
Apple proliferation (AP) 'Ca. P. mali' European stone fruit yellows '*Ca*. P. prunorum' Pear decline '*Ca*. P. pyri' Spartium witches' broom (SpaWB) '*Ca*. P. spartii' Black alder witches' broom (BAWB(BWB))	Apple proliferation	Europe, America	X
Rice yellow dwarf '*Ca*. P. oryzae' Napier grass stunt (NGS) Sugarcane white leaf	Rice yellow dwarf	Europe, Asia, Africa	XI
Stolbur (STOL11) '*Ca*. P. solani' Australian grapevine yellows (AUSGY) '*Ca*. P. australiense' Japanese hydrangea phyllody '*Ca*. P. japonicum' Strawberry lethal yellows (SLY) '*Ca*. P. -australiense'	Stolbur	Europe, Asia, America, Africa, Australia	XII
Mexican periwinkle virescence Strawberry green petal (SGP)	Mexican periwinkle virescence	America	XIII
Bermudagrass white leaf '*Ca*. P. cynodontis'	Bermudagrass white leaf	Europe	XIV

Hibiscus witches' broom '*Ca*. P. brasiliense' Guazuma witches' broom (GWB)	Hibiscus witches' broom	America	XV
Sugarcane yellow leaf syndrome '*Ca*. P. graminis'	Sugarcane yellow leaf syndrome	America	XVI
Papaya bunchy top '*Ca*. P. caricae'	Papaya bunchy top	America	XVII
American potato purple top wilt '*Ca*. P. americanum'	American potato purple top wilt	America	XVIII
Chestnut witches' broom '*Ca*. P. castaneae'	Chestnut witches' broom	Asia	XIX
Rhamnus witches' broom '*Ca*. P. rhamni'	Rhamnus witches' broom	Europe	XX
Pinus phytoplasma '*Ca*. P. pini'	Pinus phytoplasmas	Europe	XXI
Lethal yellow disease Mozambique '*Ca*. P. palmicola' Cape St. Paul wilt disease			XXII
Buckland valley grapevine yellows			XXIII
Sorghum bunchy shoot			XXIV
Weeping tea witches broom			XXV
Sugarcane phytoplasma D3T1			XXVI
Sugarcane phytoplasma D3T2			XXVII
Derbid phytoplasma			XXVIII

Cassia witches' broom '*Ca*. P. omanense'	Cassia witches' broom	Asia	XXIX
Salt cedar witches' broom '*Ca*. P. tamaricis'	Salt cedar witches' broom	Asia	XXX
Soybean stunt '*Ca*. P. costaricanum'	Soybean stunt	America	XXXI
Malaysian p. virescence '*Ca*. P. malaysianum' Malayan oil palm phytoplasma	Malaysian periwinkle virescence and phyllody	Asia	XXXII
Allocasuarina phytoplasma '*Ca*. P. allocasuarinae'	Allocasuarina muelleriana phytoplasma	Australia	XXXIII

1.1.4 Phytoplasma diseases and symptoms

Phytoplasmas are associated with several hundred diseases in wild and cultivated plant species belonging to diverse families and result in economically important epidemics worldwide (Marzachi et al., 2004). Many crop plants are affected by phytoplasmas such as grapevines, fruit trees, ornamentals, vegetables, and cereals; the most economically important plant diseases in Europe that are caused by phytoplasmas are pear decline, apple proliferation and European stone fruit yellows (Ahmad et al., 2013, Seemüller and Schneider, 2004).

Phytoplasmas substantially indistinguishable based on 16S rDNA sequences can be found associated with diseases inducing different symptoms and/or affecting different plant species; in addition, different phytoplasmas can develop similar symptoms in the same plant species or in different plant hosts after infection. For instance, the phytoplasma associated with a lethal yellowing-type disease (LYDM) of coconut (*Cocos nucifera* L.) in Mozambique shared 100% identity with a comparable sequence derived from a phytoplasma strain (LDN) responsible for Awka wilt disease of coconut in Nigeria, and shared 99.0–99.6% identity with 16S rRNA gene sequences from strains associated with Cape St Paul wilt (CSPW) disease of coconut in Ghana and Côte d'Ivoire (Bertaccini et al., 2014, Harrison et al., 2014). And Aster yellows phytoplasmas are

associated with different diseases in different plant species worldwide (Lee et al., 2004).

For more accurate discrimination between isolates, molecular markers that are ideally associated with pathogenicity features of phytoplasmas could in the future be identified, along with the use of multi-locus sequence typing (MLST). This will aid in further studies of phytoplasma host ranges and insect vectors in the different agricultural or forestry ecosystems. At the same time, it will help to avoid assumptions of phytoplasma identity based on just symptoms and host species (Bertaccini et al., 2014).

Inside the phytoplasma infected plant there will be profound disturbances in the normal balance of plant hormones or growth regulators, to account for the array of symptoms that are exhibited in the plants in different forms. These include phyllody, which is where the floral parts of the plant develop into leafy structures, the development of green flowers and the loss of normal flower pigments known as virescence, proliferation of auxiliary or axillary shoots known as witches'-broom, sterility of flowers, abnormal elongations of internodes resulting in slender shoots, small flowers and leaves and shortened internodes that are considered as general stunting. Furthermore, leaf curling or cupping, discolorations of leaves or shoots, the bunchy appearance of growth at the ends of the stems, and also stunting, dieback of twigs or unseasonal yellowing or reddening of the leaves are selected as a

general decline (Lee et al., 2000). Some other plant species may also be symptomless or exhibit mild symptoms when infected by a phytoplasma, presumably because such plants are tolerant to phytoplasma infections. Additionally, in the diseased plants different phytoplasmas at different stages of infection can induce different symptoms (Lee et al., 2000, Bertaccini and Duduk, 2009, Hogenhout et al., 2008).

Sometimes phytoplasma can be used as a beneficial tool in commercial production. For example, in the case of phytoplasma-infected poinsettia plants, the shortened internodes due to phytoplasma infection make it possible to grow them in pots (Bertaccini and Duduk, 2009).

1.2 Transmission of phytoplasmas

Phytoplasma transmission between diseased and healthy plants has been done naturally by insects (leafhoppers or psyllids), or they are transmitted by grafting, and also through parasitic plants or dodder (*Cuscuta* spp.). Additionally, phytoplasmas have been spread by vegetative propagation through cuttings, rhizomes, storage tubers or bulbs (Gasparich, 2010, Kamińska and Korbin, 1999, Marcone et al., 1999a). Furthermore, phytoplasma transmission by seed has been investigated and was first suspected in the spread of coconut lethal yellowing. Some studies have provided further evidence that phytoplasmas might be transmitted by seed such as in alfalfa (*Medicago*

sativa) in Oman cultivations, and also lime (*Citrus aurantiaca*) and tomato (*Lycopersicum esculentum*) from Oman and Italy respectively, which were tested at several growth stages and after germination under sterile conditions, following which some of the seeds contained phytoplasmas belonging to ribosomal groups 16SrI, 16SrXII and 16SrII (Bertaccini and Duduk, 2009).

1.2.1 Phytoplasma transmission by insect vectors

The single most successful order of insect phytoplasma vectors that have been shown to effectively transmit phytoplasmas in the natural ecosystem is *Auchenorrhyncha.* The nymphs and the adults can transmit phytoplasmas, as they are in the same physical location and feed similarly; this feature makes them more efficient vectors of phytoplasmas. These vectors feed specifically in phloem cells and while they are feeding on infected plants, phytoplasmas are acquired passively through the feeding into the vectors body. There are some factors that influence the ability of the insect vector to transmit phytoplasmas such as the genus, sex, and age of the insect (Weintraub and Beanland, 2006).

A sufficient titre of phytoplasma needs to be acquired by the insect vectors during their feeding on infected plants; this is known as the acquisition access period (AAP), and it is possible to be completed

between a few minutes to hours. The chance of successfully transmitting depends on the duration of AAP, with a longer APP increasing the chance of transmission (Weintraub, 2007). The period of time that has elapsed from initial acquisition to the ability to transmit the phytoplasma is known as the latent period (LP) and is sometimes referred to as the incubation period. During the latent period, the phytoplasmas start to move through and replicate in the competent vector's body.

Three taxonomic groups of phloem-feeding insects are confirmed as vectors of phytoplasmas; Cicadellidae, Fulgoromorpha (in which four families of vector species are found), and two genera in the Psyllidae (Weintraub, 2007, Rojas-Martínez, Weintraub and Beanland, 2006). Table 1.2 shows some confirmed vectors, taxonomy, pathogen association with their host ranges and distribution area.

Table 1.2 Insect vectors of phytoplasmas, taxonomy, pathogen association with host plant and victor distribution area, adapted from Weintraub (2007).

Vector Species	**Disease Association/ Phytoplasma group**	**Host Plants**	**Distribution**
Deltocephalinae			
Circulifer tenellus (Baker)	Columbia basin potato purple top	Beets, potatoes, weeds	Oregon, Washington USA
Fieberiella florii Stål	Apple proliferation/	Apple	Italy
Macrosteles sp.	yellows disease	Watercress,plantain,lettuce	Hawaii, USA
Neoaliturus fenestratus (Herrich-Schäffer)	Lettuce phyllody, wild lettuce phyllody	Lettuce, wild lettuce, periwinkle, sowthistle	Iran
Orosius cellulosus Lindberg	Cotton phyllody	Cotton	Africa
Orosius lotophagorum (Kirkaldy)	Little leaf disease	Bellvine	Australia
Orosius lotophagorum (Kirkaldy)	Witches' broom of sweet potato	Sweet potato	Japan
Orosius orientalis (Matsumura)	Garden beet witches' broom	Beets	Iran
Yamatotettix flavovittatus (Matsumura)	Sugarcane white leaf disease	Sugarcane	Thailand
Fulgoridea cixiidae			
Reptalus panzeri (Löw)	Maize redness	Maize	Serbia

1.2.2 Phytoplasma transmission by Dodder

The parasitic plant *Cuscuta* spp. (dodder) can be employed as a natural way to transmit phytoplasmas from infected plant host species into healthy plants, and this has been developed into an effective way to maintain a wide range of phytoplasmas for further research. The Madagascan periwinkle plant, *Catharanthus roseus,* is the most commonly used experimental host plant for maintaining phytoplasmas in greenhouses and tissue culture collections. By contrast, herbaceous plants that start dormant seasons in natural conditions and deciduous tree species are not easy to use as experimental plants (Marcone et al., 1999a, Přibylová and Špak, 2013). Using dodder bridges it is possible to transmit phytoplasmas from an infected plant to a healthy plant in both the same or in a different species. For instance, an experiment has confirmed that a dodder plant has been used for phytoplasma transmission from infected poinsettia cultivar Jolly Red plants to healthy periwinkle plants. After 30 days the dodder shoots were detached from the periwinkle plants, and then after 60 days, branches of the periwinkle plants were grafted onto other healthy periwinkle plants, and after another 60 days the graft-transferred periwinkle plants exhibited symptoms (Lee et al., 1997).

1.2.3 Phytoplasma transmission by grafting and vegetative propagation

Phytoplasmas can also be transmitted through vegetative propagation such as cuttings, storage tubers, rhizomes, or bulbs. Vegetative propagation and grafting have played an important role in the spread of phytoplasmas between plants and also for maintenance of phytoplasma collections for research purposes.

1.3 Detection of phytoplasma

Detection of phytoplasmas is different from the majority of microorganisms because they are uncultured and have to be maintained in infected plants or insects. Initially, the identification and classification of phytoplasmas mainly depended on traditional methods such as accurate symptoms of infected plants, range of plant hosts and relationships with insect vectors. More recently, use of molecular-based biotechnology has resulted in an alternative way to gain information about phytoplasmas and has been developed into a system for identification and classification of these microorganisms (Lee et al., 2000).

Different approaches have been used for phytoplasma detection, such as staining of the pathogen nucleic acid with DNA-specific dyes, electron

microscopy observations, grafting on indicator plants and serological-based diagnosis, but the last one lacks sensitivity especially when the pathogen titre is low (Marzachi, 2006). Over the past 25 years major efforts have been made to develop nucleic acid-based tools, such as hybridization probes and PCR assays; consequently, nucleic acid-based techniques (mainly PCR) are usually used for phytoplasma diagnosis nowadays (Marzachì et al., 1998, Marzachi, 2006). However, in woody plants phytoplasma detection is difficult due to their low concentration, but in the case of insect vectors, possibly due to the high titre of the bacteria, sometimes diagnostic PCR can produce acceptable results even when total DNA is prepared with a quick boiling procedure (Boudon-Padieu et al., 2003, Marzachi, 2006). Furthermore, electron microscopic studies show that phenolic compounds accumulate and cell walls collapse in phloem sieve tubes containing high numbers of phytoplasma cells (Boudon-Padieu et al., 2003). Also, HonetŜlegrová et al. (1996) reporred that micro-scopic observations had confirmed the presence of phytoplasma associated with strawberry green petals disease in the Czech Republic, results are confirmed with nested PCR too.

For phytoplasma detection the first stage is to prepare a total DNA extraction from the plant or insect; for that reason, different protocols have been reported and the success of each protocol depends on the plant host species as well as on the sampling procedures or storage conditions of collected samples (Gibb and Padovan, 1994). The preparation of good quality DNA with a consistent yield from

phytoplasma-infected hosts is essential for PCR amplification. Plant variety, the age of infection and date of sampling significantly influences the titre and distribution of phytoplasmas in different parts of the plant (Berges et al., 2000, Bosco et al., 2002). Conventional PCR has become one method of choice for detection and diagnosis of phytoplasma, but real-time PCR assays are becoming even more important. These have been developed for both generic and specific phytoplasma detection, and these assays have the advantage of being more easily automated and less labour intensive than conventional PCR, such that appropriate controls can be conducted more easily (Hodgetts et al., 2009, Bekele et al., 2011).

1.3.1 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) has become a versatile tool for detecting phytoplasmas and has been demonstrated as an identification technique for these pathogens in their insect and plant hosts (Smart et al., 1996).

Detection of phytoplasmas by the PCR technique often requires a nested PCR approach to get a clear and reliable result, because a single round of PCR with the universal primer pair can not detect low-titre phytoplasma infection. Therefore, a first round PCR is applied with a universal primer pair followed by a second round (nested PCR) with a

primer pair that targets primer sites within the first round PCR product. Furthermore, nested PCR increases the sensitivity of detection 100 fold, and it has proved very effective for detection of phytoplasmas from known insect vectors and is also useful for investigation of potential insect vectors (Smart et al., 1996, Srivastava et al., 2006, Hodgetts et al., 2008, Gundersen and Lee, 1996, Liu et al., 1996, Lorenz et al., 1995, Deng and Hiruki, 1991, Lee et al., 1995).

1.3.2 Real-time quantitative PCR

Recently, developments in the field of real-time quantitative PCR have led to a wide acceptance of this technique in research due to its sensitivity and accuracy. Amongst the advantages are the short cycle time, making the technique quicker than conventional PCR, and the use of sensitive fluorescence detection equipment that completes the process without referring to gel electrophoresis for post-PCR detection. Furthermore, because amplification and detection of samples occur in a single tube, this decreases the chances of carry-over contamination in comparison to nested PCR assays. At the same time, it can be used as a method to quantify levels of DNA. Real-time PCR assays have been employed for universal phytoplasma detection based on the 16S rRNA gene and on the 23S rRNA gene (Hodgetts et al., 2009, Nejat and Vadamalai, 2010, Mehle et al., 2013, Bertaccini et al., 2014).

Real-time quantification of phytoplasma DNA can be applied to study several aspects of phytoplasma infection in both plant and vector. This method is important in screening plants for tolerance against phytoplasmas. also it can be used to calculate different phytoplasmas in their insect vectors, and to measure phytoplasma multiplication in vectors (Galetto et al., 2009, Christensen et al., 2013). Also, real-time PCR can be used to measure the amount of cellular RNA and this helps to determine that to what extent that particular gene is express (Schmittgen and Livak, 2008).

1.3.3 Loop-mediated isothermal amplification (LAMP)

Phytoplasmas can also be detected and specific groups determined by the loop-mediated isothermal amplification (LAMP) assay, which is a relatively novel method for amplifying nucleic acid at a single constant temperature. In LAMP, four or six primers are used simultaneously which recognize six or eight regions on the target nucleic acid as shown in Figure 1.2. For this reason, it is possible to do detection in the LAMP method without referring to gel electrophoresis afterwards.

The LAMP method is also a highly efficient amplification method in which a large amount of DNA can be synthesized in a short time (Mori et al., 2004). With the real-time LAMP technique, the likelihood of sample contamination and the reaction time have been reduced; the method

combines the sensitivity and specificity of nucleic acid-based methods with simplified equipment. These features make the method potentially suitable for on-site use, and also this technique can be carried out without the need for any gel electrophoresis as there is a visual display to see the results instantly (Tomlinson et al., 2010, Hodgetts et al., 2011, Bekele et al., 2011).

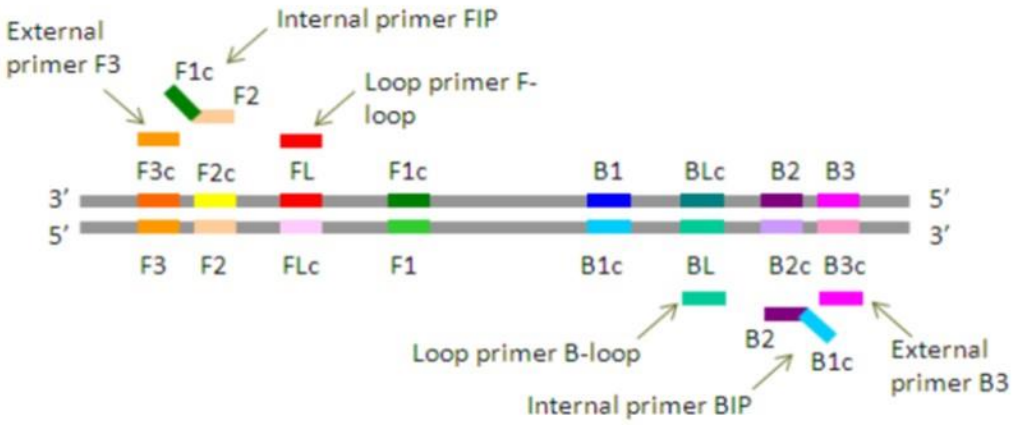


Figure 1.2 Schematic representation of LAMP assay shows primers and their sites which include external primers (F3 and B3), internal primers (FIP and BIP), and loop primers (loopF and loopB) (Tomlinson, 2013).

1.4 Control of phytoplasma-associated diseases

Phytoplasma diseases are very difficult to control. Disease-tolerance host plants (when available) and pesticide application to control insect vectors have been used to control them with limited success (Gasparich, 2010). Applications of tetracycline into infected plants has been done in some crops but is too costly and environmentally damaging since resistance to antibiotics in other microbial populations may develop, not to mention potential entry into the human food chain. Genetically engineered host plants have also been developed to try and deter insect vector feeding or to interfere with phytoplasma replication (Gasparich, 2010). Spraying of various insecticides to control insect vectors with the removal of symptomatic plants in the infected fields are the most common mechanisms that use for phytoplasmas disease control (Hogenhout et al., 2008). The primary concern about phytoplasma control is mostly prevention rather than treatment. For instance, planting clean stocks (disease-free plants) or disease-resistant varieties with controlling insect vectors. Breeding of disease-resistant cultivars can be useful and produce a more direct and efficient way to control many devastating phytoplasma diseases; whilst, the identification of resistance genes in crop plants or their close relatives is difficult and introducing these new genes to cultivated crops through traditional breeding is very time-consuming (Lee et al., 2000).

1.5 Aims and objectives

The aims of this thesis were to apply new techniques to diagnose phytoplasma infections and develop improved methods to quantify these pathogens in infected plants at different stages of development and following the application of different control strategies on infected plants.

For that reason, the objectives are:

- To further develop the use of periwinkle plants as a host plant to maintain isolates, using techniques including cuttings, dodder plants, grafting, and to examine transmission between periwinkles and other plants by dodder bridges. This is addressed in Chapter 3.
- To design new primers for LAMP assays and qPCR for detection and quantification of phytoplasmas with both LAMP and qPCR techniques. This is addressed in Chapter 2.
- To compare the sensitivity and reliability of LAMP and qPCR assays for detecting and quantifying phytoplasma. This is addressed in Chapter 4 and 5.
- To evaluate the methods for reducing the phytoplasma titre in infected plants, including the use of altered nitrogen fertilization regimes, treatment with salicylic acid, and UVC treatments. This is addressed in Chapter 4 and 5.

Chapter 2. Design of new LAMP assays for phytoplasma detection and quantification

2.1 Introduction

Phytoplasmas are plant pathogenic, Gram-positive, wall-less prokaryotes that are associated with a number of severe plant diseases worldwide. These microorganisms infect more than 700 plant species and are transmitted by insect vectors (Hogenhout et al., 2008, Oshima et al., 2011, Sugawara et al., 2012). The majority of crops are susceptible to phytoplasma diseases; consequently, these pathogens cause significant damage to agricultural crops in many areas. Up to date, the most common ways to protect the crops from phytoplasma infection are to remove infected plants and control insect vectors (Hogenhout et al., 2008). Therefore, it is important to develop a rapid, accurate diagnostic method to detect phytoplasma infection in their hosts (Sugawara et al., 2012).

Various techniques have been developed for phytoplasma diagnostics, and PCR-based methods, in particular, have become the most common methods, replacing other techniques such as DNA hybridization and electron microscopy. Amplification of phytoplasma DNA using PCR is more sensitive and specific, and can be used as a generic method for

detecting the presence of a phytoplasma, or as a more specific method in which RFLP analysis or sequencing of the PCR product can be used to assign the phytoplasma to a 16Sr group, or group-specific primers can be used in a nested PCR analysis. However, Loop-Mediated Isothermal Amplification (LAMP) assays have the potential to be more rapid than PCR and are easier to use in the field, because they do not need to be connected with other bulky equipment and conducted in laboratories (Kogovsek et al., 2015). For instance, PCR-based techniques require generally a thermal cycler and an electrophoresis apparatus. The LAMP technique has therefore become a popular diagnostic tool for plant pathogens since it is fast and requires minimal equipment to obtain the result.

Loop-mediated isothermal amplification techniques for phytoplasmas have been shown to achieve amplification within 30 minutes (Hodgetts et al., 2011, Sugawara et al., 2012, Yankey et al., 2011). According to Hodgetts et al. (2011), a new protocol for LAMP-based diagnostics has been developed that can be carried out in the field and applied to a range of phytoplasmas, and with this technique a diagnosis can be provided within 1-hour of DNA extraction. Providing the result in a short time with less equipment to conduct the protocol makes the method more simple and quicker than ordinary PCR method and easy to undertake. However, one disadvantage of the current method is that the rapid DNA extraction method used in the field results in DNA that will not remain reliable if stored long-term (Hodgetts et al., 2011).

This rapid real-time group-specific LAMP assay uses enzymes and equipment supplied by OptiGene UK. This method uses real-time based on the detection of a fluorescent dye incorporated into double-stranded DNA generated during the amplification to analyse the results (Bekele et al., 2011, Dickinson, 2015). The reactions are then run on a Genie II or Genie III machine, followed by an anneal analysis of the product, which this was confirmed that the annealing temperatures for the test samples were the same as those for the reference strains. The LAMP technique can also be used for RNA target detection, which needs the addition of reverse transcriptase and a short incubation at a lower temperature approximately 50–55°C prior to the amplification step (Bekele et al., 2011, Dickinson, 2015).

Quantitative approache could be useful for biological aspects of the relationships between phytoplasmas and their hosts, such as susceptibility of different plant genotypes to competition of different '*Ca*. Phytoplasma' species infecting the same host plant or insect vector. Quantification of phytoplasma concentration in different parts of the vector could be useful to elucidate the fate of the phytoplasma in the vector and therefore to understand what makes an insect a vector of phytoplasmas. Apply of high-throughput, rapid, sensitive and quantitative techniques can provide a monitor to understand how phytoplasmas exploit their unique ecological niches, also can be useful to the evaluation of phytoplasma tolerance levels (Torres et al., 2005, Galetto et al., 2009).

The aim of this chapter, a new set of phytoplasma LAMP assay primers based on the leucyl-tRNA gene were designed, with the aim to detect specific phytoplasmas from 16S ribosomal groups 16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI, and 16rSX. The specific primers for selected groups of phytoplasma to assess them for detection and specificity. Additionally, methods were developed to test the capacity of the LAMP assays for phytoplasma quantification.

2.2 Materials and Methods

2.2.1 Phytoplasma strains in this study:

All the phytoplasmas used in these experiments are listed in (Table 2.1). Madagascan periwinkle plants (*Catharanthus roseus*) were used as the host plant to maintain the phytoplasma strains throughout the experiments and were grown in a glasshouse with 16 hour day time at 28°C and 8 hour night time at 20°C. All the selected groups of phytoplasma used in this study were maintained through the use of new cuttings from a mother periwinkle plant, and healthy periwinkle plants were used as controls to compare with diseased plants (Figure 2.1).

The method for producing new cuttings from infected and healthy periwinkle plants was as described in Hodgetts et al. (2013). Phytoplasma-infected periwinkles and healthy periwinkle plants were selected, then a young non-flowering shoot was cut from both plants using a sterile scalpel blade. The shoots cuttings were approximately 2-3 mm in diameter and approximately 6-8 cm in length. The shoots were prepared for planting by carefully cutting away all the leaves, flowers, and buds if present, except the top three pairs. After that, the stem was dipped into a hormone rooting compound. Seed compost and 25% perlite was prepared in a seed tray and the stem was inserted into the seed tray and covered with a propagator lid. The new cuttings were watered twice a week in the glasshouse, and after 30 to 45 days the plants were moved into a single pot and watered daily. When the plants had grown enough to take samples, leaf samples were excised for DNA extraction.

Table 2.1 Different groups of phytoplasmas that been used in this study.

Phytoplasmas name	Acronym	16Sr group	Source
Rehmannia glutinosa	RG	I-B	University of Nottingham
Soybean phyllody	SP	II-C	University of Nottingham
Vinca coconut phyllody	VCP	II-C	University of Nottingham
Plum leptonecrosis	LNI	III	University of Nottingham
Elm yellow	EY	V	University of Nottingham
Potato witches' broom	PWB	VI	University of Nottingham
Apple proliferation	AP	X-A	University of Nottingham

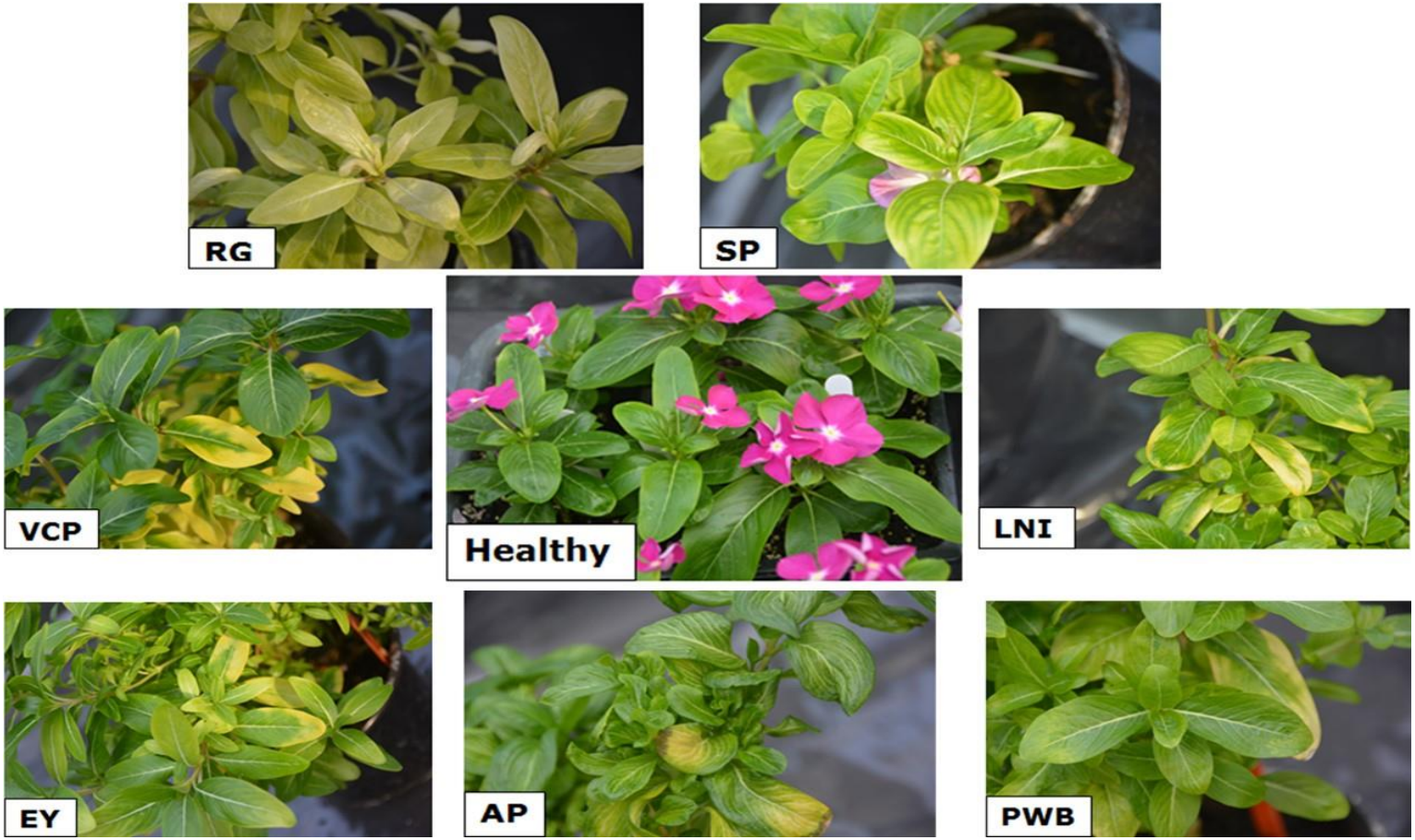


Figure 2.1 Different disease symptoms on periwinkle plants. RG: Rehmannia glutinosa, SP: Soybean phyllody, VCP: Vinca coconut phyllody, LNI: Plum leptonecrosis, EY: Elm yellows, PWB: Potato witches' broom, AP: Apple proliferation and healthy periwinkle.

2.2.2 DNA extraction

After the new plants had grown for about one and a half months, leaf samples were collected from the top, middle and bottom of plants for DNA extraction. Approximately, 200 mg of leaf was used for DNA extraction. DNA was extracted using approximately 3.0 ml CTAB extraction buffer (20 mM EDTA pH 8.0, 100 mM Tris-HCL pH 8.0, 2% CTAB, 1.4 m NaCl, 2-mercaptoethanol in sterile distilled water) which was added to each extraction bag (filter bags from BIO-RAD Laboratories UK.) containing the leaves. Samples were homogenised by manually crushing the leaves using an homogenizer tool at room temperature to extract the sap into the CTAB buffer. Subsequently, 800 µl solutions were collected and transferred from each bag to an Eppendorf tube at room temperature and incubated in a water bath for 30 min at 65 ºC, after which 500 µl of Phenol: Chloroform: Isoamyl alcoholwas added to each tube. Tubes were vortexed vigorously for 1 min and centrifuged for 10 min at 2375 g in a bench-top centrifuge. The supernatant (500 µl) was added into a tube which contained 500 µl of isopropanol, mixed and then placed in the freezer -20 ºC for at least 2 hours.

Following centrifugation for 15 mins at 16060 g, the solution was discarded and the pellet was washed with 70% ethanol and centrifuged for 2-3 sec. The ethanol wash was removed and the pellet dried for 30

min and dissolved in 30 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and finally stored at -20ºC until use.

2.2.3 DNA sample validation with universal 16S rRNA gene primers

After DNA extraction, all the different DNA templates were tested by PCR using universal primers to validate that the correct phytoplasma was present and to confirm the absence of phytoplasmas in healthy plant DNA. Universal primers for the 16S rRNA gene were applied in a nested PCR assay, using primers P1 (5'-AAGAGTTTGATCCTGGCTCAGGATT-3') (Deng and Hiruki, 1991) and P7 (5'-GGATGGATCACCTCCTT-3') (Schneider et al., 1995) followed by nested PCR with primer pair R16F2n (5'- GAA ACG ACT GCT AAG ACT GG - 3') and R16R2 (5' - TGA CGG GCG GTG TGT ACA AAC C - 3') (Gundersen and Lee, 1996). To confirm the presence of phytoplasma DNA, PCR products were visualized on 1% agarose gels stained with ethidium bromide. The efficiency of extraction for DNA carried out on RG phytoplasma, results are shown in (Table 2.2).

Table 2.2 Phytoplasma titre of group 16SrI (RG) in leaves taken from different parts of diseased periwinkle plant.

Plant section	Amplification time (mean)	Calculated phytoplasma cells per 1 gram of leaf sample
Upper part leaves	17.25	8.1×10^4 a
Middle part leaves	17.13	9.6×10^4 a
Lower part leaves	17.17	9.1×10^4 a

Data are mean of amplification time with phytoplasma cell number of 3 replicates (leaf). Cell number with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

2.2.4 PCR, cloning and sequencing of the leucyl-tRNA synthetase gene

PCR amplification for DNA samples was carried out with seven isolates of phytoplasma groups to obtain sufficient DNA template that could be cloned and used for developing the quantification standard curves. For the PCR reactions, a master mix (25 µl) was prepared which consisted of 12.5 µl Mangomix (MangoTaqTM DNA polymerase), 9.5 µl sterile water, 1 µl (10 µM) forward primer and 1 µl (10 µM) reverse primer per PCR reaction. To each PCR reaction, 1 µl DNA was added from the genomic DNA extracts.

The universal leucyl-tRNA synthetase gene primer sets used in this study were as described in (Abeysinghe et al., 2016), which amplify approximately 1 Kb of the *leu* gene and which have been validated on a broad range of phytoplasma taxonomic group (Abeysinghe et al., 2016). The primer sequences are presented in (Table 2.3). Amplifications were conducted with an initial denaturing temperature of 94ºC for 2 min, followed by 35 cycles of 94ºC for 30 sec, 53ºC for 1 min and 72ºC for 1 min, and a final extension at 72ºC for 10 min. For more sensitivity, nested PCR was used with dilutions of the first round product 1:40 (1 µl of PCR product and 39 µl sterile water); all other PCR conditions were as before. PCR products were visualized on 1% agarose gels stained with ethidium bromide.

Subsequently, the templates from the nested PCRs were gel purified with the QIAquick® Gel Extraction Kit (QIAGEN Ltd., United Kingdom) following the manufacturer's instructions, and the products were ligated into the pGEM®T vector (Promega UK). A 2.5 µl aliquot of the ligation mixture was used to transform high-efficiency competent cells of *Escherichia coli* DH5-alpha and plated on Luria - Bertani (LB) medium containing 100 µg/ml ampicillin, 80 µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and 0.05 mM/ml IPTG (Isopropyl-β-D-thiogalactopyranoside). Clone inserts were then amplified from transformant colonies and mixed into 25 µl of PCR reaction containing 1x concentration of MangoMix™, 100 ng of the primers M13 forward (5'-GTAAAACGACGGCCAGT-3') and M13 reverse (5'-

CAGGAAACAGCTATGAC-3'). The PCR conditions were: denaturation at 94°C for 30 sec, annealing at 56°C for 1 min and extension at 72°C for 1 min for 30 cycles.

The cloned PCR products were purified using a Qiaquick PCR Purification kit (Qiagen) before sending for sequencing. The sequencing was done by Eurofins MWG Operon, the UK. Using both forward and reverse strands. Sequences were checked and edited using GAP4 (StadenPakage, USA). Consensus sequences from reverse and forward were also combined using the same software.

Table 2.3 List of PCR primers based on leucyl-tRNA used in this chapter for phytoplasmas detection.

Primer name	Sequence (5'_3')
Leufor1	GATATGTTTCCTTATCCTTC
Leurev1	TACCAAGARCTTCCWGC
Leufor2	CATCCTTTTGGTTGGGATTC
Leurev2	CTSCCCAATATCTTTGRCG

2.2.5 Plasmid preparation and assay sensitivity

Representative recombinant clones containing partial leucyl-tRNA synthetase gene sequences were cultured in LB liquid media containing 0.1 mg/ml ampicillin and were then incubated at 37°C in an incubator shaker for 16 hours. Plasmid DNA was isolated using the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich Co.) and additionally purified with the QIAquick® Gel Extraction Kit (QIAGEN Ltd., United Kingdom) following manufacturer's instructions. Plasmid DNAs were quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.) and used to prepare 10-fold serial dilution series in 200 ng/µl healthy periwinkle DNA solution.

For the LAMP reactions 1 µl from all serial dilutions was used in three replications; subsequently, the starting amplification times were identified by using GraphPad Prism® software (Prism 7) version 7.01 (GraphPad Software, Inc.) and the standard curves were created.

To evaluate the sensitivity of the primers, plasmid DNA mixed with healthy periwinkle DNA solution (200 ng/µl) with dilutions prepared up to (10^{0} – 10^{-6}) in sterile distilled water.

2.2.6 LAMP testing

The loop-mediated isothermal amplification (LAMP) primers were designed using the Optigene LAMP designer program based on the sequences of the leucyl-tRNA synthetase genes. Each set of primers consisted of external primers F3 and B3, internal primers FIP and BIP, and loop primers F-Loop and B-Loop. All the primers designed and used in this study are listed in).

Within this method, LAMP reactions were performed according to Tomlinson et al. (2010) and Bekele et al. (2011) with modifications, using a 1x concentration of Isothermal Mastermix (Genesys Ltd.), 1 µl of DNA sample, 200 nM external primers, 1 µM internal primers and 1 µM loop primers. The reactions are then run on a Genie II or Genie III machine (OptiGene Ltd. UK), with reactions held at 63 ^{0}C for 30 min. The serial dilutions (10^{0} – 10^{-6}) were used with master mix and tested, three replicates were performed for each dilution.

Table 2.4 List of LAMP primers for detection and identification of specific phytoplasma groups.

Target Phytoplasma	Primer set	Sequence (5'-3')	Specific groups
RG	F3	AACTCAATATGCAGACAGACTT	I
	B3	CGTAAAAACACCTGTTTTGTCT	
	FIP(F1c+F2)	AGGGCGAGTGGTGAAAGTTTTATTGGCAAAAATCAAGGTGC	
	BIP(B1c+B2)	TTAGTAATAGCGCCCGAACACGGTTAACTGCTTGTTGGTATTCG	
	LoopF	TCAGAAACTGGAAAAGAGACGA	
	LoopB	TAGCCTTACAACTCACCAAACC	
SP	F3	TGGTTTGCCTACAGAACG	II
	B3	CATTTCCTTAATAGACTCTGGC	
	FIP(F1c+F2)	CCGAAGTCGCTAATTCTCTACTACAGGTCAACATCCTTCTTATG	
	BIP(B1c+B2)	GCTGTTTTGCAAGATGTTGAGGGATGACCACCACGTTCTG	
	LoopF	CCAATCAATACCTTTTCCAAGC	
	LoopB	ACAGTATTAGCGAATGAAGAGG	
	F3	TGGTTTGCCTACAGAACG	

VCP			II
	B3	CATTTCCTTAATAGACTCTGGC	
	FIP(F1c+F2)	CCGAAGTCGCTAATTCTCTACTACAGGTCAACATCCTTCTTATG	
	BIP(B1c+B2)	GCTGTTTTGCAAGATGTTGAGGGATGACCACCACGTTCTG	
	LoopF	CCAATCAATACCTTTTCCAAGC	
	LoopB	ACAGTATTAGCGAATGAAGAGG	
LNI	F3	CCAGATCGTTGGAATGAAGT	III
	B3	ACAGCGATAATTTTACGATTCG	
	FIP(F1c+F2)	AAGGAACGGTCATAATGGCTCCTGCTATTAACTTCGCTAATCAA	
	BIP(B1c+B2)	ACCTGAAGAAACCCAAAATTCTCTTCTTCATAATCAAGACCGTT	
	LoopF	AATCAGCGATCCAAATAGGGAT	
	LoopB	AGAAATTAAGGTGGAAGACGGT	
EYC	F3	CCCAATATCTTTGGCGAGAA	V
	B3	TTGGATATCTGATTATGTCCTT	
	FIP(F1c+F2)	ACGGTCTTAATGTTGAAGAAGCAGCCAATCACGCATTTGA	
	BIP(B1c+B2)	AGCGAAACAAAAATCTCTTTCACCTTCTTATGCTACAGGAGC	

	LoopF	TTAGGTCATGTTCATTATGCTT	
	LoopB	TCACAAAAAGGAACCAACATAA	
PWB	F3	GCTTCTTCAAAAGTAAATCCGT	VI
	B3	GGAAGTTTTTACGACAAAACCA	
	FIP(F1c+F2)	CGGTGTTGTTATGTCTGTTCCTGCATCATTAGTGGATTCGGA	
	BIP(B1c+B2)	AGGATGAATAGCATAACTACCAACAGATTTTGTCGAAGGTGT	
	LoopF	TGAAAGAGATTTTGCTTTTGCT	
	LoopB	ACTCCTGTTTGATCTTTGTTCA	
AP	F3	AACTGGCAACAATCCTAGAA	X
	B3	TCCAATCCAATTACGTTGAATT	
	FIP(F1c+F2)	ACAGTTCCTAATCCTTCACAAATTAGCTACTTCTGATAACGATT	
	BIP(B1c+B2)	CCGAAAGAGGCGATTTTCCGAAGCCTTTCTGCATAATCAGT	
	LoopF	TCTTGAGAATCCATTGTGTCCA	
	LoopB	ACCTTAAAACAGTGGGTTTTGA	

2.3 Results

2.3.1 Validation of DNA samples using 16S rRNA PCR

Based on the validation using PCR amplification with universal primers, it was confirmed that all periwinkle plants used in this study are phytoplasma diseased plants, also a healthy periwinkle plant (negative control) has shown no amplification (Figure 2.2).

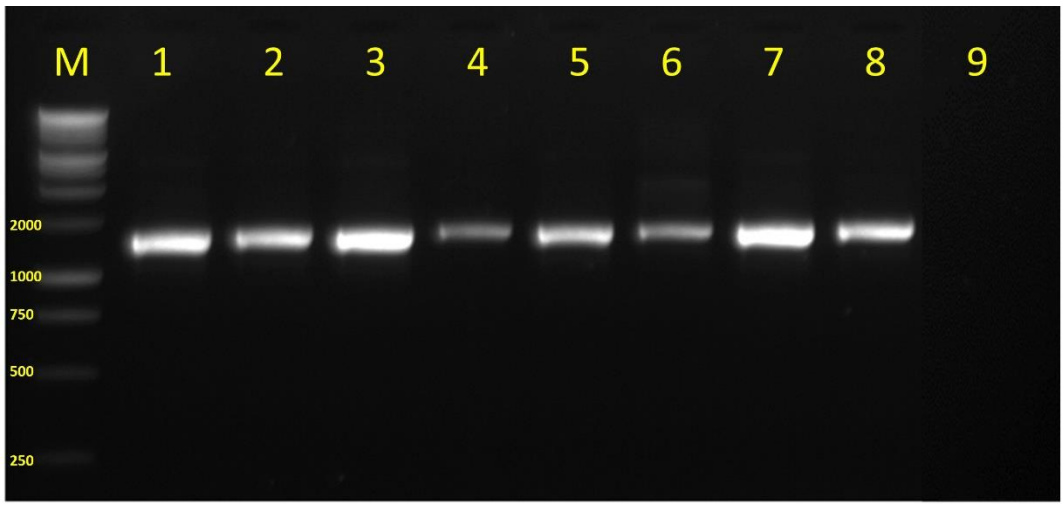


Figure 2.2 Representative agarose gel electrophoresis of PCR products from infections showing the results of nested PCR amplification with primers P1 / P7 followed by R16mF2 / R16mR1, generating a 1,250 bp product. Lane layout: M. 1 kb marker, 1. *Rehmannia glutinosa*, 2. Soybean phyllody, 3. Vinca coconut phyllody, 4. Plum leptonecrosis, 5. Elm yellows, 6. Potato witches' broom, 7. Apple proliferation, 8. Positive control, 9. Negative control (healthy periwinkle plant).

2.3.2 Amplification and sequencing of leucyl-tRNA synthetase gene

PCR amplification of leucyl-tRNA synthetase of all six groups (7 isolates) (RG 16SrI, SP and VCP 16SrII, LNI 16SrIII, EY 16SrV, PWB 16SrVI, and AP 16SrX) produced PCR fragments of approximately 1000 bp, also a healthy periwinkle plant was tested as a negative control (Figure 2.3). Each fragment has approximately 1100 bp nucleotides sequences based

on the sequencing result. All seven sequences were subjected to phylogenetic analyses using maximum likelihood method with Tamura Nei Model and the phylogenetic relationship of the phytoplasma in this study based on leucyl-tRNA synthetase gene were as presented in (Figure 2.4).

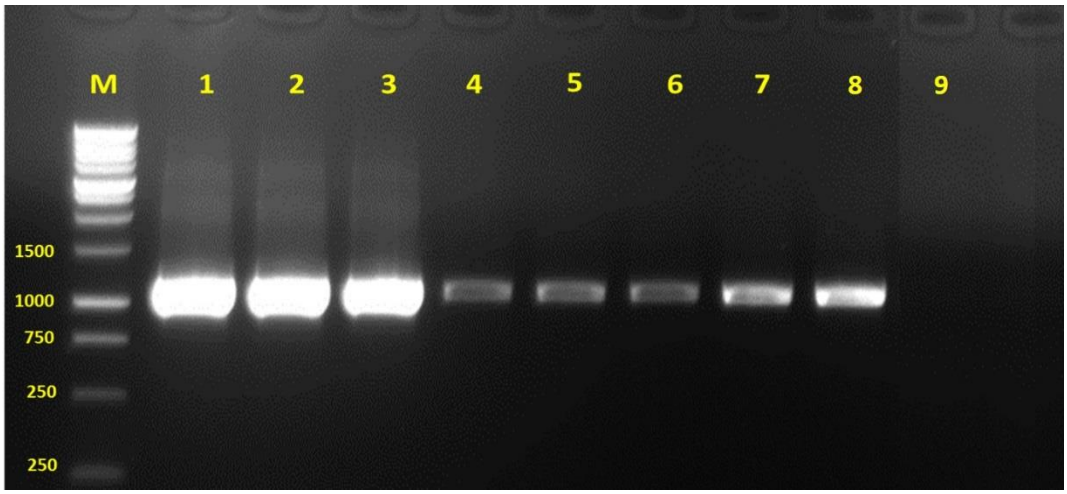


Figure 2.3 Nested PCR product obtained using the leucyl-tRNA synthetase gene primers. . Lane layout: M. 1 kb marker, 1. *Rehmannia glutinosa*, 2. Soybean phyllody, 3. Vinca coconut phyllody, 4. Plum leptonecrosis, 5. Elm yellows, 6. Potato witches' broom, 7. Apple proliferation, 8. Positive control, 9. Negative control (healthy periwinkle plant).

2.3.3 Designing LAMP primers for specific groups

For validation of the primers, all sets were tested on various phytoplasma DNAs in LAMP assays in which all reactions were held at 63^{o}C for 30 min. LAMP results confirmed that the sets of primers worked on the phylogenetic group that they were designed against and not against phytoplasmas from other phylogenetic groups (ie they had the correct specificity). There was also no cross reactivity with the bacterium

E. coli. The amplifications of different primers and their specificities are indicated in Table 2.5 (Figure 2.5, Figure 2.6 and Appendix 2a – 2c).

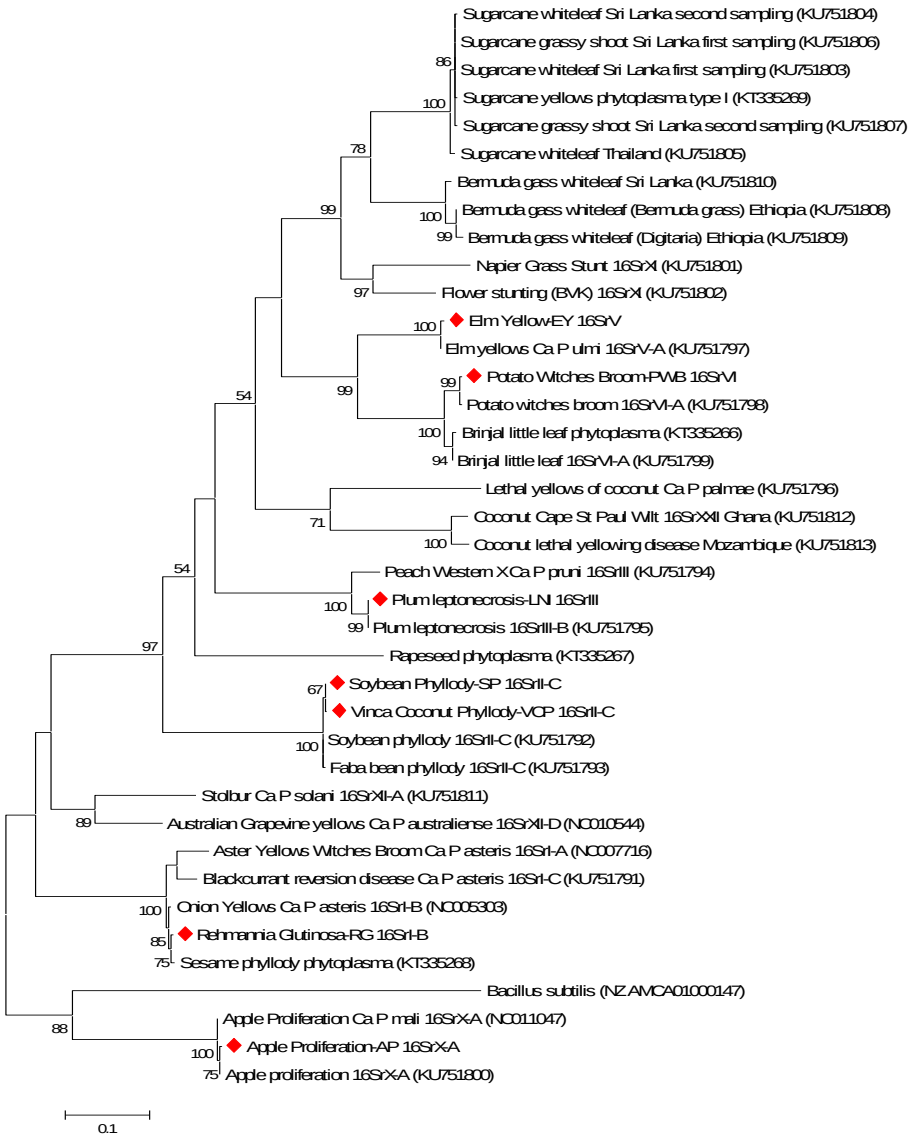


Figure 2.4 Phylogenetic tree of the phytoplasma groups used in this study (marked with red) with some more strains taken from NCBI GenBank (accession no. are in bracket) based on partial leucyl tRNA synthetase gene sequences. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The percentage of trees in which the associated taxa clustered together (bootstrap of 1000 replicates) is shown next to the branches. The analysis involved 39 nucleotide sequences with were a total of 421 positions in the final dataset. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. The evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

Table 2.5 Amplification of specific groups of phytoplasmas with specific LAMP primers based on the leucyl-tRNA synthetase gene.

Isolate and acronym	16Sr group	Amplification with specific group LAMP primers (Leucyl-tRNA)					
		Leu I	Leu II	Leu III	Leu V	Leu VI	Leu X
Rehmannia glutinosa (RG)	I	+	-	-	-	-	-
Soybean phyllody (SP)	II	-	+	-	-	-	-
Vinca coconut phyllody (VCP)	II	-	+	-	-	-	-
Plum leptonecrosis (LNI)	III	-	-	+	-	-	-
Elm yellows (EY)	V	-	-	-	+	-	-
Potato witches' broom (PWB)	VI	-	-	-	-	+	-
Apple proliferation (AP)	X	-	-	-	-	-	+
E. coli		-	-	-	-	-	-

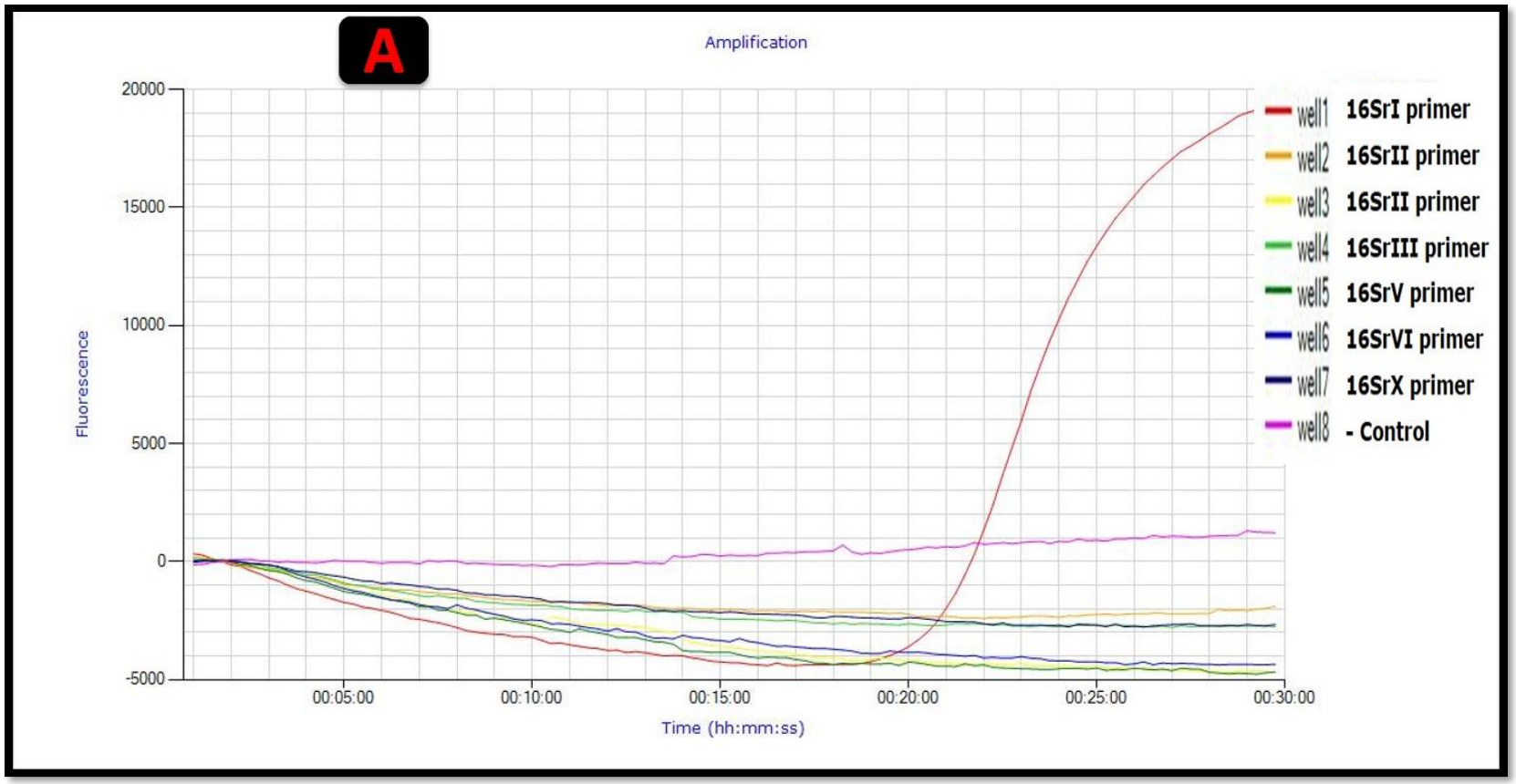


Figure 2.5 Validation of specific LAMP primer to detect specific phytoplasmas. A: Only RG DNA is amplified with the assay for 16Sr group I.

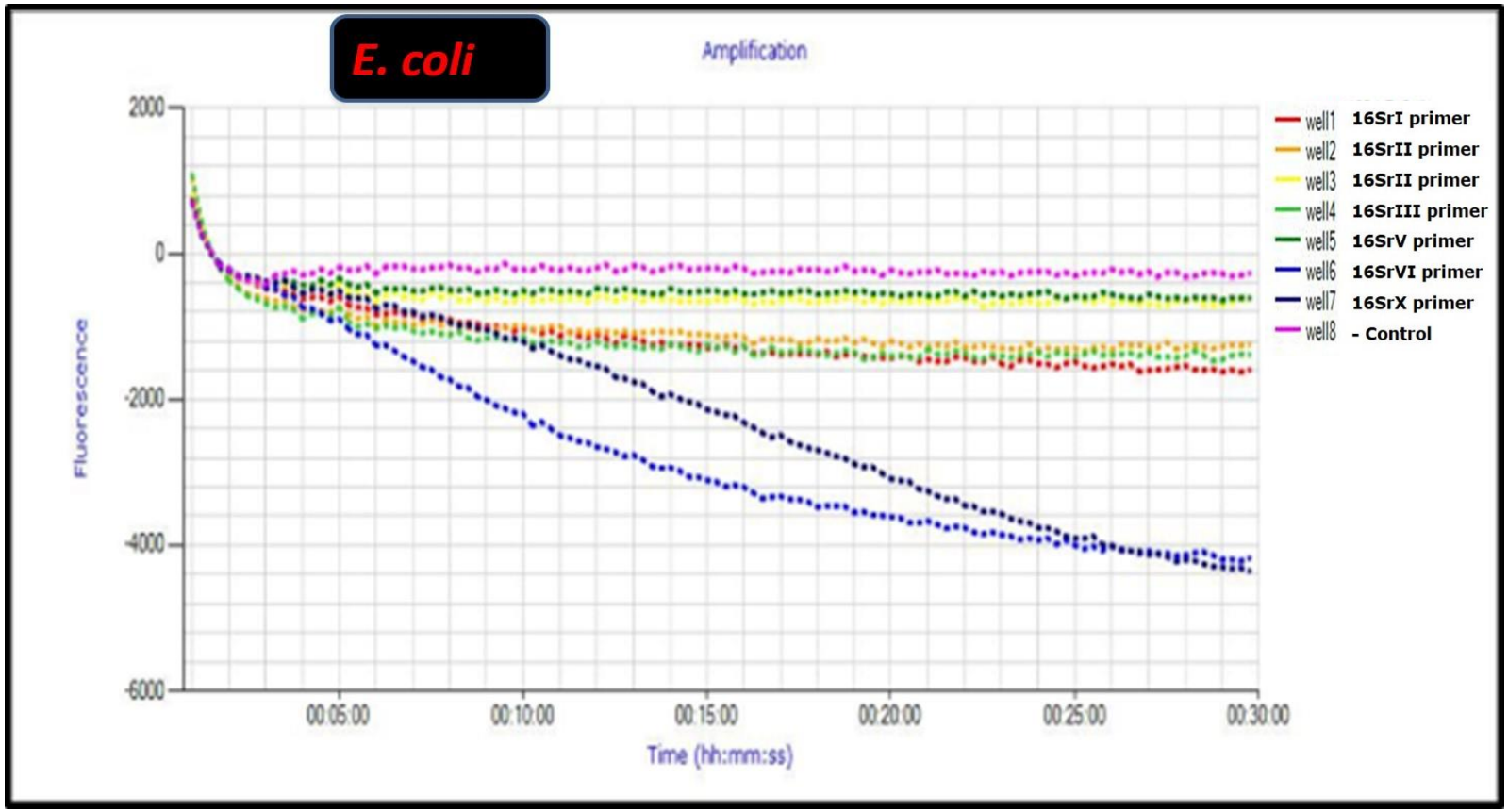


Figure 2.6 Validation of specific LAMP primer to detect specific phytoplasmas. DNA of *E. coli* tested with all the primers (each colour (well) presents a different assay) confirming that it was not amplified with any of them.

2.3.4 Standard curves

Amplification standard curves of 10 fold dilution series using all seven groups were successfully prepared (Figure 2.7, Appendix 3a & 3b). The regression squares (R^2) acquired were reliable and were at least 0.99. Also, the amplification factor is 4.44 and E value is 344.29%. which means the LAMP assay provides more product throughout the amplification process. The serial dilution results are used to generate a standard curve.

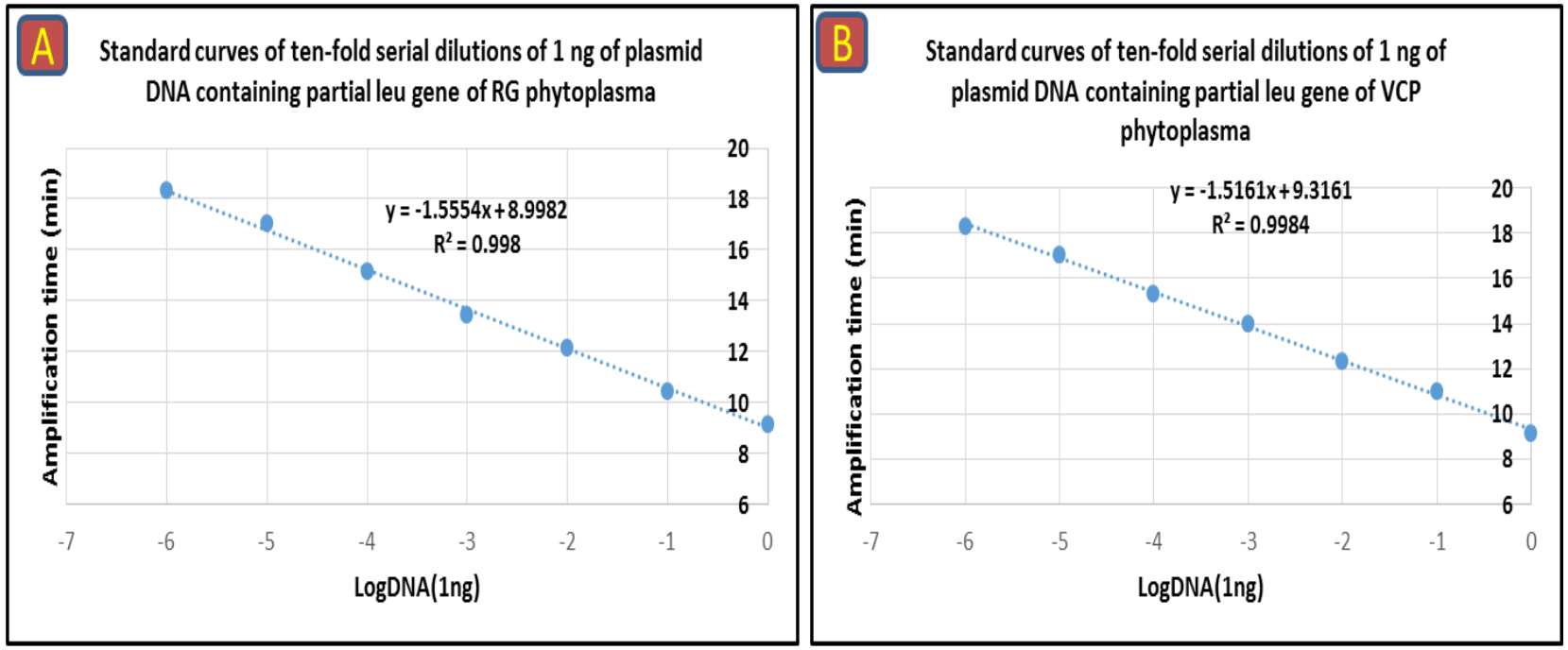


Figure 2.7 Standard curve of ten-fold serial dilutions (10^{0}-10^{-6}) of 1.0 ng of plasmid DNA containing partial leucyl-tRNA gene of (A) RG, and (B) VCP.

2.3.5 Sensitivity evaluation

It was observed that LAMP amplification using the primers can amplify the DNA samples at concentration ranging from 1.0 ng/ml to $1x10^{-6}$ ng/ml (Table 2.6), with no amplification of negative controls (water), also no amplification after $1x10^{-6}$ fold dilution. The LAMP assay was conducted both at 63°C and 65°C to see the optimal isothermal temperature. which various amplification temperatures indicated 63 ^{0}C is optimal for amplification. High and low concentrations of tested samples can be showed by early and late amplification time respectively on the Genie machine. Based on the test, 63 ºC was shown to give the better amplification curve with evenly spaced with serial dilutions compared to 65 ºC (Appendix 1a - 1b). After 10^{-6} dilutions the primers showed no more amplification.

Table 2.6 Results of LAMP test for a series dilution plasmid DNA in healthy periwinkle plant DNA solution.

Serial dilutions (ng)	Amplification time of different isolate (Plasmid DNA) (mm:ss)					
	RG	**SP**	**LNI**	**EYC**	**PWB**	**AP**
1.0	9:15	9:15	8:00	8:30	7:15	9:00
0.1	10:45	11:00	9:30	10:00	08:45	10:30
0.01	12:15	12:30	11:00	11:15	10:15	12:00
0.001	13:45	14:00	12:30	12:45	11:45	13:30
0.0001	15:15	15:30	14:10	14:30	13:15	15:00
0.00001	17:00	17:00	15:45	15:15	14:40	16:30
0.000001	18:30	18:30	17:30	17:15	16:15	18:10
0.0000001	na	na	na	na	na	na
Negative control	na	na	na	na	na	na

Furthermore, through checking the sensitivity of the assays and primers to use for quantification in the diseased plants, ten-fold serial dilutions (10^{0}-10^{-6}) of 1.0 ng of plasmid DNA containing the partial leucyl-tRNA gene of LNI phytoplasma diluted with plant DNA and dH_2O separately were used. Both serial dilutions were tested with LAMP assays individually with the same conditions of 63°C for 30 min. The results indicated that when the phytoplasma DNA was diluted with plant DNA amplification started earlier than when it was mixed with dH_2O (Table 2.7). This suggests that plant DNA has an influence on amplification time and this will affect quantification as well. As a consequence, this confirmed that when using LAMP assays to quantify phytoplasmas within infected plants the amount of infected plant sample needs to be known to make it possible to estimate the number of phytoplasma cells per gram of infected plant weight (Table 2.7 and Appendix 4).

Table 2.7 LAMP amplification of phytoplasma LNI plasmid mixed with dH2O and plant DNA.

	Plasmid LNI diluted with dH2O		**Plasmid LNI diluted with plant DNA**	
Concentration	Amplification time (mm:ss)	Annealing temperature(°C)	Amplification time (mm:ss)	Annealing temperature(°C)
$1x10^{0}$	09:00	83.8	09:15	83.4
$1x10^{-1}$	11:00	83.9	10:45	83.5
$1x10^{-2}$	12:45	83.7	12:15	83.5
$1x10^{-3}$	14:45	83.6	13:45	83.4
$1x10^{-4}$	16:45	83.7	15:15	83.5
$1x10^{-5}$	17:45	83.7	17:15	83.7
$1x10^{-6}$	21:15	83.9	17:45	83.9

2.4 Discussion

The isothermal amplification method in these experiments has shown evidence that the LAMP assay technique can be used successfully to quantify phytoplasmas due to its sensitivity and also its ability to be used for identification of different phytoplasma strains through using specific LAMP primers. Furthermore, it does not take a lot of time to get the result, as the process can be completed in a shorter time than PCR. For this reason, LAMP assays are suitable for quantification by analysis of the amplification times.

The most common method for phytoplasma detection that has been used in the past is PCR based on the 16S rRNA and 23S rRNA gene. Also, PCR has provided a fast and sensitive method for screening for vectors too by direct PCR (Smart et al., 1996, Marzachì et al., 1998, Hodgetts et al., 2008, Lorenz et al., 1995). Marzachì et al. (1998) have suggested using direct PCR for phytoplasma detection, except in cases of the low titre of phytoplasma DNA where nested PCR might still be useful. The detection and identification by PCR had offered several advantages, such as relative simplicity, specificity, versatility with high sensitivity, and also this method has more sensitivity than microscopic, serological, and hybridization methods (Lorenz et al., 1995).

In this study, the leucyl-tRNA synthetase gene has been selected particularly to design specific primer for detection based on LAMP assays. LAMP has been developed as a technique in the last 16 years and can be used as a sensitive and accurate method for detection of pathogens (Notomi et al., 2000). In this method of detection, a set of four or six primers are designed which recognize a total of six of eight distinct sequences, and the method uses as does PCR on the target DNA and the result only takes 30 to 45 min to obtain. Furthermore, the use of a real-time method has the advantage that the result can be seen instantly without wasting time and doing gel electrophoresis (Parida et al., 2008).

So far LAMP assays have been used primarily for detection, but in this chapter, a new set of primers has been designed and used for detection and discrimination between 16Sr groups of phytoplasmas based on the leucyl-tRNA synthetase gene, and then developed for quantification as well. All the primers have been tested with the same conditions and temperature against six different 16Sr groups of phytoplasmas applied on the LAMP machine (Genie II and Genie III), and only the specific group DNA was amplified with the specific group primers. Moreover, the primers were validated on *E. coli* bacteria and none of the primers gave positive results with it. Specificity and differentiation between different primers have been shown in Table 2.5, Figure 2.5, Figure 2.6 and Appendix 2a – 2c. Furthermore, the copy number of *leu* gene in phytoplasma genomes is only one copy, which makes the calculation of phytoplasma cell number straightforward such that it is possible to

develop precise quantifications from the standard curves that were obtained, as will be discussed further in the following chapters.

The quantification assay can be useful for the pre-symptomatic diagnosis disease, identification, and epidemic studies. Also, it can be used for monitor phytoplasma kinetics such as the progress of an infection, to estimate the number of copies carried by vectors, or the evaluation of phytoplasma tolerance levels (Torres et al., 2005, Trivedi et al., 2009).

Chapter 3. Transmission of phytoplasmas by dodder bridges (*Cuscuta* spp.)

3.1 Introduction:

Transmission of phytoplasmas from infected plants (host species) to *Catharanthus roseus* (Madagascar periwinkle) by using the parasitic plant *Cuscuta* spp. (dodder) is one of the most effective ways to maintain a wide range of phytoplasmas for further research (Přibylová and Špak, 2013). *Catharanthus roseus* (L.) G. Don, family Apocynaceae, has been used in plant pathology as an experimental host for phytoplasmas due to its suitability as a convenient host for an extremely wide range of phytoplasmas. The ability to transmit these pathogens by dodder and insects can be used as a means of confirming association of the pathogen with disease (Bertaccini et al., 2014) and as a convenient means of maintaining collections of different phytoplasmas from different host species – ie the same glasshouse / growth conditions can be used to maintain the entire collection.

In addition, the maintenance of phytoplasmas in the experimental host periwinkle has several other advantages, especially for work at the molecular level. For example, the best source for phytoplasma nucleic acids, proteins, and other immunogens is periwinkle plants, especially

when compared to natural hosts such as trees, because the concentrations of phytoplasmas are typically higher in the periwinkle plants. This makes the development of phytoplasma detection, characterization and differentiation systems much simpler (Marcone et al., 1997, Přibylová and Špak, 2013).

Marcone et al. (1999b) demonstrated that when phytoplasma transmission is carried out by dodder plants, the success of the procedure depends on both type of phytoplasma and *Cuscuta* vector; for instance, *Cuscuta compestris* and *C. europea* could transmit Rubus stunt and Cotton phyllody phytoplasma at a high rate to the periwinkle plants, whereas the other phytoplasmas such as Pear decline, European stone fruit yellows, and *Picris echioides* yellows could only be transmitted at a low rate by *C. reflexa* and *C. odorata* to periwinkle plants.

In vitro conditions phytoplasmas can not be culture, also their maintain in the field is not as easy as other pathogens, and there is also the possibility that several factors in addition to phytoplasma infection may contribute to the symptom severity. For example, time of infection, growth conditions and colonization of phytoplasma may affect symptom development (Kamińska and Korbin, 1999).

The main aim of this chapter was to study the transmission of phytoplasma by dodder, to aid the development of further research approaches on these pathogens. Also to transmit phytoplasmas between

diseased and healthy plant to maintain phytoplasma on different host plants.

3.2 Materials and methods:

3.2.1 Plant materials

3.2.1.1 Dodder plant (*Cuscuta compestris*) growth

To obtain dodder plants, dodder seeds need to be sown with the seeds of a host plant such as *Medicago sativa* (alfalfa); therefore, *Cuscuta compestris* and alfalfa seeds were sown together near the surface of the compost in the same seed tray as described previously (Přibylová and Špak, 2013). They were kept in a controlled environment in a glasshouse at 25°C with 70–90% relative humidity and supplementary intensive light (14 hour light, 10 hours dark). After approximately three weeks the dodder and alfalfa plants had grown together and the seedlings of *M. sativa* parasitized with dodder shoots were transferred to the same pots that diseased plants were planted in and kept in the glasshouse next to the healthy plants.

3.2.1.2 Healthy plants

All fresh healthy periwinkle plants were propagated from a healthy mother periwinkle plant and maintained in a glasshouse with 16 hour day time at 28°C and 8 hour night time at 24°C. The cutting method was as described by (Hodgetts et al., 2013). After 5 weeks all the new cuttings were transferred to an individual pot and were checked with nested PCR based using secA gene primers (Bekele et al., 2011, Hodgetts et al., 2013) to confirm that they were healthy and uninfected with phytoplasmas. Finally, the plants were ready to be used for phytoplasmas transmission with dodder.

Healthy tomato plants (*Solanum lycopersicum*) were kept in the glasshouse and five different cultivars were used in this study, they are Ailsa Craig, cherry tomato, White cherry, M82D, and Green Zebra. Tomato seeds were sown in a Seed tray filled with seed compost. After 4 weeks all the seedlings were transferred to individual pots with compost and 25 % perlite. The plants were ready to be used after 6 weeks. Before starting the transmission experiments all the tomato plants were checked with nested PCR using *secA* gene primers to confirm that all the tomato plants were uninfected.

Oil palm (*Elaeis guineensis)* seedlings (Dura x Pisifera) from Malaysia were used in this study, and the seeds were sown in a seed tray filled with a mixture of soil (Levington F2 Seed & Modular Compost) and perlite

at the ratio of 8:1 in a glasshouse at 28°C daytime and 22°C night time. The photoperiod was 14-16 hours a day with watering every day during summer and on alternate days during winter. After three months, the seedlings were grown enough to be transferred into larger 5 litre pots. The seedlings at three months old were used in the transmission experiments. The oil palm seedlings were confirmed as healthy plants prior to transmission.

3.2.1.3 Phytoplasma infected stock plants

Fresh infected periwinkle plants (*C. roseus*) were taken from mother plants which were previously shown to be infected with different phytoplasmas from the groups 16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI and 16SrX (Table 3.1). All the plants were confirmed to be infected with particular phytoplasma 16Sr groups by nested PCR which amplified target DNA from 16S rRNA gene.

All the infected Napier grass (*Pennisetum purpureum*) stunt (NGS) plants were kept in a glasshouse, as an original host plant to maintain the 16SrXI phytoplasma strain. In order to check that the original host plants were infected with the 16SrXI phytoplasma, 200 mg leaf samples were taken from the plants, and *secA* gene primers were used in nested PCR, followed by gel electrophoresis.

3.2.2 Transmission by dodder

After approximately 2-3 weeks the seedlings (alfalfa with dodder) were transferred adjacent to the infected and healthy plants and allowed to continue growing and attach to the infected and healthy plants to make a bridge between them, through which the phytoplasmas transmission can potentially occur. Transmission of phytoplasma by dodder was carried out for various plant as follows:

1. from infected periwinkle to healthy periwinkle plants

2. from infected Napier grass (original host) to healthy periwinkle plants

3. from infected periwinkle to healthy tomato plants

4. from infected periwinkle to healthy oil palms

Samples of the leaf of all plants were taken from different positions in the plant and checked by PCR after a week to confirm plant status as healthy or infected, and subsequently every week the samples are checked with PCR for the assay with Napier grass, and every week for the assays with tomato and oil palm too. For that reason, 200-300 mg plant material were taken from leaves of all the plants followed by DNA extraction and PCR amplification based on secA primers.

3.2.3 DNA extraction and PCR

For the DNA extractions, the CTAB method was employed to obtain genomic DNA. DNA extraction of all the samples was carried out using approximately 3.0 ml CTAB extraction buffer which was added to each extraction bag (filter bags from BIO-RAD Laboratories UK.) containing the leaves. Samples were homogenised by manually crushing the leaves using an homogenizer tool at room temperature to extract the sap into the CTAB buffer. Subsequently, 800 µl solutions were collected and transferred from each bag to an Eppendorf tube at room temperature and incubated in a water bath for 30 min at 65 ºC, after which 500 µl of Phenol: Chloroform: Isoamyl alcohol was added to each tube. Tubes were vortexed vigorously for 1 min and centrifuged for 10 min at 2375 g in a bench-top centrifuge. The supernatant (500 µl) was added into a tube which contained 500 µl of isopropanol, mixed and then placed in the freezer -20 ºC for at least 2 hours. Following centrifugation for 15 mins at 16060 g, the solution was discarded and the pellet was washed with 70% ethanol and centrifuged for 2-3 sec. The ethanol wash was removed and the pellet dried for 30 min and dissolved in 30 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.6) and finally stored at -20ºC until use.

The DNA samples were amplified by nested PCR using primers secAfor1 (5’ – GARATGAAAACTGGRGAAGG - 3’) and secArev3 (5’ – GTT TTR GCA GTT CCT GTC ATN CC – 3’) (Hodgetts et al., 2008) as the first primer

pair and primers secAfor5 (5’ – ASTCGTGAAGCTGAAGG – 3’) + (5’ - AGCTAAAAGAGAATTTGAAGG – 3’) and secArev2 (5’ – CCNTCRCTAAATTGNCGTCC – 3’) (Bekele et al., 2011) as the nested primer pair to test all phytoplasmas which were in 16SrI group including Rehmannia glutinosa (RG), 16SrII group including Soybean phyllody (SP), 16SrIII group including Plum leptonecrosis (LNI), 16SrV group including Elm yellow (EY), 16SrVI group including Potato witches’ broom (PWB), 16SrX group including Apple proliferation (AP), and Napier grass. In the PCR reactions 1x concentration of the MangoMix™ (Bioline Ltd.), 1 µl of DNA sample and 400 nM of each primer were used. The PCR conditions used for the first and nested round were as follow: denaturation at 94°C for 30 sec, annealing at 53°C for 1 min and extension at 72°C for 1 min for 30 cycles, followed by gel electrophoresis to confirm that phytoplasmas are transmitted successfully as a first step. Healthy plant and distilled water were used for negative control, sometimes only master mix with primers tested as a negative control too.

After that, the PCR products were purified using GenElute™ PCR Clean-Up Kit (Sigma-Aldrich Co. LLC) following the manufacturer’s instructions. Subsequently, the purified samples were sequenced by Eurofins MWG Operon, Germany. Nucleotide sequences were analysed by BLAST at NCBI website (http://blast.ncbi.nlm.nih.gov/).

In addition, LAMP technique was also used to test all the plants to confirm the transmission of phytoplasma from infected to healthy plants. LAMP reactions were performed, following Tomlinson et al. (2010) and Bekele et al. (2011) with modifications, using a 1x concentration of Isothermal Mastermix (Genesys Ltd.), 1 µl of DNA sample, 200 nM external primers, 1 µM internal primers and 1 µM loop primers. LAMP assays were performed with a Genie® II Instrument (Genesys Ltd.) by incubating the reactions at 63°C for 30 min and results were analysed based on Tp values (the time taken to generate a positive result) and annealing temperature of amplicons.

Table 3.1 List of the phytoplasmas used for dodder transmission from infected to healthy plants.

Phytoplasmas name	**Acronym**	**16Sr group**	**Origin source**
Rehmannia glutinosa	RG	I-B	Univeristy of Nottingham UK
Soybean phyllody	SP	II-C	Univeristy of Nottingham UK
Plum leptonecrosis	LNI	III	Univeristy of Nottingham UK
Elm yellow	EY	V	Univeristy of Nottingham UK
Potato witches' broom	PWB	VI	Univeristy of Nottingham UK
Apple proliferation	AP	X-A	Univeristy of Nottingham UK
Napier grass stunt	NGS	XI	Univeristy of Nottingham UK

3.3 Results

3.3.1 Dodder transmission

After 14 to 21 days the dodder and alfalfa plants had grown together. This method allowed the dodder stems to attach easily to the infected plants as shown in Figure 3.1. Afterwards, the dodder bridges were kept unbroken until symptoms developed on the healthy selected plants (Azadvar et al., 2011, Přibylová and Špak, 2013). All the plants (healthy, infected and dodder) were arranged next to each other for every specific 16Sr groups that were used in this study (Table 3.1), and PCR assays were applied every week with secA primer pairs to confirm the presence of phytoplasmas from donor and recipient plants.

3.3.2 Phytoplasma transmission from infected periwinkle to healthy periwinkle plants

Phytoplasma transmission by dodder shoots takes a short time when all conditions are optimal. *Cuscuta* spp. grows rapidly and has the ability to attach itself to nearby plants that are grown in the same field and parasitizes this plant. Dodder shoots attach to both infected and healthy periwinkle plants by making bridges between them, as shown in (Figure 3.1). After 10 – 21 days of bridges, phytoplasmas were transmitted into

healthy periwinkle plants, and results were confirmed by PCR and LAMP assays. First, the healthy periwinkles (*C. roseus*) were infected by phytoplasma through dodder shoots, followed by the appearance of symptoms on the healthy periwinkle plants after approximately 5-6 weeks of transmission. Mostly, yellowing and little leaves were noticed on the majority of the plants. With RG and SP the symptoms were yellowing and mottling with small flowers. LNI showed elongation of leaves with yellowing leaf symptoms, while EY showed symptoms such as witches' broom and mottling. PWB plants showed virescence with elongation of petals, with AP plants showing small leaves, leaf roll, and yellowing. These various symptoms are shown in Figure 3.2. The results from nested PCR of the plant leaf samples using secA primers and gel electrophoresis showed that all the phytoplasmas selected for this study could be transmitted to healthy periwinkle plants by dodder shoots (Figure 3.3).

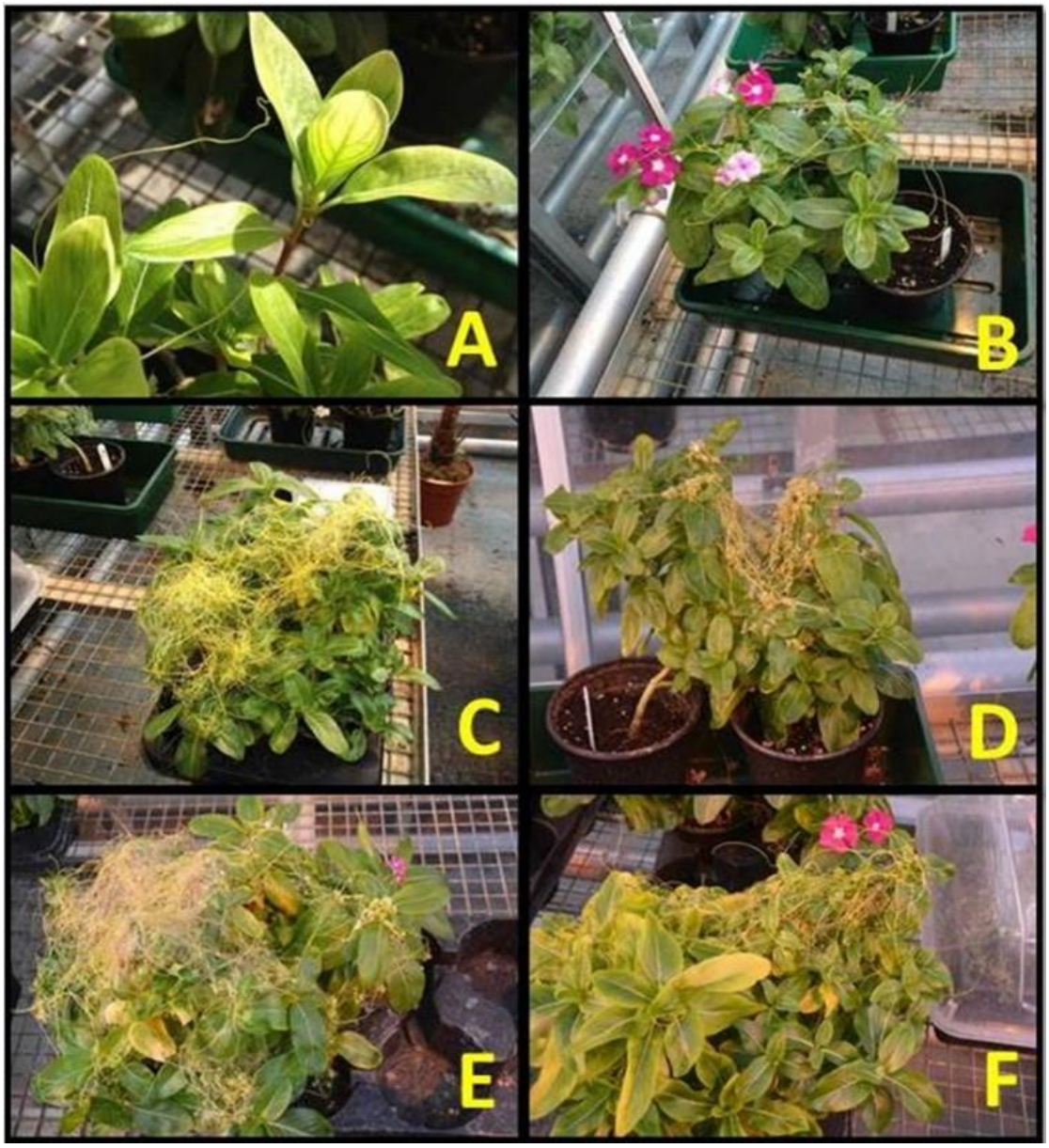


Figure 3.1 Phytoplasma transmission between periwinkle plants by dodder (*Cuscuta* spp.). The *Cuscuta* spp. produced bridges between diseased and healthy periwinkle plants. A & B: Dodder shoots started growing and attaching infected and healthy periwinkle plants, C, D, E and F: Healthy periwinkle plants are changed to diseased plants due to phytoplasma transmission by parasitic dodder.

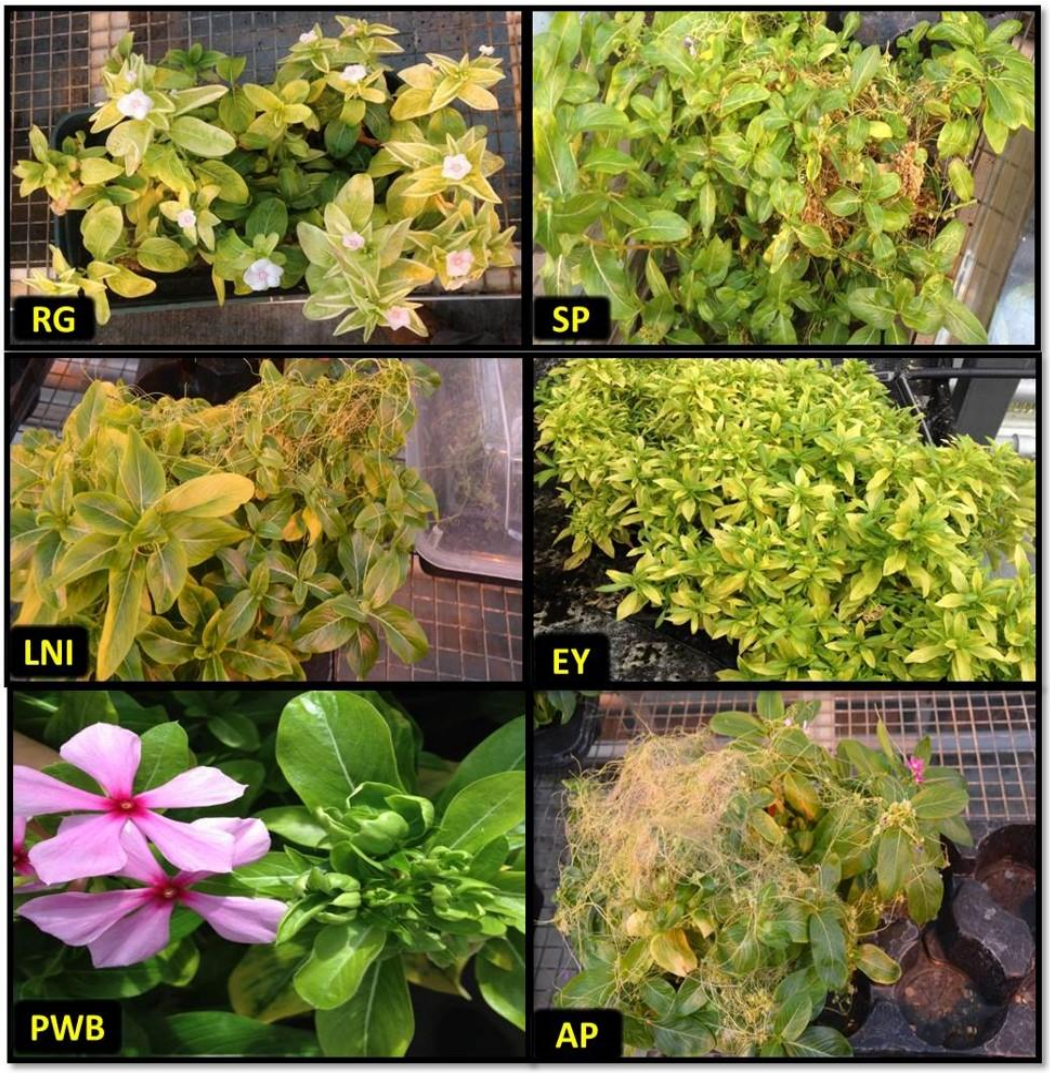


Figure 3.2 Periwinkle plants showing different symptoms with different phytoplasma strains after infection through dodder plant bridges.

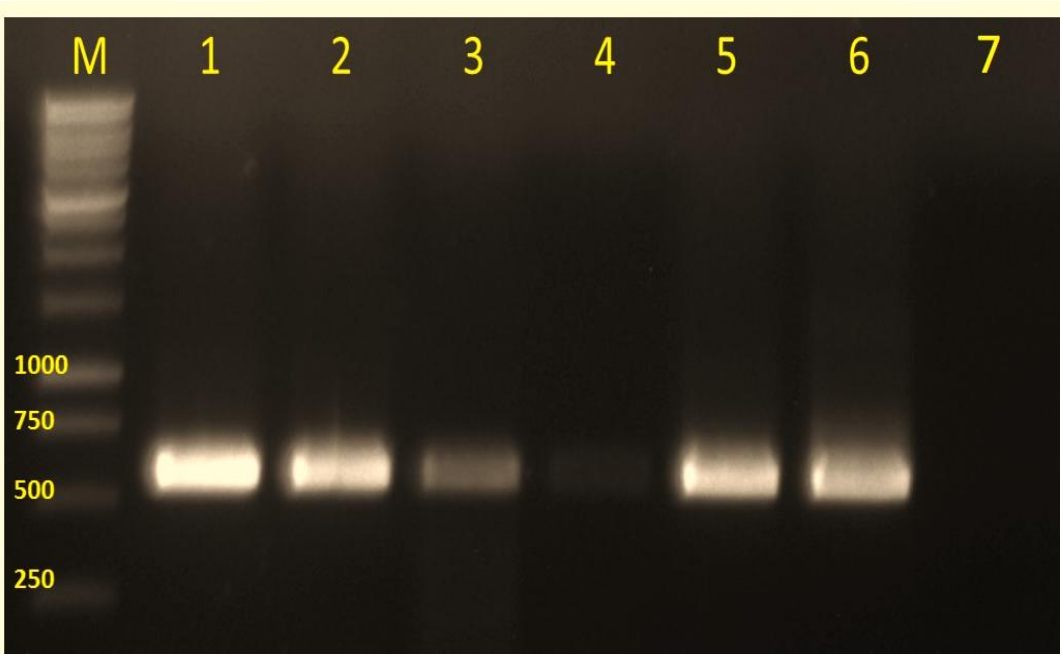


Figure 3.3 Various phytoplasmas were transmitted from diseased periwinkle into healthy periwinkle plant effectively by dodder *Cuscuta* spp. Results were confirmed by PCR with secA primers, DNA ladder (M): 1kb; the nested PCR result of amplified DNA samples from plants subject to phytoplasma transmission with; 1:RG, 2:SP, 3:LNI, 4:EY, 5:PWB, 6:AP, and 7: Negative control.

3.3.3 Phytoplasma transmission from infected Napier grass (original host) to healthy periwinkle plants

Phytoplasma transmission from an original host plant to a healthy plant in a different species is not easy to achieve unless it is happening naturally (for example through the natural vector species). In this study, attempts were made to transfer the 16SrXI Napier grass stunt phytoplasma from its original host (Napier grass) to a healthy periwinkle plant through dodder shoots. Nine weeks after attempted transmission the plants were tested. Gel electrophoresis confirmed the results that phytoplasma can be transferred from an original host plant such as Napier grass to a healthy periwinkle plant in a different species after almost 9 weeks of transmission as shown in Figure 3.4.

Data analysis provided more evidence that transmission had occurred successfully. Disease symptoms on the transferred plants were observed after 10 weeks as shown in (Figure 3.6), which showed different symptoms such as yellowing and some typical symptoms such as mosaics. Results confirmed the presence of the specific phytoplasmas within the infected plants.

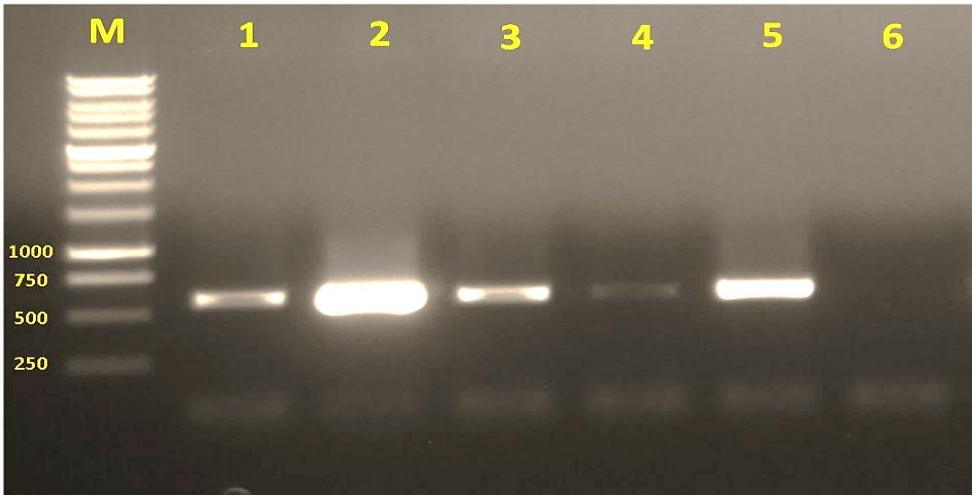


Figure 3.4 Detection and identification of phytoplasma with nested PCR based on secA primers after phytoplasma transmission from Napier grass stunt to healthy periwinkle plants through dodder plant shoots.

Lane M : 1 kb ladder/marker

Lane 1 : Dodder plant shoots

Lane 2 : Napier grass stunt phytoplasma 16SrXI (Host plant)

Lane 3 : First infected periwinkle plant with phytoplasma stunt (16SrXI) 9 weeks post transmission.

Lane 4 : Second infected periwinkle plant with phytoplasma stunt (16SrXI) 9 weeks post transmission.

Lane 5 : Third infected periwinkle plant with phytoplasma stunt (16SrXI) 9 weeks post transmission.

Lane 6 : A healthy periwinkle plant

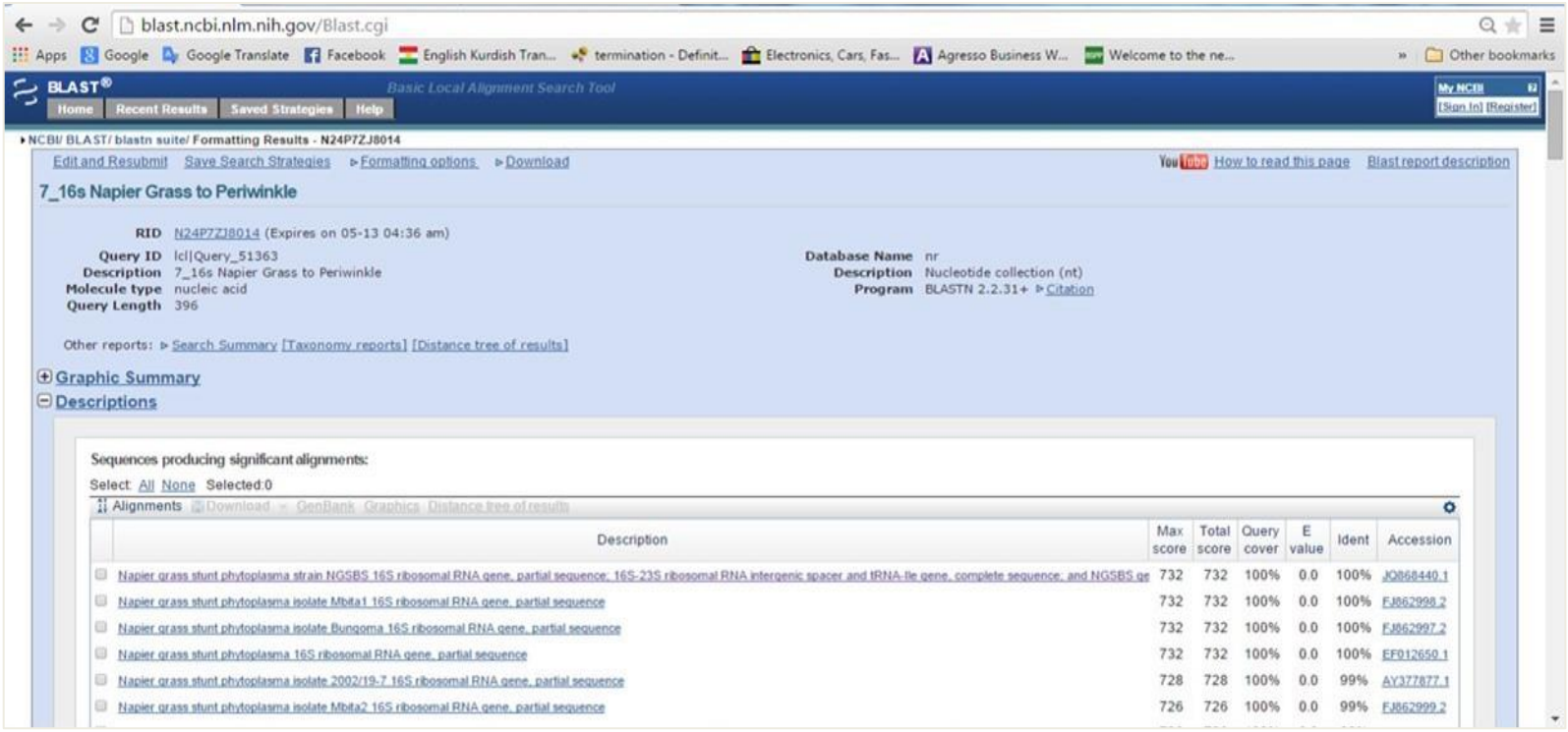


Figure 3.5 Blast result of the sequence obtained from a periwinkle plant after infection with Napier grass stunt through dodder shoots. The results were obtained after nested PCR amplifications based on secA primers, the purified samples were sequenced by Eurofins MWG Operon, Germany. Nucleotide sequences were analysed by BLAST. The sequences showed 100% identity with Napier grass stunt phytoplasmas 16S ribosomal RNA gene.

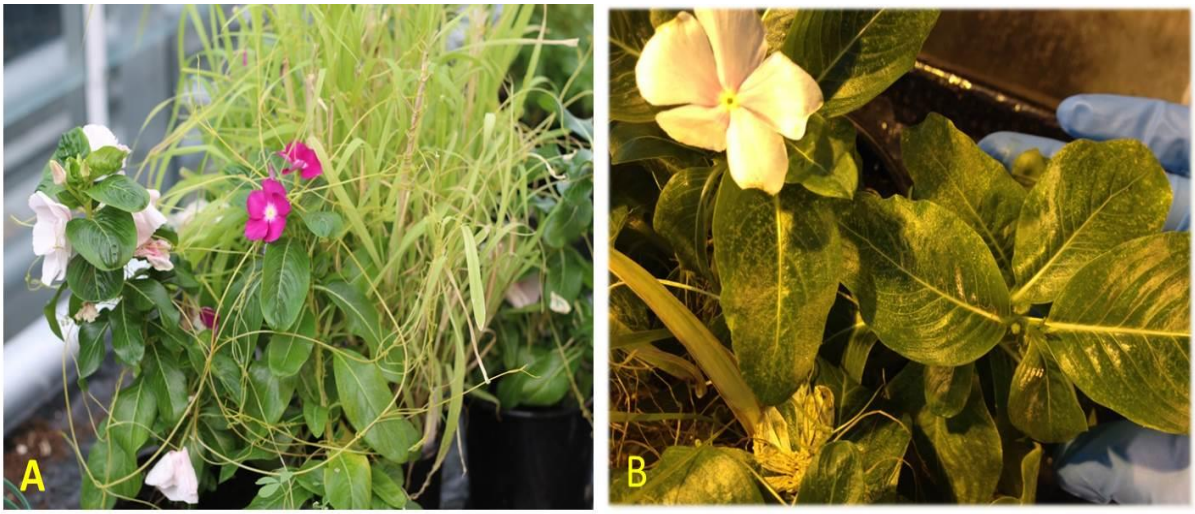


Figure 3.6 Symptoms on a healthy periwinkle plant after infection with Napier grass stunt 16SrXI from the original host plant, Napier grass, through dodder

3.3.4 Phytoplasma transmission from infected periwinkle to healthy tomato plants

Six strains of phytoplasmas from 16Sr taxonomic groups (16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI and 16SrX) were tested to see whether they could be transferred from infected periwinkle plants to the healthy tomato plants using dodder shoots. *Cuscuta compestris* and *M. sativa* (host plants) were joined to both plants to produce a bridge between them to potentially transfer the phytoplasmas (Figure 3.7). Tomato plants infected with phytoplasmas showed symptoms after approximately 8 months including yellowing, leaf roll, reduced leaf size, and mottling.

Monthly checking of leaf samples with PCR of some tomato plants shown positive amplification after 7-8 months confirming the plants to have been infected with phytoplasma through the dodder shoots. Transmission of phytoplasma of RG, SP and LNI groups from periwinkle to cherry tomato was confirmed with nested PCR using secA primers. However, no amplification was observed for RG and LNI using LAMP assays, which might be due to technical error, since samples of tomato leaves following transmitted using other groups, SP, EY, PWB, and AP showed a positive LAMP amplification even though no amplifications were achievable using nested PCR, probably because of low phytoplasma titre in the plants (Table 3.2). Transmission of phytoplasma groups RG, LNI and AP to tomato cultivar M82D was also confirmed with LAMP assays.

Table 3.2 Tomato species used for transmission against six different 16Sr groups of phytoplasma with their PCR and LAMP amplification results.

Strain	16Sr group	White cherry		Green zebra		cherry		M82D		Ailsa Craig	
		PCR result	LAMP result	PCR result	LAMP result	PCR result	LAMP result	PCR result	LAMP result	PCR result	LAMP result
RG	I-B	-	-	-	-	+	-	-	+	-	-
SP	II-C	-	-	-	-	+	+	-	-	-	-
LNI	III	-	-	-	-	+	-	-	+	-	-
EY	V	-	-	-	-	-	+	-	-	-	-
PWB	VI	-	-	-	-	-	+	-	-	-	-
AP	X-A	-	-	-	-	-	+	-	+	-	-



Figure 3.7 Dodder bridges around infected periwinkle and healthy tomato plants during transmission of phytoplasmas through growing dodder shoots.

3.3.5 Phytoplasma transmission from infected periwinkle to healthy oil palms

SP, EY, and PWB infected periwinkle plants were also attached to healthy oil palms by dodder to test for transmission from the infected periwinkle into the healthy oil palm (Figure 3.8). After checking for 12 months, the results were negative indicating no transmission through the dodder bridge.



Figure 3.8 A healthy oil palm with an infected healthy periwinkle plants were connected with dodder shoots in attempt of transmitting phytoplasmas from diseased periwinkle to healty oil palm.

3.4 Discussion

The aim of this chapter was to develop and validate methods for transmitting phytoplasmas by dodder between plants within the same plant species and between plants from different species. Phytoplasma distribution is irregular in woody plants, and this makes the appearance of symptoms variable. Additionally, the time taken for transmission can be variable within and between different plant species (Přibylová and Špak, 2013).

Transmission by dodder is a practical and applicable method for moving phytoplasmas between plant species and has been used for transmission from infected lily 'Casablanca' to Alstroemeria seedlings (Kamińska and Korbin, 1999), from young pear, apricot, and peach seedlings, blackberry plants (*Rubus fruticosus*), *Picris echioides* and cotton plants to periwinkle plants (Marcone et al., 1999a), and from redcurrant plants (*Ribes* sp.) to periwinkle plants (Přibylová and Špak, 2013).

In this current study, transmission of phytoplasmas from six different phylogenetic groups, 16SrI RG, 16SrII SP, 16SrIII LNI, 16SrV EY, 16SrVI PWB and 16SrX AP to healthy periwinkle plants was demonstrated (Table 3.1). The results based on this demonstrated that the protocol used here is a robust and reliable way to use *Cuscuta* spp. to transmit phytoplasmas from diseased to healthy periwinkle plants *Catharanthus roseus* plants are convenient hosts to use for phytoplasma

diseases, particularly as they are often propagated by cuttings, such that it is possible to maintain them as clonal lines. Therefore it is possible to introduce phytoplasmas from different phylogenetic groups and hosts into periwinkles of an identical genotype so that the different symptoms observed can be assumed to be due to the phytoplasmas and not to any genetic variability within the host.

Whilst transmission of some phytoplasmas from their natural hosts to periwinkle can be achieved by grafting, often this is not the case because the graft unions are incompatible / unsuccessful. For example, it is not possible to graft Napier grass to periwinkle, nor to use the method developed by Praphat Kawicha in our laboratory (Kawicha, 2014), in which sections of Napier grass tissue were embedded into the vascular system of periwinkles (a method that was demonstrated to work successfully for transmission between periwinkles). Therefore, methods such as dodder are required, and in this study napier grass stunt phytoplasma has been transferred by dodder plant from infected host Napier grass to healthy periwinkle plants. It is worthy that within the same plant species, phytoplasma transmission from infected plants to healthy plants through dodder appears to occur more quickly than transmission between different plant species. For example, transmission between diseased and healthy periwinkle plants was obtained within approximately two weeks after starting the transmission (as demonstrated by PCR detection), whilst transmission from infected

Napier grass stunt to healthy periwinkle plants took more than two months.

Symptom development can be affected by growth conditions, time of infection and colonization by phytoplasmas (Kamińska and Korbin, 1999). Symptoms in the plants infected from an infected periwinkle were observed after 5-6 weeks, with the typical symptoms of yellowing, mottling, leaf roll, reduced leaf size, small flowers, witches' broom and virescence. The symptoms were different on periwinkle plants when they were infected with Napier grass stunt, which developed yellowing on leaves, mottling, and mosaics.

Transmission of phytoplasmas to tomato was also demonstrated, and the symptoms on infected tomato plants, which were observed approximately 8 months after transmission, were yellowing with leaf roll.

Phytoplasma transmission through dodder plants to periwinkle plants has several advantages: periwinkle plants are better sources than trees for phytoplasma nucleic acids, proteins, and other immunogens, due to the phytoplasma titres being usually higher in the periwinkle plants and phytoplasma components are easier to obtain from periwinkle plants (Marcone et al., 1999a). Also, propagation and growing these plants is easier as they can be propagated through infected cuttings. One of the problems with maintaining phytoplasmas in plants such as tomato or arabidopsis is that plants generally have to be propagated from seed, so

in order to maintain collections, the phytoplasmas have to be transferred to new plants as they mature from the old infected plants.

Experimental infection of periwinkle with phytoplasma can be performed by grafting or transmitting through dodder plants. Grafting method needs to be performed carefully as any mistake can result in no infection, and also some plants are not easy to be used for grafting because of the differences between donor and recipient plants. Using dodder plants for phytoplasma transmission can be performed easily, by attaching the dodder plants to both donor and recipient plants, as shown in this study.

Chapter 4. Quantification of phytoplasmas using LAMP in plants grown under altered nitrogen fertilization regimes

4.1 Introduction

Fertilizers are commonly used commercially in farming to maximize crop yield and plant growth when water and light conditions are sufficient (Veresoglou et al., 2013). The improvement of (N) use efficiency is a major goal of crop improvement and use of the nitrogen fertilizer supplied is improving crops and also producing higher yields with better protein content. This might be achieved, at least in part, through a better understanding of nitrogen meta-bolism and its regulation, also through identifying likely target genes for manipulation by either direct gene transfer or marker-assisted breeding (Miflin and Habash, 2002). Nitrogen fertilization also has an influence on the interactions between pathogens and host plants; nitrogen fertilisation can affect the plant susceptibility to disease, which has stated that plant growthat high nitrogen (N) availability may result in increased plant susceptibility to pathogens as a result of increased foliar nitrogen concentrations. Nevertheless, plant pathogen dynamics in response to nutrient availability are believed to be more complicated than this.

Although evidence from some plants such as *Solanum* spp. showed that nitrogen fertilization can decrease disease severity and could potentially be used to control the spread of infection outbreaks (Veresoglou et al., 2013). Nitrogen is one of the two nutrients (with potassium) that is most commonly used to limit plant growth; this suggests that their limited availability can be used as a valuable technique for the plant in terms of disease resistance (Veresoglou et al., 2013).

Different nitrogen sources and different amounts can have different effects on plant disease; some nitrogen forms increase plant disease, and some other sources decrease plant disease, due to the inability of pathogens to use a specific form of nitrogen. For example, in the case of root rot disease, the nitrate form (NO_3) of nitrogen decreases plant disease, whilst the ammonium form (NH_4) increases plant disease (Huber and Watson, 1974). Shaner and Finney (1977) found that high rates of nitrogen provided optimal conditions for recognition of slow-mildewing resistance in Knox wheat. To date, there has been no published research on the influence of various nitrogen source or various amount of nitrogen on phytoplasma diseases.

Molecular techniques, such as real-time PCR have been developed and used in the past in attempts to quantify levels of phytoplasmas in plants (Bertaccini and Duduk, 2009, Berges et al., 2000, Torres et al., 2005). More recently, we have also been developing the LAMP technique for phytoplasma quantification in periwinkle plants. Phytoplasma titre in a

plant may vary with plant susceptibility, the symptoms expressed, the phytoplasma strain, plant age at the onset of infection and climatic conditions. Moreover, different plant organs may sustain different population densities (Saracco et al., 2006).

In this chapter, results from the validation of the real-time LAMP technique for quantification of phytoplasmas will be presented along with the use of this technique for investigating the effects of nitrogen fertilization on the disease to find out the impact of nitrogen on phytoplasma levels. Specific composts have been made and mixed with different sources of nitrogen–ammonia versus nitrate and then the phytoplasma infected plants of each group have been transplanted into these different composts for more than 6 months. Samples have been taken every month to detect levels of phytoplasmas using quantification by the LAMP technique.

The hypothesis of this chapter is the nitrogen fertilizer suplimented in the plant media (compost) will reduce the phytoplasma titre in diseased periwinkle plants. Our aim particulary was to find which source of nitrogen (Ammonium nitrate, Calicium nitrate, and Ammonium sulphate) has impact on phytoplasma quantity, also to measure the concentrate of nitrogen element in the solutions.

4.2 Materials and methods:

4.2.1 Compost preparation

The compost used in the control treatments was Levington Pot and Bedding Compost M3, which is a high nutrient fertilizer with a nitrogen level of 204 mg/L, phosphorus 104 mg/L, and potassium 339 mg/L. The concentration of 204 mg/L of nitrogen was therefore prepared as standard for the other sources used, based on the concentration of nitrogen in commercial compost M3. Nitrogen free compost was prepared as the base compost to be used in the assay by adding nitrogen elements to define final concentrations, also the same levels of phosphorus and potassium were added to the nitrogen free compost as in commercial compost M3 control. To test the effect of different nitrogen sources to phytoplasma titre in phytoplasma, nitrogen was added to the nitrogen free compost at three different final concentrations as follow:

1. 204 mg/L (the same as normal commercial compost)

2. 102 mg/L (half strength concentration)

3. 408 mg/L (double concentration)

4.2.2 Different sources of nitrogen

(Three distinct nitrogen sources), Ammonium nitrate (NH_4NO_3), Ammonium sulphate ($(NH_4)_2SO_4$), and Calcium nitrate ($Ca(NO_3)_2$) were selected based on different forms of nitrogen such as Ammonium and nitrate, also these sources are commonly used as nitrogen fertilizers. The sources are determined to be used in this study to see if the nitrogen source also effecting phytoplasma in the periwinkle plant. In addition, to find out which source has more impact on phytoplasma quantity (whichever decrease or increae phytoplasma amount) in diseased perwinkle plants. Final concentration of each nitrogen source was as mentioned above. Commercial compost M3 acted as a control.

4.2.3 Infected periwinkle plants

New cuttings were taken from diseased periwinkle mother plants which contained phytoplasmas from four taxonomic groups, 16SrIII (LNI), 16SrV (EY), 16SrVI (PWB) and 16SrX (AP). All the new cuttings were rooted in the different mixtures of composts that had been prepared in advance. In summary, for each specific group of phytoplasma, ten different treatments were prepared in 3 replicates. The treatments are as follow:

1. Commercial compost (control) with a nitrogen concentration of 204 mg/L

2. Ammonium nitrate compost with a nitrogen concentration of 408 mg/L

3. Ammonium nitrate compost with a nitrogen concentration ratio of 204 mg/L

4. Ammonium nitrate compost with a nitrogen concentration of 102 mg/L

5. Ammonium sulphate compost with a nitrogen concentration of 408 mg/L

6. Ammonium sulphate compost with a nitrogen concentration of 204 mg/L

7. Ammonium sulphate compost with a nitrogen concentration of 102 mg/L

8. Calcium nitrate compost with a nitrogen concentration of 408 mg/L

9. Calcium nitrate compost with a nitrogen concentration of 204 mg/L

10. Calcium nitrate compost with a nitrogen concentration of 102 mg/L

4.2.4 Healthy periwinkle plants

New cuttings from healthy periwinkle plants were taken and used as controls in the different composts. All the plants were maintained in the same glasshouse with daily watering.

4.2.5 DNA extraction

Plant samples were taken monthly for six months and DNA extracted using the CTAB method as described in section 2.2.2. All the DNA samples were kept in the freezer at -20°C. After collecting all the samples, all DNA was diluted 1:10 prior to carrying out the quantification test. In such a sensitive technique DNA needs to be diluted, diluted sample gives a more accurate measurement, also will eliminate the impact of inhibitors in the reactions.

To assess the efficiency of DNA extraction, leaf samples were taken from the top, middle, and bottom of the same plant, all the samples are extracted seperately and tested with LAMP technique.

4.2.6 Real-time LAMP reactions

Preparing LAMP reactions and running samples with LAMP assays was as described by (Tomlinson, 2013, Dickinson, 2015). LAMP reaction mixes were prepared from 1x concentration of isothermal master mix (OptiGene, UK, product number ISO-001) with 1 µl of DNA sample, 200 nM external primers, 1 µM internal primers and 1 µM loop primers which were based on the leucyl tRNA synthetase gene. From the master mix, 20 µl was aliquoted into each of the 8 × 0.2-ml tubes in a strip and 1 µl diluted DNA was added to the tubes. Tubes 1 - 6 were used for different DNA samples and tube 7 was a positive control and tube 8 was the negative control. The lids were closed and the tubes placed in the Genie II machine. Samples were run using the manufacturer's instructions at 63°C for 30 min and results were analysed based on T_p values (the time taken to generate a positive result) and annealing temperature of amplicons as described by (Bekele et al., 2011, Kawicha, 2014).

4.2.7 Specific LAMP primers for quantification

LAMP reactions were performed with a particular primer set for each 16Sr group based on the leucyl-tRNA synthetase gene, all the primers are shown in Chapter 2, Table 2.4. The selected primers for phytoplasma

detection and quantification were 16SrIII (LNI), 16SrV (EY), 16SrVI (PWB), and 16SrX (AP).

4.3 Results

All the primers for each group, 16SrIII, 16SrV, 16SrVI, and 16SrX, were tested on dilutions of plasmid constructs from each group by LAMP to develop standard curves. Plasmid copy number in the LAMP reactions was calculated by using the formula: (plasmid amount, ng) x (6.022 x 10^{23}) / ((plasmid length, bp) x (1x10^{9}) x 650). source: http://cels.uri.edu/gsc/cndna.html. From this calculation, phytoplasma cell number can be estimated in an infected plant based on the finding that there is just one copy of the leucyl-tRNA synthetase gene in the phytoplasma genome.

It is found that there was no significant differences in phytoplasma titre in the leaves taken from differnet locations of the same diseased periwinkle plant. For this experiment 15 leaves were teasted on a plant, which were 5 leaves from upper, 5 leaves from middle and 5 leaves from lower part of the same plant (Table 4.1).

Table 4.1 Phytoplasma titre of group 16SrVI (PWB) in leaves taken from different parts of diseased periwinkle plant.

Plant section	Amplification time (mean)	Calculated phytoplasma cells per 1 gram of leaf sample
Upper part leaves	15.68	2.8×10^4 a
Middle part leaves	15.58	3.3×10^4 a
Lower part leaves	15.30	5.1×10^4 a

Data are mean of amplification time with phytoplasma cell number of 3 replicates (leaf). Cell number with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

4.3.1 Plum leptonecrosis (LNI) phytoplasmas quantification

Quantification of plum leptonecrosis (LNI) phytoplasma, group 16SrIII, in infected periwinkle plants using LAMP was performed based on the equation and standard curve as shown in Chapter 2, Figure 2.7. The value of R^2 was 0.99 for LNI which is greater than 0.95, indicating that this curve is valid to use for quantification. After 2 months of planting with the compost with different concentration of ammonium nitrate (NH_4NO_3), it was observed that the phytoplasma titer in plant planted in composted with nitrogen supplement at 102 mg/L, 204mg/L and 408

mg/L using ammonium nitrate as nitrogen source were significantly reduced compared to control, which was planted in normal commercial compost (Table 4.2). The highest reduction was observed in plants treated with 204 mg/L nitrogen in the soil and was significant compared with the other treatments. The phytoplasma continued to show a reduction in each subsequent month until 6 months in all treatments but the reduction was not significant when compared to controls except in the treatment with 204 mg/L nitrogen, suggesting the superiority of using 204 mg/L nitrogen in soil from ammonium nitrate in reducing phytoplasma titre compared to other concentrations. Although, the 204 mg/L nitrogen of treatment compost has the same amount of nitrogen in the control compost it is not clear which source of nitrogen has been used in the commercial compost.

Table 4.2 Ammonium nitrate -AN- (NH_4NO_3) – phytoplasma group 16SrIII (LNI), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L, and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (AN)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	4.4×10^5 a	3.5×10^5 ab	8.9×10^6 b	8.4×10^5 b	2.1×10^5 b	1.1×10^6 c	7.4×10^5 b
102	3.7×10^5 a	8.9×10^5 b	1.3×10^5 a	2.4×10^5 ab	2.9×10^5 b	0.6×10^5 ab	2.9×10^5 ab
204	4.8×10^5 a	0.4×10^5 a	1.0×10^5 a	0.9×10^5 a	0.3×10^5 a	0.4×10^5 a	0.6×10^5 a
408	9.1×10^5 a	2.7×10^5 ab	2.8×10^5 a	1.7×10^5 ab	8.5×10^5 b	3.0×10^5 bc	1.3×10^6 b
P value (ANOVA)	0.3261	0.2016	0.0341	0.2042	0.0169	0.0071	0.0230

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

With ammonium sulphate $(NH_4)_2SO_4$, all concentration of 102 mg/L, 204 mg/L and 408 mg/L appeared to significantly decrease phytoplasma titre in periwinkle compared to controls at two and three months after planting. However, there was no reduction in phytoplasma titre at the fourth, fifth, and sixth months compared to controls for plants treated with 204 and 408 mg/L of ammonium sulphate. On the other hand, 102 mg/L nitrogen was still shown to significantly reduce phytoplasma titre up until six months as shown in (Table 4.3).

With calcium nitrate (CN) $Ca(NO_3)_2$ the 102 mg/L nitrogen had a significant effect on phytoplasma titre and decreased the amount of the phytoplasma in diseased plants starting from the second month after applying calcium nitrate. 204 mg/L nitrogen also showed a significant reduction in the levels of the LNI phytoplasma as shown in (Table 4.4). However, the 408 mg/L nitrogen did not affect phytoplasma levels.

Table 4.3 Ammonium sulphate -AS- $(NH_4)_2SO_4$ – phytoplasma group 16SrIII (LNI), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L, and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (AS)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	4.4×10^5 a	3.5×10^5 a	9.0×10^6 b	8.4×10^5 c	2.1×10^5 b	1.1×10^6 b	7.4×10^5 b
102	1.2×10^5 a	4.4×10^5 a	1.1×10^5 a	1.9×10^5 b	0.2×10^5 a	0.5×10^5 a	0.3×10^5 a
204	0.8×10^5 a	0.9×10^5 a	0.5×10^5 a	0.2×10^5 a	0.5×10^5 ab	2.9×10^5 ab	5.6×10^5 b
408	0.9×10^5 a	4.4×10^5 a	0.9×10^5 a	0.5×10^5 ab	1.4×10^5 ab	5.6×10^5 b	3.5×10^5 b
P value (ANOVA)	0.3685	0.6625	0.0560	0.0015	0.1527	0.0177	0.0049

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

Table 4.4 Calcium nitrate -CN- ($Ca(NO_3)_2$) – phytoplasma group 16SrIII (LNI), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L, and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (CN)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	4.4×10^5 a	3.5×10^5 a	9.0×10^6 b	8.4×10^5 c	2.1×10^5 ab	1.1×10^6 b	7.4×10^5 b
102	3.3×10^5 a	1.0×10^5 a	0.9×10^5 a	0.2×10^5 a	1.1×10^5 ab	1.0×10^5 a	0.3×10^5 a
204	2.3×10^5 a	1.5×10^5 a	1.2×10^5 a	0.3×10^5 a	0.5×10^5 a	0.3×10^5 a	6.5×10^5 ab
408	1.0×10^5 a	3.6×10^5 a	2.4×10^5 ab	1.7×10^5 b	2.8×10^5 b	19.7×10^5 b	12.3×10^5 b
P value (ANOVA)	0.5590	0.5321	0.0802	0.0003	0.1023	0.0011	0.0915

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

4.3.2 Elm yellows (EY) phytoplasma quantification

Quantification of elm yellows (EY) phytoplasma group 16SrV was performed using the same approach as described above. The equation for making a standard curve for this group was 0.99, indicating that using LAMP for quantification was valid. The results showed that the quantity of phytoplasma group 16SrV (EY) can be affected and decreased significantly with a high rate of nitrogen when using 408 mg/L nitrogen from ammonium nitrate (AN) and ammonium sulphate (AS), but significant reduction only observed after 4 months of planting (Table 4.5 and Table 4.6). However, in the case of calcium nitrate, it was the normal rate of nitrogen that resulted in a decrease in phytoplasma cell numbers in the infected periwinkle plants (Table 4.7).

Table 4.5 Ammonium nitrate -AN- (NH_4NO_3) – phytoplasma group 16SrV (EY), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (AN)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	1.1×10^6 a	6.4×10^5 a	8.8×10^5 a	1.1×10^6 a	1.9×10^6 b	5.6×10^5 b	2.5×10^6 b
102	1.5×10^6 a	8.0×10^5 a	4.4×10^5 a	9.7×10^5 a	4.4×10^5 ab	1.7×10^5 ab	3.9×10^6 b
204	1.9×10^6 a	0.6×10^5 a	1.6×10^6 a	3.3×10^5 a	5.1×10^5 ab	7.7×10^5 b	1.0×10^6 ab
408	4.2×10^5 a	0.7×10^5 a	2.0×10^6 a	1.6×10^5 a	$1.1 \times 10^{5*\#}$ a	0.2×10^5 a	0.2×10^5 a
P value (ANOVA)	0.7158	0.0897	0.6988	0.3774	0.2065	0.0983	0.0719

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

Table 4.6 Ammonium sulphate -AS- $(NH_4)_2SO_4$ – phytoplasma group 16SrV (EY), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (AS)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	1.1×10^6 a	6.4×10^5 a	8.8×10^5 b	1.1×10^6 a	1.9×10^6 b	5.6×10^5 b	2.5×10^6 b
102	2.9×10^5 a	5.9×10^5 a	4.5×10^5 ab	1.7×10^5 a	6.1×10^5 b	9.1×10^5 b	1.7×10^6 ab
204	2.9×10^5 a	0.3×10^5 a	3.3×10^5 ab	2.6×10^5 a	1.7×10^6 b	8.7×10^5 b	2.0×10^6 b
408	4.5×10^5 a	0.8×10^5 a	0.4×10^5 a	0.9×10^5 a	0.6×10^5 a	0.3×10^5 a	1.7×10^5 a
P value (ANOVA)	0.5675	0.3149	0.0940	0.2092	0.0126	0.0164	0.1027

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

Table 4.7 Calcium nitrate -CN- ($Ca(NO_3)_2$)– phytoplasma group 16SrV (EY), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (CN)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	1.1×10^6 a	6.4×10^5 a	8.8×10^5 b	1.1×10^6 b	1.9×10^6 b	5.6×10^5 b	2.5×10^6 b
102	5.8×10^5 a	4.1×10^5 a	9.5×10^5 b	9.4×10^5 b	6.1×10^5 b	3.1×10^5 b	1.6×10^6 ab
204	3.6×10^5 a	0.6×10^5 a	0.2×10^5 a	0.3×10^5 a	0.4×10^5 a	0.6×10^5 a	1.0×10^5 a
408	5.3×10^5 a	3.5×10^5 a	1.1×10^6 b	6.2×10^5 b	1.6×10^6 b	8.9×10^5 b	4.4×10^6 b
P value (ANOVA)	0.5514	0.2933	0.0027	0.0033	0.0067	0.0117	0.0915

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

4.3.3 Potato witches' broom (PWB) phytoplasma quantification

Quantification of potato witches' broom (PWB) phytoplasma group VI was performed in the same way, using specific *leu* gene primers. Over the six-month, the results showed that ammonium nitrate (AN) at 102 mg/L significantly reduced the amount of phytoplasma after two months, and this reduction was sustained for the rest of the time course. 204 mg/L ammonium nitrate (AN) also started to reduce phytoplasma amount at the fifth month, but the reduction was not as great as occurred with 408 mg/L AN as shown in (Table 4.8).

In the case of applying ammonium sulphate (AS), results indicated that a low concentration of nitrogen into the compost had a significant impact on phytoplasma quantity on the same plants. AS at 102 mg/L showed a significant reduction of phytoplasma titre at the first and second months, and this reduction continued during the fourth, fifth and sixth months as well. AS at 204 mg/L also showed a significant reduction in phytoplasma quantity in the first month; however, the results are showing low titre at subsequent months against 204 mg/L, but statistical analysis of the results indicated a nonsignificant effect, as shown in (Table 4.9).

The effect of nitrogen as calcium nitrate (CN) was different from ammonium sulphate as the calculation results indicated that the double rate of CN reduced phytoplasma quantity in the fifth and sixth months,

whilst the low rate of the same nitrogen source had no impact on phytoplasma titre in the infected periwinkle plants (Table 4.10). Calcium nitrate (CN) at 204 mg/L significantly reduced the phytoplasma amount only at the sixth-month stage.

Table 4.8 Ammonium nitrate -AN- (NH_4NO_3) – phytoplasma group 16SrVI (PWB), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (AN)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	2.5×10^5 a	2.4×10^5 a	6.7×10^5 b	0.4×10^5 a	1.8×10^5 a	2.3×10^5 b	2.5×10^5 b
102	4.2×10^5 a	5.8×10^5 a	4.1×10^5 b	0.04×10^5 a	2.4×10^5 a	1.4×10^5 ab	2.3×10^5 b
204	1.7×10^5 a	1.8×10^5 a	3.9×10^5 b	0.4×10^5 a	0.1×10^5 a	0.2×10^5 a	0.1×10^5 a
408	4.4×10^5 a	0.9×10^5 a	0.2×10^5 a	0.04×10^5 a	0.2×10^5 a	0.2×10^5 a	0.05×10^5 a
P value (ANOVA)	0.7244	0.3884	0.0287	0.1209	0.1926	0.0921	0.0042

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

Table 4.9 Ammonium Sulphate -AS- $(NH_4)_2SO_4$ – phytoplasma group 16SrVI (PWB), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L, and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (AS)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	2.5×10^5 a	2.4×10^5 b	6.7×10^5 b	0.4×10^5 a	1.8×10^5 b	2.3×10^5 b	2.5×10^5 b
102	2.8×10^5 a	0.09×10^5 a	0.03×10^5 a	0.03×10^5 a	0.09×10^5 a	0.07×10^5 a	0.5×10^5 a
204	2.8×10^5 a	0.1×10^5 a	3.8×10^5 b	0.05×10^5 a	0.4×10^5 ab	0.2×10^5 ab	0.9×10^5 ab
408	3.4×10^5 a	1.5×10^5 b	0.3×10^5 ab	0.2×10^5 a	0.2×10^5 ab	0.4×10^5 ab	1.0×10^5 ab
P value (ANOVA)	0.7678	0.0078	0.0290	0.2460	0.1513	0.2025	0.1406

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

Table 4.10 Calcium nitrate -CN- ($Ca(NO_3)_2$ – phytoplasma group 16SrVI (PWB), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (CN)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	2.5×10^5 a	2.4×10^5 a	6.7×10^5 a	0.4×10^5 a	1.8×10^5 a	2.3×10^5 b	2.5×10^5 b
102	3.3×10^5 a	1.5×10^5 a	9.6×10^5 a	0.1×10^5 a	0.6×10^5 a	1.1×10^5 ab	3.6×10^5 b
204	2.2×10^5 a	0.9×10^5 a	3.4×10^5 a	0.3×10^5 a	1.2×10^5 a	0.4×10^5 ab	0.2×10^5 a
408	3.7×10^5 a	2.4×10^5 a	0.6×10^5 a	0.6×10^5 a	0.2×10^5 a	0.09×10^5 a	0.2×10^5 a
P value (ANOVA)	0.7708	0.8815	0.4130	0.8086	0.4621	0.1483	0.0160

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

4.3.4 Apple proliferation (AP) phytoplasma quantification

Following application of the different sources of nitrogen at different concentrations into the compost for apple proliferation phytoplasma, the calculations showed varied results. There was an increase in phytoplasma quantity in the infected plants in the first month when 204 mg/L ammonium sulphate (AS) was used for the compost. However, by the fifth and sixth month, the phytoplasma levels reduced using this nitrogen source and rate. The other concentrations of AS had no significant impact on apple proliferation phytoplasma titre as shown in (Table 4.12).

With the nitrate forms of ammonium nitrate (AN) and calcium nitrate (CN), there were significant reductions in apple proliferation phytoplasma titre with 204 mg/L nitrogen. The influence of the nitrate forms started to become significant in the third month and was continued over the subsequent months. Furthermore, ammonium nitrate at double rate produced some phytoplasma titre reduction at the fifth and sixth months but the impact was less than 204 mg/L nitrogen for the same source at the same time, as shown in both Table 4.11 and Table 4.13. Although, there was evidence of phytoplasma reduction in the periwinkle plants with different nitrogen source in the planting compost using DNA quantification, there were no obvious changes in physiological symptoms to the naked eye for any assay.

Table 4.11 Ammonium Nitrate -AN- (NH_4NO_3) – phytoplasma group 16SrX (AP), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L, and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (AN)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	2.6×10^7 a	2.6×10^6 a	1.0×10^7 a	2.2×10^7 b	6.4×10^7 b	2.7×10^7 b	6.3×10^6 b
102	2.1×10^6 a	6.6×10^6 a	3.1×10^6 a	6.4×10^6 ab	1.3×10^7 ab	1.8×10^7 b	3.5×10^7 b
204	5.7×10^5 a	8.0×10^6 a	6.0×10^6 a	1.7×10^6 a	1.7×10^6 a	9.7×10^5 a	3.7×10^5 a
408	8.4×10^6 a	8.3×10^6 a	1.1×10^7 a	8.9×10^6 ab	9.3×10^6 ab	2.1×10^6 a	4.4×10^5 a
P value (ANOVA)	0.6606	0.5860	0.7038	0.2168	0.0275	0.0024	0.0051

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

Table 4.12 Ammonium Sulphate -AS- $(NH_4)_2SO_4$ – phytoplasma group 16SrX (AP), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L, and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (AS)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	2.6×10^7 a	2.6×10^6 a	1.0×10^7 a	2.2×10^7 a	6.4×10^7 a	2.7×10^7 a	6.3×10^6 a
102	4.8×10^6 a	4.3×10^6 a	2.8×10^6 a	8.5×10^6 a	3.3×10^7 a	2.7×10^7 a	2.3×10^7 ab
204	4.9×10^6 a	3.0×10^7 b	3.5×10^7 a	1.2×10^7 a	7.5×10^7 a	5.9×10^8 b	1.4×10^8 b
408	4.8×10^6 a	1.1×10^7 ab	9.2×10^6 a	1.5×10^7 a	2.2×10^7 a	2.2×10^7 a	1.9×10^7 ab
P value (ANOVA)	0.3797	0.0536	0.8846	0.7448	0.3219	0.0032	0.0823

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

Table 4.13 Calcium nitrate -CN- ($Ca(NO_3)_2$) – phytoplasma group 16SrX (AP), Control compost nitrogen 204 mg/L with (N102 mg/L, N204 mg/L and N408 mg/L).

Concentration of nitrogen (mg/L)	Calculated phytoplasma cells per 1 gram of leaf sample (CN)						
	Day 0	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6
Control	2.6×10^7 a	2.6×10^6 a	1.0×10^7 ab	2.8×10^7 b	6.4×10^7 b	2.7×10^7 b	6.3×10^6 b
102	4.9×10^7 a	7.9×10^6 a	9.7×10^7 b	1.8×10^7 b	2.3×10^7 b	5.3×10^7 b	7.5×10^6 b
204	4.8×10^6 a	1.9×10^6 a	5.3×10^6 a	1.0×10^6 a	1.1×10^6 a	3.1×10^6 a	7.0×10^5 a
408	3.1×10^6 a	1.6×10^7 a	7.7×10^6 ab	2.7×10^7 b	1.7×10^7 b	2.5×10^7 b	1.2×10^7 b
P value (ANOVA)	0.2711	0.3185	0.1486	0.0057	0.0272	0.0121	0.0358

Note: Data are mean of 3 replicates (plant). Mean value with the same alphabet are not significantly different according to Fishers Least Significant Difference (LSD) test with P=0.05, degree of freedom=3.

4.4 Discussion

The aim of this study was to provide evidence that the real-time LAMP method, using specific primers, could be used for phytoplasma quantification and to show that using altered nitrogen sources could influence the levels of phytoplasmas in infected plants. Development of all these primers was as described in chapter two, and all the primers were designed based on the leucyl tRNA synthetase gene. Primers only amplified from the particular phytoplasma group that they had been designed for and not for any other groups.

Phytoplasma diagnostics can be performed using polymerase chain reaction as a versatile, and rapid method for detection of these microorganisms (Smart et al., 1996, Marzachì et al., 1998). More recently, LAMP has also been developed and used for phytoplasma detection as a rapid technique, including for use in the field (Dickinson, 2015, Tomlinson, 2013). However, to date, quantification of these pathogens has generally been carried out using the approach of quantitative real-time PCR (Saracco et al., 2006).

In this chapter, the LAMP technique with specific primers sets has been used to quantify the levels of phytoplasmas in plants that have been grown under different nitrogen fertilizer regimes. The data indicate that the real-time LAMP method can be used successfully for phytoplasma

quantification. Standard curve produced 0.99% R-squared value which can be used for measurement. One of the advantages of this technique is that quantification can be completed in a short time of less than one hour once the DNA samples have been prepared.

A disadvantage is that using the Genie II technology, only 16 samples can be run simultaneously with the LAMP technique, which therefore requires more time if a large number of samples are to be tested, although recent work has shown that is also possible to undertake real-time LAMP assays on real-time PCR machines, which enables much faster throughput. It is also important to use the same extraction protocol for obtaining the DNA template, and the same amount of buffers and timings in all steps. If the same method and same weight of plant sample are used, it is then possible to compare predicted quantifications of phytoplasma titres between samples. However, an alternative method that can also be used is to use the second set of primers that amplify from the host plant DNA, and undertake quantification of plant DNA in each sample so that amounts of phytoplasma DNA relative to plant DNA can be calculated for each sample. Specific primers such as cox primers are needed to make standard curves for plant DNA for this approach.

In these experiments, it was decided not to use quantification of plant DNA as the control, but instead to ensure that the DNA template for quantification was derived from a known standard amount of infected plant sample; hence, the quantification of phytoplasma cells can be

estimated per gram of plant weight. This method of validation, using known amounts of plant DNA, had also been used previously in real-time PCR approaches to measure phytoplasma cell per gram of selected plant weight (Christensen et al., 2013).

Nitrogen is one of the most important nutrients that is a component of fertilizers along with phosphorus and potassium. There are different views about the use of high or low rates of nitrogen to affect the ability of plants to resist pathogens and/or sometimes make plants stronger (Shaner and Finney, 1977, Veresoglou et al., 2013). In this study, the various concentration of nitrogen used was 102 gm/L (half strength), 204 mg/L (normal strength), and 408 gm/L (double strength). The concentration of 204 mg/L (normal strength) is the amount of nitrogen currently recommended for planting according to the commercial compost Levington M3.

The quantity of phytoplasma cells changed over time using different nitrogen sources and the results showed that in most cases it was the higher concentrations of nitrogen (204 mg/L or 408 mg/L) that were best as reducing the phytoplasma quantity. However, in one case the ½ rate of 102 mg/L increased phytoplasma quantity. Despite the reduced levels of phytoplasmas in some nitrogen regimes, the use of different nitrogen sources as fertilizer did not provide any evidence that this could be used as a reliable means of attempting to control phytoplasma quantity completely. However, some forms of nitrogen at particular

concentrations can be applied to reduce phytoplasma titres, and also some other forms can be avoided due to their impact on increasing phytoplasma titre. Also, different phylogenetic groups of phytoplasmas appeared to respond in different ways to the different nitrogen fertilizers. For example, when ammonium sulphate (AS) was applied at the normal rate on apple proliferation phytoplasmas 16SrX, there was a significant increase in phytoplasma titre, whilst the same source with low concentration applied on potato witches' broom phytoplasmas 16SrVI significantly reduced the phytoplasma titre in infected periwinkle plants. According to LAMP measurement results, ammonium nitrate (AN) in almost all of the situations increased phytoplasma amounts significantly when applied at a high dose rate.

In the case of using calcium nitrate with elm yellows and apple proliferation phytoplasmas, normal nitrogen level treated plants developed a significant reduction of phytoplasma titre for both phytoplasma groups. However, on potato witches' broom it was the 408 mg/L fertilizer that produced the most significant reduction, whilst with plum leptonecrosis phytoplasma the 102 mg/L application reduced the phytoplasma titre significantly. Ammonium nitrate (AN) was different since the low level at 102 mg/L reduced phytoplasma levels in both plum leptonecrosis and potato witches' broom, whilst the 204 mg/L fertilizer reduced the phytoplasma quantity for elm yellows.

This study has indicated that the LAMP technique has the capability of quantifying phytoplasma DNA with specific primers and can be used for different groups of the same pathogen. According to the quantification analysis, normal levels or higher levels of nitrogen as ammonium nitrate can be used to reduce phytoplasma quantity in infected periwinkle plants for LNI, EY, PWB and AP phytoplasmas. Calcium nitrate can be used at the same levels as ammonium nitrate, whereas ammonium sulphate is recommended to be used mainly at low levels.

Chapter 5. Quantification of phytoplasmas by real-time PCR in plants following treatments with salicylic acid or UV light.

5.1 Introduction

Numerous approaches have been used in the past to try to control plant diseases. UV light is one of the treatments that has been applied as an alternative technique to conventional control methods such as resistance breeding and agrochemicals, to reduce disease severity by stimulating defence mechanisms in the treated plants or through damaging the microorganisms on the exposed surface of the crops (Stevens et al., 1999, Marquenie et al., 2003). In addition, salicylic acid has been used in some studies in attempts to protect plants from phytoplasma diseases by inducing plant resistance and enhancing the plant defence against phytoplasma infections (Sánchez-Rojo et al., 2011, Wu et al., 2012).

Disease control, particularly of phytoplasmas, is not simple and is made more difficult because microorganisms and insect vectors can become resistant after overuse of pesticides. For that reason, induced plant resistance is one of the potential ways to protect plants from diseases and their casual agents. For example, 50% of the tomato plants treated with salicylic acid for protection against infection by the potato purple

top phytoplasma were free from symptoms with no signs of phytoplasma infection detected by diagnostic PCRs (Marquenie et al., 2003, Wu et al., 2012). Also, potato plants were protected from phytoplasma infection and tuber photosynthate assimilation improved more after they were treated with a low concentration of salicylic acid (Sánchez-Rojo et al., 2011). In addition, resistance was induced in sweet potato storage roots against fusarium root rot after they were treated with a low dose of UV-C exposure (Stevens et al., 1999). UV-C hormesis has been described as the application of potentially harmful radiation at low doses to living organisms in order to induce beneficial stress responses (Jagadeesh et al., 2011).

Quantification of these pathogens, as a means of determining what effect a control technique has had, can be carried out by real-time PCR, which is the most efficient and precise method for phytoplasma detection and quantification. Furthermore, real-time PCR assays can provide a higher detection sensitivity compared to nested PCR, and this technique is less sensitive to contamination and is less labour intensive such that a large number of samples can be tested simultaneously (Christensen et al., 2004, Galetto et al., 2009, Christensen et al., 2013). Real-time PCR is the most suitable method for quantifying the nucleic acids of many plant pathogens, and screening plants for resistance against phytoplasmas can be achieved through quantification.

Due to the high sensitivity of this technique, it is possible to do the quantitative analysis during the very early phases of infection (Saracco et al., 2006, Galetto et al., 2009, Yvon et al., 2009, Christensen et al., 2013). SYBR Green I®, a highly specific, double-stranded DNA binding dye has been used to detect phytoplasma-specific PCR product that accumulates during real-time PCR cycles; however, SYBR Green I® dye chemistry can detect all double-stranded DNA, including non-specific reaction products, so a well-optimized reaction is essential to achieve accurate results by running a melting curve analysis (Galetto et al., 2009).

The aim of this chapter was to quantify phytoplasmas in infected periwinkle plants, using SYBR Green qPCR, to test the impact of UV light or salicylic acid (individually) on phytoplasma titre in infected periwinkle plants, and whether the UV light or salicylic acid can cause any significant reduction or increase in phytoplasma quantity.

5.2 Materials and methods

5.2.1 Periwinkle plants

All the phytoplasma-infected periwinkle plants were taken by cutting's from the same mother plant with the same genetic background as

described in section 2.2.1. All the young plants were kept in the glasshouse and after 6 weeks they were transferred into individual pots and were ready for the start of UV-C pulse and salicylic acid treatments.

5.2.2 Phytoplasma strains used in this study

5.2.2.1 Phytoplasma groups treated with salicylic acid

The phytoplasma 16Sr groups used in this study and treated with salicylic acid were: 16SrI (RG), 16SrIII (LNI), 16SrVI (PWB) and 16srX (AP). All the phytoplasmas were maintained within periwinkle plants from which samples were taken and checked by conventional PCR of the 16S rRNA gene using universal primer pair P1 / P7 (Deng and Hiruki, 1991, Smart et al., 1996) and nested PCR was performed with primer pair R16F2 / R16R2 (Gundersen and Lee, 1996) to confirm the presence of phytoplasma DNA.

5.2.2.2 Phytoplasma groups treated with ultraviolet light (UV)

Ultraviolet light UV-C pulses were applied on infected periwinkle plants containing phytoplasmas from four taxonomic groups, 16SrI (RG), 16SrII (VCP), 16SrIII (LNI), and 16SrVI (PWB), were these also tested first as described in section 2.2.1.

5.2.3 Salicylic acid (SA) application ($C_7H_6O_3$)

Eighty phytoplasma-infected periwinkle plants were prepared for treatment with salicylic acid solutions; the plants were sprayed (only foliage) every alternative day with one of four different solutions with (100-150 ml) for approximately six weeks. Watering of plants was carried out every other day (when no spraying was applied). For each group of phytoplasma, four different solutions were applied with five replicates of infected periwinkle plants; the salicylic acid solutions were at pH 5.6–5.7 and were applied as follow:

1. 20 plants were sprayed with 0.1 mM-SA.
2. 20 plants were sprayed with 0.01 mM-SA.
3. 20 plants were sprayed with 0.001 mM-SA.
4. 20 plants were sprayed with distilled water (controls).

Control plants were also prepared (healthy periwinkle) to determine any negative affect on plant growth.

5.2.4 UV treatment

In this study different intensities of UV-C light with different pulses were applied. Five KJ/m^2 and 1 KJ/m^2 were used with different timings, and also 5, 10, 20, and 30 pulsed treatment were used. Plants were kept in the dark for 100 mins prior to the UV treatment and after treatment,

they were kept in the dark overnight between 16-24 hours. Control plants without UV treatment were kept in the same conditions as the treated plants. In the case of pulse measurement, 3.7 KJ/m^2 of conventional UV source can be achieved in 8 pulses. All the UV pulses for plant treatments were performed with the plants kept at 30 cm from the high-intensity UV source. Pulsed UV treatment was applied twice a week for approximately a month. All the equipment is shown in Figure 5.1.





Figure 5.1 A: Pulsed source which produces 3 pulses per second, B: Conventional UV source in the lab which produces a minimum UV light intensity of 1000 mw/cm^2 and maximum intensity of 5000 mw/cm^2.

5.2.5 Collecting samples and DNA extractions

Every week, 500 mg leaf samples were taken from infected plants followed by DNA extraction using the CTAB method as described in section 2.2.2. Following DNA extraction, all the samples were suspended in 35 µl of TE buffer and stored in the freezer at -80°C.

5.2.6 Syber Green real-time quantification method

Quantification of each phytoplasma was performed using the real-time PCR technique using a LightCycler® 480 Instrument (Roche Diagnostics Ltd.) and SYBR® Green JumpStart™ Taq ReadyMix™ (1.25 units *Taq* DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 3.5 mM $MgCl_2$, 0.2 mM dNTP, stabilizers, JumpStart Taq antibody and SYBR Green I) (Sigma-Aldrich, Inc.). Universal phytoplasma primers were used to quantify all the phytoplasmas in this experiment; forward primer (5' – CGT ACG CAA GTA TGA AAC TTA AAG GA – 3') and reverse primer (5' – TCT TCG AAT TAA ACA ACA TGA TCC A – 3') (Christensen et al., 2013). The concentration of each primer was 300 nM, and the thermal cycling parameters were: initial denaturation at 94°C for 2 min and 40 cycles of denaturation at 94°C for 15 sec and annealing, extension and fluorescence read at 62°C for 1 min. The quantity of each phytoplasma

before and after the treatments was analysed by using IBM SPSS statistics 23, and the means were compared by using the LSD method.

Quantification of phytoplasma titre was carried out from the C_T values using the equation Y = -3.2714x + 8.9014 for RG, Y = -3.1707x + 9.3121 for VCP, Y = -3.1575x + 9.1561 for LNI, Y = -3.3164x + 8.6364 for PWB, and Y = -3.1714x + 8.64 for AP. Essentially, satisfactory qPCR validation is based on the correlation coefficient (R^2) of the linear regression and amplification efficiency (E). R^2 values should be more than 0.99.

5.3 Results

5.3.1 Standard curve preparation

Standard curves were obtained using ten-fold serial dilutions of 0.5 ng of the partial rDNA gene of RG, VCP, LNI, PWB, and AP phytoplasmas. For this, phytoplasma DNA was amplified with conventional PCR and the nested PCR products were quantified by using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.) and subsequently used to prepare the 10-fold serial dilutions. The serial dilutions of each particular phytoplasma group were then amplified with the qPCR technique to develop standard curves using the universal qPCR primers

designed by (Christensen et al., 2013). Results are shown in Figure 5.2, Figure 5.3, Figure 5.4 and Appendix 5.

Once the cycle threshold (C_T) values had been identified, the standard curves were created by the interpolation of the $\log^{10}$ of the amplified product of each standard plotted on the x-axis with their individual C_T values on the y-axis. The formula: $E=10_{(1/-slope)}$-1x100 was used to calculating amplification efficiency (E) from the slope of the standard curve. The melting temperature of qPCR amplicons was determined by analysing generated melting curves of each particular phytoplasma reaction.

For phytoplasma quantification, copy number of partial rDNA genes in the PCR product was calculated by using the formula: ((PCR product, ng) x ($6.022x10^{23}$)) / ((PCR product length, bp) x ($1x10^{9}$) x 650) and divided by two since there are 2 copies of the rRNA operons in a phytoplasma genome or cell. From this, the number of phytoplasma cells per one gram of plant weight could be calculated.

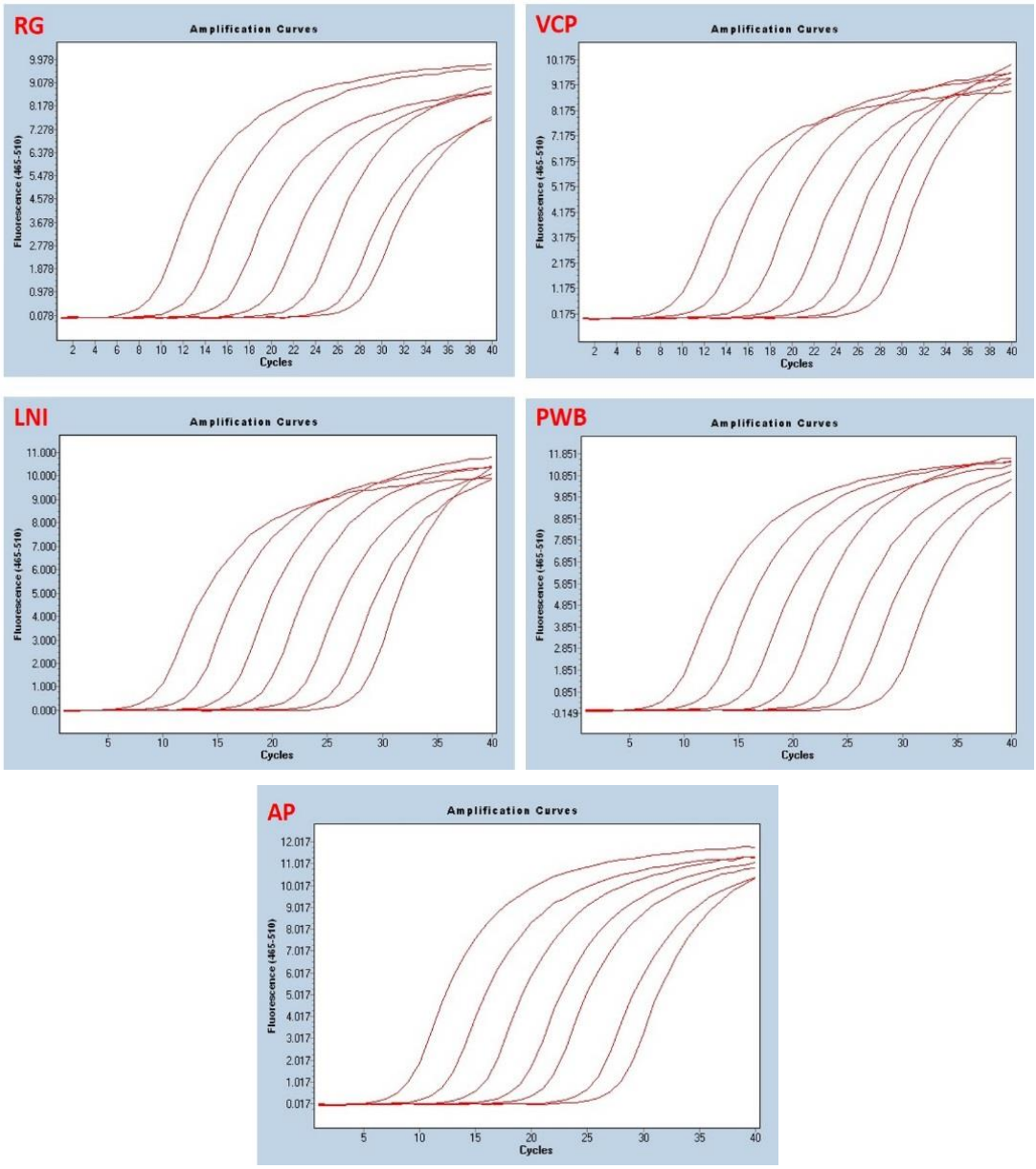


Figure 5.2 Amplification curves for ten-fold serial dilutions of 0.5 ng of PCR products containing partial rDNA genes of particular phytoplasmas: RG, VCP, LNI, PWB, and AP. Universal primers are applied for qPCR

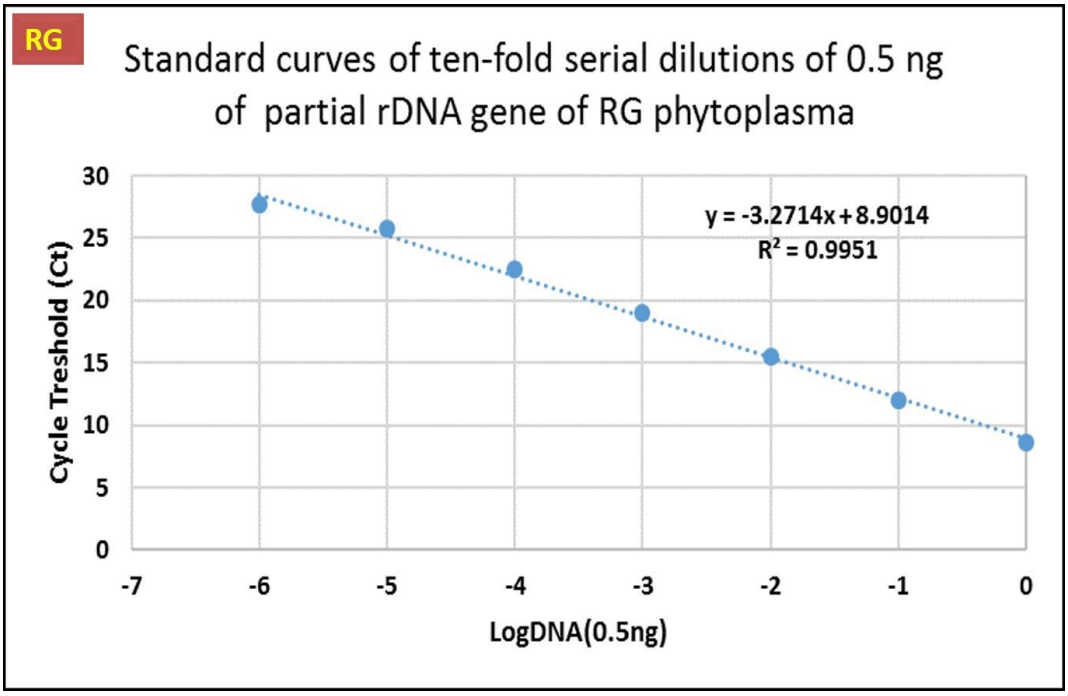
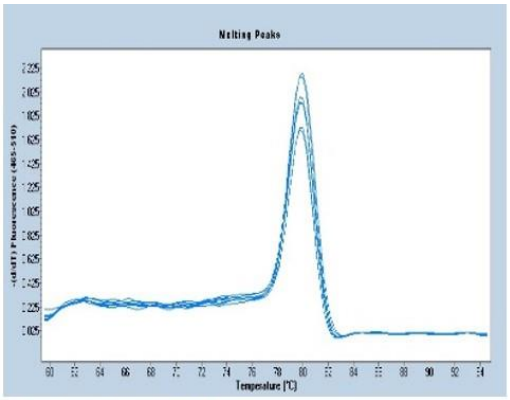
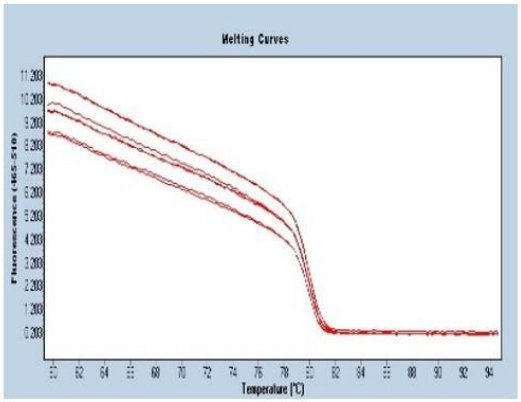
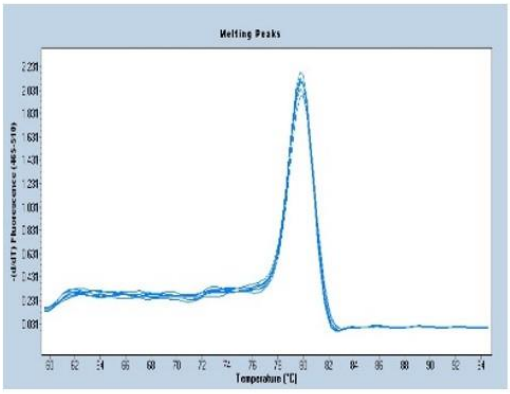


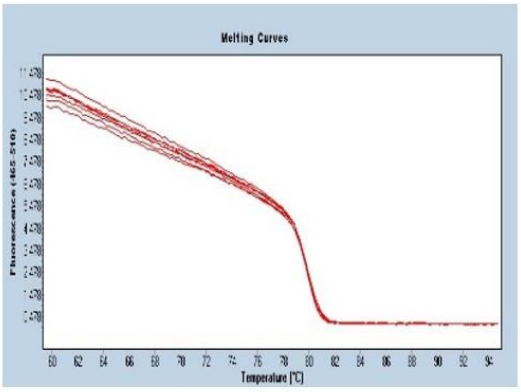
Figure 5.3 Standard curves of ten-fold serial dilutions (10^{0}-10^{-6}) of 0.5 ng of PCR products containing partial rDNA genes of particular phytoplasmas (RG) using qPCR technique.



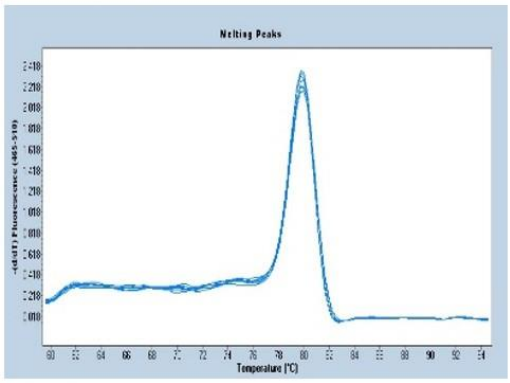


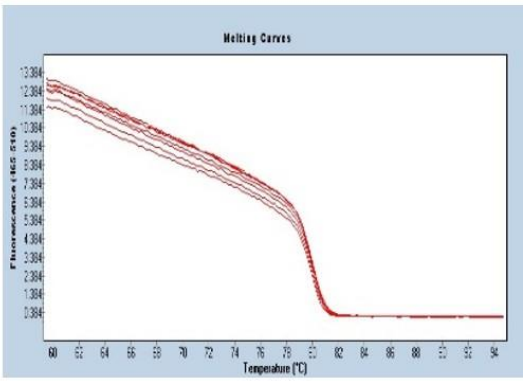
A: RG, Tm~79.9 °C



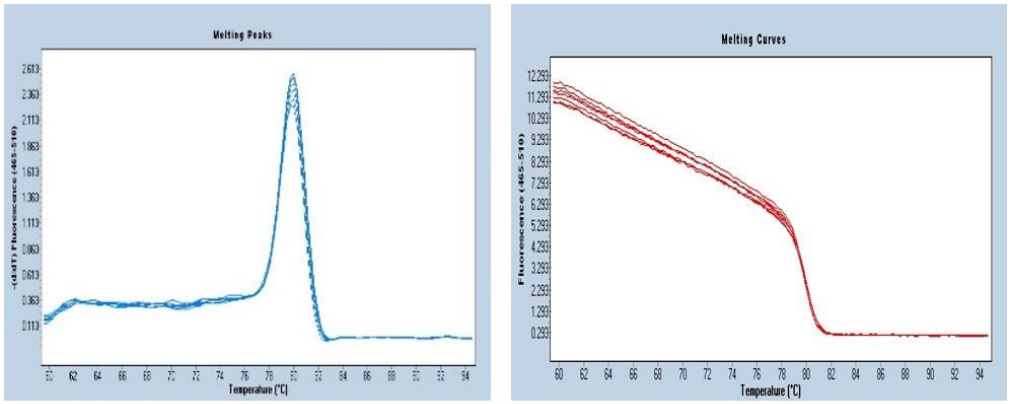


B: VCP, Tm~79.8 °C

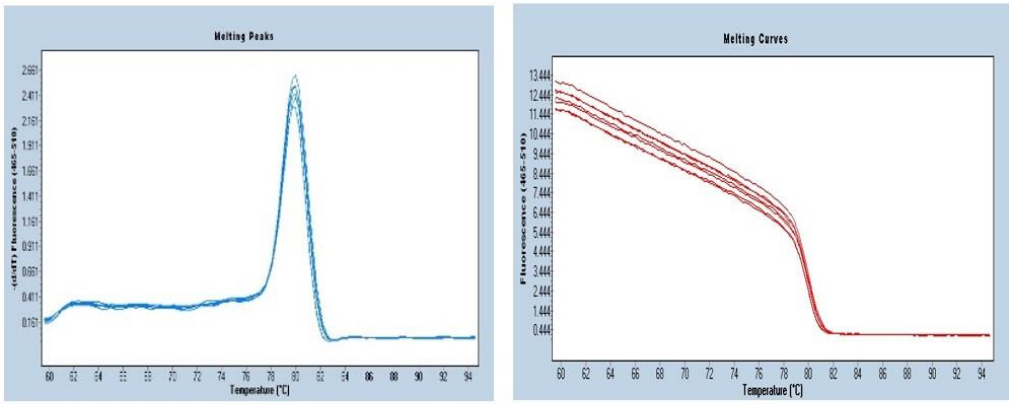




C: LNI, Tm~79.9 °C



D: PWB, Tm~79.9 °C



E: AP, Tm~79.9 °C

Figure 5.4 Melting curves and peaks obtained from 10-fold serial dilutions (10^{0}-10^{-6}) of 0.5 ng of PCR product containing partial rDNA genes of RG, VCP, LNI, PWB, and AP phytoplasmas.

5.3.2 Salicylic acid results

Salicylic acid is one of the chemicals that can be used to activate plant defence responses against phytoplasma attack and reduce the symptoms of infection. Spraying infected periwinkle plants for a period

of six weeks indicated that low concentrations of SA solutions gave the optimal significant effect on phytoplasma quantity and reduced infection on diseased periwinkle plants. However, a high concentration of the same solution could produce a negative effect and phytotoxicity on periwinkle plants as shown in (Figure 5.5).

Periwinkle plants infected with RG, LNI, PWB, and AP were sprayed every alternative day with SA concentrations of 0.001 mM, 0.01 mM, 0.1 mM or distilled water dH_2O as a control replicate for 6 weeks. The results showed that the lowest concentration (0.001 mM) of SA application significantly reduced phytoplasma quantity in infected periwinkle plants and resulted in a good quality of plant growth in comparison with control plants sprayed with distilled water under the same conditions.

RG phytoplasma titre was significantly reduced after spraying infected plants with 0.001 mM of SA compared with control plants and 0.01 mM or 0.1 mM of SA solution. However, 0.01 mM SA produced a nonsignificant effect and 0.1 mM increased phytotoxicity in treated plants significantly (Figure 5.5 and Figure 5.6). For the LNI phytoplasma, there was a significant reduction in phytoplasma titre with both 0.001 mM and 0.01 mM SA application. However, 0.1 mM SA treated plants showed phytotoxicity.

PWB and AP phytoplasmas both had significant reductions in titre with 0.001 mM of SA. However, 0.01 mM and 0.1 mM of SA resulted in nonsignificant differences in the quantity of phytoplasma, and 0.1 mM

produced phytotoxicity on treated plants. However, AP phytoplasma titre appeared to be reduced with 0.01 mM SA application, but the differences were not statistically significant when compared with control treatments.

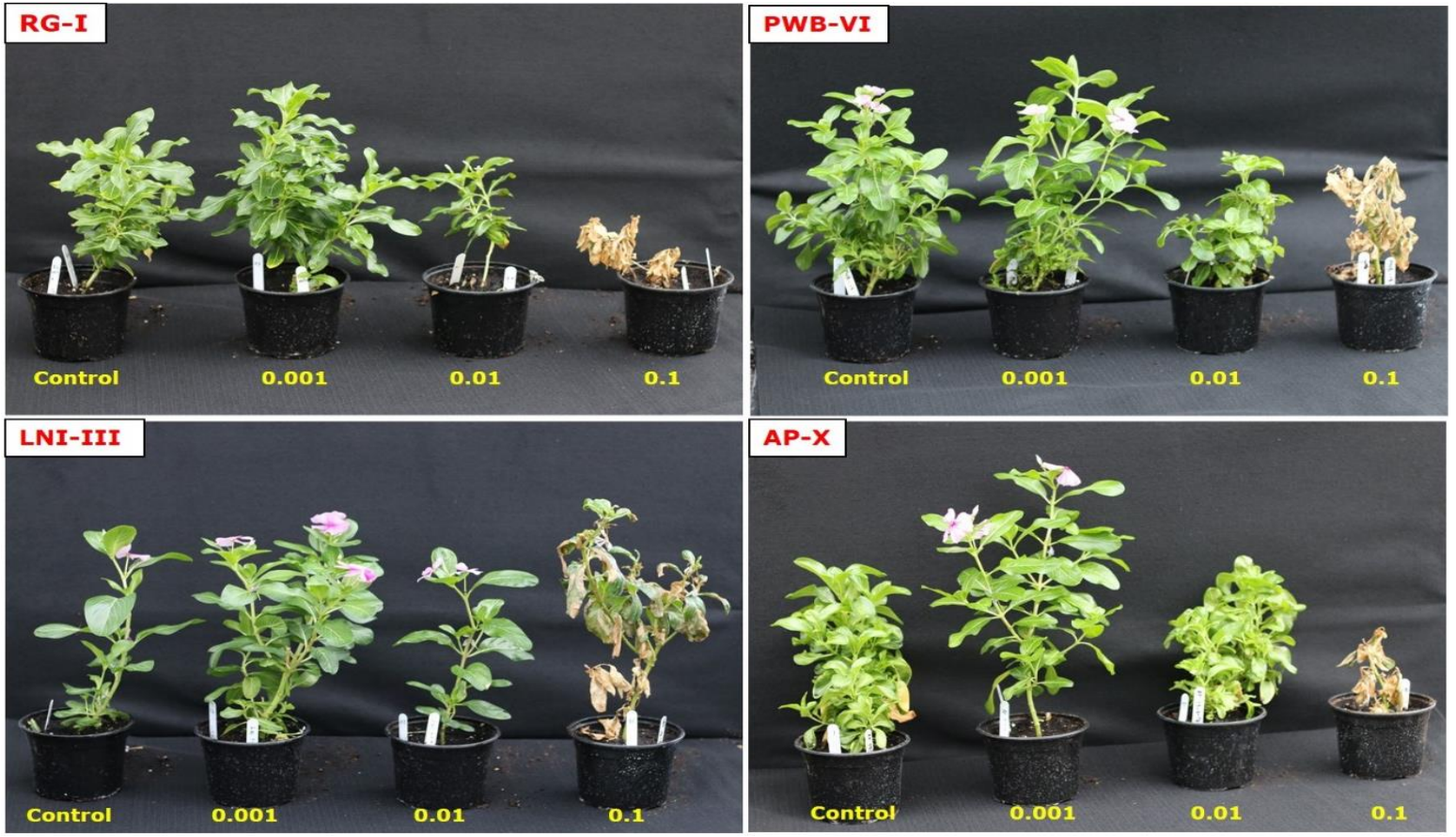


Figure 5.5 Application of salicylic acid (0.001, 0.01, and 0.1 mM) with dH2O to induce resistance on periwinkle plants against phytoplasma groups 16SrI (RG), 16Sr III (LNI), 16SrVI (PWB), and 16SrX(AP). The control plants are phytoplasma infected plants were treated with dH2O.

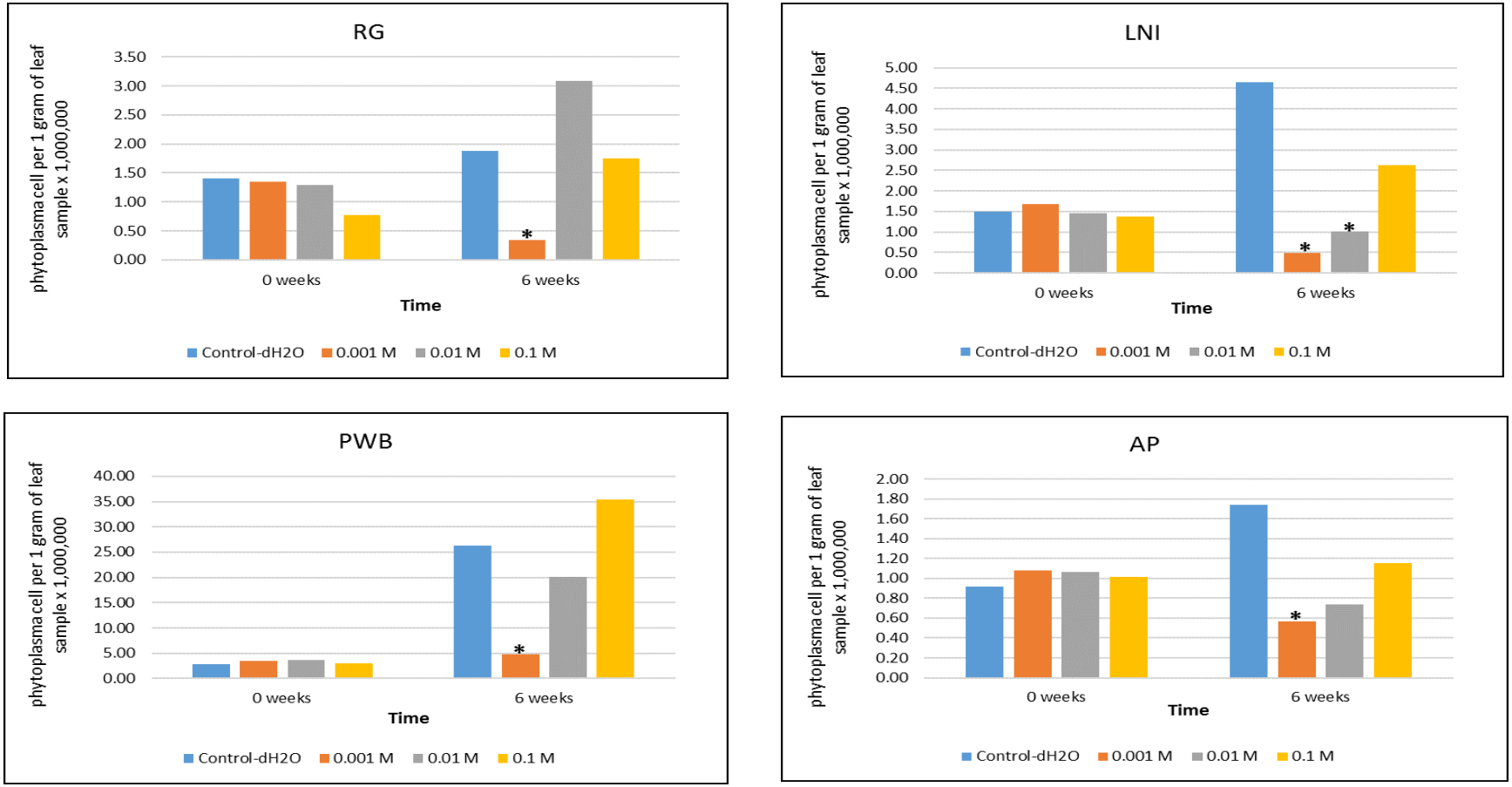


Figure 5.6 Quantity of phytoplasma during 6 weeks of spraying salicylic acid solutions on infected-periwinkle plants with RG, LNI, PWB, and AP. *: Shows significant reduction of phytoplasma titre in comparison with control according to Fishers Least Significant Difference (LSD) test with P=0.05.

5.3.3 UV treatment results

UV treatment was performed with both conventional UV and pulsed sources. Minimum and maximum intensity of conventional UV were 1 KJ /m^2 and 5 KJ /m^2 applied in the lab as shown in Figure 5.1. The treated plants with 5 KJ /m^2 developed some damage on the plant leaves and some of the plants 15% died after 2 – 3 weeks of treatment. In addition, 1 KJ /m^2 at 50 seconds produced leaf damage on the periwinkle plants. At 10 seconds with 1 KJ/m^2 UV intensity, the plants were free of damage, but after more than one month of treatment, there were no significant differences on the treated plants in phytoplasma quantity.

The pulsed UV source was also applied on the healthy periwinkle plants and initial experiments were undertaken to determine the best distance and number of pulses to protect plants from any damage. After analysing a pulse intensity on the healthy periwinkle plants for approximately 4 weeks, the best distance and lowest number of pulses that resulted in no obvious physical damage were 30 cm with no more than 50 pulses. Based on this, UV pulse light was applied with 5, 10, 20, and 30 pulses at a distance of 30 cm for phytoplasmas RG, VCP, LNI, and AP.

For the period of four weeks of pulse UV treatment on infected periwinkle plants with RG phytoplasmas, no damage was observed on the plants. From the quantification of phytoplasma data prior to and after the

treatment, the results indicated that 10 pulses significantly reduced phytoplasma titre in the infected plants in comparison to control plants (no UV applied) (Figure 5.7). Some reduction was noticed with 20 pulses of UV; however, the differences were not significant. Similarly, 30 pulses of UV showed no significant effect (Figure 5.7).

For LNI phytoplasma quantification after treatment with pulsed UV, there was again a significant reduction in the quantity of phytoplasma with 5 pulses of UV in comparison with control plants (no pulsed UV) (Figure 5.7). The other UV pulses produced non-significant differences with all replications of treated plants. Furthermore, plants infected with VCP and PWB showed no significant differences with any of the various pulsed UV intensity treatments.

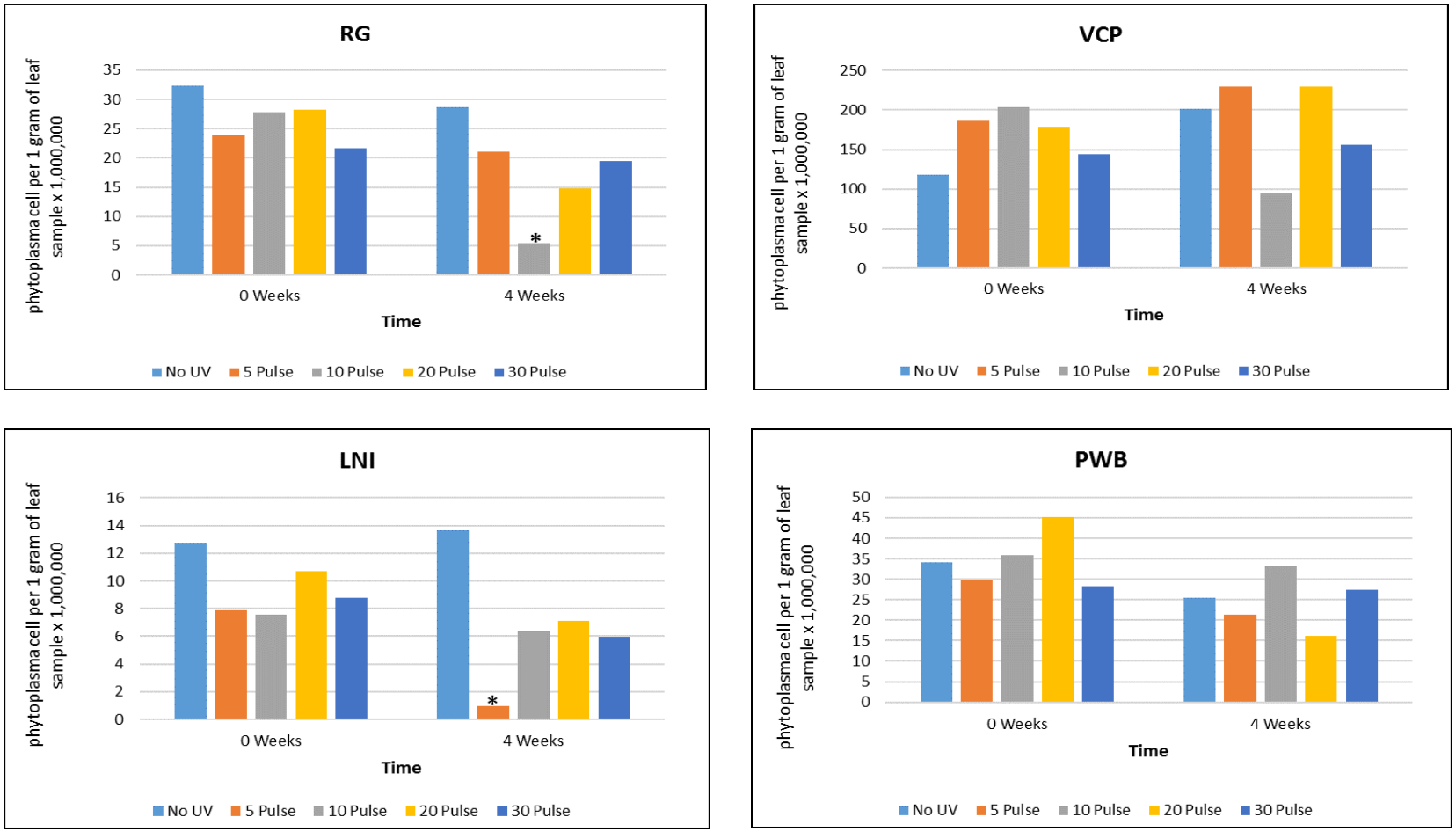


Figure 5.7 Pulsed UV impact on phytoplasma quantity of RG, VCP, LNI, and PWB. During four weeks of treatment using infected periwinkle plants to mainatain different phytoplasma as a host plant. *: Shows significant reduction of phytoplasma titre in comparison with control according to Fishers Least Significant Difference (LSD) test with P=0.05.

5.4 Discussion

This chapter has provided evidence for an efficient quantification of phytoplasmas in plants using SYBR green real-time PCR, and has also provided evidence for the best concentration of salicylic acid solutions to reduce the titre of phytoplasmas in infected plants. Additionally, UV treatments have been tested as a new technique to reduce the phytoplasma titre and enhance plant resistance.

Phytoplasma disease control is difficult and these microorganisms can be associated with diseases of many cultivated and wild plant species. Phytoplasmas can cause significant disease outbreaks, for instance, serious outbreaks of potato purple top disease caused significant yield losses and a reduction in tuber processing quality in the Columbia Basin of Washington and Oregon and Yakima Valley (Munyaneza et al., 2007, Munyaneza et al., 2010, Wu et al., 2012).

Phytoplasma quantification by qPCR can be used as an approach to study their titre in diseased plants. Salicylic acid solutions have been used effectively as a pretreatment by Wu et al. (2012) to enhance plant defence against potato purple top phytoplasma and reduce infection symptoms on tomato plants. Also, Sánchez-Rojo et al. (2011) discovered that low SA concentration (0.001 mM) significantly enhanced alleviation of symptoms and tuber production on potato plants.

Additionally, salicylic acid has been used to induce plant resistance in tobacco plants against viral infection (Malamy et al., 1990, Gaffney et al., 1993, Yalpani et al., 1993, Maurhofer et al., 1998).

In this chapter, the qPCR quantification technique was used to assess the influence of salicylic acid and UV treatment on phytoplasma titre. Real-Time quantification indicated that SA had optimal effects at low concentrations with all phytoplasma infected plants treated with SA, which were RG, LNI, PWB, and AP. A significant reduction was obtained with 0.001 mM of SA solution sprayed on infected plants. However, a higher concentration of SA was phytotoxic to the treated plants, particularly on plants sprayed with a 0.1 mM SA.

In this study, pulsed UV treatment was also tested to discover whether it reduced or increased phytoplasma titre. The technique of applying UV has been used in other studies to enhance resistance and reduce infection with other pathogens such as fungi and bacteria. For instance, Stevens et al. (1999) used UV-C hormesis to induce resistance of sweet potato against fusarium root rot and Marquenie et al. (2003) tried to use pulsed white light with UV-C to inactivate conidia of *Botrytis cinerea* and *Monilia fructigena*. However, so far no one has used UV light or UV pulses for phytoplasma treatment. In this study, four different phytoplasma groups were treated with UV pulses.

Quantification of phytoplasma titre through the qPCR technique identified that LNI phytoplasma titre was significantly reduced in infected

periwinkle plants after treatment with 5 pulses of UV light at 30 cm from the plant. Phytoplasma-infected plants with RG also showed significant reduction of phytoplasma titre after the plants were treated with 10 pulses of UV. However, VCP and AP phytoplasma titres were not significantly reduced by UV pulses. UV treatments have shown a positive impact on other diseases, and also within this chapter it has been indicated that UV pulses can be used to reduce the titre of some phytoplasmas, presumably by inducing plant resistance against phytoplasmas. There is therefore some scope to expand these studies further in the future and test different ways of applying and different wavelengths of UV as a tool to reduce infection and symptoms caused by phytoplasmas, and to investigate the underlying mechanisms by which the treatments induce resistance against phytoplasmas and whether this can be expanded to other host species.

In term of comparing LAMP and real-time PCR quantification, there are differences between these two techniques in the results after amplification. In the qPCR the amount of PCR product perfectly double during each cycle, however; it is difficult to calculate the amount of LAMP for each cycle. Also, in the qPCR the amplification factor is 2 but in the LAMP is 4.44 this makes E value 344.29% for LAMP and from this calculation we can interpret that LAMP assay provide much more product than qPCR, which is exactly right as the amplification process of LAMP and qPCR is different.

Subsequently, to compare the sensitivity between both techniques, the analytical sensitivity of the LAMP and qPCR assay can be showed using serial dilutions of DNA.

Chapter 6. General discussion

Phytoplasma diseases are a serious threat to many plant species worldwide and these pathogens can cause severe diseases on economically important plants (Lee et al., 1998, Munyaneza et al., 2007, Munyaneza et al., 2010, Wu et al., 2012). Phytoplasmas can be transmitted between plants by insect vectors, which makes their control more difficult, and to achieve effective control probably requires an integrated approach that involves both insect control and control of infections in the plants (Weintraub and Beanland, 2006, Weintraub, 2007, Hogenhout et al., 2008).

In this research, diagnostic methods have been improved by developing new methods of phytoplasma detection and quantification. Transmission of phytoplasmas using dodder, both within and between plant species has also been tested, and new approaches have been used in attempts to reduce phytoplasma titres in infected periwinkle plants through induced plant resistance or construction of a negative environment for the phytoplasmas.

New LAMP primer sets based on the leucyl-tRNA synthetase gene were designed for six different phylogenetic groups: 16SrI, 16SrII, 16SrIII, 16SrV, 16SrVI, and 16SrX. For validation of the primers, all the primers were tested on various phytoplasma DNAs in LAMP assays in which all

reactions were held at 63ºC for 30 min, and the LAMP results confirmed that the sets of primers worked on the phylogenetic groups that they were designed for. They were also tested on *E. coli* to confirm that they gave no amplification from other unrelated bacteria. This confirmed that the primers can be used to detect the particular groups that they were designed for, with the advantage that the real-time LAMP technique takes less than one hour to get the results. Moreover, it was shown that these primers can be used for quantification of phytoplasmas as well. For this, 10-fold serial dilutions from 10^0 to 10^7 were taken from 0.5 ng of plasmids for each group, and amplifications were performed by LAMP to obtain standard curves through different amplification time against different concentrations. The correlation coefficient (R^2) of the linear regression was 0.99 for all groups tested, which is greater than the 0.95 that is usually taken as sufficient for such quantification analyses, confirming that the primers and the LAMP technique can be used for quantification.

As yet, phytoplasmas can not be reliably or routinely cultured *in vitro* in cell-free media, so model host plants such as periwinkles (*Catharanthus roseus* L.) are used as the most effective way to maintain these microorganisms for research purposes (Lee et al., 2000, IRPCM, 2004, Ayman et al., 2010, Nejat et al., 2010). Transmission of phytoplasmas between plants is important to study infection symptoms and also to study the differences in infections between different phytoplasma groups. Dodder transmission of phytoplasma within the same plant

species and between plants from different species has been carried out in this research, and transmission was successful between periwinkle plants in a short period of time, and also from periwinkle to tomato plants and from Napier grass to periwinkle plants as well, although in these cases, transmission took longer. Symptoms were observed on the healthy plants after transferring phytoplasmas from diseased plants. Phytoplasma transmission through dodder bridges is a practical and applicable method, which can work where methods such as grafting are not possible (ie between grasses and periwinkles) and can be performed simply by placing dodder plants between both plants (healthy and infected). The only issue in this technique is that the plants have to be kept in a separate and safe area away from other plants due to the rapid growth of dodder plants and their shoots, which could otherwise easily attach to other surrounding plants and potentially cause disease in these or multi-infections.

The LAMP primers that have been designed in this study were shown in and were used to quantify the phytoplasmas LNI, EY, PWB, and AP within infected periwinkle plants. Altered nitrogen fertilization regimes were applied on periwinkle plants for the period of six months to discover which source of nitrogen (ammonium nitrate, ammonium sulphate, or calcium nitrate) could impact the levels of phytoplasmas in diseased plants. Furthermore, the effects of different concentrations were analysed to determine which was more effective to reduce the phytoplasma concentration in diseased plants. Phytoplasma quantity

was calculated every month using LAMP, which is believed to be the first time that the real-time LAMP technique has been used successfully for phytoplasma quantification. The LAMP technique was recently developed as a new rapid and reliable assay that can be used for phytoplasmas detection, and the technique is reported to be 10 to 20-fold more sensitive than nested PCR (Obura et al., 2011, Sugawara et al., 2012, Tomlinson, 2013, Dickinson, 2015). In this research, the LAMP method was further developed for quantification rather than just detection.

The investigation of nitrogen fertilizers on phytoplasma titre showed, using the real-time LAMP for quantification, that nitrogen at higher concentrations of 204 mg/L or 408 mg/L resulted in a reduction of phytoplasma level in the infected periwinkle plants. However, observations of phytoplasma titres within infected plants that were treated with different sources of nitrogen showed no evidence that this could be used as a reliable means of attempting to completely control phytoplasma levels. Applying some forms of nitrogen at particular concentrations did reduce phytoplasma titre, suggesting that the use of some fertilizers might be recommended for use over others, and also some other forms appeared to result in increases in phytoplasma titre, suggesting that these should be avoided for use as a fertilizer. However, it was noted that different phylogenetic groups of phytoplasmas showed different responses to the different nitrogen fertilizers, although the reasons for this were not investigated.

Two further approaches were also used to influence phytoplasma titre in infected periwinkle plants, salicylic acid, and UV-C light. Low concentrations of salicylic acid (0.001 mM) applied to potato plants by Sánchez-Rojo et al. (2011) resulted in reduced symptoms of infection and improved tuber production on potato plants. Also, SA application was used by Gaffney et al. (1993), Yalpani et al. (1993), and Maurhofer et al. (1998) to protect tobacco plants by inducing resistance in the plants against viral infections. In this current study, the different SA concentration used were 0.001 mM, 0.01 mM, 0.1 mM applied by spraying on the infected periwinkle plants every alternate day. The real-time PCR provided evidence that the best concentration of salicylic acid solutions to reduce the titre of phytoplasmas in infected plants was 0.001 mM, and the results also provided evidence that a higher concentration of SA can be phytotoxic to the treated plants, particularly on plants sprayed with 0.1 mM SA.

UV-C hormesis has been used in other plant-pathogen interactions as an alternative mechanism to induce resistance, for example in sweet potato against fusarium root rot (Stevens et al., 1999), and pulsed white light with UV-C was used by Marquenie et al. (2003) to inactivate conidia of *Botrytis cinerea* and *Monilia fructigena*. However, this is the first time that UV-C pulses have been used for phytoplasma treatments. Pulsed UV-C was applied on periwinkle plants for the period of four weeks to discover the effects of UV-C on phytoplasma titre within infected plants

and to enhance the resistance of the plants against phytoplasma infections.

Real-time PCR was used for quantification before and after UV treatment to assess the changes in the quantity of phytoplasmas during the treatment. RG, VCP, LNI, and PWB phytoplasma were treated with 5, 10, 20, and 30 pulses at a distance of 30 cm from the UV-C light. The quantification results provided by SYBR green real-time PCR with universal primers indicated that LNI phytoplasma titre in infected periwinkle plants was reduced significantly after infected plants were treated with 5 pulses of UV light. Also, RG phytoplasma within treated infected plants with 10 pulses showed significant reduction of phytoplasma titre. These experiments, therefore, indicate that UV-C pulse treatments can be used to reduce the titre of some phytoplasmas, presumably by inducing plant resistance against phytoplasmas.

The quantification methods used in this research and the results provided by comparing the real-time LAMP with the SYBR green real-time PCR method has led to the following conclusions:

1. The LAMP technique is more rapid, and easy to apply for quantification, whilst the real-time PCR needed more time for preparation and running of samples.
2. Real-time LAMP technique is convenient if only a small number of samples need to be analysed at a time. However, if more than 16 samples need to be run, the process can become time-consuming since

it is necessary to wait for the machine to cool down after the annealing analysis, otherwise, this will affect the quantification time for the next samples. Furthermore, for running a high number of samples with the LAMP technique, control sample needs to be included every time with a new strip of 16 samples, whilst with real-time PCR, 384 samples can be run at a time.

3. The LAMP technique when using the Genie platform required less bulky equipment than real-time PCR, and also, the LAMP machine is portable, so can potentially be used for analysis in remote locations. In addition, the LAMP machine is cheaper in comparison to the real-time PCR machines, so might be a viable alternative for quantification in facilities that are unable to afford real-time PCR equipment.

In conclusion, phytoplasma diagnostics can be carried out with different methods and techniques, and also different sets of primers can be used with nested PCR, LAMP, and real-time PCR to detect and quantify these pathogens. Furthermore, we have demonstrated that transmission of phytoplasmas between different host plants can be performed with relative simplicity using dodder shoots. Phytoplasma control requires further research. The results have demonstrated that different sources and ratios of nitrogen can influence the titre of phytoplasmas, but this varies for different phytoplasmas. Further work is required to determine if these are 16Sr group-specific responses and whether certain fertilizers work better on particular 16Sr groups and also to determine the

mechanisms through which the reduction in titre occurs. It will also be important to translate these studies back into crop plants to confirm that the same affects occur as in periwinkle plants.

Salicylic acid and UV-C treatment of plants have also been demonstrated to provide a reduction in phytoplasma titre in infected plants. It is likely that this is due to induced resistance, but further experiments are required to confirm this and to determine whether the two methods work by similar molecular mechanisms or are able to complement each other to provide enhanced control. Again, such studies are easiest to perform initially in model systems such as periwinkles, but once optimal treatments have been identified, these should ultimately lead to field trials in crop systems. Currently, there is a lack of effective control methods for phytoplasma-infected plants, with the removal of infected plants where this is practical and/or insect control being the main approaches used. Research to find reliable alternative methods is, therefore, a priority.

This study help us to understand that:

1- Maintain phytoplasmas on infected plants or transmit them through dodder plants can be used to keep different phytoplasma groups through out year to do more experiments.

2- Using qPCR assay for phytoplasma diagnostics is sensetive and accurate, also LAMP assay is fast, highly specific, and similarly sensitive as qPCR. Detection and quantification of phytoplasmal diseases at early

stages can be useful to scan and predict any disease prior become untreatable matter, also it is useful economically (Kogovšek et al., 2017).

3- More researches can be done on salycilic acid and UV treatments with associate to other effctive methods to achieve a better technique to control the phytoplasmal diseases.

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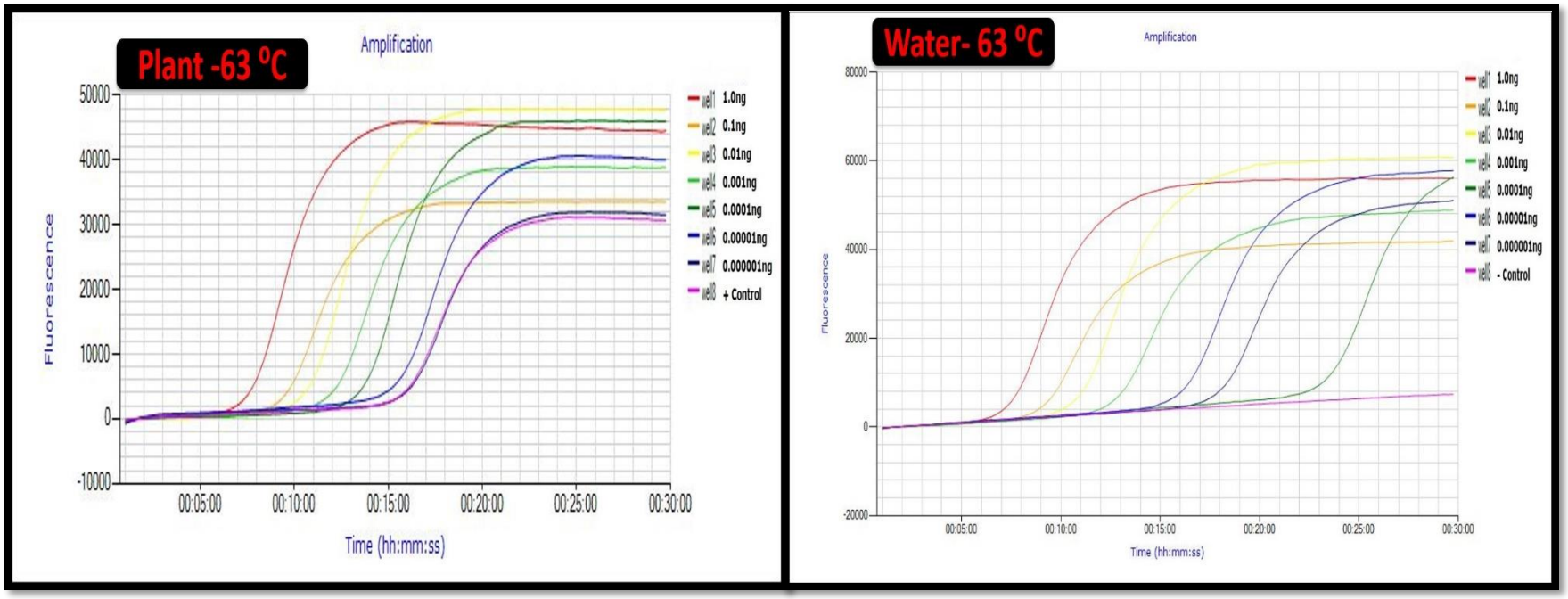
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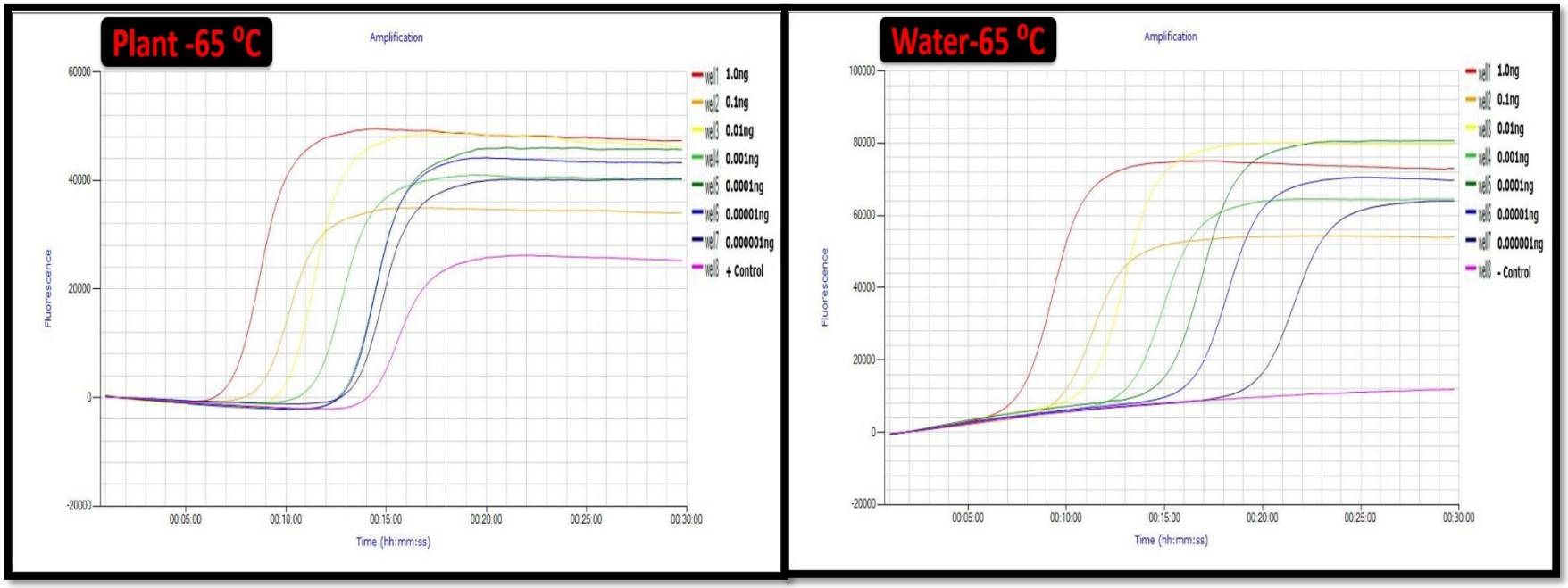
APPENDIX

Appendix 1a:



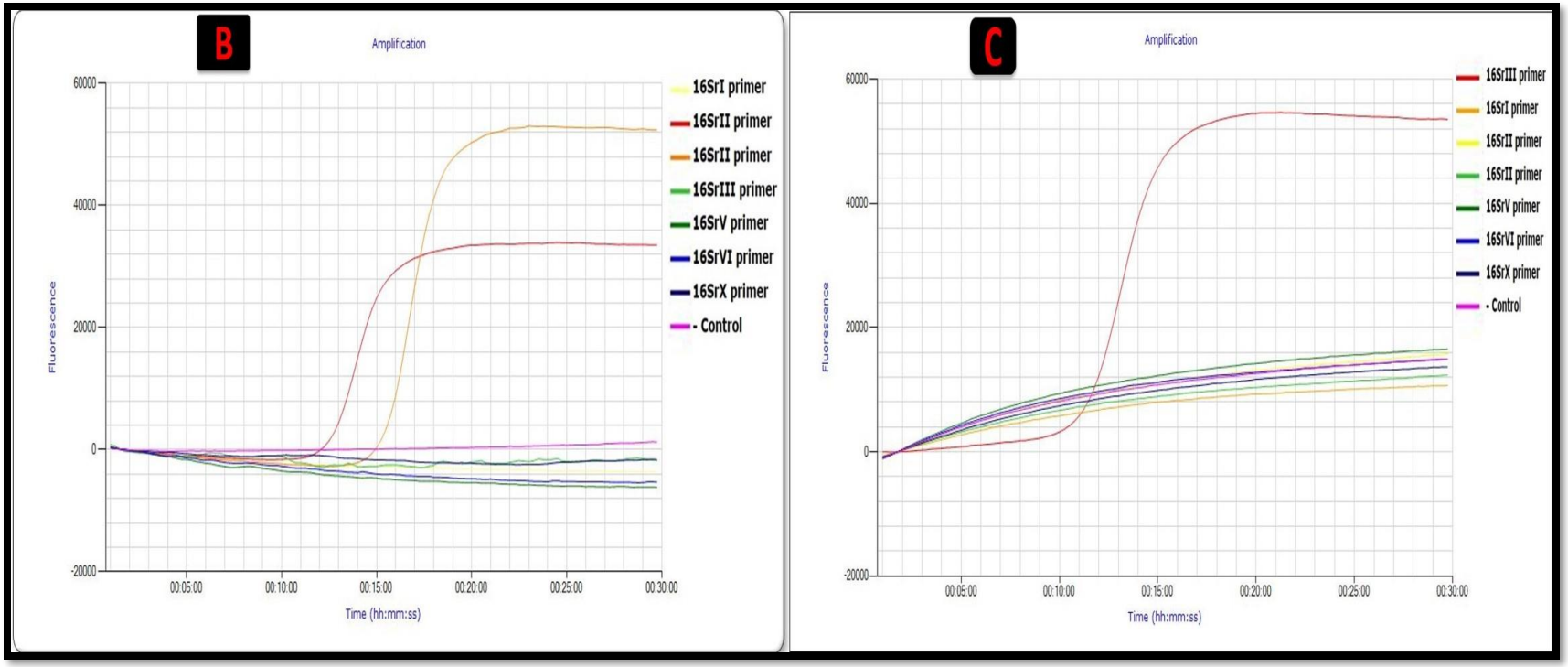
Serial dilutions plasmid DNA of phytoplasmas are prepared in plant DNA (left) and water (right) at different amplification time based on LAMP assay which conducted at 63ºC.

Appendix 1b:



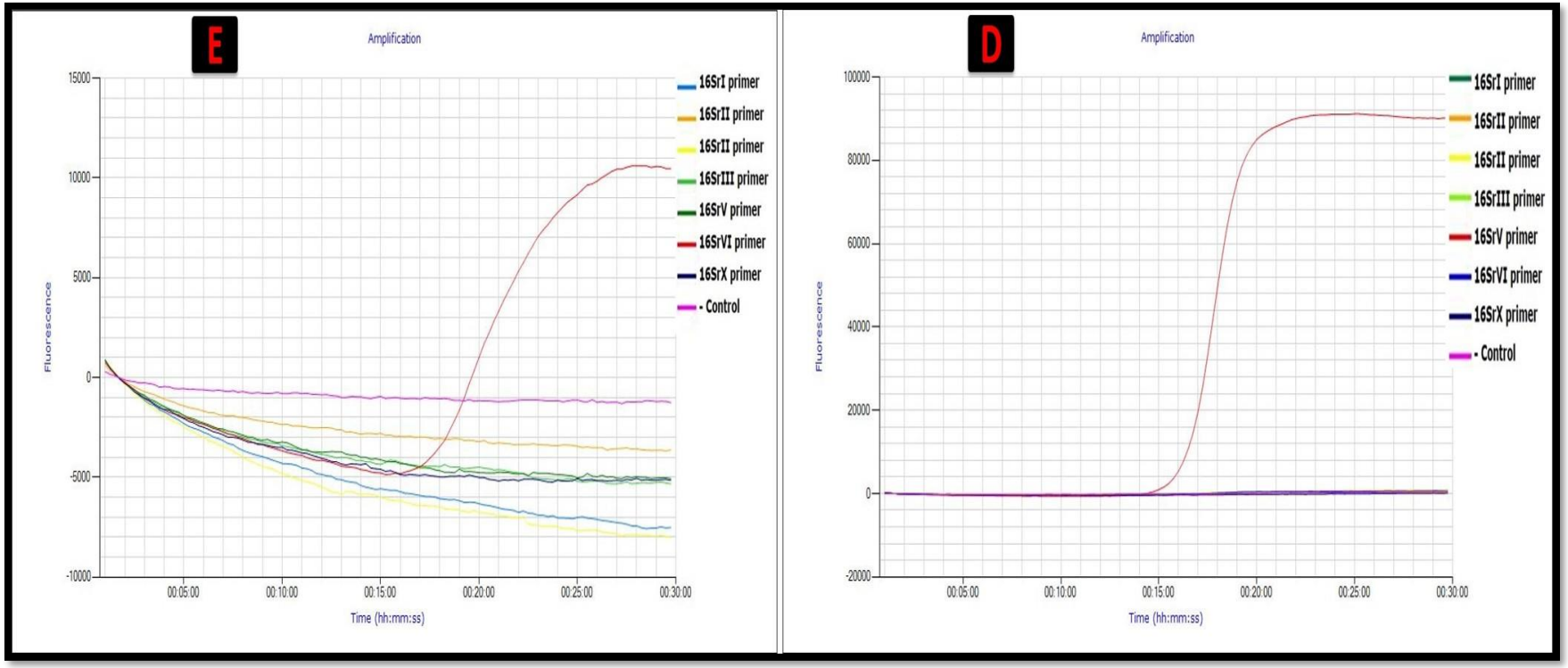
Serial dilutions plasmid DNA of phytoplasmas are prepared in plant DNA (left) and water (right) at different amplification time based on LAMP assay which conducted at 65°C.

Appendix 2a:



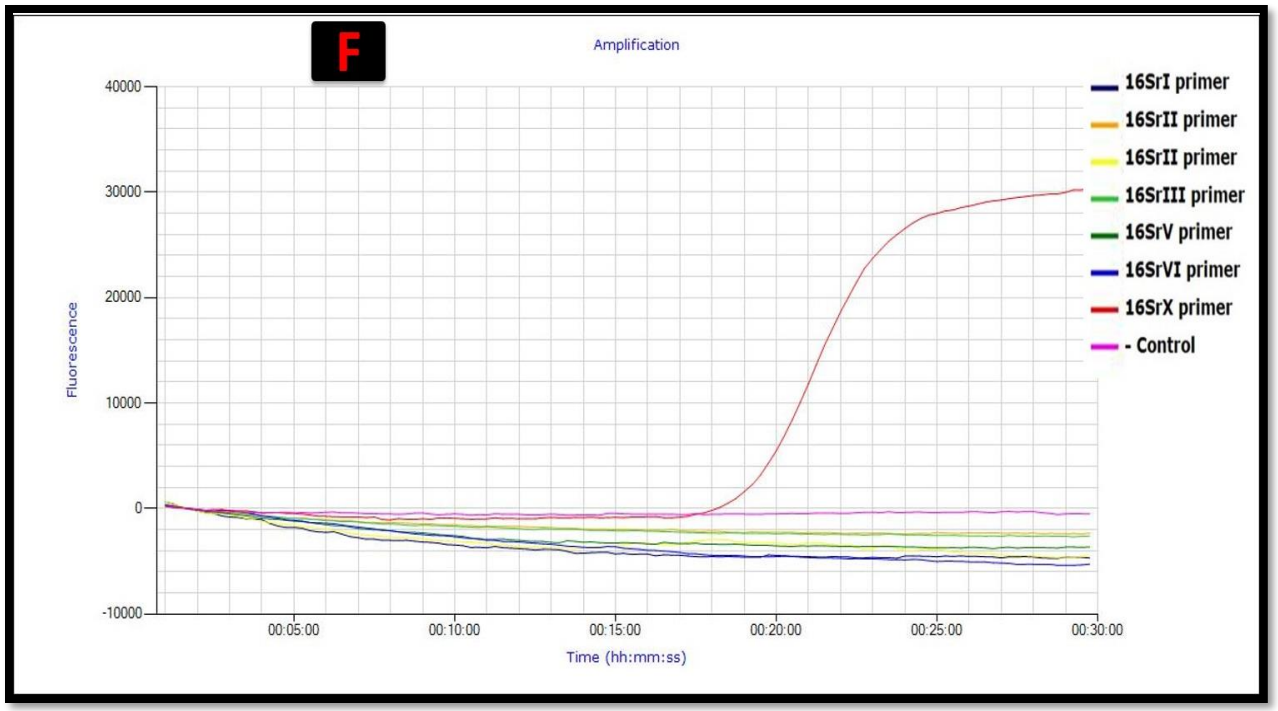
Validation of specific LAMP primers to detect specific phytoplasmas. B: SP and VCP DNAs are amplified with primer group II, C: Only LNI DNA is amplified with primer group III.

Appendix 2b:



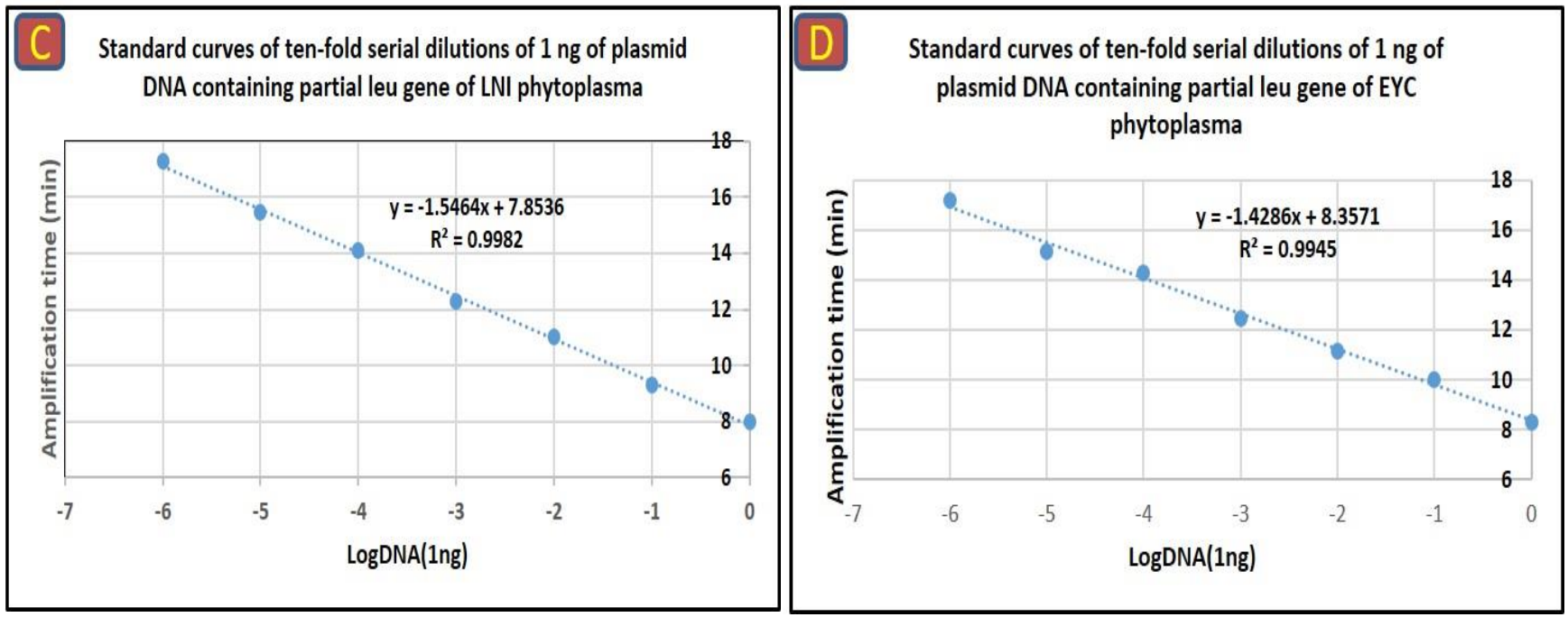
Validation of specific LAMP primer to detect specific phytoplasmas. D: Only EY DNA is amplified with primer group V, E: Only PWB DNA is amplified with primer group VI.

Appendix 2c:



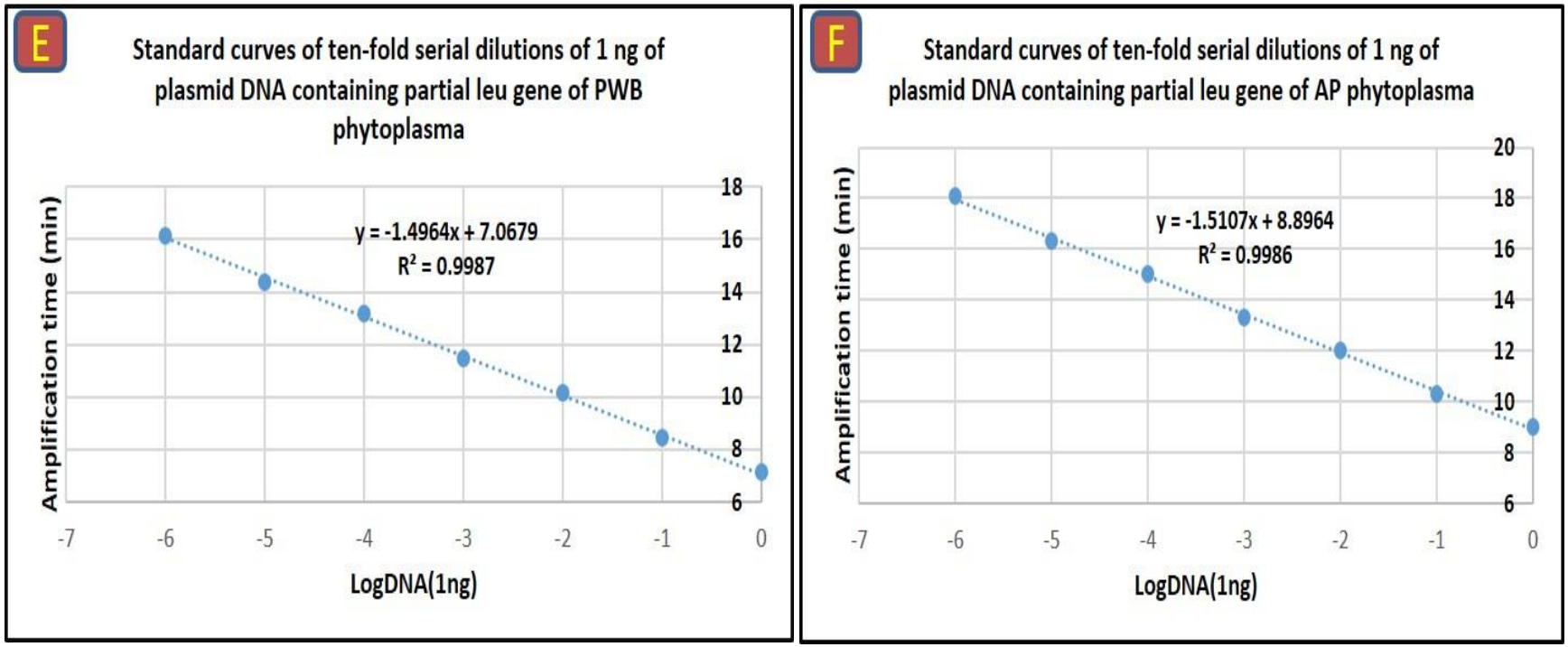
Validation of specific LAMP primer to detect specific phytoplasmas. F: Only AP DNA is amplified with primer group X.

Appendix 3a:



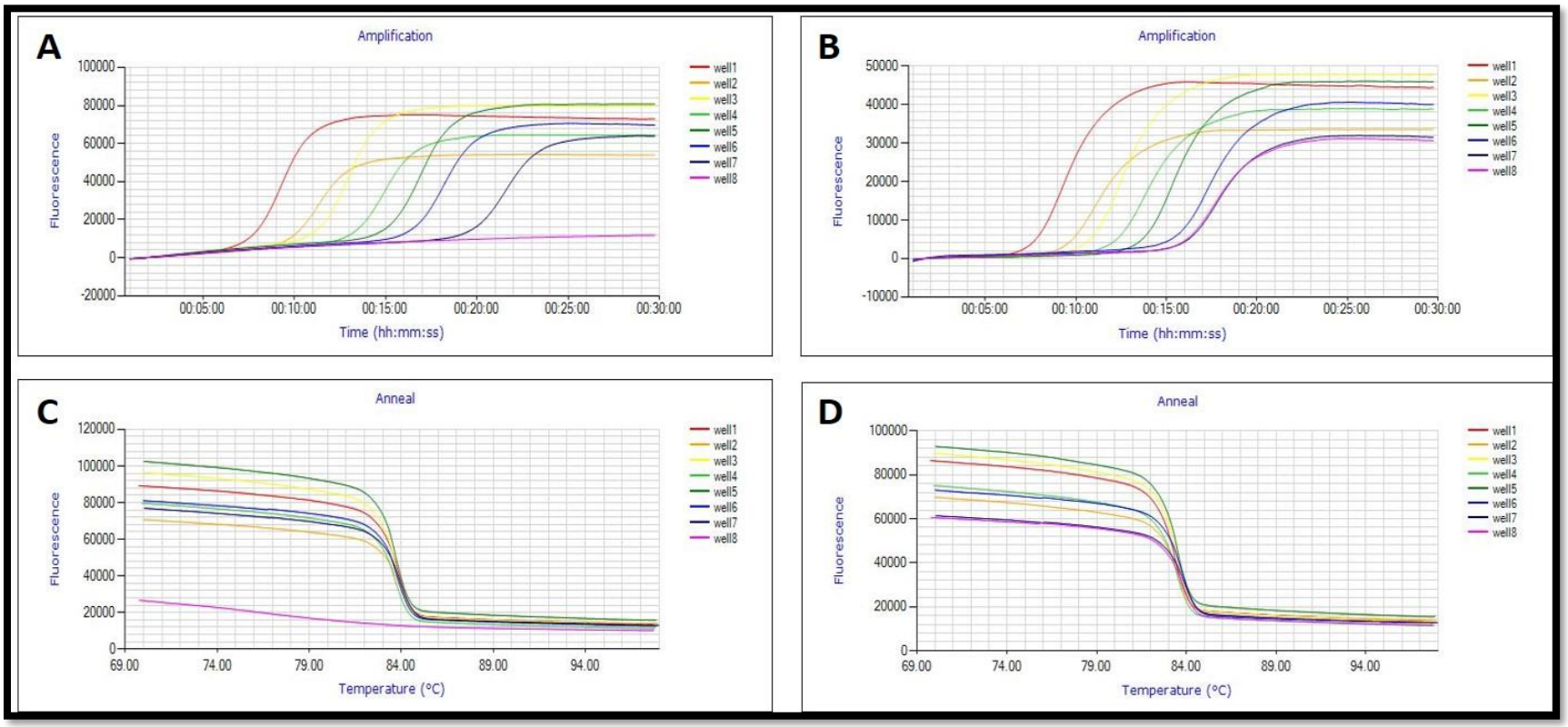
Standard curve of ten-fold serial dilutions (10^{0}-10^{-6}) of 1.0 ng of plasmid DNA containing partial leucyl-tRNA gene of (C) LNI, and (D) EYC using LAMP method.

Appendix 3b:



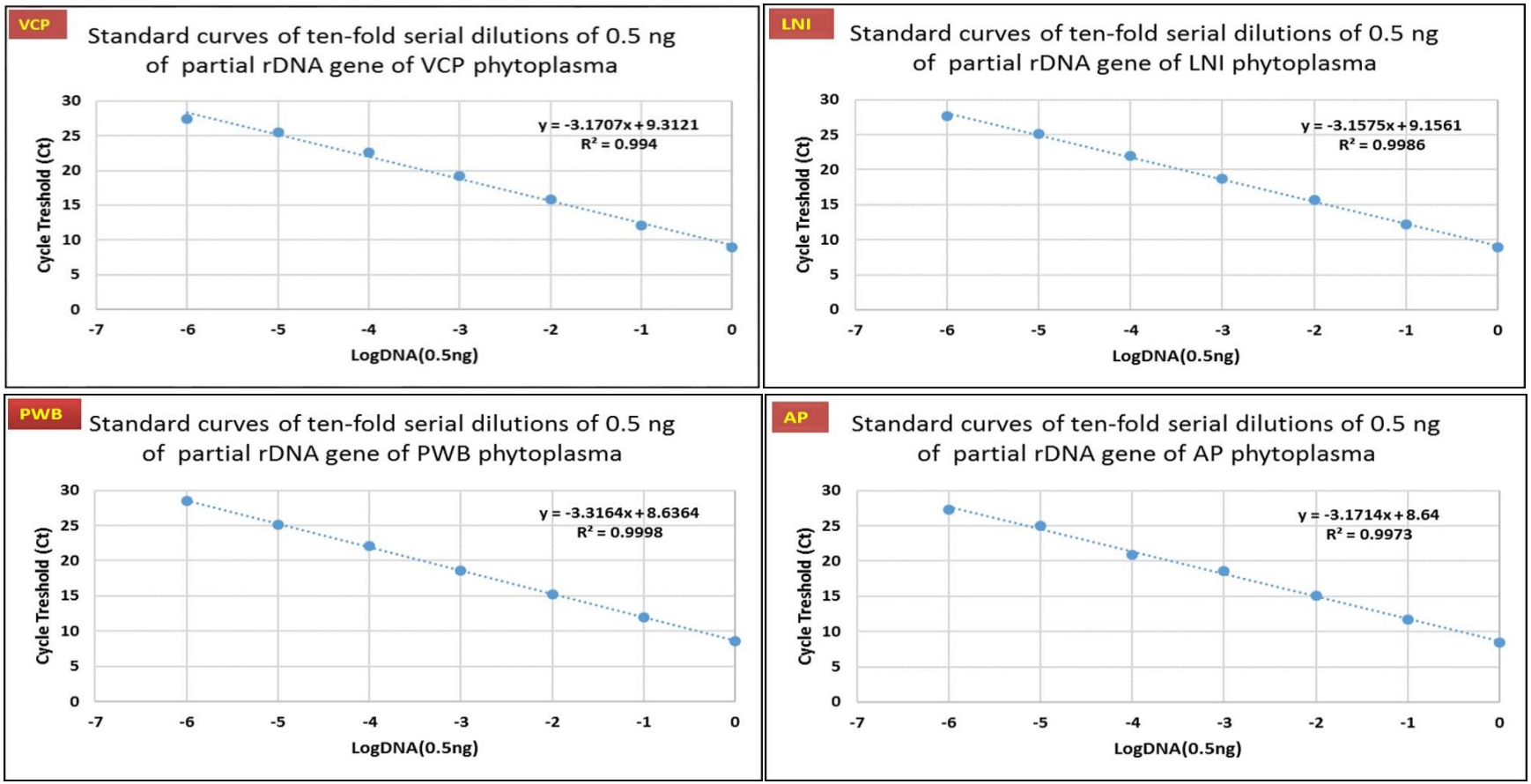
Standard curve of ten-fold serial dilutions (10^{0}-10^{-6}) of 1.0 ng of plasmid DNA containing partial leucyl-tRNA gene of (E) PWB, and (F) AP.

Appendix 4:



Ten-fold serial dilutions (10^0-10^{-6}) of 1.0 ng of plasmid DNA containing partial leucyl-tRNA gene of LNI phytoplasma by using LAMP assay. A: Amplification curve of LNI plasmid diluted with dH2O, B: Amplification curve of LNI plasmid diluted with 200 ng healthy periwinkle plant DNA, C: Annealing curve at 83ºC of LNI plasmid with dH2O, D: Annealing curve at 83ºC of LNI plasmid with plant DNA.

Appendix 5:



Standard curves of ten-fold serial dilutions (10^{0}-10^{-6}) of 0.5 ng of PCR products containing partial rDNA genes of particular phytoplasmas: VCP 16SrII, LNI 16SrIII, PWB 16SrVI, and AP 16SrX using qPCR technique.