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FINAL REPORT
FOR 2012

PROGRAMME & PROJECT LEADER INFORMATION

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PROJECT INFORMATION

Project number	UP KK 01
Project title	Transmission of Aster yellows to grapevine by insect vectors
Project Keywords	Phytoplasma, vector, Cicadellidae, leafhoppers, *Mgenia fuscovaria*

Industry programme	**CFPA**	
	Deciduous	x
	DFTS	x
	Winetech	x
	Other	

Fruit kind(s)	Wine grapes, table grapes and raisins
Start date (dd/mm/yyyy)	15/01/2009
End date (dd/mm/yyyy)	15/01/2012

FINAL REPORT

1. Executive summary

The project was initiated to identify vector(s) of Aster yellows phytoplasma (AY) in grapevine in South Africa. Controlled transmission experiments with field-collected *Mgenia fuscovaria* (Stål, 1855) (Hemiptera: Cicadellidae) demonstrated that this species can transmit AY to grapevine. A field trial was undertaken to determine the time of AY infection in the field using grapevine and periwinkle as bait plants and relate time of infection with leaf-/planthopper species present and their abundance in the field. A comparison of the time of AY infection of bait plants with leaf- and planthopper abundance suggests that AY transmission was due to *M. fuscovaria*, because it was the most abundant species, and in some instances the only species found on the traps in the weeks AY-infected bait plants were recorded. The results of the controlled transmission experiment together with those of the field trial indicate that *M. fuscovaria* is a vector of AY in grapevine in South Africa. Monitoring data suggest that *M. fuscovaria* has at least two generations per year with a relatively high abundance in January and February and from May/June to September/October. To identify alternative host plants of AY, 572 crop and weed plants belonging to 13 families and 31 species were collected from AY-infected vineyards. Four plant species, maize, triticale, blackjack and periwinkle, tested positive for AY. In controlled transmission experiments with field-collected *M. fuscovaria* using plants grown from seed under insect-free conditions, maize, wheat and triticale tested positive for AY. Experiments have been carried out to determine transmission characteristics of AY by *M. fuscovaria*.

2. Problem identification and objectives

Rationale

Phytoplasmas (formerly mycoplasma-like organisms (MLOs)) are single-celled organisms that have been associated with more than 700 plant diseases in wild and cultivated plants (Weintraub & Beanland, 2006). In 2006, Aster yellows phytoplasma (AY) was recorded for the first time from grapevine in South Africa (Engelbrecht *et al.*, 2010). It has since spread in the Vredendal, Waboomsrivier and Robertson areas of the Western Cape. Aster yellows phytoplasma has a wide host range and has been reported to infect both several economically important fruit and vegetable crops, and weeds in a number of countries (Lee *et al*., 2004; Hogenhout *et al*., 2008). Aster yellows is a destructive disease that can cause major economic losses. Symptoms of AY infection may include stunting, yellowing and virescence (excessive greening of floral tissue) and phyllody (development of leaf-like floral structures) (Seemüller *et al*., 1998). In grapevine, grapes drop before ripening and infected vines may decline and die, resulting in severe yield losses.

Phytoplasmas are restricted to the phloem tissue of plants and can be transmitted through grafting of infected plant material, parasitic plants, seed and phloem-feeding insect vectors (leafhoppers and related species) (Weintraub & Beanland, 2006). Insect vectors include Cicadellidae, as well as species belonging to several other hemipteran families (Cixiidae, Delphacidae, Derbidae, Flatidae, and Psyllidae). A number of vector species have been identified in other parts of the world. However, the vectors of AY in South Africa are not known. Therefore, the identification of insect vectors is of paramount importance if the disease is to be managed. A survey of Hemiptera (e.g. leafhoppers) occurring in vineyards in the Western Cape prior to this project was carried out and leafhoppers and related insects have been tested for the presence of AY (CA de Klerk; ADK1). However, positive results only suggest that a species might be a vector of AY and controlled transmission experiments have to be done to determine if a species is a vector.

Objectives

The objectives of the study were to:
1. establish a diagnostic PCR reliably to test insects and plant material for AY at the University of Pretoria,
2. carry out controlled transmission of AY by leaf-/planthoppers to identify vectors,
3. determine time of AY transmission in the field to identify vectors and assist with the development of management strategies,
4. develop AY transmission protocol for identification of leaf- and planthopper vectors,
5. determine transmission characteristics (acquisition access period (AAP), latent period, inoculation access period (IAP)), and
6. identify alternate host plants of AY to assist with the development of management strategies.

3. Workplan (materials & methods)

3.1 Establish a diagnostic PCR reliably to test insects and plant material for Aster yellows phytoplasma at the University of Pretoria

Background

The aim of this aspect was to establish a diagnostic PCR at the Department of Zoology & Entomology at the University of Pretoria accurately and reliably to detect Aster yellows phytoplasma in plant material as well as in leafhoppers. This diagnostic tool is of importance for determining the presence of phytoplama in plant and insect samples to identify vectors of phytoplasma in South Africa. The two methods adopted to detect phytoplasma include a universal phytoplasma real-time PCR that allows detection of a wide range of phytoplasmas (Christensen *et al.,* 2004) and an Aster yellows specific real-time PCR that detects only the Aster yellows phytoplasma (Angelini *et al.,* 2007). Real-time PCR is the most sensitive detection method to date.

Materials and Methods

Reliable DNA extraction method for plant material
- A total of seven grapevine plants were sampled from a heavily phytoplasma-infected vineyard in the Vredendal region. Five of the seven grapevine plant samples showed clear symptoms of phytoplasma. Two grapevine plant samples showed no symptoms and were collected as a negative control.
- DNA was extracted from field collected grapevine plant material using a 3 % cethyl-trimethyl-ammonium bromide (CTAB) extraction method (Angelini *et al.* 2007).
- Extracted DNA samples were diluted 1:10.
- Stock (concentrated) and diluted DNA were stored at -70 ºC for later analysis using real-time PCR.

Aster yellows real-time specific PCR
- To test the real-time PCR, 2 µl of stock DNA extract and 2 µl of 1:10 diluted DNA extract were used for PCR. Stock and dilutions were used to ensure detection of low concentrations of phytoplasma.
- A positive control was obtained from Prof. Gerhard Pietersen.
- Real-time PCR was performed using the LightCycler® TaqMan® Master kit. The LightCycler technology and the protocol from Angelini *et al.* (2007) have been adapted for detection of Aster yellows.

Reliable DNA extraction method for leafhoppers

- DNA was extracted from leafhoppers collected from phytoplasma-infected vineyards using a 2 % CTAB extraction method (Angelini *et al.* 2007). Adaptations to the 2 % CTAB extraction method were kindly supplied by Dr. Elisa Angelini.
- Extracted DNA samples were stored at -70 ºC for later analysis using real-time PCR.

Test Aster yellows specific real-time PCR for leafhoppers

- Real-time PCR was performed using the LightCycler® TaqMan® Master kit. The LightCycler technology and the Aster yellows protocol from Angelini *et al.* (2007) were optimized for detection of phytoplasmas in leafhoppers.

3.2 Controlled transmission experiments

Background

Based on fieldtrips to Vredendal and Waboomsrivier in February 2009 together with the identification results from Mr M. Stiller (ARC-Plant Protection Research Institute (ARC-PPRI), Biosystematics Division), the leafhopper *Mgenia fuscovaria* (Stål, 1855) (Hemiptera: Cicadellidae) was considered to have potential as a vector. This species was present in field-collected samples from both regions. Furthermore, the largest number of *M. fuscovaria* were collected from grapevines and very few from weeds. In the Vredendal region, *M. fuscovaria* was collected from vineyards that showed severe symptoms of phytoplasma infection, and moreover this leafhopper was found to be the predominant leafhopper in these vineyards. In addition, *M. fuscovaria* individuals tested positive for AY in four independent molecular laboratories in South Africa (CA de Clerk (ADK1); this project). The phytoplasma was detected in adults and nymphs. However, the presence of phytoplasma only indicates that the species feeds on infected plants and may therefore potentially be a vector; to confirm the status as a vector, these insects must be used in controlled transmission experiments with the pathogen.

For the identification of the insect vector(s) of AY in grapevine field-collected insects instead of insects from laboratory cultures were used because of the difficulties experienced in establishing cultures. The rationale for using field-collected insects was that insects collected from highly-infected vineyards are likely to be infected with phytoplasma and when transferred to phytoplasma-free plants for the transmission of the pathogen, the insect species that are vectors would infect the plants.

Materials & Methods

Trial location

- Adult leaf- and plant hoppers were collected in AY-infected vineyards in Vredendal.

Plants

- Grapevine (cv. Chardonnay) propagated at Vititech in Paarl (Western Cape, South Africa) and *Nicotiana benthamiana* grown from seed at the University of Pretoria (Gauteng, South Africa) served as recipient and negative control plants.
- The plants were transferred to, and maintained at, the Vredendal region under insect-proof conditions.
- Recipient and control plants were tested before experiments with real-time PCR using a protocol adapted from Angelini *et al*. (2007) to confirm their phytoplasma-free status.
- Leaves from phytoplasma-infected grapevine plants from the field were collected and served as positive controls.

Insects

- Leaf- and planthoppers were collected from grapevines and weeds in a vineyard showing phytoplasma symptoms.
- Collected insects were identified to morphospecies (typological species distinguished solely on the basis of morphology) level with the assistance of photographs kindly supplied by Mr. M. Stiller.
- *Mgenia fuscovaria* was collected from grapevines in a vineyard highly infected with phytoplasma with pooters (aspirators) and sweep-netting from grapevine during the growing season and in winter from weeds.
- Sub-samples of *M. fuscovaria* were collected, preserved in 95 % EtOH, and sub-samples sent for confirmation of identification to Mr M. Stiller.

Transmission experiment

- *Nicotiana benthamiana*: field-collected leaf- and planthoppers were grouped according to morphospecies level and then transferred to single *N. benthamiana* plants in groups ranging from one to 11 individuals per plant. Insects were left on single plants until they died. Plants/cages were inspected daily for dead insects which were collected and preserved in 95 % EtOH.
- Grapevine: field-collected adult *M. fuscovaria* were transferred in groups of 20 to 10 recipient plants each (Fig. 1). In general, insects are not able to transmit phytoplasmas immediately after feeding on an infected plant because the pathogen needs to multiply and establish in the insect (known as the latent period, which can range from days to weeks) (Welliver, 1999; Hill & Sinclair, 2000). Therefore, *M. fuscovaria* adults were given an inoculation access period (IAP) until they died. Chardonnay plants not exposed to insects served as negative controls.
- After completion of the IAP plants were treated with imidacloprid (Confidor®) to prevent re-infestation by insects.
- Plants were tested for the presence of AYP using real-time PCR (Angelini *et al.,* 2007).

Leafhopper identifications

- To confirm the identity of leaf- and planthopper species used in the transmission experiment, sub-samples were submitted for identification to Mr M. Stiller and *M. fuscovaria* was tested for presence of AY phytoplasma.

DNA extraction from plants

- Leaf material from negative controls and recipient plants was collected starting five weeks after transmission experiments and stored at -70 °C.
- DNA was extracted from plant material and insects stored in 95 % EtOH using a 3 % CTAB extraction method from Angelini *et al.* (2007), with a few minor adaptations and the NucleoSin Plant II kit (buffer PL 2) (Macherey Nagel).
- Extracted DNA was stored at -70 °C for later analysis with real-time PCR.

Real-time PCR of plant DNA extract

- Real-time PCR was performed using the LightCycler® TaqMan® Master kit, the LightCycler technology and the protocol developed by Angelini *et al.* (2007).

3.3 Determine time of Aster yellows transmission in the field

Background

The objective of this aspect is to determine the time of AY infection in the field and relate it to the insect populations present at the time of infection. The rationale is that bait plants placed

weekly in the field will become infected with AY by attracting AY-infected vectors. The time of AY-infection together with monitoring leaf- and planthopper populations, also on a weekly basis, will assist with determining the time of AYP transmission in the field and identifying potential vectors. If plants from a particular week test positive for AY, this can then be related to the leaf-/planthopper populations present during that week and assist in identifying additional vectors. An added benefit will be that determining the time of year of AY infection in the field will assist growers in developing control strategies.

Material and Methods

Field trial location

- One block each in two vineyards was selected in the Vredendal region for the field trial.
- One block in vineyard 1 was selected because it showed severe AY symptoms and a high abundance of *M. fuscovaria*.
- One block in vineyard 2 was chosen as it showed some symptoms of AY and had a high abundance of leaf- and planthopper species in December 2008.

Bait plants

- Grapevine plants (cv. Cabernet franc, Cabernet sauvignon, Chardonnay and Chenin blanc), propagated at Vititech in Paarl and Ernita Nursery in Wellington (Western Cape, South Africa).
- Periwinkle (*Catharanthus roseus* (L.) G. Don (Apocynaceae)) grown from seedlings collected in a garden in Pretoria (Gauteng, South Africa).
- Leaf samples of each bait plant were collected prior to placing plants in the field trial and stored at -70 ℃ for later analysis using real-time PCR to confirm their AY-free status.
- Leaves collected from AY-infected plants from the field served as positive controls.

Field trial

- The field trial commenced on 20 October 2009 and continued until 7 December 2010.
- 10 grapevine bait plants were placed in each vineyard.
- In addition, when available, 10 periwinkle bait plants were placed next to the grapevine plants in vineyard 1, starting on 9 November 2009.
- To attract potential vectors to the bait plants, yellow key holders were attached to each bait plant in the field.
- To monitor insects, yellow sticky traps (Chempac (Pty) Ltd) were placed in the vicinity of each bait plant in each vineyard, i.e. 10 yellow sticky traps per vineyard.
- The bait plants were placed in the highly AY-infected block of vineyard 1 for 46 weeks (20 October 2009 to 8 June 2010, 24 August 2010 to 14 September 2010 and 28 September 2010 to 7 December 2010) (433 grapevine bait plants and 260 periwinkle bait plants). From 8 June 2010 the number of grapevine plants placed in the field varied between four and 10, depending on availability. These were placed in the vineyard 2 block for 28 weeks (20 October 2009 – 4 May 2010) (280 bait plants). Placing of plants in the block of vineyard 2 was discontinued in May because of the low number of leaf-/planthoppers trapped and the limited number of plants available for the field trial, because the trial was added to the project at short notice in 2009. For the same reason, periwinkle plants were used only in vineyard 1.
- Ten sticky traps each were placed in both blocks for 59 weeks (1180 sticky traps).
- All the bait plants and sticky traps were collected and replaced with new bait plants and sticky traps weekly. Replacing the bait plants and sticky traps on a weekly basis gave potential vectors an inoculation access period (IAP) of seven days and allowed for accurate monitoring of the leaf-/planthopper species present in each vineyard during each week.
- Plants received water and fertilization through drip irrigation.

- After removal from the field, plants were treated with imidacloprid (Confidor®) to prevent infestation by insects.
- Plants were maintained in insect-proof tents in Vredendal.
- Yellow sticky traps were placed in the insect-proof tents to monitor insects. The yellow sticky traps were replaced weekly.

Leafhopper identifications
- Leafhoppers collected on the yellow sticky traps were counted and the mean number of leafhoppers per week was determined.
- The yellow sticky traps were sent to Mr M. Stiller for identification of leaf- and planthoppers to species/genus level.

DNA extraction from plants
- Leaf material from bait plants was collected at five- to eight-week intervals after field exposure and stored at -70 °C.
- DNA was extracted from plant material using the NucleoSpin Plant II kit (buffer PL 2) (Macherey Nagel).
- To ensure that the extraction method was successful, DNA was extracted from a positive control grapevine (extraction method control) each time DNA was extracted from the grapevine and periwinkle bait plants.
- Extracted DNA was stored at -70 °C for later analysis with real-time PCR.

Real-time PCR
- Real-time PCR was performed using the LightCycler® TaqMan® Master kit, the LightCycler technology and the protocol developed by Angelini *et al.* (2007).

3.4 Establish Aster yellows phytoplasma transmission protocol

Background

The aim of this aspect was to establish an AY transmission protocol using field-collected leafhoppers.

Materials & Methods

The protocol was developed based on initial transmission experiments carried out.

3.5 Determine transmission characteristics

Background

The aim of this aspect has been to determine the number of *M. fuscovaria* adults required to transmit AY to healthy grapevine plants and the acquisition access period, the latent period and the inoculation access period.

Materials and Methods

Trial location
- Adult *M. fuscovaria* were collected from AY-infected and AY-free vineyards in Vredendal.

Plants
- Grapevine cv. Chardonnay (Vitaceae), propagated at Vititech in Paarl (Western Cape, South Africa), served as recipient and negative control plants. Recipient and control

plants were tested before experiments with real-time PCR using a protocol adapted from Angelini *et al*. (2007) to confirm their phytoplasma-free status. The plants were transferred to, and maintained at the Vredendal region under insect-free conditions.

Insects

AY-infected insects

- AY-infected insects*: M. fuscovaria* collected with a pooter (aspirator) and sweep-netting from grapevine plants from a highly AY-infected vineyard
- Sub-samples of *M. fuscovaria* were collected and sent for confirmation of identification to Mr M. Stiller.
- Sub-samples of insects were collected for testing for AY presence using real-time PCR (Angelini *et al*., 2007).
- To confirm the identity of *M. fuscovaria* used in the transmission experiment, sub-samples were sent for identification to Mr M. Stiller and tested for presence of AY phytoplasma.

DNA extraction from plants

- Leaf material from negative controls and recipient grapevine (cv. Chardonnay) plants were collected starting five weeks after transmission experiments and stored at -70 ℃.
- DNA was extracted from plant material and insects stored in 95 % EtOH using the NucleoSpin Plant II kit (buffer PL 2) (Macherey Nagel).
- Extracted DNA was stored at -70 ℃ for later analysis with real-time PCR.

Real-time PCR of plant DNA extract

- Real-time PCR was performed using the LightCycler® TaqMan® Master kit, the LightCycler technology and the protocol developed by Angelini *et al.* (2007).

Determining AAP

- Eight different feeding times were tested to determine the minimum acquisition access period (AAP).
- Transfer adult *M. fuscovaria* from the AY-free culture in groups of 10 to AY-positive grapevine plants for AAPs of 15 min, 30 min, 1 h, 2 h, 4 h, 12 h, 24 h and 48 h, respectively. After each AAP, the insects from each feeding time were transferred to recipient plants for an inoculation access period (IAP) of 24h.
- Plants were treated with imidacloprid (Confidor®) after transmission experiments to prevent re-infestation by insects.
- Recipient plants not exposed to insects served as negative controls.
- Plants were tested for the presence of AYP using real-time PCR (Angelini *et al*., 2007).

Determining IAP

- Eight different feeding times were tested to determine minimum IAP.
- Groups of 10 field-collected *M. fuscovaria* were transferred to separate recipient plants for IAPs of 15 min, 30 min, 1 h, 2 h, 4 h, 12 h, 24 h and 48 h, respectively.
- Plants were treated with imidacloprid (Confidor®) after transmission experiments to prevent re-infestation by insects.
- Recipient plants not exposed to insects served as negative controls.
- Plants were tested for the presence of AYP using real-time PCR (Angelini *et al*., 2007).

Determining latent period

- Adult *M. fuscovaria* were transferred from the AY-free plants to AY-positive grapevine plants for an AAP of 24h to determine the latent period. After completion of the AAP, *M. fuscovaria* adults were transferred in groups of 10 to recipient plants each, for a period of 7, 14, 21 and 28 days, respectively.
- Plants were treated with imidacloprid (Confidor®) after transmission experiments to prevent re-infestation by insects.

- Recipient plants not exposed to insects served as negative controls.
- Plants tested for the presence of AYP using real-time PCR (Angelini *et al*., 2007).

Transmission efficiency
- One, 5, 10 and 20 field-collected *M. fuscovaria* adults were transferred from a highly AY-infected vineyard to separate AY-free recipient grapevine plants for 48 hours to determine the number of insects required to transmit AYP.
- Plants treated with imidacloprid (Confidor®) after transmission experiments to prevent re-infestation by insects.
- Recipient plants not exposed to insects served as negative controls.
- Plants tested for the presence of AYP using real-time PCR (Angelini *et al*., 2007).

3.6 Identify alternate host plants of Aster yellows phytoplasma

Background

The aim of this aspect was to determine alternate host plants of AY to identify and reduce sources of inoculum in and around vineyards. Identifying alternate host plants of AY will aid in developing control strategies for AY. The aspect was done in collaboration with Mrs Roleen Carstens (ARC- Infruitec/Nietvoorbij).

Materials and Methods

Alternative host plants
- Weed and crop plant samples were collected in several localities in and within the vicinity of vineyards heavily infected with AY.
- Plants were collected in October 2009, March 2010, April 2010, February 2011, July 2011, December 2011, February 2012 and March 2012.
- Leaf material collected from plants has been stored at -70 °C for later analysis with real-time PCR.
- Subsamples of plant species collected were submitted to Dr Johan Fourie (Soil Science Division, ARC Infruitec-Nietvoorbij) or the National Botanical Institute (Pretoria) for species identification.

Phytoplasma detection using real-time PCR
- DNA was extracted from plants using the NucleoSpin Plant II kit (buffer PL 2) (Macherey Nagel).
- To ensure that the extraction method was successful, DNA was extracted from a positive control grapevine (extraction method control) each time DNA was extracted from potential alternate AY host plants.
- DNA extractions were stored at -70 °C for later analysis using real-time PCR.

Real-time PCR
- Real-time PCR was performed using the LightCycler® TaqMan® Master kit. The LightCycler technology and the protocol were taken from Angelini *et al.* (2007).

4. Results and discussion

Milestone	Achievement
1. Establish diagnostic PCR reliably to test insects and plant material for Aster yellows phytoplasma at the University of Pretoria	Two real-time PCR assays, one with phytoplasma universal and one with Aster yellows phytoplasma (AYP) specific primers, have been established in the molecular laboratory at the Department of Zoology and Entomology, University of Pretoria.
2. Controlled laboratory transmission of Aster yellows to identify vector(s)	The leafhopper *Mgenia fuscovaria* was identified as a vector of Aster yellows phytoplasma in grapevine (Douglas-Smit *et al*., 2010; Krüger *et al*., 2011).
5. Determine time of Aster yellows transmission in the field	Transmission of AY in the field coincided with the presence of *M. fuscovaria,* supporting results of controlled laboratory transmission experiments that *M. fuscovaria* is a vector of AY in grapevine in South Africa (Krüger *et al*., 2011).
3. Establish Aster yellows phytoplasma transmission protocol	A protocol has been developed and used in this project (UP/KK1) and a new project on determining vectors other than *M. fuscovaria* (UP/KK2).
6. Determine transmission characteristics (acquisition access period (AAP), latent period, inoculation access period (IAP))	Transmission experiments to determine transmission characteristics have been carried out.
4. Identify alternative host plants of Aster yellows phytoplasma	572 plants belonging to 13 families and 31 species were tested with real-time PCR for the presence AY. Maize, tricicale, blackjack and periwinkle tested positive for the phytoplasma. In addition, AY was transmitted by *M. fuscovaria* to maize, triticale, and wheat in controlled laboratory transmission experiments.

4.1 Diagnostic PCR reliably to test insects and plant material for Aster yellows phytoplasma at the University of Pretoria

Universal phytoplasma real-time PCR
Real-time PCR was performed using the LightCycler® TaqMan® Master kit, the LightCycler v1.5 technology and the protocol developed by Christensen *et al.* (2004) and adapted by Prof. Gerhard Pietersen. The probe was labeled at the 5'-end with the fluorescent dye FAM as reporter and at the 3'-end with TAM fluorescent dye as quencher. The reaction mixture contained 16 µl water (PCR grade), 7.5 µM of each primer and 5 µM of probe and 4 µl TaqMan® Master Mix and 2 µl DNA extraction. The amplification reaction conditions were 95 ºC for 10 min, followed by 45 cycles of 95 ºC for 1s, 60 ºC for 40s and 72 ºC for 1s.

Aster yellows specific real-time PCR
Real-time PCR was performed using the LightCycler® TaqMan® Master kit, the LightCycler v1.5 technology and the protocol developed by Angelini *et al*. (2007). The Aster yellows

primers and probe designed by Angelini *et al*. (2007) were used. The probe was labeled at the 5'-end with the fluorescent dye FAM as reporter and at the 3'-end with BHQ-1 fluorescent dye as quencher. The reaction mixture contained 13 µl water (PCR grade), 10 µM of each primer and 10 µM of probe and 4 µl TaqMan® Master Mix and 2 µl DNA extraction. The amplification reaction conditions were: 95 °C for 10 min, followed by 45 cycles at 95 °C for 15s, 60 °C for 1 min and 72 °C for 1s.

4.2 Controlled transmission experiments

Mgenia fuscovaria

Five weeks after the first day of inoculation some grapevine (cv. Chardonnay) plants started to show AY symptoms. Plants were tested 5 to 11 weeks after transmission for the presence of AY. Three out of 10 plants tested positive for AY, demonstrating that transmission with *M. fuscovaria* was successful (Fig. 1). Control plants not exposed to insects and kept under the same conditions as recipient plants tested negative for AY. All 10 recipient plants died several weeks after transmission, whereas none of the negative control plants died. The plants used for transmission experiments were very young non-woody plants and the death of the plants may be related to AY infection or *M. fuscovaria* feeding. In addition, Chardonnay is very sensitive to AY and it is possible that the death of the plants could be due to phytoplasma infection.

The *M. fuscovaria* positive control had a low CP value, hence a high concentration of AY phytoplasma (Fig. 1). The high concentration suggests that the phytoplasma multiplied in the insect and did not occur in the gut content only.

Voucher specimens of *M. fuscovaria,* consisting of two males and two females, were deposited in the National Collection of Insects (ARC-Plant Protection Research Institute (ARC-PPRI), South Africa). The accession number is CCDL21650.

To our knowledge, this is the first report of a vector of AY phytoplasma in South Africa and of *M. fuscovaria* as a vector of AY phytoplasma.

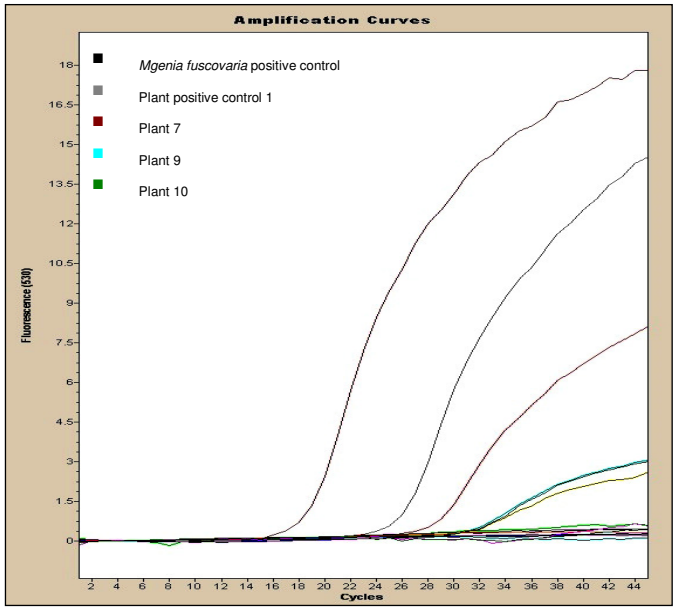

Fig. 1. LightCycler™-assisted real-time PCR of grapevine and leafhopper DNA extractions. The samples showing no amplification are the negative controls.

Leaf- and planthoppers

For AY transmission experiments 287 leaf- and planthoppers (one to 11 individuals per plant) were transferred to 115 *N. benthamiana* plants. Sixteen species/morphospecies (msp.), including *Afrosteles distans*, *Austroagallia* msp., *Cicadulina* msp., *Circulifer* msp., *Exitianus* msp., *Mgenia fuscovaria, Peragallia* msp., *Sogatella* msp. and *Toya* msp., were used in transmission experiments. The number of replicates per species/morphospecies ranged between one and 19. The number of insects available limited the number of replicates and insects exposed to individual recipient plants. All *N. benthamiana* DNA extractions tested negative for Aster yellows or phytoplasma in general, using both the Aster yellows-specific and universal phytoplasma real-time PCR. The negative result could be due, for example, to the inability of the taxa tested to transmit AY, insects not being infected with AY, or the low number of insects exposed to individual plants.

4.3 Time of Aster yellows transmission in the field

AY transmission to periwinkle plants occurred not only when numbers of *M. fuscovaria* were high but also when they were relatively low, for example in weeks 14 and 15 in April 2010 (Fig. 2). Positive bait plants were recorded from December 2009 until end of April 2010. No positive bait plants were recorded between August and December 2010. The field trial provided some insight into the biology of *M. fuscovaria*. Mr M. Stiller has identified at least four different *M. fuscovaria* nymphal instars. The field data suggest that there is more than one generation of *M. fuscovaria* per year because there were three peaks for nymphs.

Vineyard 1

Leaf-/planthoppers collected on sticky traps during the 59-week period of the field trial have been counted. Mr M. Stiller identified 25 leaf-/planthopper species and one cixiid species. *Mgenia fuscovaria* was collected in large numbers (1121 nymphs and 1171 adults) and was the most abundant species in this study (Table 1). *Mgenia fuscovaria* abundance started to increase in December 2009 and reached a peak during January 2010 (Fig. 2). During this time, the majority of *M. fuscovaria* individuals trapped were nymphs (Fig. 2). The number of adult *M. fuscovaria* only started to increase when the nymph population started to decline. There were three peaks for *M. fuscovaria* adults, one from January to February 2010, a second in June/July 2010 and a third in August 2010. Nymphs peaked in January, June and December in 2010.

Three of the grapevine bait plants tested positive for AY (Fig. 2). Each of the negative control grapevine samples tested negative for AY, indicating that no contamination occurred confirming that the grapevine bait plant was AY-positive.

A large number of the periwinkle bait plants did not survive in the field. DNA was extracted from the remaining periwinkle bait plants using the NucleoSpin Plant II kit (buffer PL 2). Fourteen periwinkle bait plants tested positive for AY. The periwinkle bait plants exposed in December 2009 in weeks 50 and 51, January 2010 in week 3, February 2010 in weeks 7 and 8, March/April in weeks 13 to 15 and April/May in week 18 tested positive for AY (Fig. 2). The 14 AY-positive periwinkle bait plants all tested negative for AY phytoplasma prior to the field trial.

Table 1. Leafhopper and planthopper species and number of adults recorded on sticky traps in vineyard 1 from October 2009 to December 2010.

No.	Family	Species	Number of individuals over 59 weeks
1.	Cicadellidae (leafhoppers)	*Acia lineatifrons*	54
2.		*Aconurella* sp.	3
3.		*Austroagallia nigrasterna*	5
4.		*Austroagallia* sp.	33
5.		*Balclutha* sp.	17
6.		*Cicadulina mbila*	67
7.		*Circulifer struthiola*	11
8.		*Circulifer tenellus*	57
9.		*Empoasca* sp.	10
10.		*Exitianus* sp.	1
11.		*Exitianus taeniaticeps*	13
12.		*Glossocratus afzeli*	1
13.		*Goniagniathus agenor*	1
14.		*Hilda patruelis*	1
15.		*Maiestas* sp.	16
16.		*Mgenia fuscovaria* ADULT	1121
17.		*Mgenia fuscovaria* NYMPH	1171
18.		*Molopopterus* sp.	1
19.		*Orosius argentatus*	1
20.		*Orosius* sp.	18
21.		*Penthimiola bella*	15
22.		*Recilia cotula*	1
23.		*Recilia* sp.	1
24.	Delphacidae (planthoppers)	*Sogatella* sp.	14
25.		*Toya* sp.	6
26.	Cixiidae (primitive snout bugs)	Unidentified	1

A comparison of the time of AY infection with leaf- and planthopper abundance suggests that AY transmission was due to *M. fuscovaria, b*ecause it was the most abundant species, and in some instances the only species found on the traps in the weeks AY-infected periwinkle bait plants were recorded. Overall, the highest number of *M. fuscovaria* were recorded in September 2010 (weeks 36 and 37). The only other species recorded when bait plants tested positive for AY was the leafhopper *Penthimiola bella* with a single adult. In weeks 51 and 3, *M. fuscovaria* was the only leafhopper species recorded on sticky traps in the block in vineyard 1. In week 7, the only other species recorded apart from *M. fuscovaria* was a single adult of *Empoasca* sp. During week 8, a single *Sogatella* sp. was recorded, whereas *M. fuscovaria* occurred in large numbers.

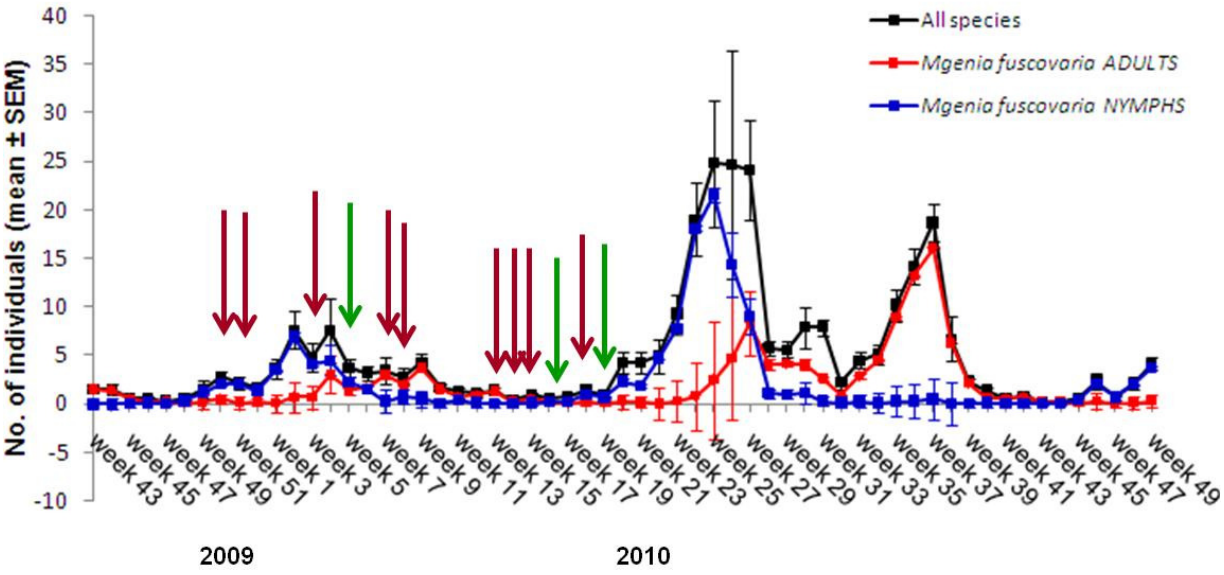

Fig. 2. Abundance of *Mgenia fuscovaria* adults and nymphs collected on sticky traps in a highly Aster yellow phytoplasma-infected block in vineyard 1 from October 2009 until December 2010. Red arrows indicate records of positive periwinkle plants and the green arrows records of positive grapevine plants.

Vineyard 2

Leaf- and planthoppers collected on the sticky traps were counted for the 59 weeks of the field trial. Mr M. Stiller identified 27 leaf-/planthopper species (Table 2). *Cicadulina mbila* and *Austroagallia* spp. were the most abundant species found on the sticky traps. A few specimens (14 adults and 6 nymphs) of *M. fuscovaria* were recorded from June to October 2010 (week 23 to week 42 in 2010) in vineyard 2 (Table 2; Fig. 3). All three species were present in lower numbers compared to *M. fuscovaria* in vineyard 1.

None of the grapevine bait plants showed AY symptoms after being exposed in the field. DNA was extracted from grapevine bait plants for the first 28 weeks of the field trial. None of the plants tested positive for AY. Each of the positive control grapevine samples tested positive for AY, indicating that the extraction method was successful and confirming that all grapevine bait plants were negative for AY.

Table 2. Leafhopper species and number of individuals recorded on sticky traps in a moderately Aster yellows-infected block in vineyard 2 from October 2009 to December 2010.

No.	Family	Species	Number of individuals over 59 weeks
1.	Cicadellidae (leafhoppers)	*Acia lineatifrons*	86
2.		*Aconurella* sp.	2
3.		*Afrosteles distans*	1
4.		*Austroagallia nigrasterna*	46
5.		*Austroagallia* sp.	187
6.		*Balclutha* sp.	21
7.		*Cicadulina anestae*	1
8.		*Cicadulina mbila*	230
9.		*Circulifer struthiola*	12
10.		*Circulifer tenellus*	42
11.		*Embolophora britmusei*	1
12.		*Empoasca* sp.	30
13.		*Empoasca* sp.1	4
14.		*Exitianus* sp.	3
15.		*Exitianus taeniaticeps*	9
16.		*Maiestas* sp.	25
17.		*Mgenia fuscovaria* ADULT	14
18.		*Mgenia fuscovaria* NYMPH	6
19.		*Orosius argentatus*	53
20.		*Orosius* sp.	6
21.		*Penthimiola bella*	1
22.		*Recilia* sp.	1
23.		*Recilia cotula*	2
24.		Typhlocybinae indet	2
25.		Typhlocybinae indet msp 1*	1
26.	Delphacidae (planthoppers)	*Sogatella* sp.	11
27.		*Toya* sp.	23

***** msp = morphospecies

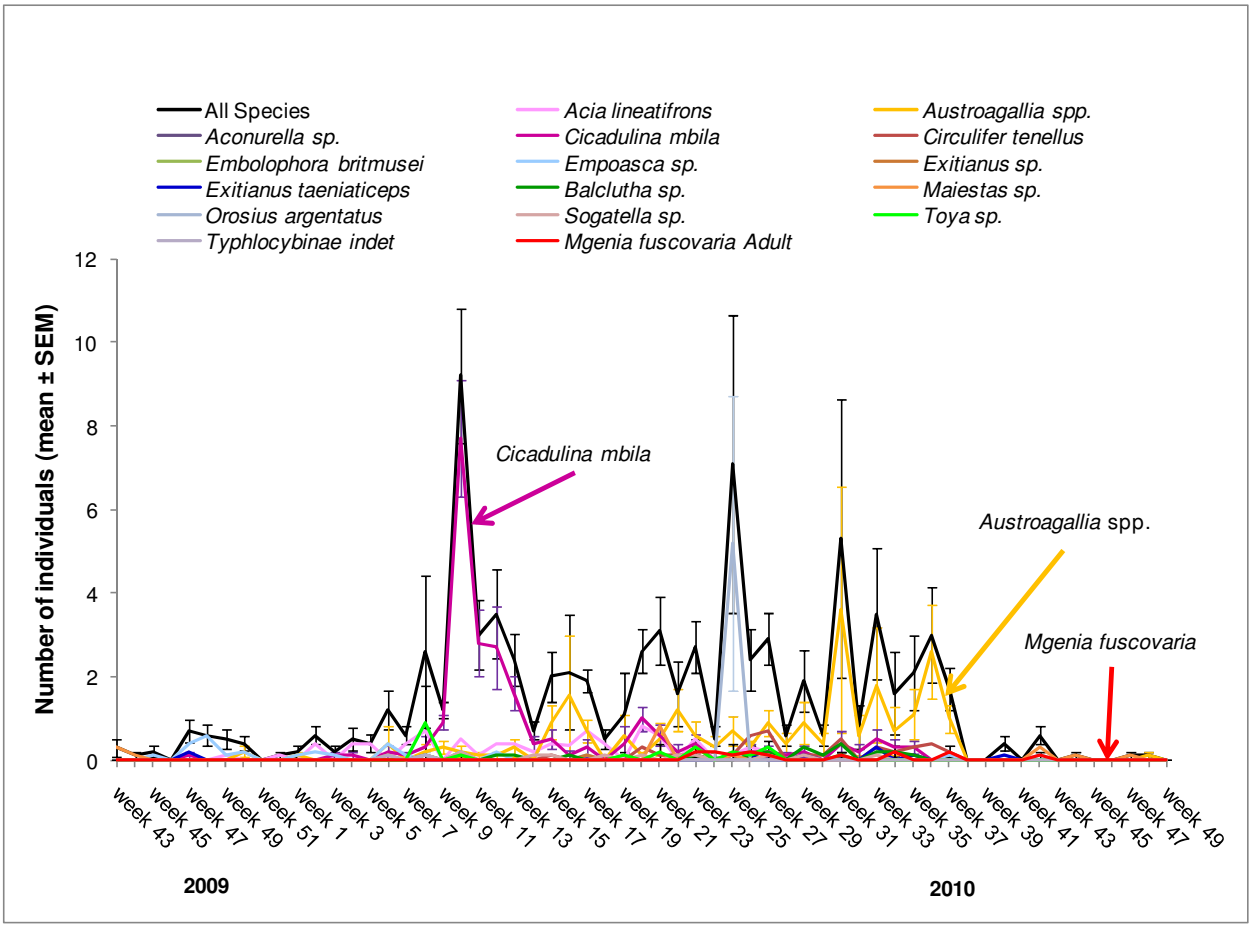

Fig 3. Abundance of leafhopper and planthopper species collected on sticky traps from October 2009 until December 2010 from vineyard 2.

Only a few grapevine plants showed AY symptoms in vineyard 2 compared to the severe AY infection in vineyard 1. Therefore, no periwinkle bait plants were used in vineyard 2 and grapevine bait plants were only placed in the field for the first 28 weeks of the field trial due to the limited number of plants available. However, sticky traps were placed in the field for the full 59 weeks of the field trial to monitor leafhopper populations.

4.4 Aster yellows phytoplasma transmission protocol

Equipment

- Stereomicroscope (binocular)
- Insect cages for recipient plants and negative controls (Fig. 4)
- Pooter (aspirator)
- Plants
  - AY source plants, e.g. grapevines from an AY-infected vineyard
  - At least 12 young plants (grapevine (e.g. Chardonnay): approximately 3-6 leaves) should be used as recipient and negative control plants
- Vectors: adult leafhoppers collected from highly AY-infected vineyards; adult leafhoppers should be used because, unlike nymphs, they can be reliably identified to species/genus level
- Insecticides: *e.g.* chlorpyrifos (immediate action) and imidacloprid (systemic action)

Fig. 4. Examples of insect cages that can be used for transmission experiments.

Transmission of AY

- Test AY (vineyard plants) source plants and sub-samples of leafhoppers to confirm AY phytoplasma presence before transmission experiments using real-time polymerase chain reaction (real-time PCR; Angelini *et al*., 2007).

- Test negative control and recipient plants to confirm AY-free status before transmission experiments using real-time PCR.

- Collect adult leafhoppers from AY-infected vineyards. Transfer leafhoppers in groups of 15 to 20 individuals each, depending on plant size, to each insect cage containing a recipient plant. The rationale is that leafhoppers collected from highly infected vineyards are likely to be infected with phytoplasma and can already transmit the pathogen when transferred to AY-free plants.

- Allow leafhoppers an inoculation access period (IAP) for up to seven days or until they die.

- Keep at least two indicator plants per transmission experiment free of leafhoppers as negative controls. Maintain negative control plants under the same conditions as the recipient plants.
- Treat recipient and control plants with an insecticide (*e.g.* chlorpyrifos and imidacloprid) to remove any survivors and to prevent re-infestation after completion of IAP. Thereafter transfer plants to an insect-free plant growth room/glasshouse.
- Test plants for AY at the earliest 5 weeks after transmission using real-time PCR. However, not all plants infected may test positive at this early stage, and plants should be tested for at least up to 12 months to ensure that the pathogen, if present, is detected in all plants.
- Depending on the region, experiments may have to be carried out and plants maintained under quarantine conditions.

4.5 Transmission characteristics

Transmission of AY by *M. fuscovaria* to healthy grapevine was achieved with 10 and 20 adults and 15 nymphs (Table 3).

Table 3. Transmission of Aster yellows phytoplasma with field-collected *Mgenia fuscovaria* adults/nymphs to grapevine.

Number of adults per plant	Number of nymphs per plant	Number positive plants/ plants tested
1	0	0/17
5	0	0/10
10	0	1/6
20	0	3/21
0	15	1/1

Experiments to determine transmission characteristics commenced in December 2011. Between 8 to 20 replicates were completed for IAPs (15 min, 30 min, 1 h, 2 h, 4 h, 12 h, 24 h, 48 h), 7 to 9 replicates were completed for AAPs (15 min, 30 min, 1 h, 2 h, 4 h, 12 h, 24 h, 48) and 8 replicates each to determine the latent period (7, 14, 21, 28 days). Thus far, none of the plants tested positive for AY. This is partially due to difficulties experienced with many plants dying shortly or several weeks after transmission and plants no longer being available for testing. Due to the premature death of test plants, which could be caused by leafhopper feeding, for example, experiments had to be repeated in February and March 2012. The plants from the latest transmission experiments have been tested for AY. Surviving plants will be tested again at a later stage as the negative results might be preliminary.

4.6 Alternative hosts of Aster yellows phytoplasma

Table 4. Field-collected weeds and crops tested as AY alternative hosts.

No.	Family	Species	Common name	Positive plants/ no. of plants tested
1.	Amaranthaceae	*Amaranthus viridus*	Slender amaranth	0/2
2.	Apocynaceae	*Catharanthus roseus*	Periwinkle	14/132
3.	Asteraceae	*Bidens bipinnata*	Spanish black jack	2/43
4.		*Erigeron bonariensis*	Flax-leaf fleabane	0/10
5.		*Erigeron Canadensis*	Horseweed fleabane	0/8
6.		*Erigeron* sp.		0/10
7.		*Lactuca serriola*	Prickly lettuce	0/9
8.		*Tolpis capensis*		0/20
9.		*Sonchus oleraceus*	Thistle	0/15
10.		*Senecio arenarius*		0/1
11.	Brassicaceae	*Brassica juncea*	Chinese mustard	0/21
12.		*Capsella bursa-pastoris*	Shepherd's-purse	0/17
13.		*Raphanus raphanistrum*		0/5
14.	Chenopodiaceae	*Chenopodium album*	White goosefoot	0/36
15.		*Chenopodium* sp. 2		0/10
16.		*Salsola kali*	Prickly saltwort	0/17
17.	Commelinaceae	*Commelina benghalensis*	Wandering Jew	0/10
18.	Convolvulaceae	*Ipomoea purpurea*	Morning glory	0/23
19.	Cucurbitaceae	*Cucurbita pepo*	Gem squash	0/10
20.	Fabaceae	*Medicago polymorpha*		0/10
21.		*Medicago sativa*	Lucerne	0/10
22.		*Melilotus alba*	White sweet clover	0/3
23.		*Phaseolus* sp.	Beans (garden bean)	0/10
24.	Lamiaceae	*Lamium amplexicaule* L.	Henbit dead-nettle	0/34
25.	Poaceae	*Setaria verticillata*	Sticky bristle grass	0/4
26.		*Triticosecale* sp.	Triticale	5/20
27.		*Zea mays*	Maize	6/10
28.	Solanaceae	*Capsicum* sp.	Pepper	0/10
29.		*Datura stramonium*	Common thorn-apple	0/1
30.		*Solanum lycopersicum* L.	Tomato	2/44*
31.	Zygophyllaceae	*Tribulus terrestris*	Devil's thorn	0/11
32.	Unidentified	Unidentified creeper		0/6

* The two plants tested only weakly positive.

Of the 572 field-collected plants belonging to 13 families, 27 genera and 31 species tested, four plant species, maize, triticale, blackjack and periwinkle, tested positive for AYP (Table 4). In controlled transmission experiments with field-collected *M. fuscovaria* and recipient plants grown from seed maize, triticale and wheat tested positive for AY (Table 5). This shows that *M. fuscovaria* is able to transmit AY to plants other than grapevine. Each of the extraction method control samples (positive control grapevine) tested positive for AY, indicating that the extraction method and PCR were successful. All negative controls tested negative throughout. Samples from two tomato plants tested weakly positive for AY and more samples will have to be tested to confirm whether tomato serves as an alternate host for AY.

Table 5. Results of controlled laboratory experiments with field collected *Mgenia fuscovaria* to determine alternative host plants.

No.	Family	Species	Common name	Positive plants/ no. of plants tested
1.	Apocynaceae	*Catharanthus roseus*	Periwinkle	0/11
2.	Asteraceae	*Bidens bipinnata*	Spanish black jack	0/1
3.	Poaceae	*Zea mais*	Maize	2/6
4.		*Triticum aestivum*	Wheat	1/4
5.		*Triticosecale* spp.	Triticale	3/5

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- Mr Michael Stiller (ARC-PPRI) for taking on the additional time-consuming tasks of identifying leaf- and planthoppers collected during the monitoring field trial;
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5. Accumulated outputs

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

Technology development, products and patents

Indicate the commercial potential of this project (intellectual property rights or a commercial product(s)).

Protocol for Aster yellows phytoplasma transmission by leafhoppers

Human resources development/training

Indicate the number and level (e.g. 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 (R)
1.	Douglas-Smit, Nicoleen (PhD)	180,000.00
2.		
3.		
4.		
5.		

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

Scientific

- Krüger K., de Klerk A., Douglas-Smit N., Joubert J., Pietersen G. & Stiller M. (2011). Aster yellows phytoplasma in grapevines: identification of vectors in South Africa. *Bulletin of Insectology* 64 (Supplement): 137-138.

Popular

- Edo Heyns, Groot deurbraak in fitoplasma vraagstuk. Wineland, March 2010, pp. 22-23.
- Lucille Botha, Vektor gevind vir vergelingsiekte. Landbou Weekblad, No. 1647, 2 April 2010, pp. 22-23.

Presentations/papers delivered

- Douglas-Smit, N., Pietersen, G. & Krüger, K. Transmission of Aster yellows to grapevine by *Mgenia fuscovaria* (Stål) (Hemiptera, Cicadellidae). 2nd Meeting, COST Action FA0807 Integrated Management of Phytoplasma Epidemics in Different Crop Systems. Sidges, Spain, February 2010. p. 47.

- Douglas-Smit, N, Krüger, K. & Pietersen, G. Transmission of Aster yellows to grapevine by insect vectors. Grapevine Virus Workshop X, Winetech. Stellenbosch, South Africa, May, 2011.

- Krüger K., de Klerk A., Douglas-Smit N., Joubert J., Pietersen G. & Stiller M. Aster yellows phytoplasma in grapevines: identification of vectors in South Africa. *Second International Phytoplasmologist Working Group Meeting*. Neustadt/Weinstraße, Germany, September 2011.

- Krüger, K., Douglas-Smit, N., Pietersen, G., Joubert, J., Stiller, M. & Carstens, R. Transmission of Aster yellows phytoplasma by *Mgenia fuscovaria* (Hemiptera: Cicadellidae). Grapevine Virus Workshop XI, Winetech. Stellenbosch, South Africa, May, 2012.

4. Total cost summary of project

	Year	CFPA	Deciduous	DFTS	Winetech	THRIP	Other	TOTAL
Total cost in real terms for year 1	2009		100,337		100,337	200,675	77,600	478,949
Total cost in real terms for year 2	2010		97,216		193,524	145,370	60,000	496,110
Total cost in real terms for year 3	2011		150,658		150,685	150,685	72,500	524,528
Total cost in real terms for year 4								
Total cost in real terms for year 5								
TOTAL			348,221		444,546	496,730	210,100	**1,499,587**