**GenUS8-2**

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

# FINAL REPORT
# 2013

## Programme & Project Leader Information

| | Research Organisation Programme leader | Project leader |
|---|---|---|
| **Title, initials, surname** | Prof JT Burger | Dr D Stephan |
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## Project Information

| | |
|---|---|
| **Research Organisation Project number** | GenUS 8/2 |
| **Project title** | The effect of antimicrobial peptides on the grapevine pathogen Aster Yellows Phytoplasma |

| | | | |
|---|---|---|---|
| **Fruit kind(s)** | Wine grapes | | |
| **Start date** (mm/yyyy) | 01/01/2009 | **End date** (mm/yyyy) | 31/12/2012 |

| | |
|---|---|
| **Project keywords** | Aster Yellows Phytoplasma, Antimicrobial peptides |

Approved by Research Organisation Programme leader (tick box) ☐

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

## Executive Summary

Give an executive summary of the total project.

This study is aimed at investigating the efficacy of antimicrobial peptides (AMPs) against Aster Yellows (AY) phytoplasma, using a transient expression system. This should lead to a more time and cost efficient approach of screening various AMPs against grapevine pathogens. Thus far, three candidate AMPs (D4E1, VvAMP1 and SN1) have been identified and D4E1 and VvAMP1 have been tested for their efficacy to reduce bacterial titres of the two bacterial grapevine pathogens *Xylophilus ampelinus* and *Agrobacterium vitis*, *in vitro* (D4E1) and *in planta* (D4E1 and VvAMP1) using the transient expression system developed in this study. In these experiments it was shown, using a semi-quantitative real-time PCR protocol, that bacterial titres were significantly reduced (by over 80%) in leaves of *Vitis vinifera* cv. Sultana when D4E1 was transiently expressed. No reduction in bacterial titre was observed when VvAMP1 was transiently expressed.

Initial difficulties to establish phytoplasma-infected grapevine plants *in vitro*, led to a new sterilization procedure being optimised, which yielded AY-infected plant material of the *V. vinifera* cv's Chardonnay and Chenin blanc successfully established *in vitro*. These plants were multiplied *in vitro* and evaluated for their AY phytoplasma status. Similarly, healthy plant material was established *in vitro*. Additionally, a VvAMP1-transgenic *V. vinifera* cv. Sultana plant was obtained from IWBT (M. Vivier) and was multiplied *in vitro* and in the greenhouse. The VvAMP1-transgenic *in vitro* plants were used in grafting experiments of AY phytoplasma-infected grapevine material onto VvAMP1-transgenic plants. To allow for the quantification of phytoplasma titres in grapevine, a qRT-PCR protocol was established. To allow infection of *Nicotiana benthamiana* with AY phytoplasma, healthy periwinkle plants were set up in a heavily AY phytoplasma-infected grapevine block in the Vredendal region.

## Problem identification and objectives

State the problem being addressed and the ultimate aim of the project.

Phytoplasmas are of increasing significance around the world, and due to the recent discovery in SA vineyards, could be highly problematic to the SA wine industry. This pathogen is known to have caused disastrous effects in vineyards in European countries, resulting in significant reductions in fruit yield and wine quality. This new emerging disease could be controlled using viticultural practices because of the low base of infection at present. However, if the disease is not controlled it could result in an infection incidence of 80-100% within certain regions and within a short period of time. Therefore, a long term approach to control through the development of resistance against the pathogen is desirable and should be investigated and implemented before phytoplasma diseases becomes established in SA vineyards. This project intends to explore the efficacy of an approach to provide resistance against the phytoplasma pathogen, and provide a mechanism to incorporate resistance within grapevine to control this devastating emerging disease.

This study aims to investigate efficiency of antimicrobial peptides (AMPs) against AY phytoplasma in grapevine, by making use of transient expression vectors. The effect of AMPs will be tested *in vivo* for phytoplasma. For *in vivo* tests, the effect of AMPs expressed by transgenic grapevine plants and AMPs expressed by a transient expression vector will be compared. Phytoplasma-infected and healthy plants will also be grafted onto VvAMP-1 transgenic plants. These grafted plants will be used to determine the effect of VvAMP-1 transgenic Sultana plants as a resistance mechanism against AY phytoplasma.

Additionally, transmission experiments are planned to infect periwinkle with AY phytoplasma. For this, periwinkle plants have already been placed in infected vineyards. Infected plants will then be grafted onto the herbaceous host *N. benthamiana*. Testing of AMP-effects in that plant species is done as an additional control to grapevine plants.

For the remaining of 2011, the project aims to validate the developed qPCR procedure for AY quantification, to determine the AY phytoplasma status of *in vitro* plantlets and finalize the cloning of the AMP Snakin1 from potato and from a grapevine homologue into the already established expression cassettes. The effect of the peptides D4E1, VvAMP1, $SN1_{potato}$ and $SN1_{vitis}$ on AY phytoplasma titres using the transient expression system will be evaluated until mid of 2012.

The *in vitro* grafting of AY phytoplasma infected material onto VvAMP1-transgenic *V. vinifera* cv. Sultana and a possible reduction in AY phytoplasma titres in transgenic plants will be evaluated until the end of 2012. As soon as phytoplasma-infected periwinkle material is available (planned October 2011), grafting experiments onto *N. benthamiana* will start.
compared.

## Workplan (materials and methods)

List trial sites, treatments, experimental layout and statistical detail, sampling detail, cold storage and examination stages and parameters.

- Candidate AMPs will be identified and their efficiency tested *in vitro* against *A. vitis* and *X. ampelinus*
- Transient expression vectors containing AMP genes will be constructed and their infectivity tested in *Nicotiana benthamiana*
- Healthy grapevine plants will be agrobacterium vacuum-infiltrated with the expression vectors and tested against *A. vitis* and *X. ampelinus* infection
- Phytoplasma infected plants will be identified and established *in vitro*
- **Phytoplasma infected plants will be agro vacuum-infiltrated with the viral vector**
    - *As it was shown in the experiments involving X. ampelinus and A. vitis, the viral expression system based on GVA is not sufficient in grapevine (phloem limitation of expression) and therefore the 35S-constructs will be used. Two additional AMPs (SN1 from potato and grapevine) will be tested using the 35S-construct instead.*
- **Existing AMP transgenic plants will be infected with *A. vitis* and *X. ampelinus***
    - *As the transient expression of VvAMP1 did not lead to a reduction of both bacteria (in contrast to D4E1-expression), the testing of transgenic expressed VvAMP1 is unnecessary. Additionally, it was shown earlier that VvAMP1 does not show an effect against bacteria in studies done at the IWBT. Nevertheless, the effect of transgene derived VvAMP1 on AY phytoplasma will still be evaluated.*
- Phytoplasma infected plants will be grafted onto the existing AMP transgenic plants
- AMPs efficiency will be tested by measuring microbial titres and disease development

## Results and discussion

State results obtained and list any industry benefits. If applicable, include a short discussion covering ALL accumulated results from the start of the project. Limit it to essential information only.

The transient expression of AMPs was successfully tested for the two AMPs D4E1 and VvAMP1. As we were only now able to establish AY phytoplasma-infected material *in vitro*, the effect of both peptides was tested against the grapevine infecting bacterial pathogens *X. ampelinus* and *A. vitis*. We observed a significant reduction in bacterial titres after D4E1 treatment *in vivo* and *in planta* of both bacterial pathogens. The effect of D4E1 was tested in vitro by a conventional plate based system and by the established transient expression approach in *V. vinifera* cv. Sultana.

When D4E1 was added to bacterial liquid cultures a significant reduction of *A. vitis* cfu on non-selective agar plates was observed, even when the peptide was applied at a concentration as low as 1.5625 µg ml$^{-1}$ (P = 0.02967). When a D4E1 concentration of 3.125 µg ml$^{-1}$ or higher was applied, no *A. vitis* cfu were observed anymore. A higher concentration of D4E1 was needed to substantially reduce *X. ampelinus* cfu on non-selective agar plates. Between the applied D4E1 concentrations of 6.25 and 12.5 µg ml$^{-1}$ the number of *X. ampelinus* cfu was significantly (P = 0.0318) reduced from over 1000 cfu to an average of 20 cfu (Fig. 1). Further reduction in *X. ampelinus* cfu was observed when D4E1 concentrations were increased to 12.5 and 50 µg ml$^{-1}$, respectively. On all control plates without D4E1 treatment, more than 1000 cfu were observed consistently. On all kanamycin treated positive controls no cfu were observed for either of the bacterial species.

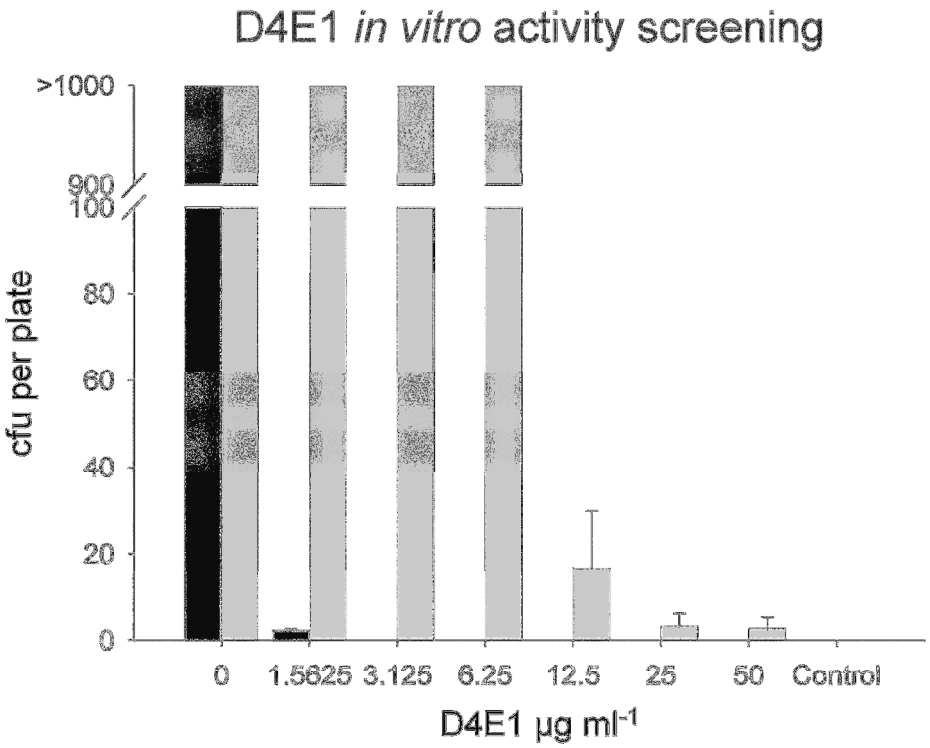


**Figure 1** Bar graph showing the average number of cfu per 100 µl of *A. vitis* ( ■ ) or *X. ampelinus* ( ■ ) liquid cultures after no peptide treatment or treatment against a dilution series of D4E1 concentration, from 1.5625 µg ml$^{-1}$ to 50 µg ml$^{-1}$. Kanamycin treated liquid cultures served as positive control.

To establish a semi-quantitative real-time PCR procedure for *A. vitis* and *X. ampelinus*, firstly, standard curves were set up for the grapevine reference gene actin. To allow semi-quantitative measurement of *X. ampelinus* titers, total extracted DNA from grapevine, containing both grapevine and bacterial DNA, was used in a dilution series from 24 ng to 0.0384 ng. A DNA dilution series of 60 ng to 0.096 ng of total extracted DNA was used to set up a standard curve for the determination of *A. vitis* titers. The Rotor-Gene Q Series Software 1.7 obtained threshold values (Ct) from the amplification curves (fluorescence against cycle numbers) by automatically setting the threshold, and plotted it on a graph against the logarithm of their relative concentrations to construct the final standard curves (Fig. 2a). The PCR reaction efficiencies (*E*) and linearity ($R^2$) of the curves were also calculated by the software and all amplifications were found all to have high efficiencies and linearity values of at least 0.97 and 0.99, respectively. Melting curve analysis indicated single products in all reactions (Fig. 2b).

The efficacy of D4E1 *in planta* was tested by comparing the bacterial titers of *X. ampelinus* or *A. vitis* in grapevine leaves pre-agroinfiltrated with either transient D4E1-expressing or non-

expressing (empty binary vector) constructs. A group-wise comparison was performed between treated and untreated groups to compare the difference in pathogen titers. This was achieved by qPCR using grapevine actin and *X. ampelinus* or *A. vitis* specific primers and comparing the titers in REST. When the obtained Ct values were processed with the REST program the average concentration of *X. ampelinus* in the treatment group was found to be 82% lower than the average concentration of the control group. This points towards a 5.5 times reduction (P = 0.004) in *X. ampelinus* concentrations as a result of D4E1 exposure resulting from peptide expression from the transient expression vector in grapevine tissue. Between the D4E1 treatment and control group, REST analysis indicated that there was no statistical difference in the average *A. vitis* concentrations of the 5 repetitions, although the average of 3 out of the 5 treated plants showed significant lower *A. vitis* concentrations (P < 0.001). In these 3 plants, the average reduction in *A. vitis* concentration was 95%.

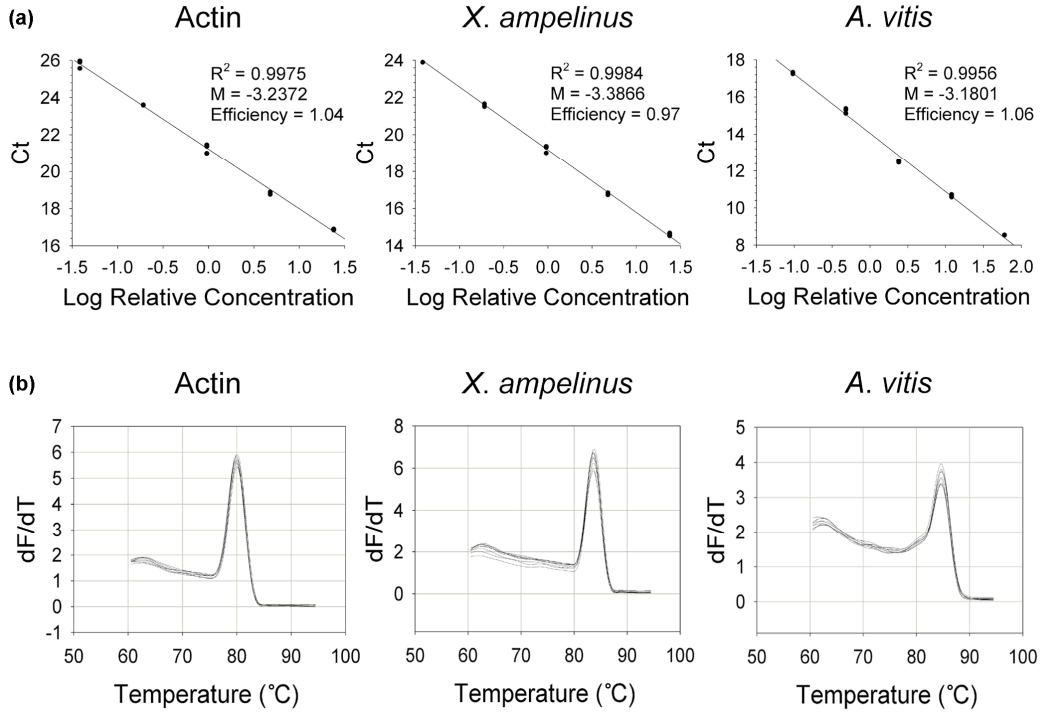


**Figure 2** (a) Standard curves for qPCR analyses of a 5-fold DNA dilution series using primers for the grapevine actin gene, *X. ampelinus* and *A. vitis* quantification. M: slope; $R^2$: linear correlation coefficient. (b) Melting curve profiles of qPCR amplification of the grapevine actin gene, *X. ampelinus* and *A. vitis*.

In order to allow for a semi-quantitative AY phytoplasma titre determination, a qPCR procedure was established. Semi-quantitative qPCR was performed using SYBR® Green I detection chemistry. Primer AY-F (5'-AAACCTCACCAGGTCTTG-3') was based on a qPCR primer described by Hollingsworth *et al*. (2008) and AY-R (5'-AAGTCCCCACCATTACGT-3') based on the AY phytoplasma-specific qPCR TaqMan probe described by Angelini *et al*. (2007), to yield an amplicon size of 172 bp from the AY 16SrDNA. In a first attempt, the qPCR procedure was compared to the conventional AY detection via nested-PCR (primers P1/P7 in the first PCR round, followed by a second PCR using primer pair R16F2n/R16R2) in a dilution series.

The qPCR primer pair AY-F/AY-R amplified the expected 172 bp fragment. Sequencing of fragments confirmed that AY phytoplasma is detected. No unspecific amplification was observed. To determine the detection threshold for these primers, in comparison to

conventional nested-PCR, both methods were used to screen a 10-fold dilution series prepared from an AY phytoplasma positive DNA sample (Fig 3). The qPCRs were able to detect the AY phytoplasma up to a dilution of $10^{-3}$ (Figure 3A, reaction 4), while the conventional nested-PCR reaction was able to detected AY phytoplasma up to a dilution of $10^{-1}$ (Figure 3B).

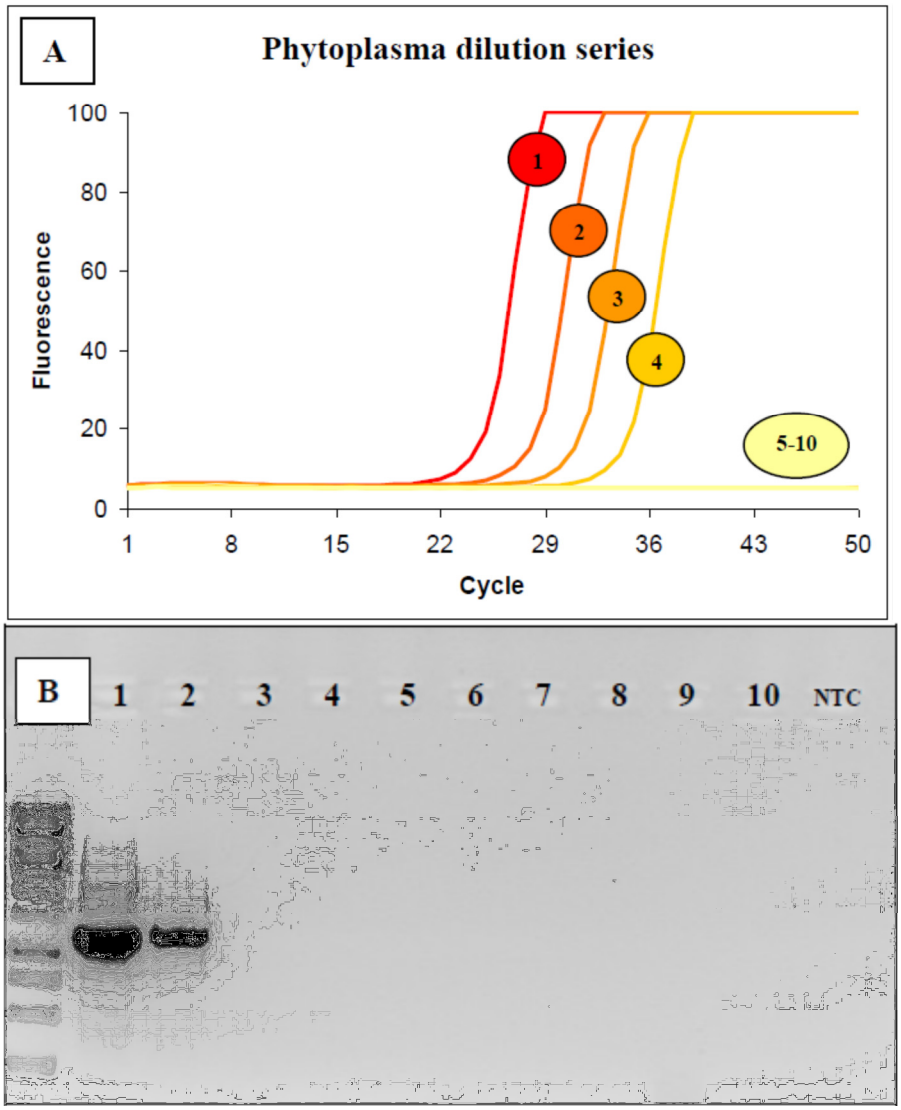


**Figure 3** Comparison of the sensitivity of qPCR vs. standard nested-PCR to detect AY phytoplasma in a dilution series. A) Real-time PCR amplification profiles and (B) agarose gel with nested-PCR products of ~1.2 kb. Reaction 1) $10^{0}$ dilution; Reaction 2) $10^{-1}$ dilution; Reaction 3) $10^{-2}$ dilution; Reaction 4) $10^{-3}$ dilution; Reactions 5-10) $10^{-4}$ to $10^{-9}$ dilutions and no template negative control (NTC).

The SYBR® Green-based qPCR procedure for AY phytoplasma detection was successfully established. This procedure will be further refined by adding a plant reference gene. Additionally, the SYBR® Green-based procedure will be compared in reliability and sensitivity to a probe-based assay.

**Additional work:**

As no AYp-infected Chardonnay material could be established and maintained *in vitro*, the question arose whether plant material might have been taken from a part of the cane where

phytoplasma titre was very low or totally absent. Therefore, the spatial distribution of AYp in five canes of an infected Chardonnay plant was investigated. Aster yellows phytoplasma was found predominantly in the nodes when compared to leaf material collected in the late season. Lastly, AYp infection was mostly detected in the upper, expanding parts of the cane when compared to lower sections. Further analysis on a bigger cohort of plants is needed to fully understand the spatial distribution of AYp throughout grapevine, taking different seasons and cultivars into consideration.

The comprehensive results of this project are available in the form of an MSc thesis, which is available at: http://hdl.handle.net/10019.1/80066

Complete the following table

| Milestone | Target Date | Extension Date | Date Completed | Achievement |
|---|---|---|---|---|
| 1. Candidate AMPs | | | | Four AMPs (D4E1, VvAMP1, $SN1_{potato}$ and $SN1_{vitis}$) were identified. |
| 2. Expression vectors | | | | 35S expression vectors were constructed for D4E1, VvAMP1 and $SN1_{vitis}$. The $SN1_{potato}$ fragment was amplified, sequenced and cloned into the 35S expression construct. |
| 3. Establishment of Phytoplasma infected plants *in vitro* | | | | AY phytoplasma infected plants of the *V. vinifera* cv's Chardonnay and Chenin Blanc were established in vitro. The AY phytoplasma infection status of in vitro plantlets was determined. |
| 4. Stock-up infected and healthy Periwinkle | | | | Healthy Periwinkle plants have been established and have been placed into an AY phytoplasma infected grapevine block in the Vredendal region. |
| 5. Stock-up infected VvAMP1-transgenic grapevine | | | | An infected VvAMP-1 transgenic Sultana plant was provided by the IWBT and has been stocked-up *in vitro* and in the greenhouse. |
| 6. Monitor disease symptoms | | | | Disease symptoms were monitored in glasshouse and *in vitro* established plants. |
| 7. Grafting of infected/healthy | | | | Compatibility of *V. vinifera* cv and Sultana plants have been tested |

| | | | | |
|---|---|---|---|---|
| grapevine onto transgenic plants | | | | and the procedure has been optimized. |
| 8. Grafting infected Periwinkle onto *N. benthamiana* | | | | Infected Periwinkle plants were established (see 4). Grafting onto *N. benthamiana* had limited success. |
| 9. Quantitative real-time PCR | | | | Optimisation has been completed for *A. vitis* and *X. ampelinus* quantification. qPCR for phytoplasma detection has been optimized, and shown to be more sensitive and reliable than conventional PCR. |
| 10. Transient expression of AMPs | | | | Expression constructs for all AMPs (D4E1, VvAMP1, $SN1_{potato}$ and $SN1_{vitis}$) were completed and are available. The effect of all four these expression vectors against phytoplasma must still be tested.<br>Tested AMP efficacy against A. vitis and X. ampelinus:<br>**D4E1 expression *in planta*:** 82% reduction of bacterial titer of *A. vitis* and<br>**VvAMP1 expression *in planta***: no significant reduction in titer for *A. vitis and X. ampelinus* |

**Accumulated outputs**

List ALL the outputs from the start of the project. The year of each output must also be indicated.

Visser, M., Snyman, M., Stephan, D., Burger, J.T. (2011) Development of a real-time PCR for semi- quantitative detection of Aster yellows phytoplasma. Second International Phytoplasmologist Working Group Meeting (IPWG), 12.-16. September, Neustadt/Weintstr., Germany.

Spinas, N.L., Snyman, M., and Visser, M, Stephan, D. and Burger J.T. (2012). Can antimicrobial peptides be used to engineer resistance against the grapevine pathogen aster yellows phytoplasma? 17th Meeting of the International Council for the Study of Virus and Virus-like diseases of the grapevine (ICVG), 7th-14th October, Davis, California, USA.

Spinas, N.L., Snyman, M., and Visser, M, Stephan, D. and Burger J.T. (2012). Attempts to establish a transient assay to test the efficiency of antimicrobial peptides against phytoplasma. COST Action FA0807 Working Group 3 Meeting, Phytoplasma Control in Crop Systems, 14th-16th November, San Michele all'Adige, Italy.

Spinas, N.L., Snyman, M., Visser, M., Stephan, D., and Burger, J.T. 2012. Can antimicrobial peptides be used to engineer resistance against the grapevine pathogen aster yellows phytoplasma? 33rd Conference of the South African Society for Enology and Viticulture, Franschoek, South Africa.

MSc thesis, N.L. Spinas:
The efficacy of the antimicrobial peptides D4E1, VvAMP-1 and Snakin1 against the grapevine pathogen aster yellows phytoplasma. (http://hdl.handle.net/10019.1/80066)

Conclusions

In this study we validated the use of transient expression systems which express antimicrobial peptides (AMPs), to study the in planta effect of these peptides against the grapevine pathogen aster yellows phytoplasma (AYp). We focused on four AMPs: Vv-AMP1, D4E1, SN1 isolated from potato and SN1 isolated from grapevine. The Agrobacterium-mediated expression system used during the current study, was successfully used for the in planta expression of D4E1 against *Agrobacterium vitis* and *Xylophilus ampelinus*, resulting in a reduction of both bacterial titres (Visser et al., 2012).
In the present work, no AYp-infected *Vitis vinifera* cv 'Chardonnay' material could be established by micro-propagation, starting from AYp-infected vineyard-growing plants as source material. Difficulties to establish and maintain phytoplasma infections in micro-propagated material has been observed in *V. vinifera* infected with Flavescence dorée, in mulberry plants infected with mulberry dwarfism phytoplasma, in sugarcane infected with sugarcane yellows phytoplasma and in Lebanese almonds infected with 'Candidatus P. phoenicium' (Gribaudo et al., 2007; Caudwell, 1961; Dai et al., 1997; Parmessur et al., 2002; Chalak et al., 2005). Factors involved in the natural recovery of phytoplasma-infected plants are not fully understood yet, although it seems reasonable that the interactions between the pathogen, the host and the environment may play a key role, as well as the involvement of grapevine bacterial or fungal endophytes (Musetti et al., 2007; Bulgari et al., 2009). As no AYp-infected Chardonnay material could be established and maintained *in vitro*, the question arose whether plant material might have been taken from a part of the cane where phytoplasma titre was very low or totally absent. Therefore, the spatial distribution of AYp in five canes of an infected Chardonnay plant was investigated. Aster yellows phytoplasma was found predominantly in the nodes when compared to leaf material collected in the late season. Lastly, AYp infection was mostly detected in the upper, expanding parts of the cane when compared to lower sections. Further analysis on a bigger cohort of plants is needed to fully understand the spatial distribution of AYp throughout grapevine, taking different seasons and cultivars into consideration.

Transmission experiments using the insect vector *Mgenia fuscovaria* were successful in establishing AYp- infected *Nicotiana benthamiana* and *Catharanthus roseus* plants. A quantitative real-time PCR assay, using SYBR-Green® I chemistry, was optimized during the current study for the quantification of AYp. When the *in planta* effects of the four AMPs were screened by the qPCR, a significant reduction of AYp titre was observed when compared to the positive control. This reduction in pathogen titre was also observed in the control treatment group. Therefore, no significant AYp titre differences could be seen in the AMP treatment group when compared to the control treatments. It is known that bacterial endophytes have an influence on the natural recovery of phytoplasma-infected plant hosts. A recent study has shown that infection by Agrobacterium spp. can increase the indole-3-acetic acid (IAA) concentration in AYp-infected plant material (Bulgari et al., 2012). This increase in IAA is known to decrease the phytoplasma concentration in plants and possibly explains why a decrease in AYp titre for both treatment groups was observed. These results show that the Agrobacterium-mediated transient expression

assay used during the current study, was possibly the wrong choice for the *in planta* screening of AMPs against the grapevine pathogen AYp.

Phytoplasmas lack an outer membrane and cell wall, making this pathogen an ideal target for AMPs. Developing alternate transient expression systems to reliably determine the effect of AMPs on AYp is therefore of great importance in future studies. The use of transient expression systems has the potential to play an important role in future disease resistant studies and in the improvement of grapevine, which is an economically important crop worldwide. To our knowledge, this study is the first to report on the distribution of AYp in infected grapevine material and serves as a pilot study for future research. Such studies could help in understanding plant-phytoplasma relationships better and help in determining efficient sampling procedures for accurate diagnostics.

## Technology development, products and patents

Indicate the commercial potential of this project, eg. Intellectual property rights or commercial product(s)

AY diagnostic assays: conventional RT-PCR and RT-qPCR
AMP-expression vectors

## Suggestions for technology transfer

List any suggestions you may have for technology transfer

GVE diagnostic tests are available

## Human resources development/training

Indicate the number and level (eg. MSc, PhD, post doc) of students/support personnel that were trained as well as their cost to industry through this project. Add in more lines if necessary.

| Student level (BSc, MSc, PhD, Post doc) | Cost to Project |
|---|---|
| 1.MSc (M Visser) | 0 |
| 2.MSc (N.L. Spinas) | 0 |
| 3.Postdoc (D Stephan) | 0 |
| 4. | |
| 5. | |

## Publications (popular, press releases, semi-scientific, scientific)

## Presentations/papers delivered

Oral presentations:

Visser, M., Snyman, M., Stephan, D., Burger, J.T. (2011) Development of a real-time PCR for semi- quantitative detection of Aster yellows phytoplasma. Second International Phytoplasmologist Working Group Meeting (IPWG), 12.-16. September, Neustadt/Weintstr., Germany.

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aster yellows phytoplasma? 17th Meeting of the International Council for the Study of Virus and Virus-like diseases of the grapevine (ICVG), 7th-14th October, Davis, California, USA.

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Spinas, N.L., Snyman, M., Visser, M., Stephan, D., and Burger, J.T. 2012. Can antimicrobial peptides be used to engineer resistance against the grapevine pathogen aster yellows phytoplasma? 33rd Conference of the South African Society for Enology and Viticulture, Franschoek, South Africa.

Total cost summary of the project

| TOTAL COST IN REAL TERMS | COST | CFPA | DFTS | Deciduous | SATI | Winetech | THRIP | OTHER | TOTAL |
|---|---|---|---|---|---|---|---|---|---|
| YEAR 1 | | | | | | 250000 | 83333 | | 333333 |
| YEAR 2 | | | | | | 275000 | 91667 | | 366667 |
| YEAR 3 | | | | | | 302500 | 100833 | | 403333 |
| YEAR 4 | | | | | | | | | |
| YEAR 5 | | | | | | | | | |
| **TOTAL** | | | | | | | | | **1103333** |