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Indicate (X) client(s) to whom this concept project proposal is submitted. Replace any of these with other relevant clients if required.

NB: The instructions in red, throughout the template, should be omitted from the final document.

FINAL REPORT
(2016)

1. PROGRAMME AND PROJECT LEADER INFORMATION

	Research Organisation Programme leader	Research Team Manager	Project leader
Title, initials, surname	Prof JT Burger	Prof JT Burger	Dr HJ Maree
Present position	Professor	Professor	Senior Researcher
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2. PROJECT INFORMATION

Research Organisation Project number	Genus 12/01
Project title	Determination of the temporal and spatial distribution of aster yellows phytoplasma in infected grapevine plants.
Short title	AY spatial and temporal distribution

Fruit kind(s)	Wine Grapes		
Start date (mm/yyyy)	01/01/2013	**End date** (mm/yyyy)	31/12/2015

Key words	Aster Yellows detection, Recovery phenotype

Approved by Research Organisation Programme leader ☐ (tick box)

THIS REPORT MUST INCLUDE INFORMATION FROM THE **ENTIRE** PROJECT

3. EXECUTIVE SUMMARY

Phytoplasmas are Mycoplasma-like organisms inhabiting plant phloem sieve elements. These pathogens lead to a myriad of diseases over many species of plants. The species of *Phytoplasma* on which this study focussed, Aster Yellows, is associated with over 100 economically important diseases globally. One disease, Grapevine Yellows, causes many devastating side effects in its plant host, *Vitis vinifera.* After initial diagnoses in South Africa in the Olifants River Valley in 2010, it has spread and led to significant losses in vineyards over the last number of years. The erratic distribution of phytoplasmas within their host has been widely observed. This uneven distribution, both spatially and temporally, greatly complicates accurate diagnosis. The main aim of this project was therefore to aid in determining the spatial and temporal distribution of Aster Yellows in grapevine plants. The optimum time point and plant organs to test were established to ensure accurate diagnosis using a triple nested Polymerase Chain Reaction assay. A secondary aim of this project was to validate the induction of a phenomenon known as the Recovery Phenotype as a permanent cure for Aster Yellows in grapevine. Regarding the optimisation of diagnostics, this study reports that the month of March is much more accurate than that of April, with phloem cane scrapings being the most accurate tissue to test when compared to petioles. The permanence in the remission in Grapevine Yellows symptoms via Recovery Phenotype induction could not be proven with full certainty in this study, but still proves plausible. This study therefore correlates spatial and temporal data of two grape growing seasons, and aids in the optimisation of a diagnosis and treatment protocol for Aster Yellows in grapevine.

4. PROBLEM IDENTIFICATION AND OBJECTIVES

Highly specific PCR-based assays to detect aster yellows phytoplasma (AY) have been developed and are continuously being refined. The reliability and accuracy of these assays however, is still a concern due to the low titre and uneven spatial distribution of the pathogen in plants.
We aim with this project to determine the spatial distribution of AY in grapevine at various time points during the growing season - both in vines displaying AY symptoms, and vines displaying the “recovery phenotype”. The result from this study will assist in the development of reliable diagnostic assays since the optimal time and tissue type will be determined. By investigating the distribution of AY in vines that were pruned back, and have seemingly recovered from phytoplasma infection (“recovery phenotype”), a possible explanation for this phenomenon, as well as the longer-term efficiency of this practice, can be put forward.

5. WORKPLAN (MATERIALS AND METHODS)

5.1 Sampling
One-hundred-and-nine grapevine plants, all sampled in Vredendal, were included in this study. Of this total, 30 originated from a Chardonnay vineyard, which was previously confirmed to contain AY positive individuals. The remaining 79 individuals originated from a Chenin blanc vineyard. Of these 79 individuals, 50 were RP plants, 11 were plants that had been negative at the time of coppicing and 18 were young plants, planted at the time of coppicing, to monitor reinfection. Therefore, if a RP plant does show signs of AY infection, the reinfection plants may indicate whether this is the original infection resurfacing, or whether a new infection was introduced by an insect vector. These 109 plants were identified in the previous study by Smyth (2014). Canes and petioles were sampled from each of the individuals in mid-March and late April. The plant material was stored at -80˚C.

5.2 Sample processing
Canes were processed by peeling of the bark, after which phloem scrapings were collected. One gram of each of the phloem scrapings and petiole samples were weighed, after which it was homogenised mechanically using liquid nitrogen.

5.3 DNA extractions
One milligram of the homogenized material was subjected to DNA extraction. The extractions were performed using the Machery-Nagel NucleoSpin® Plant II Minikit. This kit makes use of the CTAB cell-lysis method, after which the cell lysate is clarified using filters. A binding buffer allows DNA to be bound to a silica membrane in a column, after which it may be eluted using MilliQ water at 65˚C. Following the DNA extraction, 10 µL of the DNA, along with two microliters 6X Loading Dye, was run on a 1% agarose gel with a 1kb Generuler to determine whether the DNA is intact. The quality and quantity of the DNA, as well as its purity, was assessed via spectrophotometric

measurements made using a Nanodrop 2000. After extraction, the DNA was stored at -20˚C.

5.4 Individual Diagnosis

The AY status of the individual plants was determined using a triple nested Polymerase Chain Reaction (PCR) assay. Refer to Table 1 for primers and PCR conditions of each PCR-reaction. The first and second primer sets amplify regions of the DNA which are phytoplasma-specific, whereas the third primer pair amplifies the 16s rDNA region specific to AY. The DNA extracted from each sample was subjected to the above mentioned PCR protocol. The master mix for all the PCR reactions was made and aliquoted inside a Biocap, after exposing all the instruments, tubes and MilliQ water to UV radiation for a minimum of 10 minutes. This minimises the possibility of contamination producing false positives.

In the first PCR reaction, 1µL of the extracted DNA is added to the reaction, undiluted. In the second PCR reaction, the PCR1 product is diluted 30 times, and 1µL of this dilution is added to the reaction mix. Finally, the second PCR product is diluted 1:10, and 1µL of this dilution is added to the third PCR reaction mix. Three controls were used when conducting the PCR assays: one plant positive that was extracted during the previous study, a no template negative and a plant negative. After the PCR assays were conducted, 10µL of the final product, along with 2µL 6X loading dye, was run on a 1% agarose gel next to a 1kB Generuler. The absence of a band on this gel indicates that the plant sample is AY negative. A band corresponding to 1100bp will lead to a positive diagnosis for AY.

Table 1: The Nested PCR primers and reaction conditions used for AY diagnosis of the grapevine plants.

PCR reaction	**Primers**		**First hold**		**Cycle 35 X**						**Final hold**	
1	P1 (Deng *et al. 1991)*	5'-AAG AGT TTG ATC CTG GCT CAG GAT T-3'	5'	94˚C	20'	94˚C	30'	55˚C	45'	72˚C	7'	72˚C
	P7 (Schneider *et al.* 1995)	5'-CGT CCT TCA TCG GCT CTT-3'										
2	R16F2n (Gunderson and Lee1996)	5'-GAA ACG ACT GCT AAG ACT GG-3'	2'	94˚C	1'	94˚C	2'	58˚C	3'	72˚C	10'	72˚C
	R16R2 (Lee *et al.* 1994)	5'-TGA CGG GCG GTG TGT ACA AAC CCC G-3'										
3	R16(I)F1 (Lee *et al.* 1994)	5'-TAA AAG ACC TAG CAA TAG G-3'	2'	94˚	1'	94˚C	2'	50˚C	3'	72˚C	10'	72˚C
	R16(I)R1 (Lee *et al.* 1994)	5'-CAA TCC GAA CTG AGA CTG T-3'										

6. RESULTS AND DISCUSSION

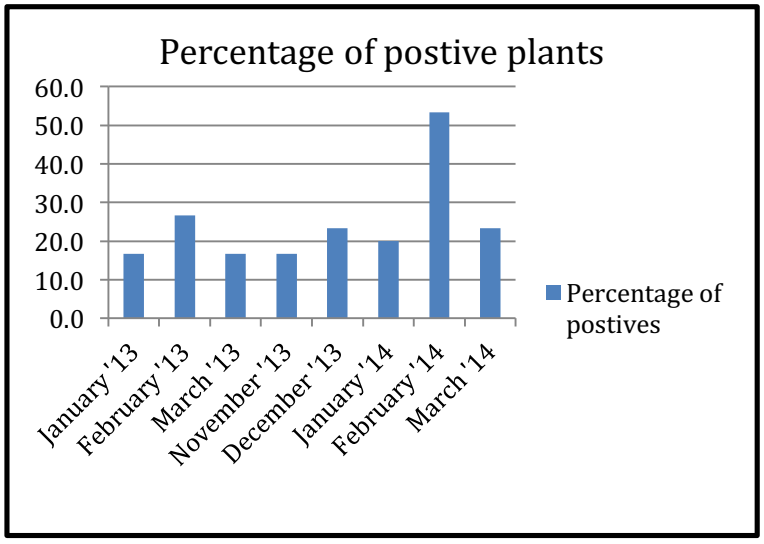
Objectives 1 and 2

Diagnostics of samples in 'recovery' as well as the 20 young plants placed in the same vineyard in January 2013 presented negative for Aster Yellows phytoplasma. This may provide further evidence to the theory of recovery phenotype through pruning. The final sampling of these plants were also done in the beginning of 2015. DNA extraction and PCR screening for AY infection are underway.

Objectives 3, 4 and 5

Temporal/seasonal distribution

Month	Number of positive plants out of a total of 30	Percentage of positive plants (%)
January 2013	5	16.7
February 2013	8	26.7
March 2013	5	16.7
November 2013	5	16.7
December 2013	7	23.3
January 2014	6	20.0
February 2014	16	53.3
March 2014	7	23.3



The months that showed the most positive samples out of the 30 collected are February 2013 and February 2014. There is a definite trend showing temporal distribution of the pathogen. Abiotic factors could be the possible cause of the variability of the infection, however it suggests that February is the time of the year where diagnosis is most accurate.

The new samples collected at the beginning of this year (2015) are being extracted for pCR screening to add temporal data for an additional season to the existing two years.

Spatial Distribution

The results of the diagnostics of the three whole plants are shown in the following tables:

Plant 1

Sample Type	Positive	Total	%	% of Total
Bark Scrapings	2	9	22%	3%
Cane Scrapings	4	12	33%	7%
Leaf	4	20	20%	7%
Petioles	1	8	13%	2%
Root	0	12	0%	0%
Total	11	61	18%	

Plant 2

Sample Type	Positive	Total	%	% of Total
Bark Scrapings	3	7	43%	7%
Cane Scrapings	3	7	43%	7%
Leaf	2	13	15%	5%
Petioles	0	3	0%	0%
Root	2	12	17%	5%
Total	10	42	24%	

Plant 3

Sample Type	Postive	Total	%	% of Total
Cane Scrapings	3	6	50%	11%
Leaf	1	8	13%	4%
Petioles	0	6	0%	0%
Root	0	7	0%	0%
Total	4	27	15%	

Cane scrapings yielded the most positive samples out of all the sample types in the three plants. Bark scrapings also yielded a few positives, however petioles, roots and leaves showed the least positives. This suggests that the cane of grapevine plants could provide more accurate diagnosis of Aster Yellows phytoplasma as this is where the pathogen titer is the highest.

7. COMPLETE THE FOLLOWING TABLE

Milestone	Target Date	Extension Date	Date completed	Achievement
1. Sampling vineyard “recovery phenotype through pruning”,	July 2013		July 2013	Completed
2. DNA extraction and AY diagnostics (samples MS1)	Jan 2014		Feb 2014	Completed

3. Identification of field plants for AY spatial and temporal distribution	March 2014		Mar 2014	Completed
4 Identification of AY infected plants, AY diagnostics and back pruning (first time)	June 2014		Jun 2014	Completed
5. DNA extraction and AY diagnostics	December 2015		Dec 2015	Completed

8. CONCLUSIONS

The erratic distribution of phytoplasmas within their host plant has been suggested by many researchers, yet the research which is available, especially concerning grapevine, is extremely limited. This study, focussing on the months of March and April, corresponds to previously attained data by Smyth (2014) and Gibb *et al.* (1999). It reports that warmer months are the most accurate time point for phytoplasma (specifically AY) diagnoses in grapevine, with a sharp decline in positive diagnoses as the months grow colder. After comparing diagnoses made using cane scrapings to those made using petiole samples, cane scrapings proved to be the most reliable plant organ to use for diagnostics. This also concurs with the findings of Smyth (2014), in which cane scrapings were the most accurate compared to petioles being the least accurate. This comparison was, however, only performed in the month of March. Future studies may need to repeat this comparison in February to say with complete certainty that the cane scrapings are indeed the most effective organ to test for the presence of AY phytoplasma. Some of the symptomatic RP plants included in this study had tested positive for AY after a coppicing event six years prior. Because some of the reinfection plants, as well as the negative plants, had also displayed symptoms and tested positive for AY, it is likely that the diagnosed infection was introduced into the vineyard by an insect vector, and is not a result of the original infection prior to coppicing. The hypothesis that induction of an RP results in a permanent recovery is therefore still viable. In order to confirm that the RP phenomenon leads to a permanent remission in AY symptoms and infection, further studies need to be conducted, if possible in the complete absence of possible insect vectors. The data obtained through this project contributes to a better understanding of both the spatial and temporal distribution of AY in grapevine, ultimately leading to advances in the accurate diagnosis, as well as insights into a possible treatment in the form of RP induction.

For more detail on project GenUS12/1 please refer to the MSc thesis of Ms N. Smyth
http://scholar.sun.ac.za/handle/10019.1/96990

9. ACCUMULATED OUTPUTS

a) TECHNOLOGY DEVELOPED, PRODUCTS AND PATENTS

A reliable PCR-based diagnostic assay for AY in grapevine, based on optimal time and tissue type for sampling.
An MSc thesis

b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

The Recovery Phenotype phenomenon needs to be investigated further

c) HUMAN RESOURCES DEVELOPMENT/TRAINING

Student Name and Surname	Student Nationality	Degree (e.g. MSc Agric, MComm)	Level of studies in final year of project	Graduation date	Total cost to industry throughout the project
Honours students					
Ane vd Vyver	RSA	BSc Hons	Honours	2015	0
Masters Students					
Natalie Smyth	RSA	MSc	Masters	2015	0
PhD students					
None					
Postdocs					
Support Personnel					
					0

d) PUBLICATIONS (POPULAR, PRESS RELEASES, SEMI-SCIENTIFIC, SCIENTIFIC)

10. BUDGET

a) TOTAL COST SUMMARY OF THE PROJECT

YEAR	CFPA	DFTS	Deciduous	SATI	Winetech	THRIP	OTHER	TOTAL
					0	0		0

*** THRIP Paid only 46% of allocation.**

b) FINAL BUDGET/FINANCIALS OF PROJECT

Please ensure that the budget is sufficiently detailed and add notes to explain all significant variations from the budget – you may submit this in an EXCEL document. Please report on the budget for the entire duration of the project. Add additional rows if required.

Project duration	Proposed budget	Actual cost incurred	Variance	Notes
TOTAL INCOME				
Industry Funding				
PHI Funding				
Other Funding				
TOTAL EXPENDITURE				
Running Expenses				
General operating costs (printing, communication, etc.)				
Local Travel				
Publication costs				
Lab Analysis				
Lab Consumables				
Other				
Running expenses SUB-TOTAL				
HR Administration and Project Management				
HR Technical				
HR Research				
Student Bursaries				
HR SUB-TOTAL				
OTHER EXPENSES				
SURPLUS / DEFICIT				

EVALUATION BY INDUSTRY

This section is for office use only

Project number	
Project name	
Name of Sub-Committee*	

Comments on project

Committee's recommendation	
• Accepted.	☐
• Accepted provisionally if the sub-committee's comments are also addressed. Resubmit this final report by_____	☐
• Unacceptable. Must resubmit final report.	☐

Chairperson_____ Date_____

*SUB-COMMITTEES

Winetech
Viticulture: Cultivation; Soil Science; Plant Biotechnology; Plant Protection; Plant Improvement;
Oenology: Vinification Technology; Bottling, Packaging and Distribution; Environmental Impact; Brandy and Distilling; Microbiology

Deciduous Fruit
Technical Advisory Committees: Post-Harvest; Crop Production; Crop Protection; Technology Transfer
Peer Work Groups: Post-Harvest; Horticulture; Soil Science; Breeding and Evaluation; Pathology; Entomology