

'Phytoplasmas and purple top disease at the global level: diagnostic and management options'

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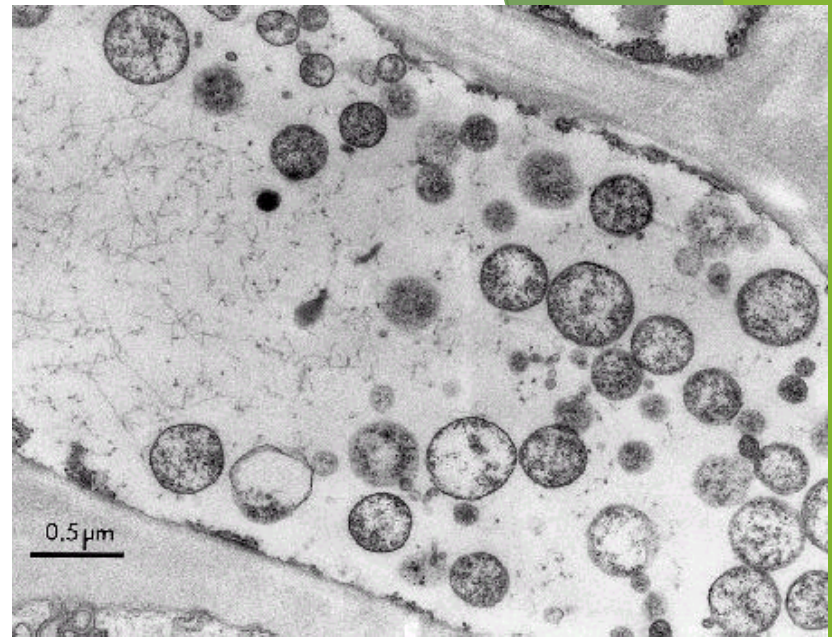


- What are phytoplasmas?
- Diseases of potato
- Diagnostics
- Management options



Phytoplasmas

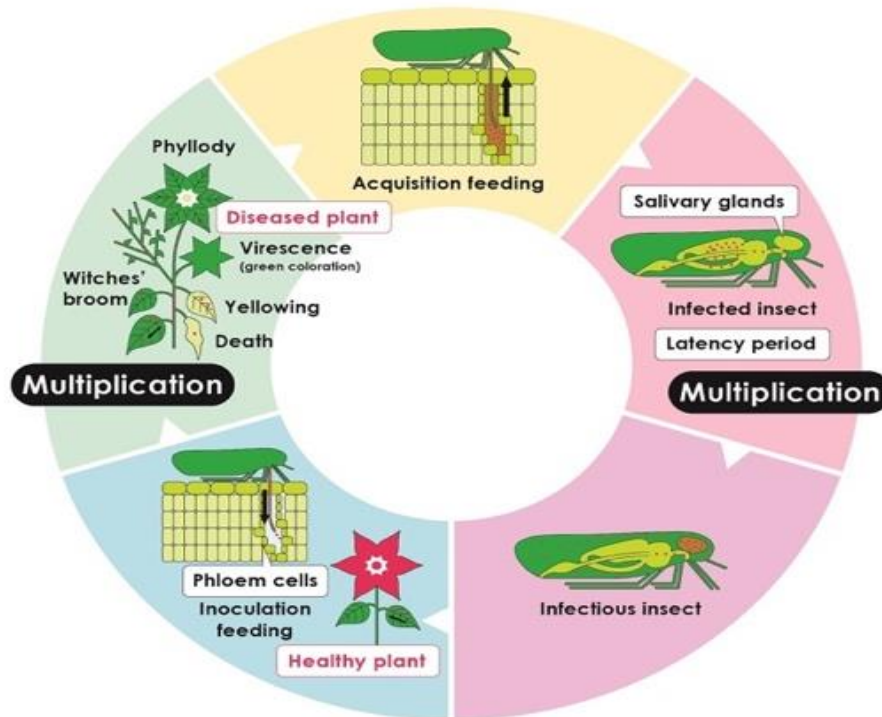
- Phloem-limited, insect-transmitted bacteria that lack a cell wall.
- Can not be grown in culture.
- Have genome sizes ranging from approx. 500 – 1200kb.
- Symptoms include yellowing, virescence, phyllody, proliferation, stunting, general decline, witches'-broom, plant death.



Transmitted primarily by members of the Auchenorrhyncha (leafhoppers and planthoppers)

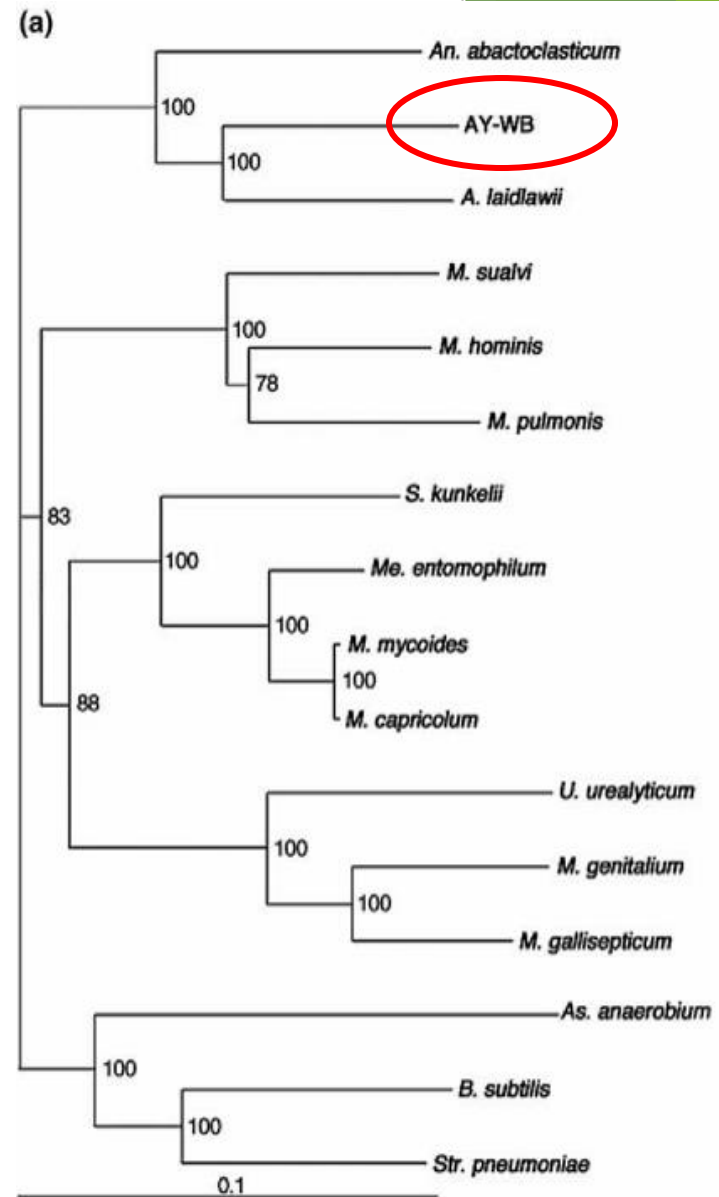


Can replicate and multiply in both plants and vectors

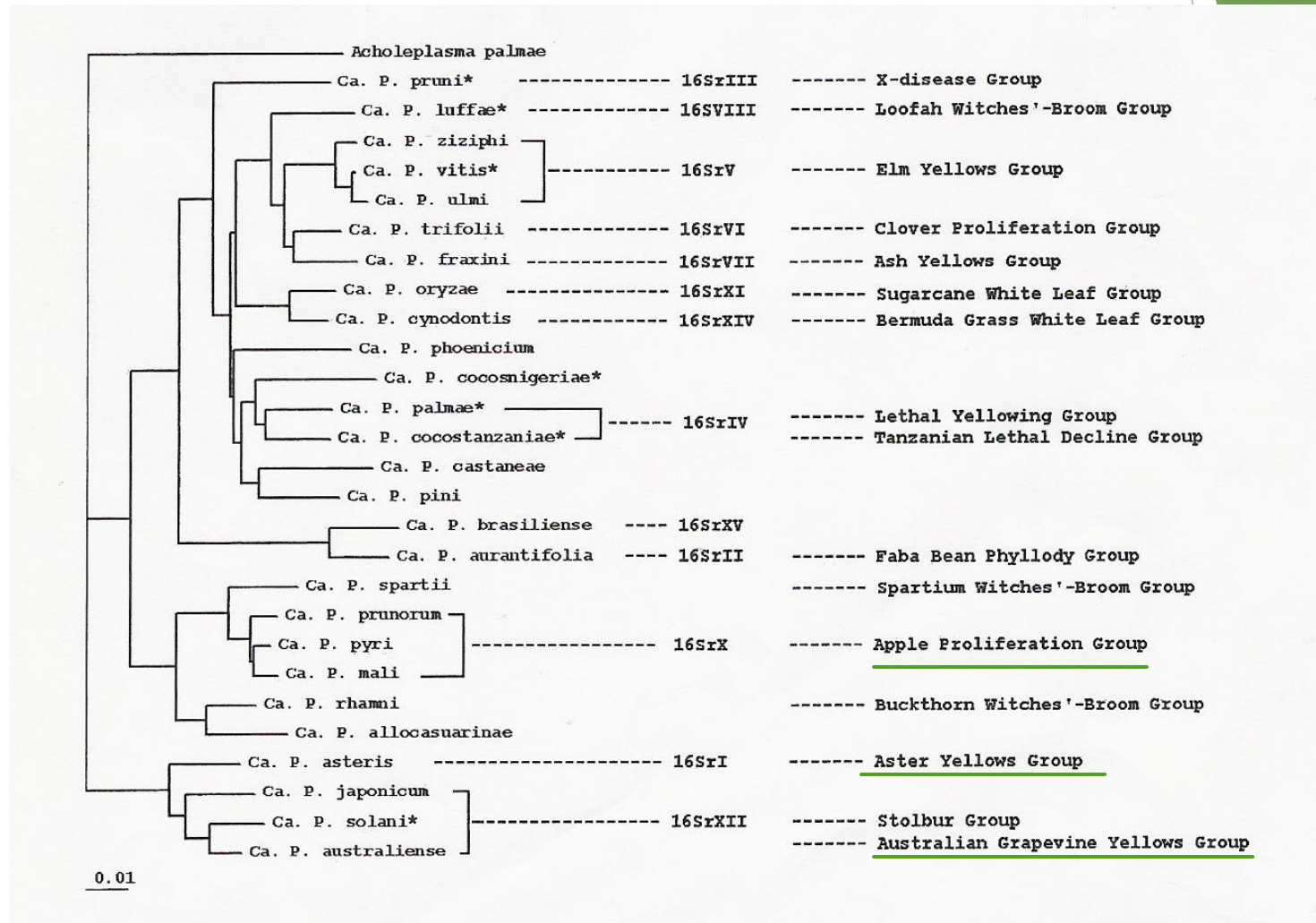


Taxonomy and genomics

- The organisms are members of the class *Mollicutes*, a branch of the Gram-positive eubacteria that lack outer cell walls.
- Other Mollicutes include the mycoplasmas, acholeplasmas, anaeroplasmas, ureaplasmas and spiroplasmas.
- They are most closely related to bacteria such as *Bacillus*, *Clostridium* and *Streptococcus*.



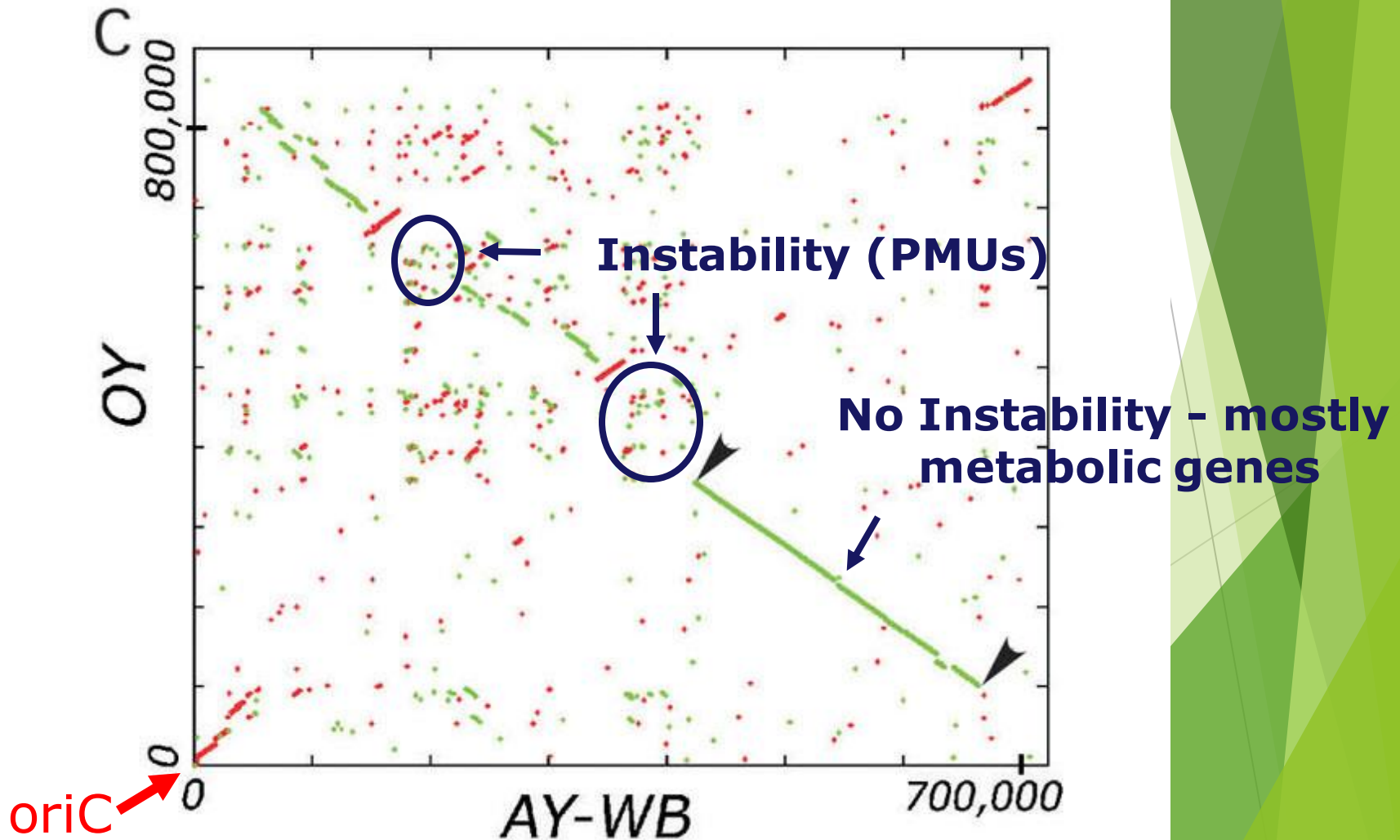
- Classified into Candidatus species and 16Sr groups, based on the sequence of the 16S rRNA gene
- Four phytoplasma genomes have been sequenced to completion



- Genomic studies indicate that whilst the main housekeeping genes are well conserved between phytoplasmas, there are other genes that are unique to specific strains.
- For example, of the 496 Apple proliferation phytoplasma genes, only 307 are also present in the aster yellows phytoplasmas.
- There is also evidence of significant rearrangements in gene organisation between different strains, even within the same '*Candidatus* species', and of potential mobile units (PMUs).

Chromosome alignment between two aster yellows type phytoplasmas reveals inversions and instability of PMU regions

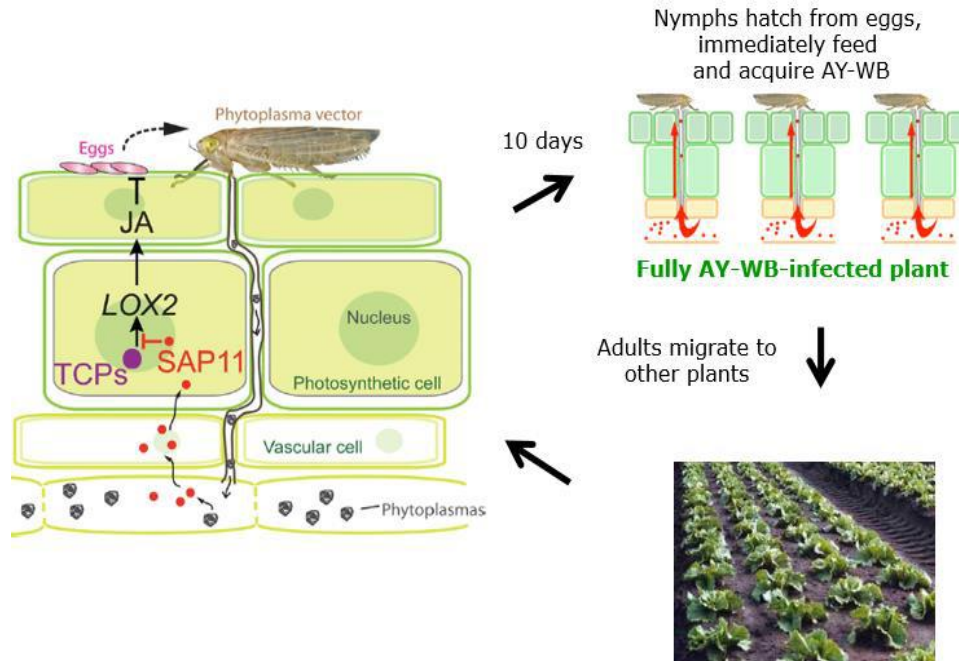
(courtesy of Saskia Hogenhout, John Innes Centre)



- Genes for 'effectors' which are believed to be the 'pathogenicity determinants' are often on these PMUs.



- These effectors are small secreted peptides that pass into cells adjacent to the phloem where they interfere with transcription factors to result in the various symptoms.



SAP11 effector protein is expressed when phytoplasma is delivered in the plant phloem. SAP11 destabilises TCP transcription factors, which lead to the downregulation of the LOX2 gene, jasmonic acid (JA) synthesis and promotion of leafhopper colonisation (Figure taken from Sugio et al., 2011).

Some phytoplasma diseases:

Aster yellows in carrot



Basil little leaf



Grapevine yellows



Coconut lethal yellowing



Sugarcane whiteleaf



Poinsettia branching factor



Many have been transferred into the Madagascan periwinkle, as an indicator plant for maintenance



ChY: Chrysanthemum yellows, RG: Rehmannia glutinosa , RIV: Ribes in vinca, SGP: Strawberry green petal, CSP: Crotalaria saltiana phyllody, SPLL: Sweet potato little leaf, VCP: Vinca coconut phyllody, PL: Plum leptonecrosis, EY: Elm yellows, PWB: Potato witches' broom, BLL: Brinjal little leaf and AP: Apple proliferation

Phytoplasma diseases in potato

Stolbur (Group 16SrXII, 'Ca. *Phytoplasma solani*')

- Found in Europe, Middle East and some other countries, but not particularly problematic on potato.
- Spread by leafhoppers, such as *Macrostoteles* sp., *Empoaca* sp. and *Hyalectes* sp.
- In the Canary Islands, the psyllid *Bactericera trigonica* has been reported as a vector of this phytoplasma in tomato.



Potato witches' broom (Group 16SrVI, 'Ca. *Phytoplasma trifolii*')

- Found in North America and results in plants with large number of small tubers that have a shortened dormancy.

Potato purple top

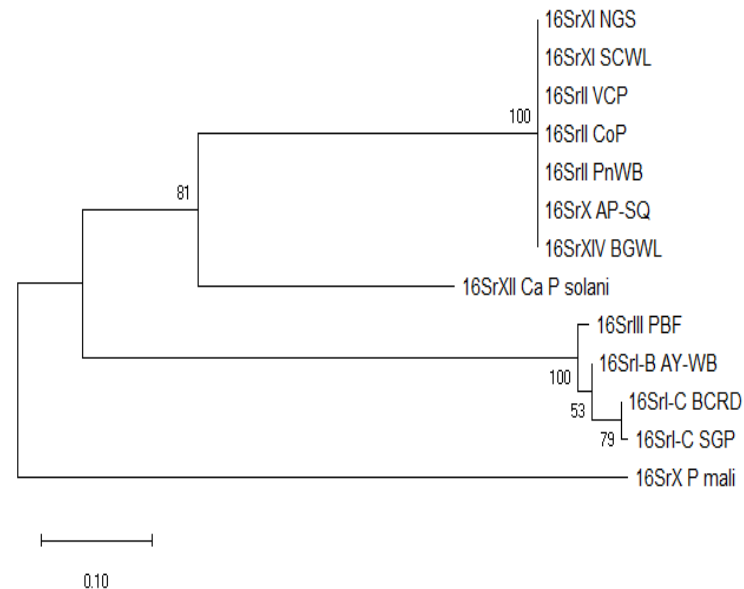
- Symptoms of potato purple top have been reported in numerous countries and different strains of phytoplasma have been found associated with the symptoms.



- In north western USA, the 16SrVI phytoplasma is associated with Columbia basin purple top disease – spread by the beet leafhopper, *Circulifer tenellus*.
- In other parts of the USA and Mexico, the 16SrI-B aster yellows type phytoplasma is associated with the disease. This phytoplasma group has a broad host range and is spread by vectors such as *Macrosteles quadrilineatus* and other *Macrosteles* species.
- In Ecuador, 16SrI-F has been detected associated with the disease (Carillo et al. 2018 Australian Plant Pathology 47, 311-315).

- In China, Saudi Arabia, Mexico and Ecuador, 16SrII, 'Ca. *Phytoplasma aurantifolia*' strains have been found associated with the disease (e.g. Caicedo et al. (2015) *New Disease Reports* 32)
- This is also a broad host range phytoplasma, spread by numerous species including *Hishimonus* sp.
- In parts of south east Asia, the 16SrIII group phytoplasmata have been found associated with potato purple top, spread by *Alebroides nigroscutellatus*.
- This situation of different phytoplasma strains causing the same symptoms in a plant species in different parts of the world is not unusual. Numerous 16Sr groups have been reported to cause grapevine yellows (16SrV and 16SrXII in Europe, 16SrI in South Africa and 16SrIII in Chile), and the lethal yellowing disease of coconut is caused by the 16SrIV group in the Americas, 16SrXXII in Africa and a new strain in south-east Asia.

- One possible reason for different phytoplasmas causing the same symptoms may be due to the effectors they contain. There is increasing evidence that the effector repertoire within a strain doesn't necessarily correspond to the 16S rRNA grouping.
- A suggestion is that effectors may get transferred by horizontal transfer between phytoplasmas on the potential mobile units (PMUs).
- Therefore the 16Sr group might determine the vector transmission / host range whilst the effector repertoire determines the symptoms.



Phylogenetic tree of SAP11 effectors from different phytoplasmas

Phytoplasmas in Peru

- In 2007, we conducted a survey with CIP on phytoplasmas present in crops including carrot, maize, coconut, papaya, potato, native potato and Shiri in different regions of Peru (Hodgetts et al. (2009) *Annals of Applied Biology* 155, 15-27).
- The 16SrI aster yellows phytoplasma was found in samples of all of these crops including a native potato with witches' broom symptoms, and 16SrII was found in a potato with yellowing, stunting and little leaf from Montaro valley.
- Interestingly, other potato samples were positive for phytoplasma in PCR from fresh samples, but nothing was detected when these samples were subsequently tested after transport back to the UK.



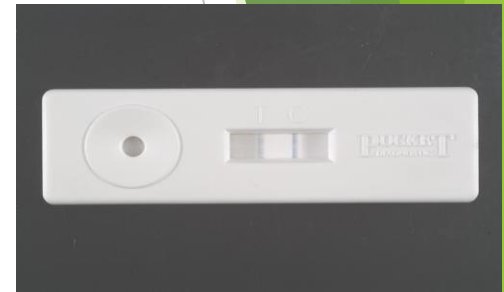
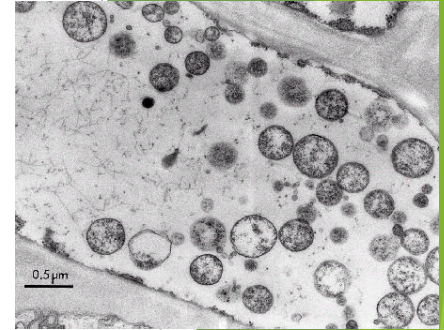
Native potato from Quillacocha, with yellowing stunting and lack of tuber production.



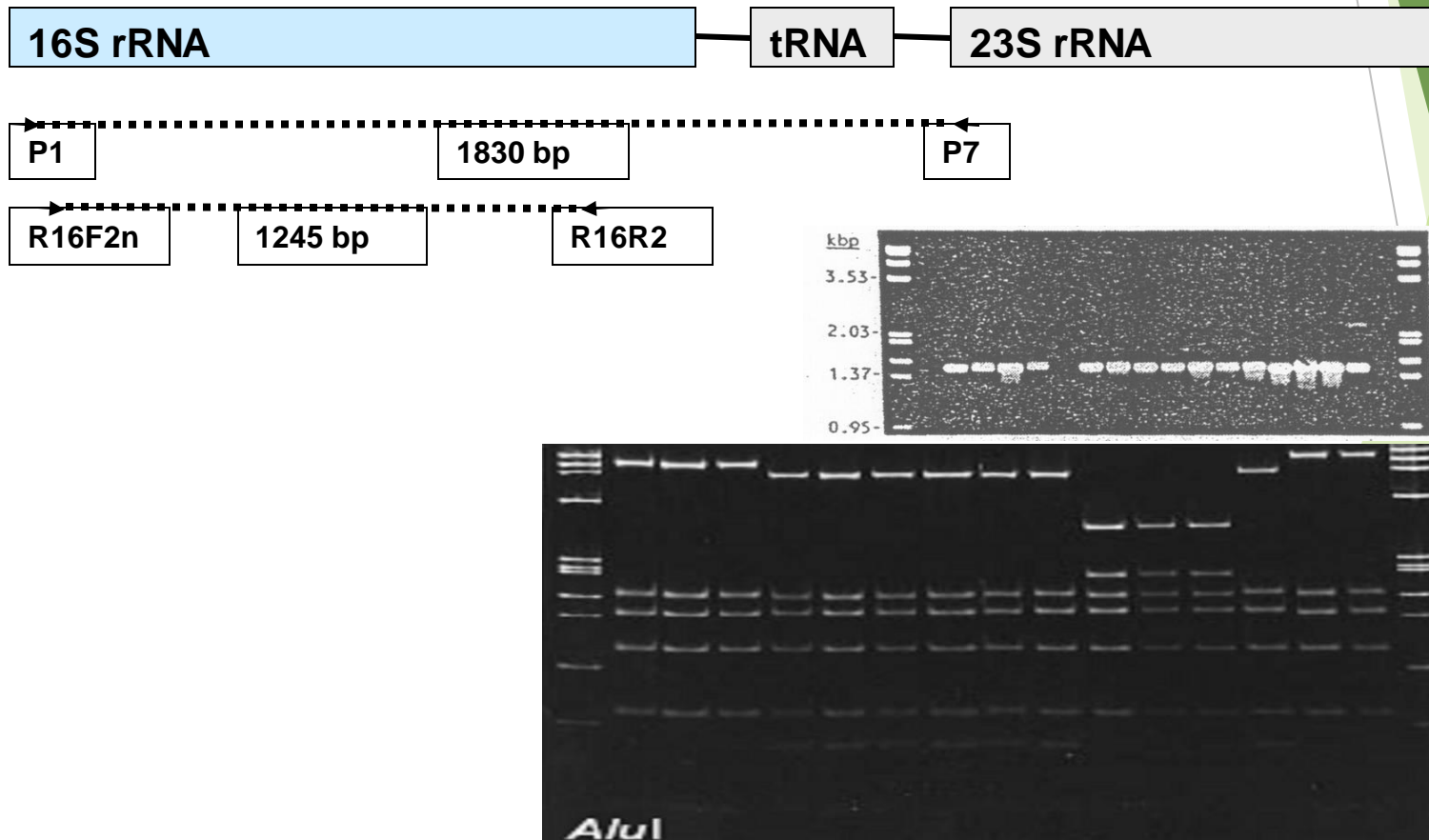
Shiri from Concepción, left symptomless and right with stunting.

Phytoplasma diagnostics

- There are no culture-based techniques or simple microscopy methods for diagnosing the presence of phytoplasmas in plants / insects.
- Attempts to develop lateral-flow type diagnostics based on antibodies have been unsuccessful due to lack of suitable target proteins and lack of sensitivity.
- PCR (polymerase chain reaction) amplification of phytoplasma DNA from infected plant extracts has been the most commonly used diagnostic method.
- However, this often requires two rounds of PCR for detection and requires gel electrophoresis to detect products.
- The combination of the time taken to purify plant DNA for PCR plus the danger of contamination of samples due to the multiple pipetting steps and opening of tubes makes this method prone to false positives and false negatives.

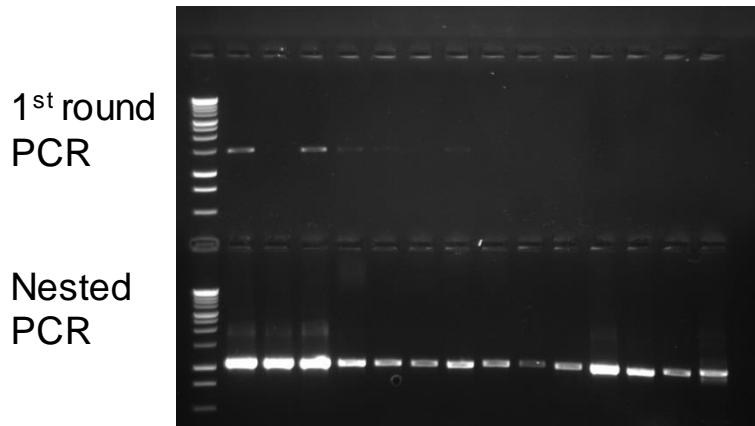


- PCR amplification of phytoplasma DNA from infected plant extracts generally uses 16S rRNA universal primers in a nested PCR approach.
- Primers can also amplify from some *Bacillus* species.
- RFLP analysis is often used as the basis of 16Sr group and sub-group classification, or sequencing of the PCR product.



Primers for non-ribosomal genes are also available for diagnostics / classification

- Primers have been designed for the *rp* operon (Martini *et al.*, 2007), and also for *tuf* and *secY*, but tend to only detect certain subgroups and are generally not universal.
- More recently we have designed universal nested primers for the *secA* and Leucyl tRNA synthetase (*leuS*) genes that appear to work on most phytoplasma groups.



Simple set of *leuS* primers designed to work on all phylogenetic groups in nested PCR – give a product of approx 1100 bp

Possible improvements in diagnostics

- Real-time PCR provides improved reliability since it is a closed diagnostic system – once reactions have been set up, the tubes don't have to be reopened.
- It is rapid, providing results within 1-2 hours, sensitive, and assays have been developed for many pathogens. Can also be used for quantification along with the more recent approach of digital PCR.
- However, it is still prone to PCR inhibitors in DNA samples extracted from plants, so false negatives can be a problem unless appropriate controls are undertaken.
- The equipment is expensive and not very portable, so it is not appropriate for 'in field' diagnostics.



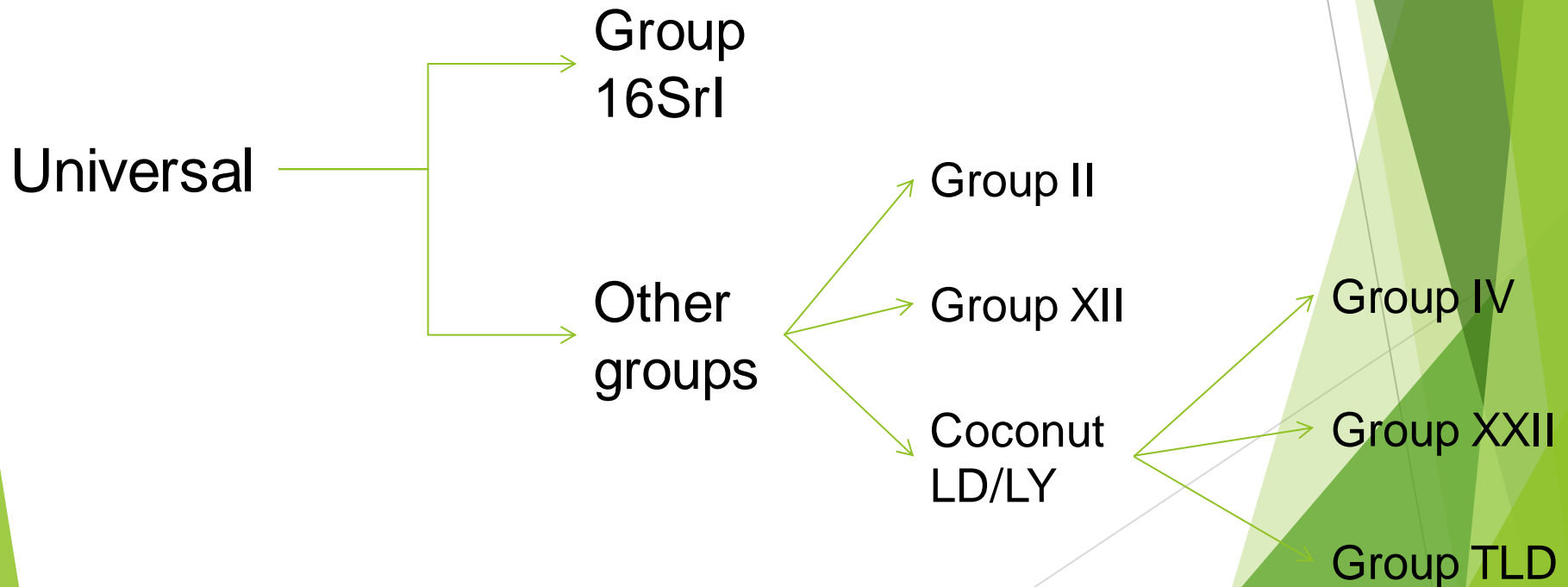
Bioline
mic  **pcr**

A new way to look at Real Time PCR



Real-Time PCR for phytoplasmas

- ▶ A number of universal and group specific assays have been developed and published.
- ▶ We have developed a range of Taqman assays based on the 23S rRNA with good specificity and sensitivity and for quantification.



Acronym	Phytoplasma Strain/Bacterial source	16Sr group	Average C _T values		
			Universal	16SrI probe	Multiplex Other groups probe
-	<i>Burkholderia gladioli: pure culture</i>	-	38.5	ND	38.1
-	<i>B. gladioli: Inoculated Allium bulb</i>	-	ND	ND	ND
CHRYM	Chrysanthemum yellows	I-A	21.8	21.1	ND
RG	<i>Rehmannia glutinosa</i>	I-B	22.0	20.0	ND
BCRD	Blackcurrant reversion disorder	I-C	32.0	26.7	ND
SGP	Strawberry green petal	I-C	20.5	19.3	ND
CLP	Cleome phyllody	II-A	15.9	ND	18.7
CX	Peach western X	III-A	17.9	ND	20.6
JR1	Poinsettia branching factor	III-H	21.8	ND	28.3
LYAM	Coconut lethal yellowing	IV-A	17.1	ND	18.7
TLD	Tanzanian lethal decline		24.2	ND	26.5
CSPWD	Ghanaian Cape St Paul wilt	XXII	23.1	ND	25.7
ULW	Elm witches broom	V-A	18.1	ND	19.7
BLL	Brinjal little leaf	VI-A	19.5	ND	21.3
ASHY-1	Ash yellows	VII-A	18.8	ND	21.3
PPWB	Pigeonpea witches' broom	IX	13.8	ND	14.6
AP-15	Apple proliferation	X-A	19.7	ND	20.8
NGS	Napier grass stunt	XI	23.3	ND	29.5
STOL	Stolbur of pepper	XII-A	16.1	ND	16.9
MPV	Mexican periwinkle virescence	XIII	12.7	ND	12.9
BGWL	Bermuda grass white leaf	XIV	13.8	ND	16.4

Have also developed Loop Mediated Isothermal Amplification (LAMP) systems

- Uses *Bst* DNA Polymerase.
- Requires 4 or 6 primers.
- Amplification occurs at a single temperature (65°C).
- Fewer problems from enzyme inhibitors in DNA extracts than PCR.
- Amplification takes 10-30 mins.
- Products can be detected through incorporation of dyes into the reaction mix, on agarose gels, or in real-time (pyrophosphate / turbidity assays or Sybr-Green type detection).
- Has been developed into a rapid field diagnostic test system.
- We have developed a range of primers for detecting different phytoplasmas and other plant diseases.

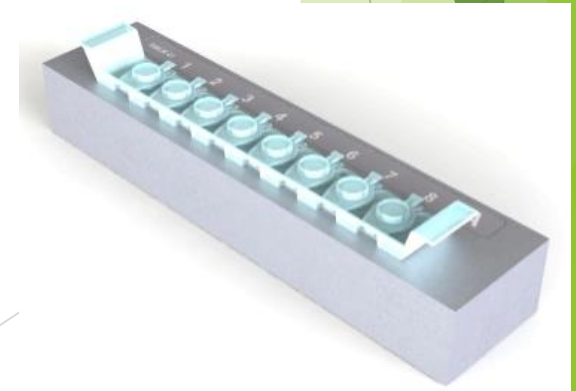
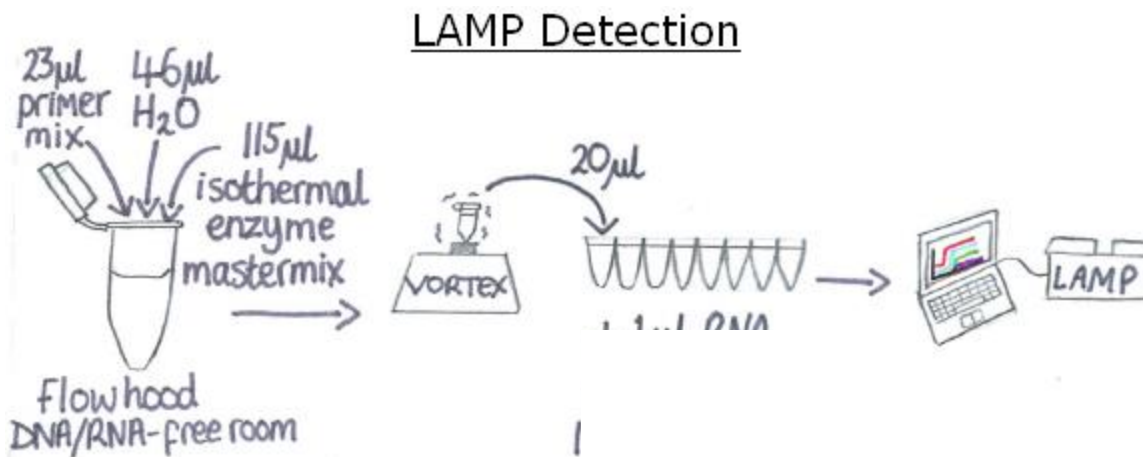
Have combined LAMP with a rapid DNA extraction method for in-field work

- A rapid DNA extraction method has also been developed that takes 2 minutes.
- Place approx 20 mg plant material into an Eppendorf tube containing 500 microlitres alkaline PEG buffer and grind with a micropestle for 30 secs. Then use 1-2 microlitres of the solution directly in the LAMP reaction mixes.
- DNA has been successfully extracted from a wide range of plant species and substrates (for coconut, trunk borings work very well).



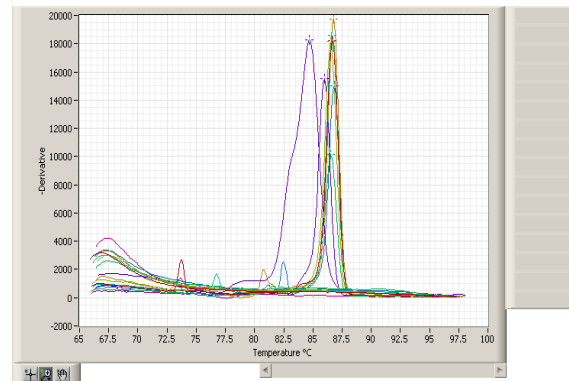
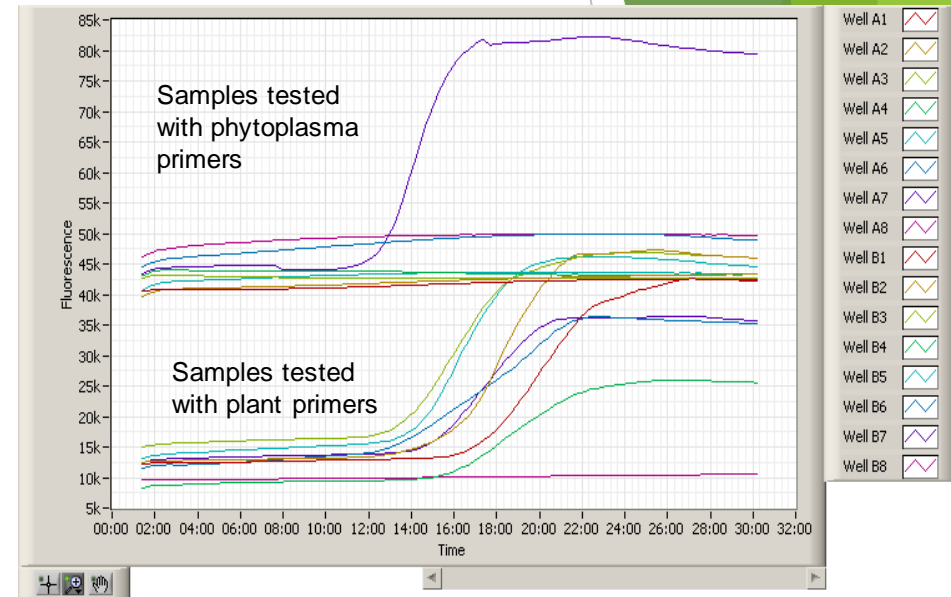
Setting up LAMP reactions in the field

- Strips of 8 tubes can be purchased from Optigene, UK, along with reagent mixes that contain the enzyme, buffer, fluorescent dye etc.
- The only additional reagent required is the primers for the particular organism to be detected.
- The reaction mixes are stable and can be stored at room temperature for at least 1 month – therefore mixes can be prepared in the lab and transported out to the field for on-site field work. The only additional requirements in the field are a pipetter and pipette tips to transfer the DNA solution into the reaction mixes, and a method for heating samples at 65°C.



REAL-TIME DETECTION SYSTEM

- Optigene have also developed a real-time machine for detection of LAMP products – battery operated, lightweight and portable.
- Detection in real-time takes 10-30 mins and melt curves can then be used to confirm products are genuine and not artefacts (unlike in real-time turbidity assays).
- Data is stored and can be transferred to a computer back in the lab.
- Can also run on a real-time PCR machine for lab-based testing.



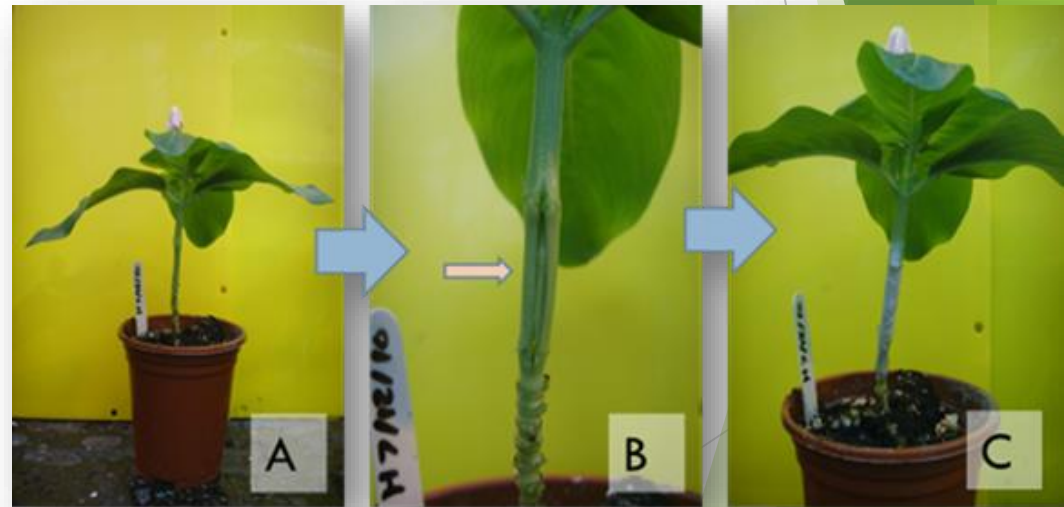
Recommendations for phytoplasma diagnostics

- Use real-time closed-tube assays (PCR or LAMP) for initial diagnostics rather than nested PCR, which is prone to contamination problems.
- Include a control for false negatives (ie some primers that amplify from plant DNA) to confirm samples don't contain inhibitors
- Once presence of a phytoplasma has been confirmed, nested PCR, using rRNA gene primers and primers from other genes can then be used to identify the group/subgroup.
- Be aware that phytoplasma titre can be quite low in plants and also unevenly distributed, so more than one sample may be required.
- Also, in our experience, some phytoplasma DNA samples appear to be unstable even when stored at -20°C – but other samples can be very stable.

- A further recommendation for working with phytoplasmas is to try transferring the phytoplasma into Madagascan periwinkle indicator plants.
- The titre is often higher and the plants can then be maintained long-term.
- Transfer can be attempted either using the parasitic dodder plant (*Cuscuta*) as a bridge from the original host to the periwinkle, or by grafting.



Use of dodder for transfer



A simple grafting technique in which a small piece of tissue from the test plant is embedded into the stem of the periwinkle

Management options for phytoplasmas

- There are no chemical treatments available to eradicate phytoplasmas from infected plants.
- In some countries tetracycline treatments have been used on high value plants, but these just reduce the titre and their use in the field is banned in many countries.
- Chemical sprays can be used to try and control insect vector populations.
- The insect vectors are often found associated with other plants such as weeds in the vicinity of the crops, so removal of weeds can help – this has been particularly effective in coconut plantations in the Caribbean for example.
- Be careful with movement of planting material from areas with phytoplasma diseases. Although there is no definitive evidence of seed or mechanical transmission, phytoplasmas can be moved through symptomless planting materials.

- For some crops, resistant / tolerant varieties have been found.
- For example, for coconut lethal yellowing in the Caribbean, Maypans (Malayan dwarf x Panama tall hybrids) have proved to be a good source of resistance, whilst in Ghana, Vanuata Tall x Sri Lankan Green Dwarf hybrids have proved to be effective.
- How this resistance works, and whether it is to the phytoplasma or the vector, is not known.
- The management approach recommended for coconut plantations is to remove and burn infected plants as soon as the phytoplasma is detected and replant with a resistant hybrid. And to keep levels of weeds low.
- It should be noted, however, that the phytoplasma groups that cause potato purple top tend to have broad host ranges, so are likely to be found in other crops and weeds in the locality .

Other approaches are being evaluated as possible phytoplasma management options, including use of salicylic acid / elicitor treatments, and biological control agents.

In addition, cross protection is being tested. For example, we have undertaken experiments to mix two phytoplasma strains within the same Madagascan periwinkle plant (by grafting) and assess competition between the strains through molecular diagnostics and symptoms.

Treatment	Phytoplasma detection(month after grafting)										Symptom	
	1	2	3	4	5	6	7	8	9	10		
➔ + RG SPLL	Green	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	RG>SPLL
RG+PL	Green	Green	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Mixed
➔ + RG EY	Green	Green	Orange	Green	Green	Orange	Orange	Green	Green	Green	Green	RG>EY
RG+PWB	Green	Green	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Mixed
➔ + SPLL PL	Green	Green	Green	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	PL>SPLL
➔ + SPLL EY	Green	Green	Green	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	EY>SPLL
SPLL+PWB	Green	Green	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Orange	Mixed

Green = no phytoplasma detected

Orange = phytoplasma detected

Competitive evidence based on symptoms



SUMMARY

- Phytoplasmas, spread by insect vectors, can be found associated with potato purple top disease.
- Phytoplasmas from different taxonomic groups have been found associated with these symptoms in different parts of the world, but this is not unusual for phytoplasma diseases.
- Initial phytoplasma diagnostics is best done using real-time, closed-tube assays, with conventional PCR then used for group identification. However, some plant materials can be difficult to extract from and there is evidence that some phytoplasma DNA samples degrade in storage.
- Good crop management, including roguing infected plants, insect and weed control, and use of clean replanting material, can be effective. In the longer term, identifying resistant planting material may be an option.

