The plum marbling conundrum:

Establishing the cause and development of a detection assay for marbling and corky flesh in Japanese plums

Hano Maree and Rachelle Bester

HORTGRO

Growing Fruit IQ

The plum marbling conundrum:

plus the

and validation

Establishing the cause ~~and~~ , development of ~~a~~ detection

~~assay~~ for marbling and corky flesh in Japanese plums

assays and their SOPs

and apricots

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Conclusions

- There is a new pathogen in stone fruit ca… …g and corky flesh
- It is spread through propagation m…
- There are multiple tests to d… …d

Questions:

- Could thi… …evented?
  - N… …remely difficult to detect and characterize, no detection method …ge in expertise
  - …t to maintain a fire station if people do not believe in fires
- Where does plant health fit into your decision model when you establish a new orchard? Trees cannot be cured from virus/viroid diseases

START CLEAN + KEEP CLEAN!

History

A pilot research project started in Nov 2017, with SAPO (Sophie Malan and Roleen Carstens) to solve the marbling in plums problem that has been present in the industry for more than 25 years.

Conclusion of the pilot project:

Marbling is a disease, associated with a novel viroid that cause marbling and/or corky flesh in plums depending on the cultivar.

Marbling task team:

Andre Smit, Rachel Kriel, Charl Stander, Andrew Hacking, Matthew Addison, Wiehann Steyn, Hugh Campbell.

Hortgro-Stone funded projects

- PL-19-USGEN-CP01: Establishing the cause of marbling and corky flesh in plums.
  - Disease characterization project
- PL-19-USGEN-CP02: Evaluation of PlVd-I transmission through top working infected trees
  - Board requested specific project
- PL-19-USGEN-CP03: Development of a hybridisation-based detection assay for PlVd-I
  - Development of a cheaper detection assay

Combined objectives of these projects

1. Determine the association of plum viroid I (PlVd-I) with marbling and corky flesh
2. Develop and validate a reliable detection assay for PlVd-I
3. Determine the possible means of spread for PlVd-I
    1. Graft transmission
    2. Seed transmission
    3. Top working, back transmission from rootstock to scion
    4. Mechanical
4. Symptom description for different cultivars
5. Develop a robust cost-effective detection assay
6. Technology transfer of detection assays to PIOs

1. Determine the association of PlVd-I with marbling and corky flesh

- HTS analyses, to construct virome profiles of symptomatic and asymptomatic plants
  - reconfirmed pilot project results
  - developed 1st generation RT-PCR assays for screening in larger surveys
- Performed surveys in two more seasons

Summary of field surveys conducted in 2017/18, 2018/19 and 2019/20

			Pos	Neg
348*	Asymptomatic	172	3	169
	Symptomatic	176	169	7

*includes 13 Apricot samples

Cultivars testing positive (commercial plantings)

African Delight
African Rose
Black Pearl
Lady Red
Laetitia
Pioneer
Purple Majesty
Ruby Crunch
Ruby Star
Ruby Sun
September Yummy
Songold
Sun Breeze

19 Farms from 8 Regions

NOTE: Observation bias due to dependency on self reporting of symptoms

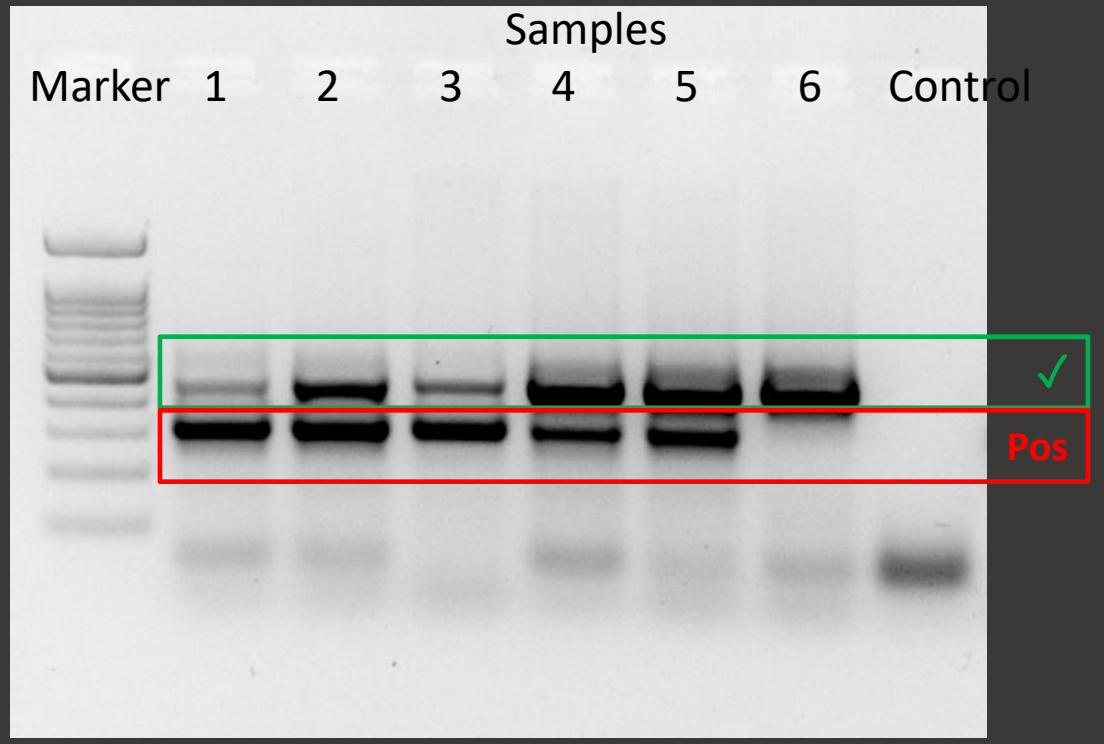
2. Develop and validate a reliable detection assay

- 2nd generation RT-PCR assay (G2) was developed and optimised using all the sequence information, HTS and Sanger.
- This assay was thoroughly validated as an endpoint RT-PCR with an internal control (G2actin).

Abilities and limitations of the assays

- 2nd generation assay has been tested and validated for the detection of 1/10 in pooled samples i.e. samples can be pooled in batches of 10. (early season)
- Cost however remains high **(~R450-R600 per test, incl. labour)**
- Good quality RNA is required for the assay

Large scale testing was never envisaged at the start of this project, but a need for more cost-effective diagnostics developed and a new focused 2-year project was started in March 2020.



3. Determine the possible means of spread

Graft-transmission?

Experimental orchard

- Half the trees inoculated with positive material (12/2018)
- Screened for PlVd-I (11/2019)
- 72/120 grafted plants tested positive
- The lack of transmission to some plants or cultivars

- 1st year fruit symptoms

Conclusion:

PlVd-I is graft transmitted

3. Determine the possible means of spread

Seed Transmission?

- Fruit from symptomatic trees (~400) were collected and seeds removed
- Seeds were germinated and 206 seedlings were sampled
- All seedlings tested negative for PlVd-I with the G1 and G2 assays

Conclusion:

PlVd-I does not seem to be seed transmitted

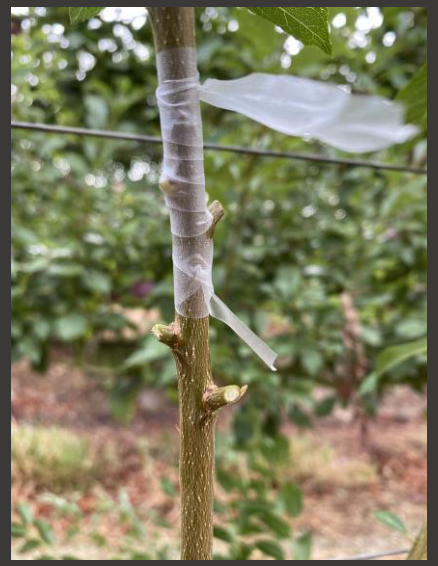
3. Determine the possible means of spread

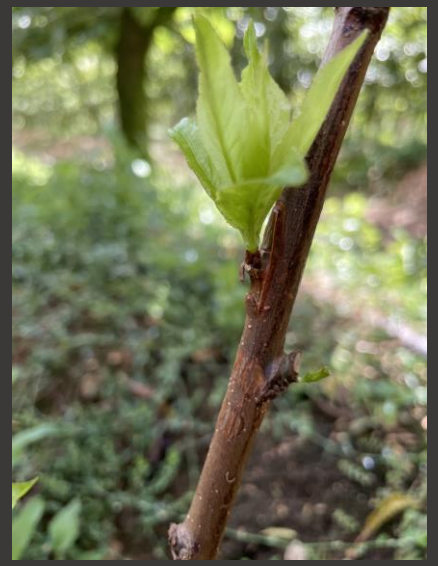
Top working, back transmission from rootstock to scion?

- Symptomatic trees in two African delight orchards were cut off below the graft union
- Regrowth was bud grafted with healthy buds
- New growth was sampled later in the season and tested for PlVd-I
- PlVd-I was detected in the new growth
  - 90% of samples tested

Conclusion:

- **Topworking PlVd-I infected material is not a viable option**
- **The top worked material will get infected**
- **RT-PCR assay (G2actin) works on rootstock material**





3. Determine the possible means of spread

Mechanical transmission?

- 25 African delight and 25 Ruby star trees were slash inoculated from an infected tree. Trees were screened before inoculation and 12 months post inoculation.
- 5 PlVd-I positive African Delight trees were planted with a healthy tree in the same pot to evaluate the probability of root grafting.
- All trees tested negative for PlVd-I.

Conclusion:

Preliminary results would indicate that mechanical transmission is a low probability event.

4. Symptom description for different cultivars

- Marbling

- Corky flesh

4. Symptom description for different cultivars

- Irregular surface

- Smaller fruit

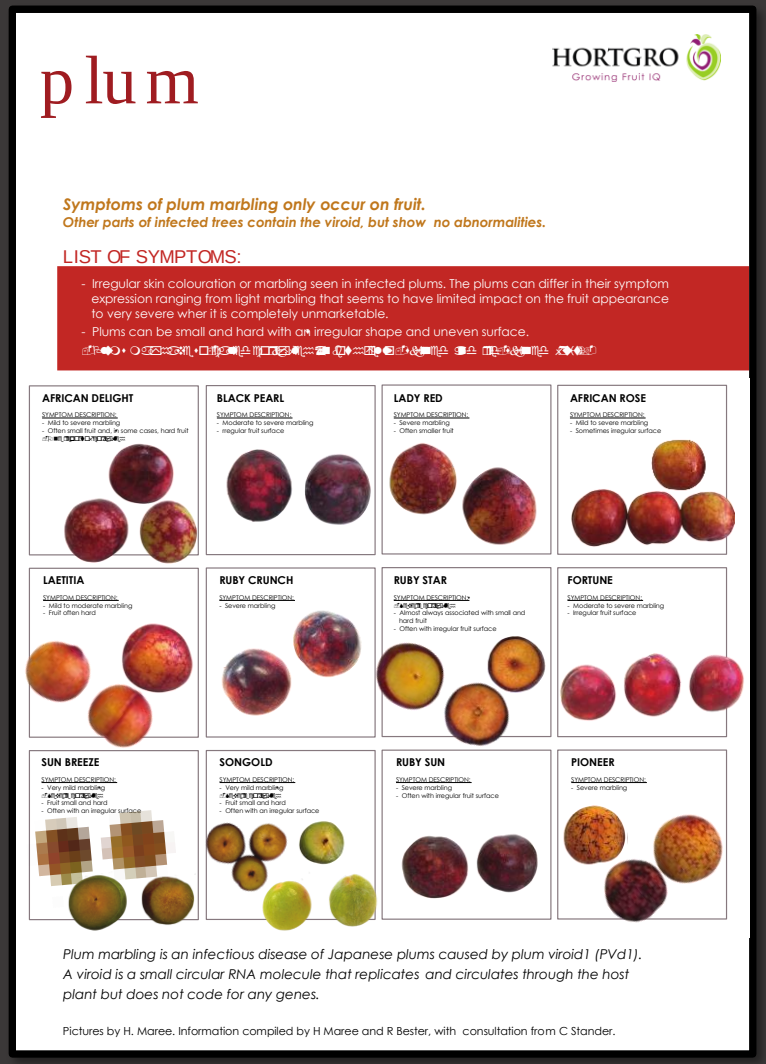
4. Symptom description for different cultivars

- **<u>Atypical symptoms</u> (Negative for Marbling)**

4. Symptom description for different cultivars

- **<u>Atypical symptoms</u> (Negative for Marbling)**

4. Symptom description for different cultivars



plum

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Symptoms of plum marbling only occur on fruit.
Other parts of infected trees contain the viroid, but show no abnormalities.

LIST OF SYMPTOMS:

- Irregular skin colouration or marbling seen in infected plums. The plums can differ in their symptom expression ranging from light marbling that seems to have limited impact on the fruit appearance to very severe wher it is completely unmarketable.
- Plums can be small and hard with an irregular shape and uneven surface.

AFRICAN DELIGHT
SYMPTOM DESCRIPTION:
- Mild to severe marbling
- Often small fruit and, in some cases, hard fruit

BLACK PEARL
SYMPTOM DESCRIPTION:
- Moderate to severe marbling
- irregular fruit surface

LADY RED
SYMPTOM DESCRIPTION:
- Severe marbling
- Often smaller fruit

AFRICAN ROSE
SYMPTOM DESCRIPTION:
- Mild to severe marbling
- Sometimes irregular surface

LAETITIA
SYMPTOM DESCRIPTION:
- Mild to moderate marbling
- Fruit often hard

RUBY CRUNCH
SYMPTOM DESCRIPTION:
- Severe marbling

RUBY STAR
SYMPTOM DESCRIPTION:
- Almost always associated with small and hard fruit
- Often with irregular fruit surface

FORTUNE
SYMPTOM DESCRIPTION:
- Moderate to severe marbling
- Irregular fruit surface

SUN BREEZE
SYMPTOM DESCRIPTION:
- Very mild marbling
- Fruit small and hard
- Often with an irregular surface

SONGOLD
SYMPTOM DESCRIPTION:
- Very mild marbling
- Fruit small and hard
- Often with an irregular surface

RUBY SUN
SYMPTOM DESCRIPTION:
- Severe marbling
- Often with irregular fruit surface

PIONEER
SYMPTOM DESCRIPTION:
- Severe marbling

Plum marbling is an infectious disease of Japanese plums caused by plum viroid1 (PVd1). A viroid is a small circular RNA molecule that replicates and circulates through the host plant but does not code for any genes.

Pictures by H. Maree. Information compiled by H Maree and R Bester, with consultation from C Stander.

FRUIT SYMPTOMS

Cultivar	*Marbling*	*Corky Flesh*	*Hard*	*Small*	*Irregular*
African Delight	*** ***	*	*	**	-
Black Pearl	***	-	-	-	***
Lady Red	***	-	?	**	-
Laetitia	***	-	**	-	-
Pioneer	***	-	-	?	-
Ruby Crunch	***	-	-	?	-
Ruby Star	-	***	***	***	**
Ruby Sun	***	-	?	?	**
Songold	***	***	***	***	**
Sun Breeze	***	***	***	***	**

*** Indicates the approximate frequency of the symptom. (* <25%, ** 25-75%, *** >75%)**
Colour indicated the severity of the symptom (Mild, Moderate, Severe)

TRANSMISSION - HOW CAN IT BE TRANSMITTED?

- Plum viriod I is not transmitted through seed. This is important as it means that it cannot be transmitted by the fruit.
- Primary spread of marbling is through the planting of infected plant material.
- Mechanical transmission of PVd1 during routine practices such as pruning is also highly probable.

CONTROL STRATEGIES

- Mark, remove and burn all infected trees.
- Disinfect pruning equipment.

Organisation.

TESTS – CONTACT POINTS

Stellenbosch University.

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Contact persons:
Lize Burger
- lize@saplant.co.za
Roleen Carstens
- roleen@saplant.co.za

Disease Clinic
Department of Plant Pathology
Stellenbosch University
Contact persons:
Sonja Coertze
- 021 808 4798 / sc2@sun.ac.za
Tammy Jensen
- 021 808 4223 / tammy@sun.ac.za

ATYPICAL SYMPTOMS
(NEGATIVE FOR MARBLING)

LAETITIA

PIONEER

ANGELENO

PlVd-I in *Prunus armeniaca* cv. Charisma

- The first observation of apricot fruit with an uneven, indented surface and irregular shape was in the 2003/2004 season.
- In the summer of 2019, representative samples were collected, and the presence of PlVd-I confirmed using the G2 assay only in the symptomatic samples.
- Apricot sample was also assayed by HTS and an additional latent virus not associated with the symptoms but highly similarity to solanum nigrum ilarvirus 1 (SnIV1) was identified.

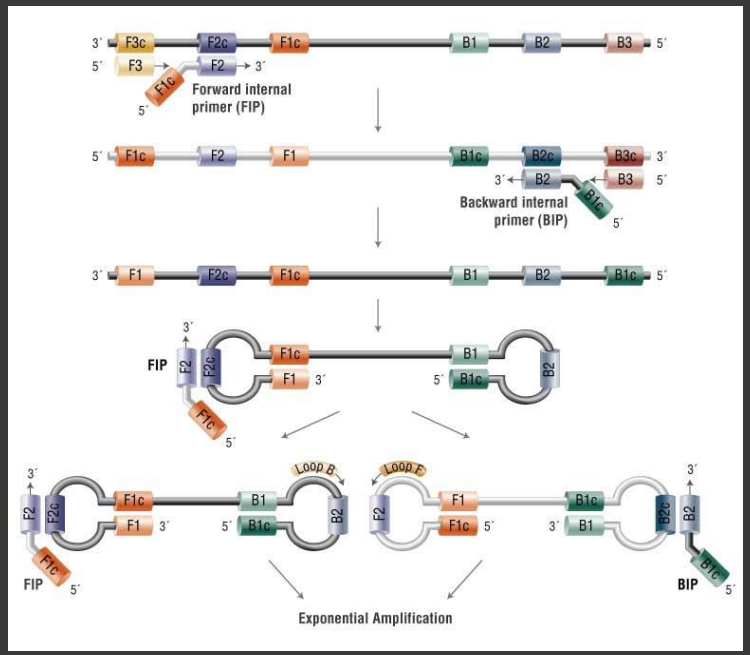
5. Develop a robust cost-effective detection assay

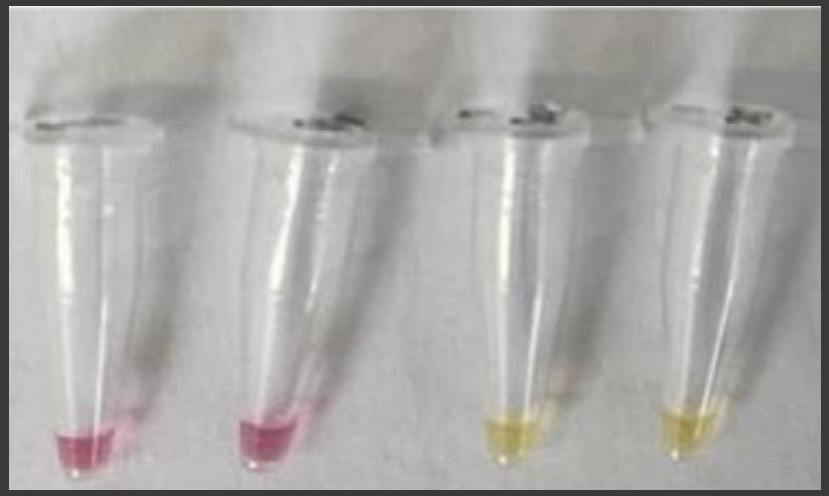
Aim: reduce cost per assay to allow for mass screening of plant material

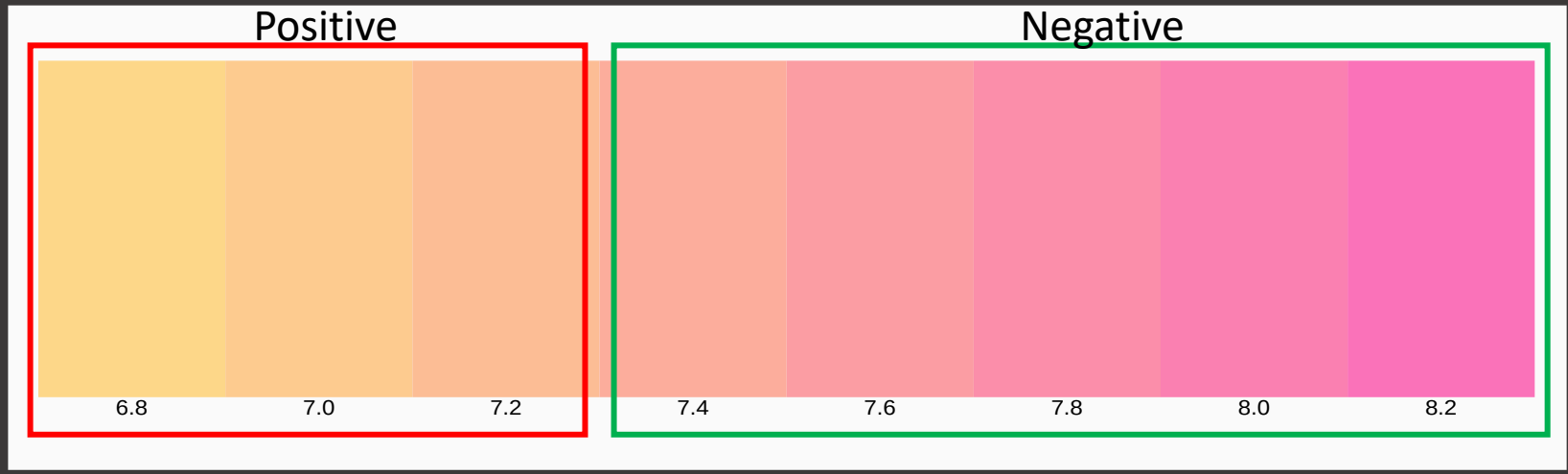
- A hybridization assay was designed. This technology was shown in the past to work on stone fruit viroids. International collaborator assisted remotely. However, the sensitivity required for a robust assay could not be achieved. Progress in this project was severely impacted by covid restrictions.
- Covid pandemic also created opportunities.
- Switch technology to LAMP

5. Develop a robust cost-effective detection assay

- Loop mediated isothermal amplification (LAMP)
  - A technique that was applied for the detection of various plant pathogens in the last two decades
  - Many successes and failures due to the technique being highly sensitive, but very prone to contamination
  - Many adaptations and tweaks has been applied, but recently New England Biolabs developed a LAMP kit that is able to directly amplify target RNA (used as Covid test)

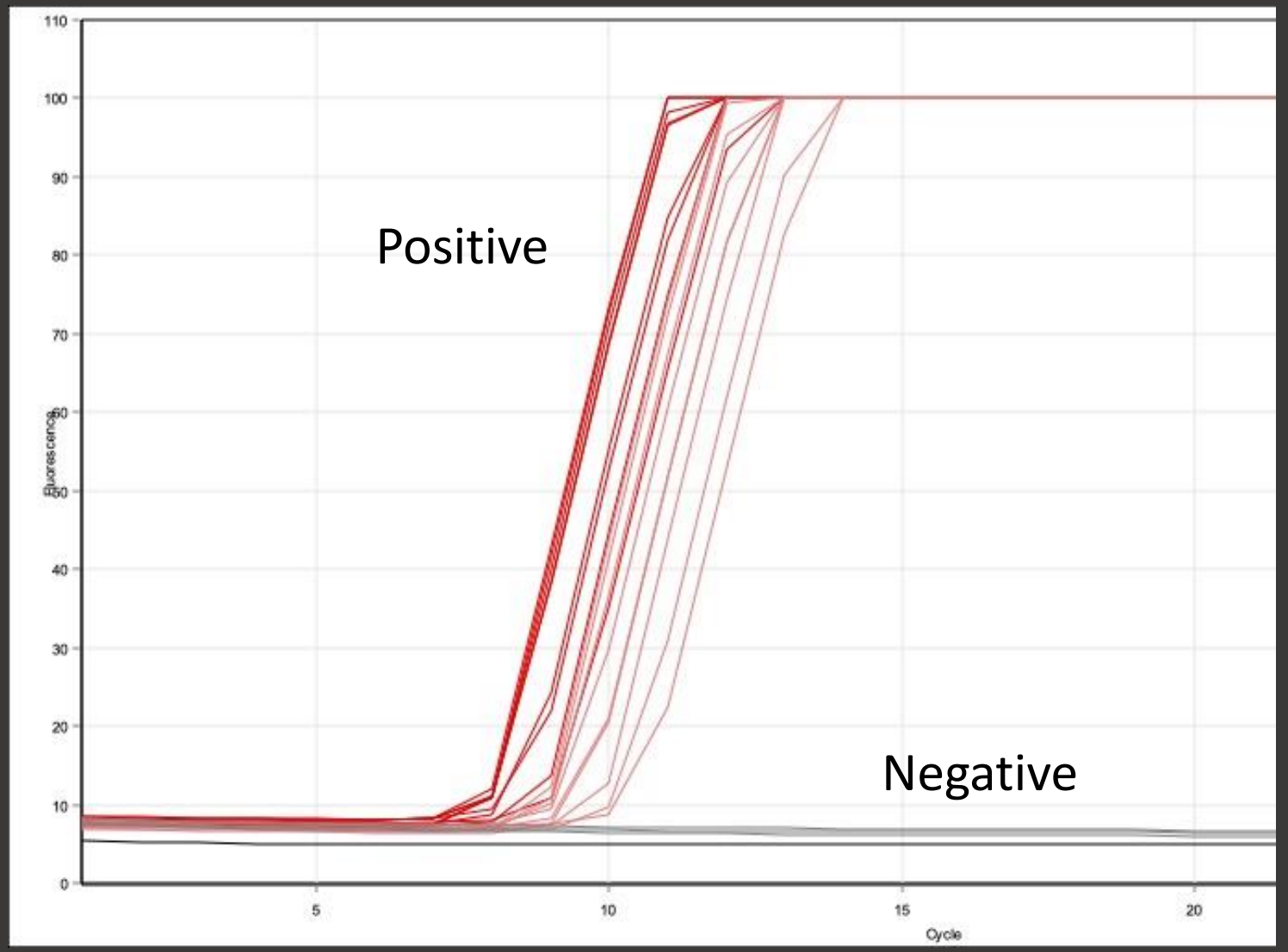






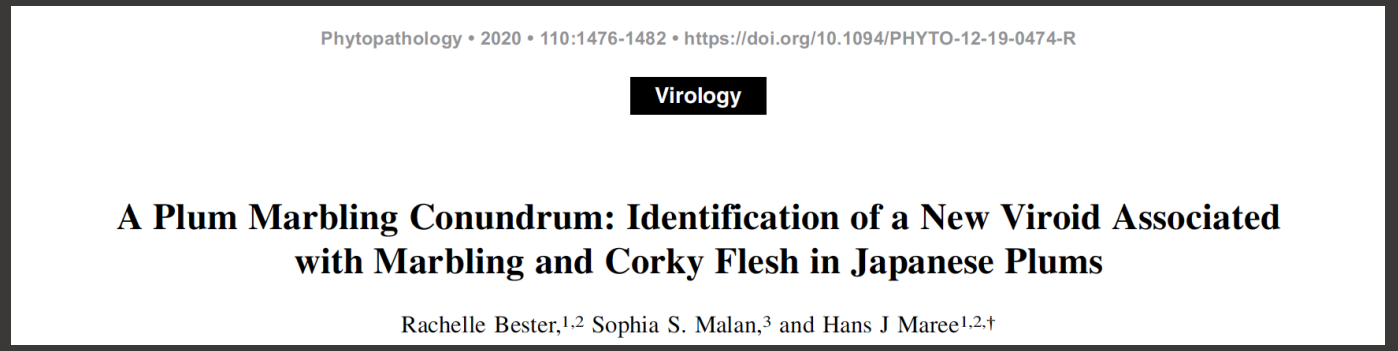
Real-time LAMP assay

- Advantages
  - Not pH dependent
  - More sensitive
- Disadvantage
  - Once-off cost of real-time PCR instrument



6. Technology transfer of detection assays to PIOs

- SOPs for the different assays were compiled and provided along with training to PIO.
- Assay G2(Actin), an RT-PCR assay, is most sensitive and accurate assay but also most expensive.
- Two LAMP assay options, colorometric or real-time. A lot cheaper than RT-PCR
  - The real-time LAMP is more sensitive. But requires real-time PCR instrument.

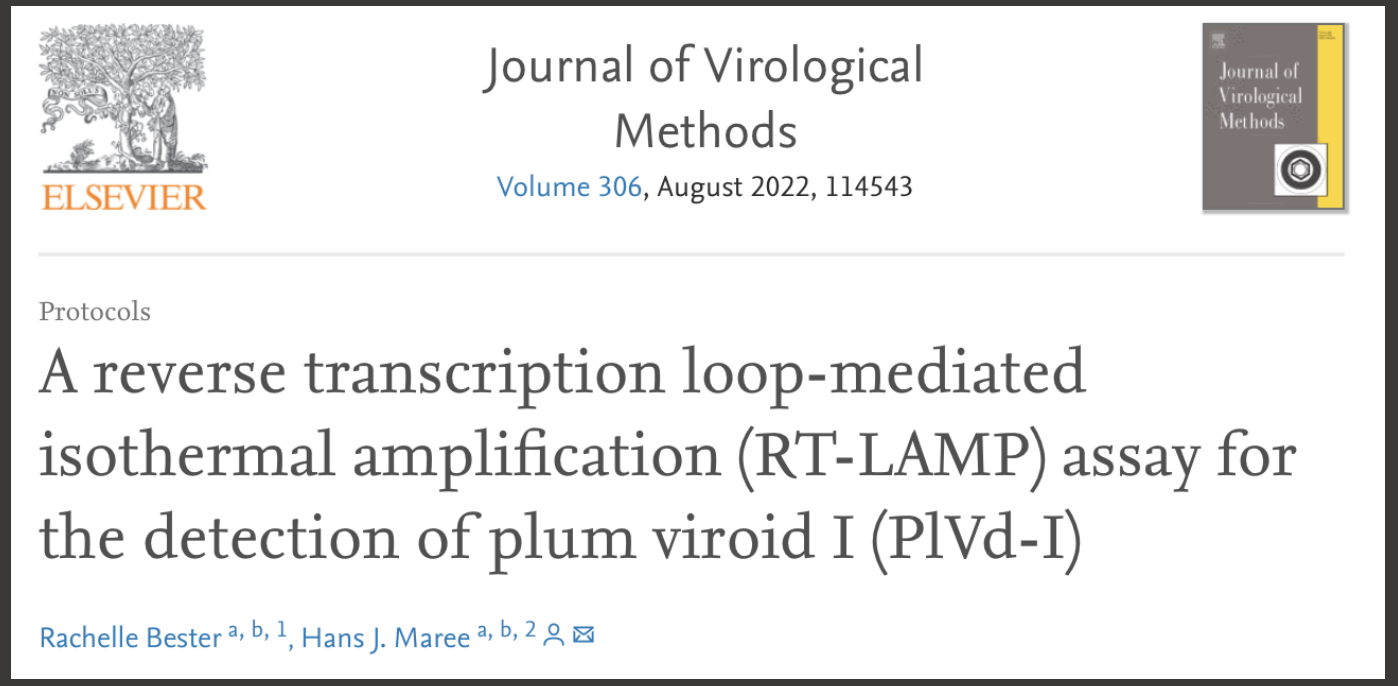


Phytopathology • 2020 • 110:1476-1482 • https://doi.org/10.1094/PHYTO-12-19-0474-R

Virology

A Plum Marbling Conundrum: Identification of a New Viroid Associated with Marbling and Corky Flesh in Japanese Plums

Rachelle Bester,[1,2] Sophia S. Malan,[3] and Hans J Maree[1,2,†]



ELSEVIER

Journal of Virological Methods

Volume 306, August 2022, 114543

Protocols

A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of plum viroid I (PlVd-I)

Rachelle Bester [a, b, 1], Hans J. Maree [a, b, 2]

Acknowledgements

- Plum producers for granting access to their orchards.
- Marbling task team: Sophie Malan, Andre Smit, Rachel Kriel, Charl Stander, Andrew Hacking, Roleen Carstens, Matthew Addison, Wiehann Steyn, Hugh Campbell.

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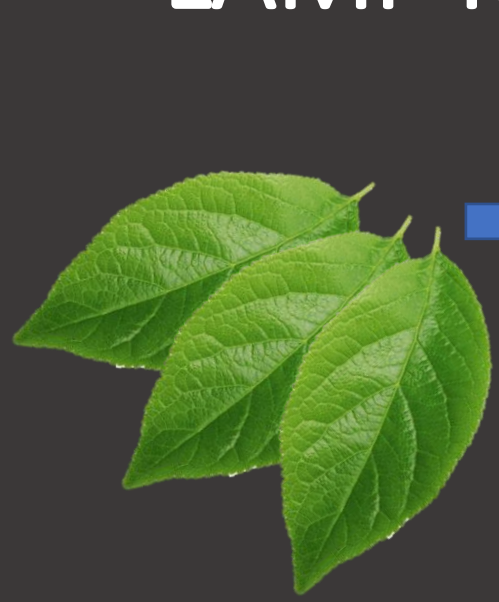
Stellenbosch UNIVERSITY IYUNIVESITHI UNIVERSITEIT

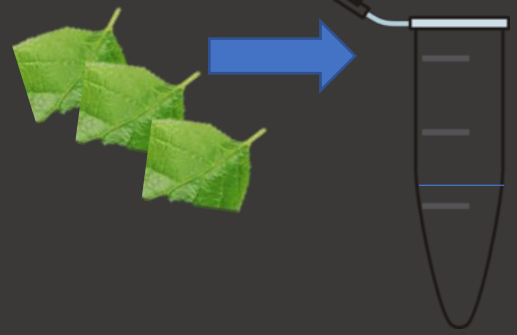
forward together
sonke siya phambili
saam vorentoe

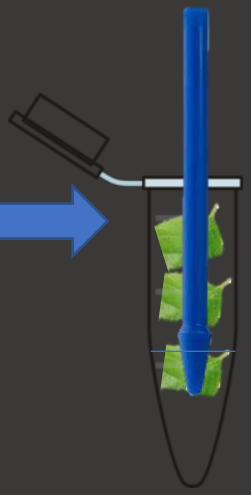
SAPO trust

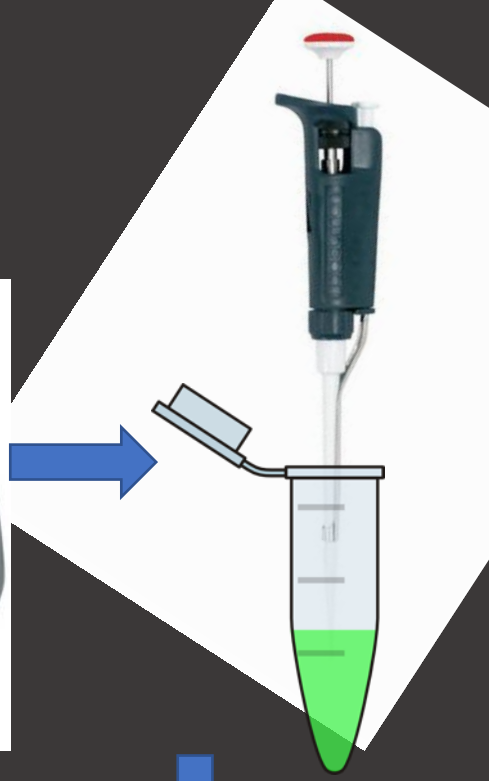
Cultivating future growth

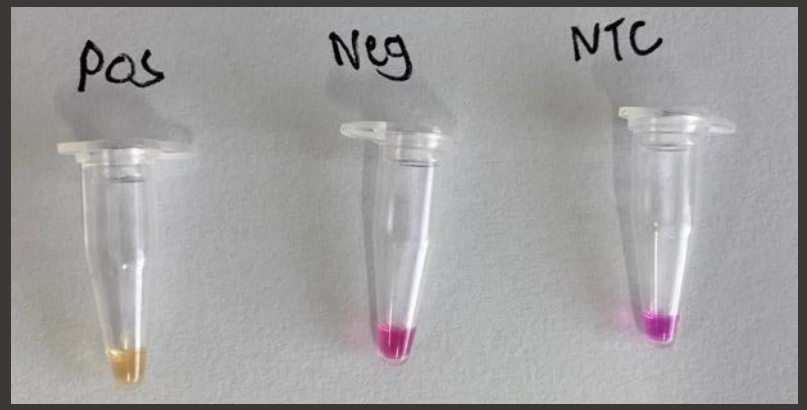
LAMP Method 1





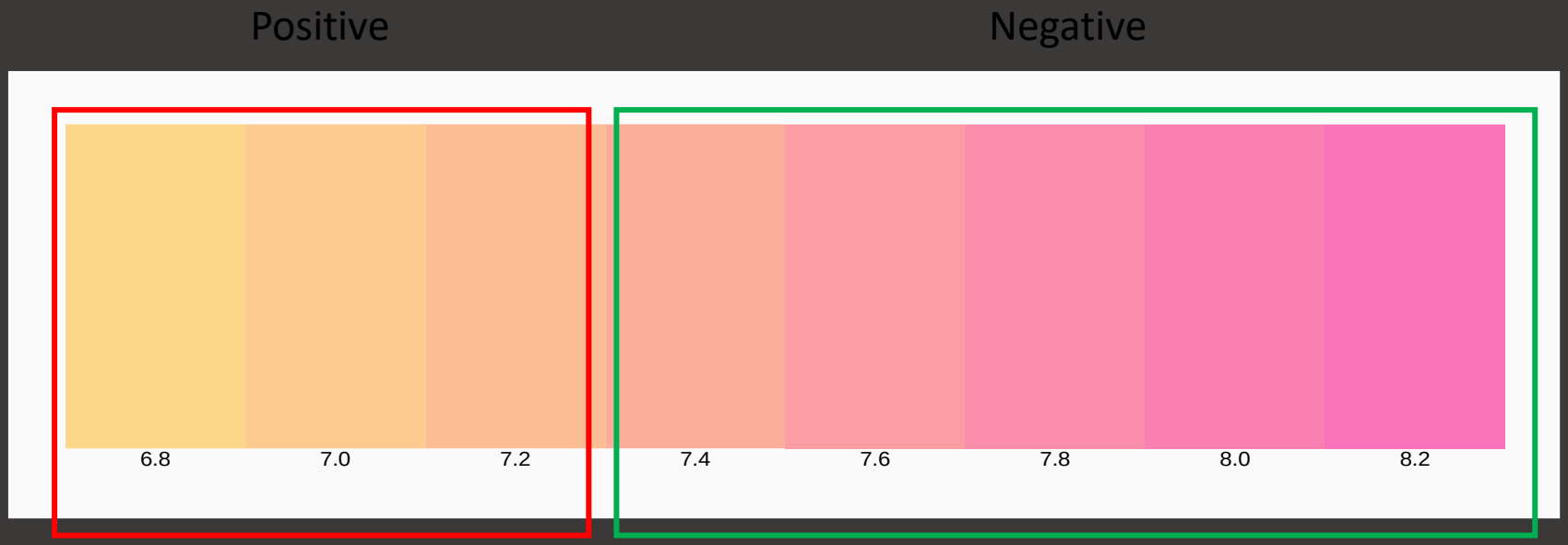




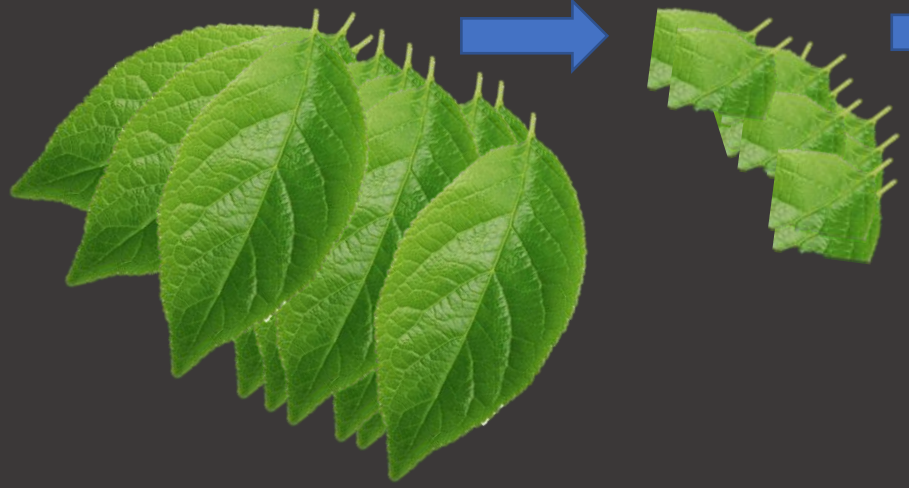


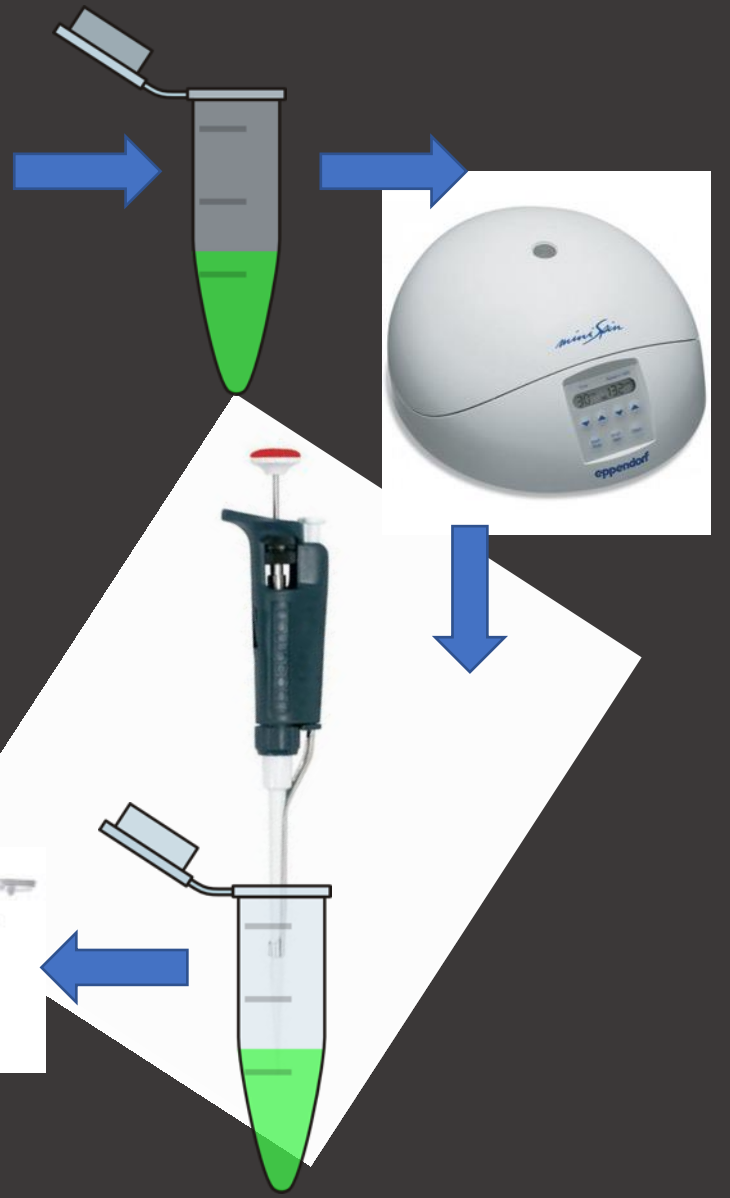
LAMP assay colour range

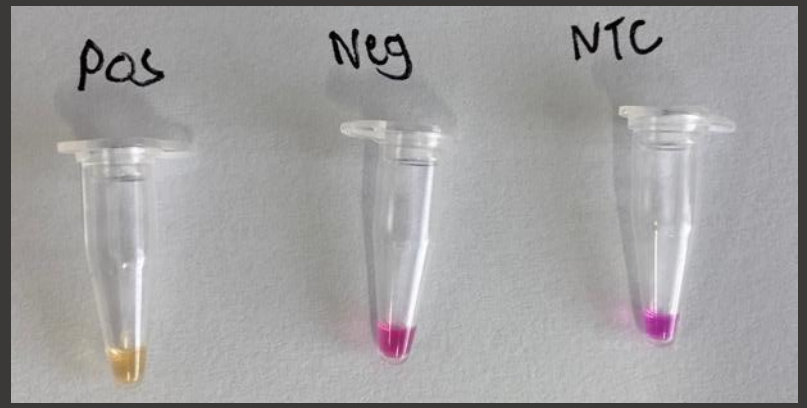
- Assay pH dependent (phenol red)
- Start as a basic solution and as more amplicons are made the pH of the solution turns acidic (DNA, Deoxyribonucleic acid)



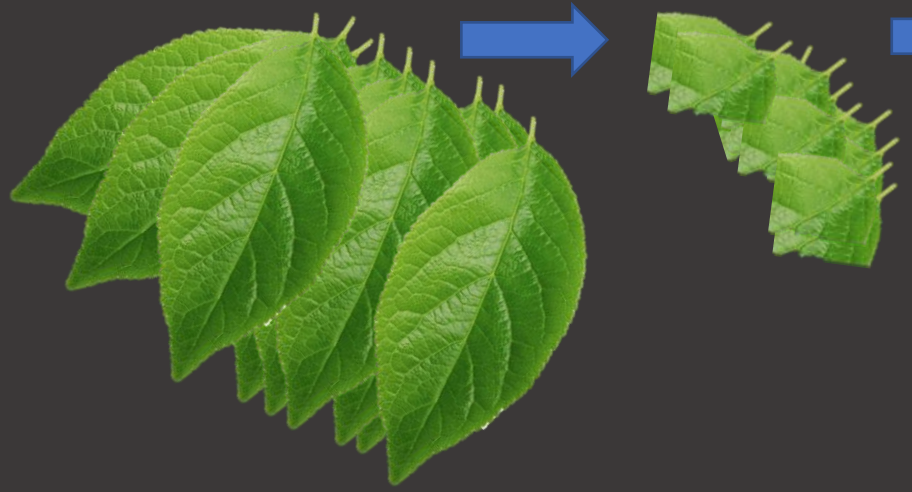
Method 2 (Pooling)

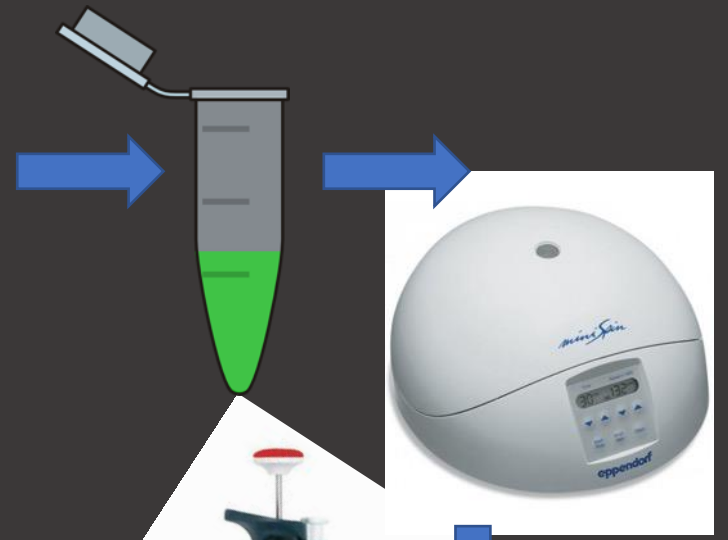


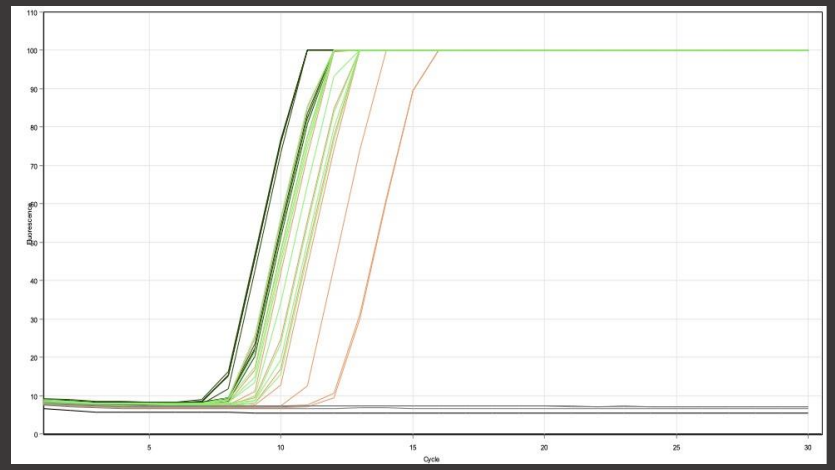




Method 3 (Real-time)







Cost per assay

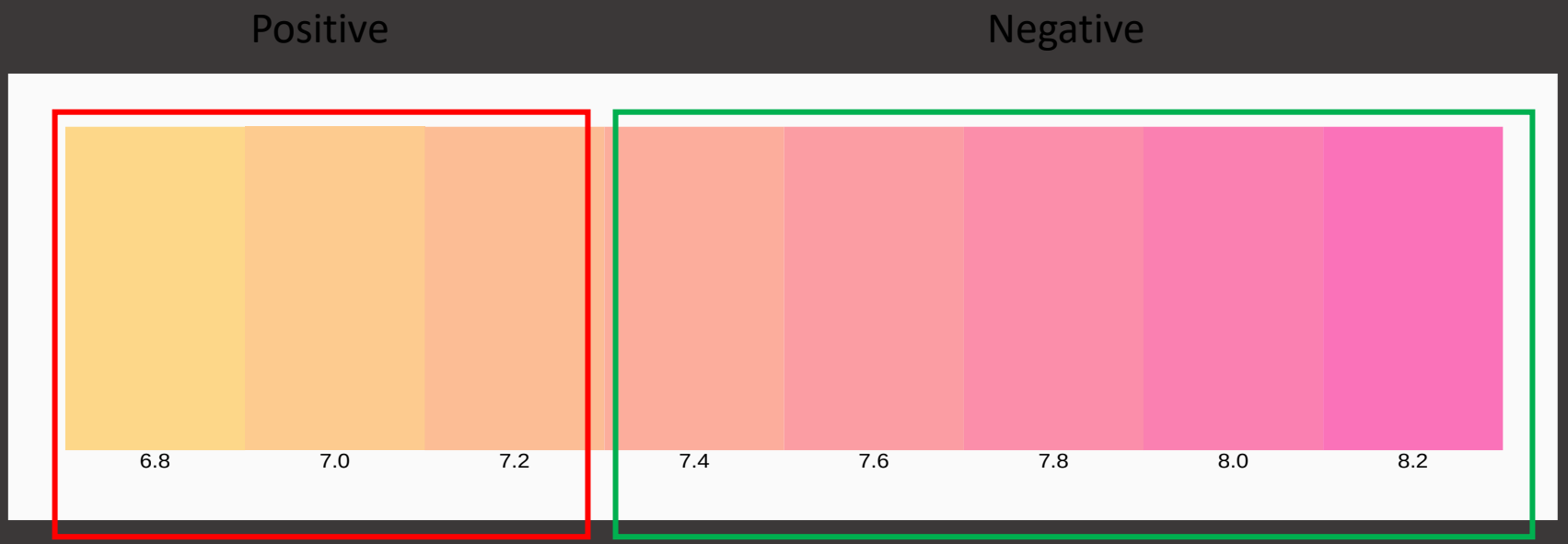
- Direct cost without labour

Method 1	Method 2	Method 2	Method 2	Method 3 (Method 1/2)
Eppi assay	Bioreba bag assay	Bioreba bag 1/10 assay	2x Bioreba bag 1/20 single assay	Real-time LAMP assay
R21/tree	R28/tree	R2.80/tree	R1.40/tree	R21 (R2.80/R1.40) + R468 534.15

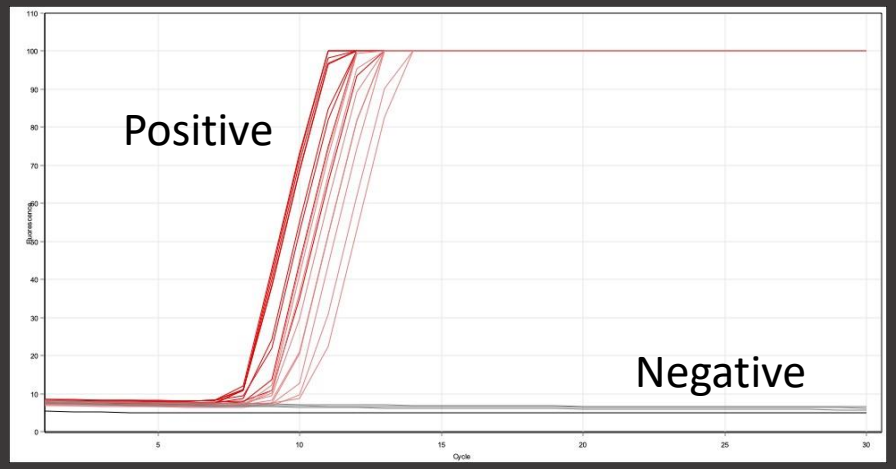
Recommendation: Assay in duplicate

Real-time LAMP assay

- Advantages
  - Not pH dependent
  - More sensitive

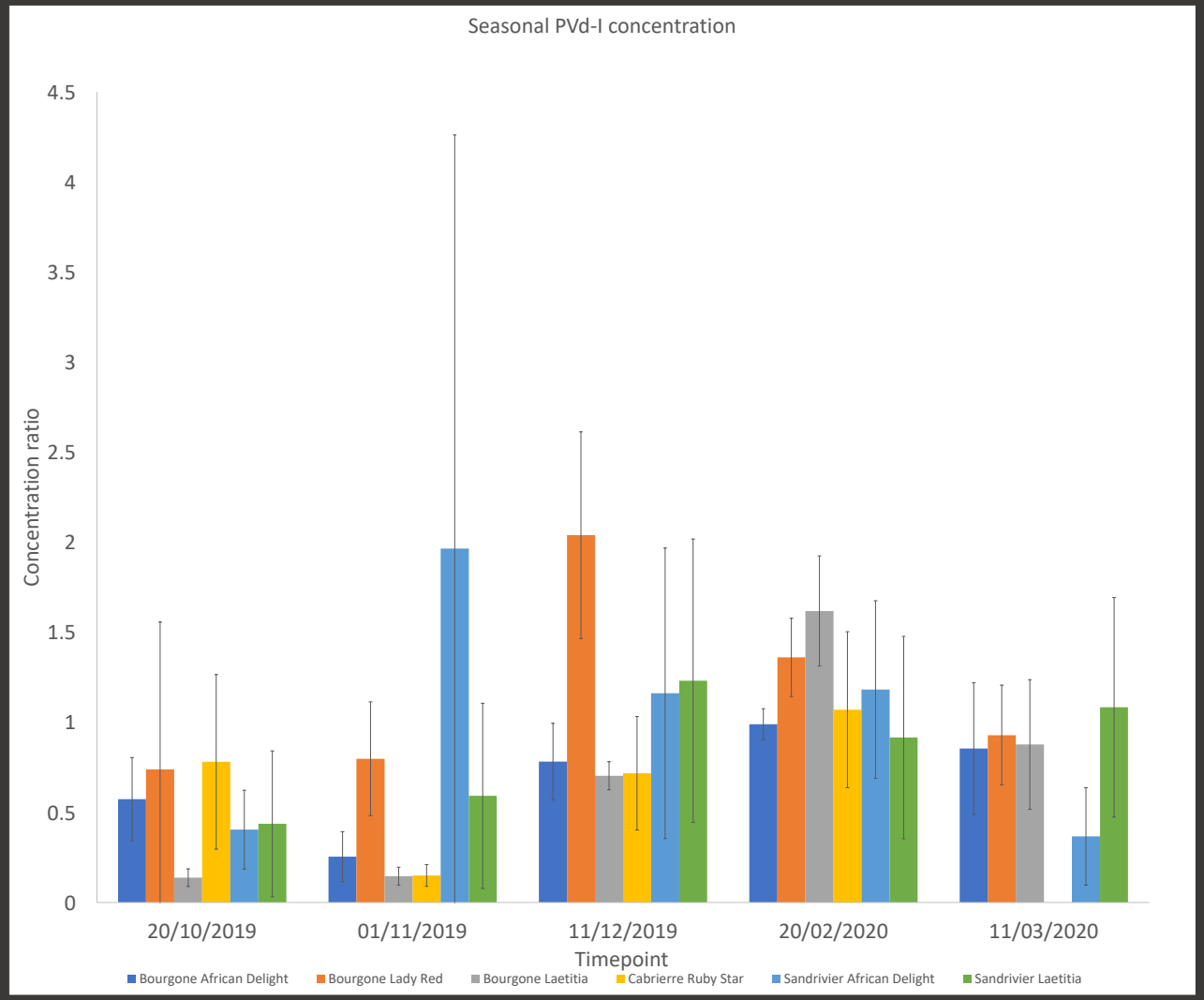


- Disadvantage
  - Once-off cost of qPCR instrument, R469 000



Optimisation of an RT-qPCR (G3) to monitor seasonal titre

- The G2 RT-PCR was converted to an RT-qPCR (G3) to monitor seasonal titre fluctuations to determine optimal time for sampling.
- PlVd-I detectable throughout the growing season
- No statistically significant titre fluctuations detected.
- RNA quality more challenging later in the season



Outputs

- International peer-reviewed scientific papers
  - Bester, R., Malan, S.S., Maree, H.J., 2020. A Plum Marbling Conundrum: Identification of a New Viroid Associated with Marbling and Corky Flesh in Japanese Plums. Phytopathology 110, 1476–1482. https://doi.org/10.1094/PHYTO-12-19-0474-R
  - Bester, R., Maree, H.J., 2020. First report of plum bark necrosis stem pitting-associated virus in Japanese plums in South Africa. Plant Disease. https://doi.org/10.1094/PDIS-04-20-0710-PDN
  - Bester, R., and Maree, H.J. 2022. A reverse transcription loop-mediated isothermal amplification (RT-LAMP) assay for the detection of plum viroid I (PlVd-I) . Journal of Virological Methods. 306, 114543. DOI: 10.1016/j.jviromet.2022.114543

  Planned
  - Bester, R. and Maree, H.J. 2021. Plum viroid I autonomous replication and mechanical transmission.
  - Bester, R., and Maree, H.J. 2021. First report of plum viroid I in apricots (Prunus armeniaca) in South Africa. Plant Disease

- Fresh NOTES
  - Hortgro fresh NOTES (170) 7 February 2020. Plum Marbling and corky flesh symptoms on plums
  - Hortgro freshNOTES (178) 16 July 2020. Plum Marbling and corky flesh symptoms on plums – Update: July 2020

- Presentations
  - HORTGRO Stone Fruit Research Feedback: Virtual Research Showcase: Establishing the cause of marbling and corky fresh in plums (R Bester) – August 2020
  - Plum Marbling poster
  - Comprehensive review of Plum Marbling project and Technology transfer workshop, 30 September 2021