A-11-USP-PM11	PHI allocated project number

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FINAL REPORT
2015

1. PROGRAMME AND PROJECT LEADER INFORMATION

	Research Organisation Programme leader	ARC Research Team Manager	Project leader
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2. PROJECT INFORMATION

Research Organisation Project number	USPP CL3
Project title	Etiology and Epidemiology of *Neofabraea* spp. on pome fruit
Short title	

Fruit kind(s)	Apple, Pear		
Start date (mm/yyyy)	01/ 10/ 2010	**End date** (mm/yyyy)	28/ 08/ 2015

Key words	Bull's eye rot, 'Cripps Pink', apple, fungicides

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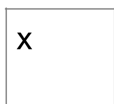

Approved by Research Organisation Programme leader (tick box)

3. EXECUTIVE SUMMARY

Objectives & Rationale

Neofabraea alba is a latent lenticel pathogen and severely affecting 'Cripps Pink' apple in South Africa. Little information is available on this disease and its control in South Africa. The information gained in this study will contribute to the development of a management strategy for *N. alba* in South Africa.
This study aimed to determine (i) if *N. alba* can be detected, using molecular analysis, on the surface of 'Cripps Pink apples and when the fungus is present in the orchard; (ii) the postharvest incidence of *N. alba* on 'Cripps Pink' apples from five growing regions in the Western Cape; (iii) *in vitro* efficacy of pre- and postharvest fungicides on *N. alba* from 'Cripps Pink' apple; (iv) the influence of climatic factors on *N. alba* disease development; and (v) the taxonomic scope of *Neofabraea* species tentatively reported on 'Packham's Triumph' pears.

Methods

1. A spore removal method and PCR-RFLP was used to screen monthly washes made of 'Cripps Pink' apples, leaves and 'Hillieri' crab apple pollinators collected during the growing season.
2. Asymptomatic fruit were collected from commercial orchards and packhouses and kept in cold storage. Thereafter evaluated for decay symptoms of *N. alba*. Isolations were made and species identity done according to morphological characteristics and with molecular analysis.
3. Mycelial plugs were inoculated on artificial medium amended with fungicide actives: fludioxonil and pyrimethanil. The radial growth was documented and the efficacy of the respective fungicide on radial growth statistically analysed.
4. Weather data was collected from the sampling sites used for spore collection and statistically evaluated for possible factors known to be beneficial for *N. alba* spore development, dispersal and infection.
5. Asymptomatic 'Packham's Triumph' pears were collected, stored and evaluated. Isolations with *Neofabraea*-like characteristics were analysed with molecular techniques.

Key Results

A spore removal method and PCR-RFLP analysis specific to the *Neofabraea* genus was developed. *Neofabraea alba*, *N. perennans* and *N. kienholzii* were detected from spore fruit washes. However, *Neofabraea alba* was the only causal agent of latent lenticel decay in postharvest 'Cripps Pink' apple. Conidia of *N. alba* were detected during summer and autumn on fruit surfaces. Disease incidences indicated that *N. alba* is present in all five growing regions evaluated. *In vitro* fungicide efficacy could not be established due to the variability of the pathogen's behaviour on artificial media. No climatic factors could be correlated with disease development in the two orchards. *Neofabraea alba* was identified from 'Hillieri' crab apples and 'Packham's Triumph' pears.

Conclusion/Discussion

Neofabraea alba is an emerging disease on 'Cripps Pink' apple in the Western Cape. Its presence on pollinators and pear fruit and the identification of two additional *Neofabraea* species indicate the need for further studies of pome fruit orchards in South Africa. Additional fungicide sensitivity testing is also required before final recommendations can be made for pre- and postharvest management strategies. However, current management strategies should focus on applying fungicides at the time points when *N. alba* was detected in the orchard.

a. **PERFORMANCE CHART**

Milestone	Target Date	Extension Date	Date completed
1. Development or rapid detection method for *N. alba* from fruit and leaf surfaces	October 2012	October 2014	August 2015
2. Compile postharvest disease incidence data on *N. alba* for 'Cripps Pink' apple	October 2013	October 2014	October 2014
3. *In vitro* fungicide sensitivity assays	October 2012	September 2015	
4. Evaluation of weather data	October 2014		November 2014
5. Identification of *Neofabraea* species on pear	October 2013	October 2014	August 2015
6. Completion of MSc Thesis	September 2014	September 2015	September 2015
7. Article: Detection and disease incidence of N. alba on 'Cripps Pink' apples in the Western Cape of South Africa - Phytopathology	January 2016		
8. Article: Neofabraea species on pome fruit in South Africa – Plant Disease	January 2016		
9. Article: Fungicide sensitivity of selected fungicides against Neofabraea alba in South Africa - Plant Pathology	January 2016		

b) WORKPLAN (MATERIALS AND METHODS)

<u>1. The development of a rapid detection method for *N. alba* on 'Cripps Pink' apple surfaces and leaves.</u>

i. Preharvest sample collection

Two 'Cripps Pink' orchards, one in the Witzenberg Valley and the other in Grabouw, were chosen for sample collection as each orchard is known to be affected by *N. alba*. Fruit and leaves from within the tree canopy were collected in 2012, 2013 and 2014 on a monthly basis during the fruit maturation stage of 'Cripps Pink', i. e. from one month post full bloom in November until harvest in April. A randomised sampling layout was followed for the collection of the fruit and tree canopy leaves. For each orchard, tree rows were numbered as well as the trees within each row. A set of 25 non-unique numbers were generated for the rows). This set excluded the first and last rows to avoid external influences from surrounding orchard blocks. An additional set of 25 non-unique numbers were generated for trees within the orchard located in the rows selected in the first number set. The first and last trees were also excluded from the sampling layout. For each month and orchard, a new row-tree number set was used. Twenty-five sample sets comprising 20 fruit and 20 leaves covering the top, middle and bottom of the tree were collected in marked brown paper bags. In 2013 and 2014, decaying leaves, weeds, pruning material were collected at random from the orchard

floor and placed in paper bags. When present, mummies were also collected. In addition, 'Hillieri' crab apples (*Malus floribunda*) were collected at the Witzenberg Valley orchard as these are used for 'Cripps Pink' pollination. All samples were stored at -0.1°C until they could be processed.

ii.Wash method for N. alba *spore removal from preharvest sample materials*

Fruit were subdivided into five manageable sets according to the tree numbers sampled from. Thus, 100 fruit from the first five trees were marked as in group one, fruit of the next five trees in the second group, and so forth. The same was done for leaves from within the tree canopy. Pruning material, decaying leaves and weeds samples were not subdivided into groups. The sonication method for removal of *B. cinerea* from grape and plum fruit by Spotts and Holz (1996) and the seed-wash method developed for seedborne pathogens by Narayanasamy (2011) were adapted and combined. First, each fruit set was processed in a sonication bath with 1 L of sterile distilled water amended with 1% Tween® 80 for 10 minutes. The fruit were discarded, the spore-fruit-wash solution poured through a clean sieve and stored at -0.1°C. This method was followed for each group until five spore-fruit-wash sets were obtained. When fruit became too large for singular processing, it was sonicated in batches recycling the same wash-water marked for that particular set. The canopy leaves, pruning material, decaying leaves and weed samples were processed the same way as mentioned above. Second, the spore-wash samples were washed and centrifuged to obtain pellets for DNA extraction. Each spore-wash set was distributed into three 250 mL Beckman jars, therefore providing three replicates per set. The replicates were spun for 20 minutes at 10.000 rpm in a J2-21 Beckman centrifuge. The supernatant was discarded and the spore-pellets transferred to 50 mL conical Falcon tubes. The tubes were filled with sterile distilled water to its maximum volume of 50 mL and briefly vortexed. Tubes were spun in a 5810 Eppendorf centrifuge for 10 minutes at 4.000 rpm. The supernatant was discarded and the wash step repeated. Resulting pellets were transferred to 15 mL Falcon tubes, filled to 15 mL with sterile distilled water, briefly vortexed and spun for 10 minutes at 10.000 rpm. The supernatant was discarded and the wash step repeated. After discarding the supernatant, the pellets were transferred to 2 mL tubes and filled to 1 mL with sterile distilled water. Tubes were spun in a centrifuge for 5 minutes at 13.000 rpm. The supernatant was discarded and the wash step repeated twice. The pellets were stored at -20.0°C.

iii.DNA extraction, primer design and polymerase chain reaction amplification

The spore-wash pellets were spun in a centrifuge for 5 minutes at 13.000 rpm and the supernatant discarded. To each pellet, approximately 0.5 g glass beads and 400 uL lysis buffer from the Wizard® Genomic DNA Purification Kit were added. Samples were homogenised in a Retsch® mill for 5 minutes at a frequency of 30 Hz. Thereafter, the lysated pellets were incubated in a 65°C water bath for 10 minutes and spun in a centrifuge at 14.000 rpm for 4 minutes. DNA was extracted and purified with a Wizard® Genomic DNA Purification Kit following the 'tissue culture cell lysates' purification method of the supplier. DNA concentration was determined on a NanoDrop® spectrophotometer. Samples were stored at -20°C.

For the identification of *N. alba* from orchard material washes, primers were designed from the partial sequences of the β-tubulin gene region published by de Jong *et al*. (2001). These sequences were obtained from GenBank, aligned and examined for conserved regions. The primer pair, NeoβtubF (5'-ACC ACTT GGT CTC CGC AGT-3') and NeoβtubR (5'-GGG ATC CAC TCG ACG AAG TA-3'), were designed in Primer3 to cover a 411 base pair (bp) fragment of the partial β-tubulin gene region.

The primer set was tested with DNA extracted from spores on BER-lesions of stored fruit and fungal colonies of confirmed *N. alba* samples collected from the Witzenberg Valley in 2009.

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These samples are STE-U 7658, 7659, 7660, 7661 and are maintained in the fungal culture bank of the Department of Plant Pathology at Stellenbosch University. A non-template control was also included. Additional testing was done with BER-representative samples of *N. alba* (CBS 304.62), *N. perennans* (CBS 453.64), *N. malicorticis* (CBS 141.22) and *N. kienholzii* (CBS 355.72, syn. *Neofabraea* sp. nov.). These isolates were obtained from the Centraalbureau voor Schimmelcultures (CBS) based in Utrecht, the Netherlands.

After successful optimisation of the PCR-conditions and reaction components, spore-washes obtained from the Witzenberg Valley and Grabouw in 2012, 2013 and 2014 were screened. Amplification was done in a GeneAmp2720 thermocycler. Each 40 µL reaction volume contained 1x reaction buffer, 1 µg/mL bovine serum albumin (BSA), 2.25 mM MgCl2, 0.2 mM dNTPs, 0.15 µM of each primer, 0.4 µL Biotaq DNA polymerase and 10-100 ng of DNA. Amplification conditions were an initial denaturing step of 3 minutes at 96°C, followed by 40 cycles consisting of 1 minute at 96°C, 1 minute at 62°C and 1 minute 30 seconds at 72°C and a final extension step of 5 minutes at 72°C. The PCR products were visualised alongside a 100-bp ladder with ultraviolet fluorescence on a 1.5% agarose gel stained with ethidium bromide.

Amplified samples were purified with a MSB® Spin PCRapace Kit following the manufacturer's instructions. For sequencing, samples were prepared with the Big Dye Terminator version 3.1 sequencing kit. Sequences were amplified in a GeneAmp® PCR System 2720 thermocycler for 5 minutes at 95°C, 30 cycles of 10 seconds at 95°C, 5 seconds at 50°C and 4 minutes at 60°C and a concluding step of 30 seconds at 60°C. Base-pairs of the amplified sequences were read in an ABI3730xl Genetic Analizer by the DNA Sequencing Unit at the Central Analytical Facility (CAF) of Stellenbosch University.

iv.Enzyme restriction analysis

The partial β-tubulin sequences generated by de Jong *et al* (2001) were aligned in Geneious and single nucleotide polymorphisms unique to *N. alba*, *N. malicorticis*, *N. perennans* and *N. kienholzii* identified in the 411-bp region amplified by the designed Neoβtub primer set. Enzymes recognising polymorphisms were selected for individual evaluation. These enzymes were BbsI, SspI, KspAI, BsuRI for *N. alba* (CBS 304.62) and HinfI for *N. malicorticis* (CBS 141.22), *N. perennans* (CBS 453.64) and *N. kienholzii* (CBS 355.72). Each enzyme digestion reaction consisted of 10 units (U) restriction enzyme, 10 x corresponding restriction buffer and 10 uL Neoβtub PCR-product. The reactions were incubated in a 37°C water bath for 3 to 16 h, as specified for the particular enzyme by the manufacturer. Fragments were separated and visualised alongside a 100-bp DNA ladder on a 3% agarose gel stained with ethidium bromide. The BsuRI enzyme was chosen for bulk screening of the spore-washes. Enzymes BsuRI and HinfI were also evaluated as a double digest in a 25 uL reaction containing 10 U of each enzyme, 10 x corresponding reaction buffer and 10 uL of PCR-product. Fragments were separated and visualised with gel electrophoresis (3% v/v).

2. Postharvest evaluation of 'Cripps Pink' apple for *N. alba*

i.Fruit collection and storage

One hundred asymptomatic 'Cripps Pink' apples were collected per producer at harvest from commercial packhouses and orchards. The producers were located in Elgin, Grabouw, Witzenberg Valley, Koue Bokkeveld and the Hemel-and-Aarde Valley in the Western Cape and sampling was done for the 2010, 2011 and 2012 seasons. Prior to collection, the fruit were not exposed to any postharvest fungicide or packhouse treatments. Collections were indiscriminate due to variable availability of fruit between producers, regions and seasons. In total, fruit were collected from 24, 42 and 42 producers distributed across the five regions for the three respective seasons. Fruit were placed on polystyrene fruit trays disinfected with Sporekill, inside commercial pome fruit boxes lined with polyethylene bags. To ensure

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favourable humidity for the development of lenticel decay, moist paper towels were placed in the base of each box. Packed fruit were stored at -0.1°C for four months in regular atmosphere (RA) and thereafter at room temperature (23°C) for 7 days to allow optimal symptom expression. Fruit were visually evaluated for bull's-eye rot symptoms and the frequency of lenticel decay per producer documented. Decay lesion morphology was also noted. Symptomatic fruit were relocated to 2°C RA for one month to allow for the development of fruiting bodies on the lenticel lesions which will be used for isolate collection.

ii. *Isolate collection and identification from stored fruit*

From each symptomatic stored fruit, an acervulus was carefully removed and crushed in 2 mL sterile distilled water. Macroconidia morphology and the presence of microconidia were noted. Spore suspensions were spread on water agar and incubated for 24h at ambient temperature. Single germinating conidia were selected and transferred to potato dextrose agar acidified with 1.25 mL of 20% lactic acid (aPDA). In the absence of fruiting bodies, fruit were surface sterilised with 70% ethanol and small sections of decaying tissue transferred to aPDA. Isolates were incubated at 23°C for 30 days. *In vitro* characteristics of the isolates were observed with regards to colony shape, colour, aerial growth and colony growth rate. In addition, the ability of colonies to sporulate on artificial medium was noted. Following colony descriptions made by (Verkley, 1999), pure cultures with *N. alba* characteristics were transferred to slanted PDA in 9 mL McCartney bottles and stored at 4°C. A total of 934 *Neofabraea* isolates were obtained in postharvest evaluations of 'Cripps Pink' apples in 2010, 2011 and 2012. Representative isolates were chosen from each region and season, cultured from storage on aPDA and incubated at to 23°C in the dark for 1 month and mycelium scraped into 2 mL tubes for DNA extraction. DNA extraction and PCR-RFLP (as described in Section 1 iii and iv) were done and sequencing performed by the DNA Sequencing Unit at the Central Analytical Facility (CAF) of Stellenbosch University. Species identity was confirmed in BLAST and comparative analysis with known *Neofabraea* species lodged in GenBank.

3. *In vitro* assay for determining fungicide efficacy of pyrimethanil and fludioxonil on *N. alba*

i. *Source of* N. alba *isolates for mycelium assay*

At April-harvest of the 2011 season, 'Cripps Pink' apples were collected from a commercial orchard located in the Witzenberg Valley, known to have high incidences of *N. alba*. The fruit were subject to commercial spray programs, but not postharvest treatments. Fruit were stored in boxes at -1°C under RA for four months. Moist paper towels were placed in each box to encourage lenticel decay development. Thereafter, symptomatic BER fruit were kept at 1°C RA for 1 to 2 months. Pure cultures were obtained by means of single spore isolation from crushed acervuli on aPDA plates. In the absence of fruiting bodies, direct tissue isolations were made from fruit surface sterilised with 70% ethanol. Cultures were incubated at incubated at to 23°C in the dark for 20 days. Isolates were identified as *N. alba* according to the description of the fungus by Verkley (1999) and PCR-RFLP protocol developed in Section 1. Isolates were transferred onto 9 mL slanted PDA in 15 mL McCartney bottles, sealed with Parafilm M (Bemis, Neenah, Wisconsin) and stored at 4.0°C. A total of 54 single-spore cultured *N. alba* isolates obtained from stored 'Cripps Pink' apple were chosen for screening in this study.

ii. *In vitro assay*

From storage, isolates were sub-cultured onto malt extract agar (MEA) and incubated at 23°C for one month in the dark. Technical grade fludioxonil, pyrimethanil, and flusilazole were dissolved in acetone and adjusted to 10 g/L stock solution. A concentration range of 0.0, 0.005, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 mg/L was prepared for flusilazole in PDA. A

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concentration range of 0.0, 0.1, 1, 10, 15, 20, 50, 100 was prepared for pyrimethanil in a minimal medium (Yourman and Jeffers, 1999) and for fludioxonil in MEA. An additional screening mycelial assay was done for 37 isolates at 0.0, 150.0 and 190.0 mg/L, the highest soluble concentration in acetone, for fludioxonil. One litre of minimal medium contained 12 g of bacteriological agar, 10 g of glucose, 3 g of $NaNO_3$, 1 g of KH_2PO_4, 1 g of K_2HPO_4, 0.5 g of $MgSO_4.7H_2O$, 0.5 g of KCl, and 0.01 g of $FeSO_4.7H_2O$, with a final pH of 6.6. The final concentration of acetone in all media, including the 0 mg/L concentration, was 0.1 mL/L.

A 4-mm-diameter plug from the margin of the colony was placed inverted on fungicide-amended plates. Each isolate was done in triplicate for each concentration. Plates were sealed with sealed with parafilm. Radial growth, or colony diameter, was measured after incubation at 23°C in the dark for 21 days. The percentage mycelial inhibition relative to the control colony was calculated for each isolate for each concentration. The effective fungicide concentration inhibiting 50% of mycelial growth or conidial germination of the population (EC_{50}) was determined by regression analysis with log^{10} concentrations of fungicides used. Higher concentrations, 150.0 and 190.0 mg/ L, were tested for 37 *N. alba* isolates to the fludioxonil concentrations range, as growth was observed at 100.0 mg/ L in the previous assay.

4. Weather data evaluation

Data was collected from weather stations located near the Witzenberg Valley and Grabouw 'Cripps Pink' orchards from which fruit were sampled for rapid detection analysis (Section 1). The data period was for seasons 2012, 2013 and 2014. Precipitation, maximum and minimum temperature and humidity were evaluated statistically by the Centre for Statistical Consultation, Stellenbosch University.

5. Identification of *Neofabraea* species causing BER of 'Packham's Triumph' pear

i. Isolate collection

Potential *Neofabraea* isolates and 3400 'Packham's Triumph' pears were collected from ExperiCo, Stellenbosch in 2013. The fruit were collected at harvest from a commercial farm located in the Witzenberg Valley of the Western Cape and treated as described in Section 2 i. Fruit were visually evaluated for bull's-eye rot symptoms. Decay lesion morphology was documented. Isolations were made onto aPDA from decaying tissue of the symptomatic fruit. The plates were incubated at incubated at to 23°C in the dark for 1 week and *Neofabraea*-like cultures transferred to fresh aPDA and incubated under the same conditions for 20 days. Thereafter, cultures were transferred to 9 mL slanted PDA and stored at 4°C in the dark.

An additional *Neofabraea* isolate from a 'Packham's Triumph' pear of the Grabouw-region was obtained from the Disease Clinic at the Department of Plant Pathology, Stellenbosch University.

ii. *Species identification*

Collected isolates were cultured from storage onto aPDA and incubated at to 23°C in the dark for 1 month. Thereafter, approximately 100 mg of mycelium was scraped into 2 mL tubes. The DNA was extracted with a Wizard® Genomic DNA Purification Kit following the same procedure for the spore pellets in Section 1 iii. Extracted DNA was amplified with the Neoβtub primers, visualised and amplified product digested with the double enzyme digestion designed in Section 1 iii). The resulting restriction patterns were confirmed by sequencing the PCR and performing a nucleotide sequence comparison search in BLAST. Comparative analysis was also done in the programme Geneious with known *Neofabraea* species lodged in GenBank.

c) RESULTS AND DISCUSSION

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1. <u>Rapid detection of *N. alba* in 'Cripps Pink' orchards</u>
The designed forward and reverse Neoβtub primer set successfully yielded the expected 411-bp fragment for *N. alba* when tested with STE-U and CBS representative isolates (Fig 4). Co-incidentally, the primer set was observed to amplify N. malicorticis, N. perennans and N. kienholzii. Therefore, the primer set was genus-specific rather than species-specific (Fig. 4). However, the double enzymes, BsuRI and HinfI, were sufficient in identifying each species. The BsuRI enzyme can be used to distinguish *N. alba*, and HinfI for *N. malicorticis*, *N. perennans* and *N. kienholzii*.

Neofabraea alba was successfully detected as early as December, two months post full bloom, in fruit washes of 'Cripps Pink' apples sampled in the Witzenberg Valley and Grabouw orchards (Table 1). However, *N. alba* was detected inconsistently, i. e. not every month or consecutively, in fruit washes for all three seasons. Ceustermans *et al*., (2015) observed variation in the level of *N. alba* infestation on 'Pinova' apples between trees in the same orchard. As a randomised sampling design was followed each month, this could have influenced detection. Also, the sampling orchards are subject to commercial practices which would have influenced the amount of inoculum present on the environmental samples tested. Summarising the detection of *N. alba* on 'Cripps Pink': the pathogen was not detected during spring (November), but frequently in summer and in autumn. This result correlates with the sporulation period observed for by Henriquez *et al*. (2006) and Burchill and Edney (1961). Precipitation is needed for the production, dispersal and germination of *N. alba* (Tan and Burchill, 1972; Edney, 1974). The period in which *N. alba* was detected corresponds with increased precipitation during the maturation phase and harvest period of 'Cripps Pink' apple cultivation in the Western Cape.

Neofabraea alba was not detected on leaves or other plant material. The PCR-RFLP method is not sensitive enough for the detection of low spore counts on the plant material. IN addition, the wash method is the best approach for removal of spores from plant surfaces, but at its best would only remove 50% of the inoculum (Spotts and Holz, 1996). Köhl *et al*., (2014) utilised fluorescent probes in a molecular TaqMan-PCR assay and found *N. alba* to be present on mummies, dead leaves, various weeds and grasses in apple and pear orchards. Yet, the presence of *N. alba* on the plant materials also varied within and between orchards during the growing season, as was observed with the fruit washes in this study, and was ascribed to orchard characteristics and the influence of management strategies

Surprisingly, *Neofabraea alba* was detected in washes of the 'Hillieri' crab apple variety used as 'Cripps Pink' pollinators in the Witzenberg Valley orchard (Table 2). In addition to N. alba, *N. perennans* and *N. kienholzii* was detected in washes made from 'Cripps Pink' apple early (January and December) in the 2013 season (Table 2). Neofabraea perennans was present in both regions, whereas N. kienholzii only in Witzenberg Valley. Sequence analysis confirmed the PCR-RFLP result. Postharvest evaluation of 'Cripps Pink' apple from these orchards found the causal agent of BER to be *N. alba*, not *N. perennans* or *N. kienholzii*.

The source of *Neofabraea* inoculum in 'Cripps Pink' orchards could not be established in this study. Cankers, the main source of inoculum for BER species, were not observed in the orchards from which fruit was sampled in this study. This suggests an alternate source from which these species could have originated. Possibly other apple or pear varieties located in nearby orchards. To our knowledge, this is the first report of *N. perennans* and *N. kienholzii* in South Africa. This is of high phytosanitary importance as pome fruit is a major export commodity of South Africa.

2. <u>Postharvest disease incidence of *N. alba* on 'Cripps Pink' apple</u>
Due to the inconsistent availability of fruit for this experiment, statistical correlations of the disease incidences between producers, regions and seasons could not be made. Fruit had typical N. alba lesions, were concentric brown and tan rings and mature lesions developed wet cream-coloured fruiting bodies (Fig 1). The decaying tissue was firm, easily separated from the healthy tissue and extended into the fruit (Fig. 2)
Bull's-eye rot occurred on fruit from all regions sampled for all three seasons evaluated (Table 3).

The occurrence of BER in 100 fruit collected per producer ranged between 0.0 to 73% in 2010, 0.0 to 6% in 2011 and 0.0 to 30% in 2012. The average disease incidence of BER

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on 'Cripps Pink' apple was 0.0 to 36.8% in 2010, 0.5 to 1.2% in 2011 and 2.0 to 6.8% in the 2012 season (Table 4). Disease incidence on fruit from producers in the Witzenberg Valley was higher than other regions, specifically producer-8. Producer-8 used the 'Hillieri' crab apple as a pollinator and the presence of *N. alba* on this cultivar could have increased the level of disease incidence observed. Also, it is possible that the climate in Witzenberg Valley is more conducive to *N. alba* disease development than other regions, but this needs to be investigated before a final conclusion can be made.

3. In vitro fungicide efficacy screening for *N. alba* towards pyrimethanil and fludioxonil

In this study, pyrimethanil EC_{50}-values were determined and had a mean EC_{50}-value of the population to be 6.69 mg/ L and ranged from 0.93 mg/ L to 58.6 mg/ L (Fig. 3). Some of the pyrimethanil isolates were capable of growing at 100 mg/ L. Fludioxonil could not inhibited *N. alba* isolates at the highest concentrations (100 and 190 mg/ L) tested (Fig. 4 and 5).

The *N. alba* population in this study has not been exposed to pyrimethanil or fludioxonil. However, the orchard from which the isolates were obtained receives commercial sprays, of which cyprodinil is applied early each season for the control of apple scab. Cyprodinil and pyrimethanil belong to the anilinopyrimidine class. Cross-resistance between pyrimethanil and cyprodinil has been observed for *B. cinerea*, *P. expansum* and *V. inaequalis* (Leroux *et al*., 2002; Li and Xiao, 2008; Larsen *et al*., 2013). It is likely, that the exposure to cyprodinil predisposed the *N. alba* population to be resistant towards pyrimethanil.

The inefficacy of fludioxonil and level of mycelial growth would suggest a genetic adaptation of the pathogen. Regarding past studies on apple pathogens *B. cinerea* and *P. expansum*, fludioxonil resistance are likely mediated by mutations in the ABC-, MFS-transporter regions and point mutations in the β-tubulin gene (Leroux *et al*., 2002; Li and Xiao, 2008; Kretschmer *et al*., 2009). This would be a prospective starting point for genetic investigating of this study's *N. alba* population for describing the absence of control for fludioxonil.

From observations made during the mycelium sensitivity screening of pyrimethanil, inconsistencies were observed in the growth of the pathogen. In some cases, the pathogen perished on the minimal medium. This could be ascribed to the nature of the pathogen and deprivation of sufficient nutrients. *Neofabraea alba* is a slow growing pathogen and survives as a saprophyte (White and Wilkinson, 1962; Spotts *et al*., 2009). Minimal medium is required for pyrimethanil to avoid interaction of the fungicide with the complex compounds normally found in a nutrient rich medium such as malt-agar (Hilbera and Schuepp, 1996). On a minimal medium the pathogen would be deprived of sufficient nutrients, resulting in poorer growth. From this study and one done by Spotts *et al*. (2009), it is evident that current methodologies are not suitable for the *in vitro* evaluation of *N. alba* sensitivity towards pyrimethanil. An alternative and reliable method would therefore be a prerequisite for future *in vitro* fungicide testing of *N. alba*.

4. Weather data

Preliminary statistical analysis found no conclusive results regarding specific climatic factors influencing disease development of *N. alba* in the two 'Cripps Pink' orchards. More in depth statistical analysis such as multivariate analysis could shed more light on this matter. Yet, little information is available regarding the sporulation patterns, distribution, infection capabilities and survival of *N. alba*, especially in South African orchards. Gaining more informative information on the epidemiology of this pathogen would be essential before climatic data could be fully analysed and a weather prediction model developed.

5. *Neofabraea* species affecting 'Packham's Triumph' pears

Neofabraea alba was successfully identified from infected tissue of 'Packham's Triumph' pears from the Witzenberg Valley. Only two fruit were affected by N. alba in the 3 400 fruit lot evaluated. *Neofabraea perennans* was isolated from a pear of the Grabouw region. Further investigation of 'Packham's Triumph' orchards from which these two Neofabraea pathogens originated were not be done in this study.

d) CONCLUSIONS

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Neofabraea alba is the only causal agent of BER on 'Cripps Pink' apple and is present in five growing regions in the Western Cape. Although a management strategy specific to this pathogen is not yet in place, information gained in this study will contribute to its development.

In this study, *N. alba* conidia was detected in washes on fruit throughout the growing season in summer and autumn, the frequency of detection increasing in the final month before harvest. A spray program should be followed the whole season, with focus in the final month before harvest as inoculum are the most abundant and fruit highly susceptible during this stage.

In this study, fludioxonil could not inhibit mycelial growth at the highest concentration. Pyrimethanil could control the population, albeit at a very high concentration. From observations made in this study and that done by Spotts *et al*. (2009), *in vitro* testing of *N. alba* towards pyrimethanil is not a suitable method for determining fungicide efficacy. *In vivo* and field trials should be done to provide a more accurate representation of fludioxonil and pyrimethanil efficacy on *N. alba* of 'Cripps Pink' orchards as laboratory results does not necessarily dictate field efficacy. For example, Bylemans and Goodwine (2004) observed excellent control of *N. alba* with a commercial formulation of pyrimethanil applied as a dip, drench and in-line spray. This is supported by dip trials of inoculated d'Anjou pears by Spotts et al. (2009). Moreover, the application of a commercial fludioxonil product as a dip and thermal-fog proved good control of *N. alba* can be obtained in the field (Spotts *et al*., 2009; Nokwasi Mbili, personal communication).

The presence of *N. kienholzii* in fruit washes of 'Cripps Pink' apples, although not a causal agent of rot in this variety, and the identification of *N. perennans* and *N. alba* from 'Packham's Triumph pears are an important phytosanitary issue. Expanding future BER research to include 'Packham's Triumph' pears and other apple varieties would be important in assessing the impact of these pathogens on the pome fruit industry in the Western Cape.

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Tables and Figures

Table 1. Seasonal detection of *N. alba* conidia on the surface of 'Cripps Pink' apples from two commercial orchards in the Western Cape.

		Month					
Region	**Season**	**Nov**	**Dec**	**Jan**	**Feb**	**Mar**	**Apr**
Witzenberg Valley	2012		*N. alba*			*N. alba*	*N. alba*
	2013			*N. alba*	*N. alba*	*N. alba*	*N. alba*
	2014						N. alba

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	2012		*N. alba*	
Grabouw	2013	*N. alba*		
	2014			*N. alba*

Table 2: Seasonal detection of *Neofabraea* species on the surfaces of 'Cripps Pink' apple and 'Hillieri' crab apples of commercial orchards located in the Witzenberg Valley and Grabouw, respectively.

Region	Season	Month					
		Nov	Dec	Jan	Feb	Mar	Apr
Witzenberg Valley	2012			'Hillieri'[a]			
	2013		Hillieri'[a], *N.perennans*	Hillieri'[a]			
	2014						
Grabouw	2012						
	2013	*N.kienholzii*	*N.perennans*				
	2014						

[a]*Neofabraea alba*.

Table 3: Incidence of bull's-eye rot, caused by *N. alba*, on 'Cripps Pink' apple collected from different growing regions in the Western Cape for seasons 2010, 2011 and 2012.

Region and assigned producer number	Season[a]		
	2010[b]	2011[b]	2012[b]
Elgin-1	2	3	0
Elgin-2	1	0	0
Elgin-3	0	0	0
Elgin-4	–	1	2
Elgin-5	0	0	0
Elgin-6	–	6	5
Elgin-7	–	0	1
Elgin-8	4	1	2
Elgin-9	–	0	3
Elgin-10	0	2	3
Elgin-11	0	0	0

	Season[a]

Region and assigned sample number for producers	2010[b]	2011[b]	2012[b]
Elgin-12	–	0	–
Hemel-en-Aarde Valley-1	3	1	12
Hemel-en-Aarde Valley-2	1	3	0
Hemel-en-Aarde Valley-3	0	1	1
Hemel-en-Aarde Valley-4	–	0	0
Hemel-en-Aarde Valley-5	0	1	1
Koue Bokkeveld-1	1	0	0
Koue Bokkeveld-2	–	0	0
Koue Bokkeveld-3	–	3	–
Koue Bokkeveld-4	–	0	1
Koue Bokkeveld-5	2	0	–
Vyeboom-1	–	0	–
Vyeboom-2	–	0	–
Vyeboom-3	1	1	2
Vyeboom-4	–	0	–
Vyeboom-5	–	0	0
Vyeboom-6	4	2	2
Vyeboom-7	4	0	2
Vyeboom-8	–	0	–
Vyeboom-9	6	0	9
Vyeboom-10	–	0	–
Vyeboom-11	–	0	–
Witzenberg Valley-1	–	0	–
Witzenberg Valley-2	15	0	3
Witzenberg Valley-3	18	0	11
Witzenberg Valley-4	–	0	1
Witzenberg Valley-5	–	0	0
Witzenberg Valley-6	–	0	1
Witzenberg Valley-7	–	0	0
Witzenberg Valley-8	73	4	30

[a] Fruit with bull's eye rot per one hundred fruit collected per producer
[b]The symbol: (-) indicate producers not sampled for the particular season.

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Table 4: Incidence of *N. alba* from stored 'Cripps Pink' apple collected from producers located across five growing regions in the Western Cape Province.

Region	Number of *N. alba* isolates[a]	Disease incidence (%)[b]		
		2010	**2011**	**2012**
Elgin	261	0.8	1.2	2.0
Grabouw	84	3.2	0.3	3.9
Hemel-en-Aarde Valley	45	1.0	1.2	2.8
Ceres	122	1.5	0.6	4.7
Witzenberg Valley	422	36.5	0.5	6.8

[a]Total number of *N. alba* isolates collected per region for the three seasons sampled.
[b]Average disease incidence of BER for each region after four months of cold storage at - 0.1 ± 1°C, regular atmosphere (RA), and thereafter at room temperature (23 ± 1°C) for 7 days.

Figure 1: Advanced *N. alba* lenticel decay, with classic concentric dark and light brown rings, a tan centre and wet, cream-coloured fruiting bodies on the lesion surface

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Figure 2: Conical-shaped *N. alba* rot extending into a 'Cripps Pink' apple.

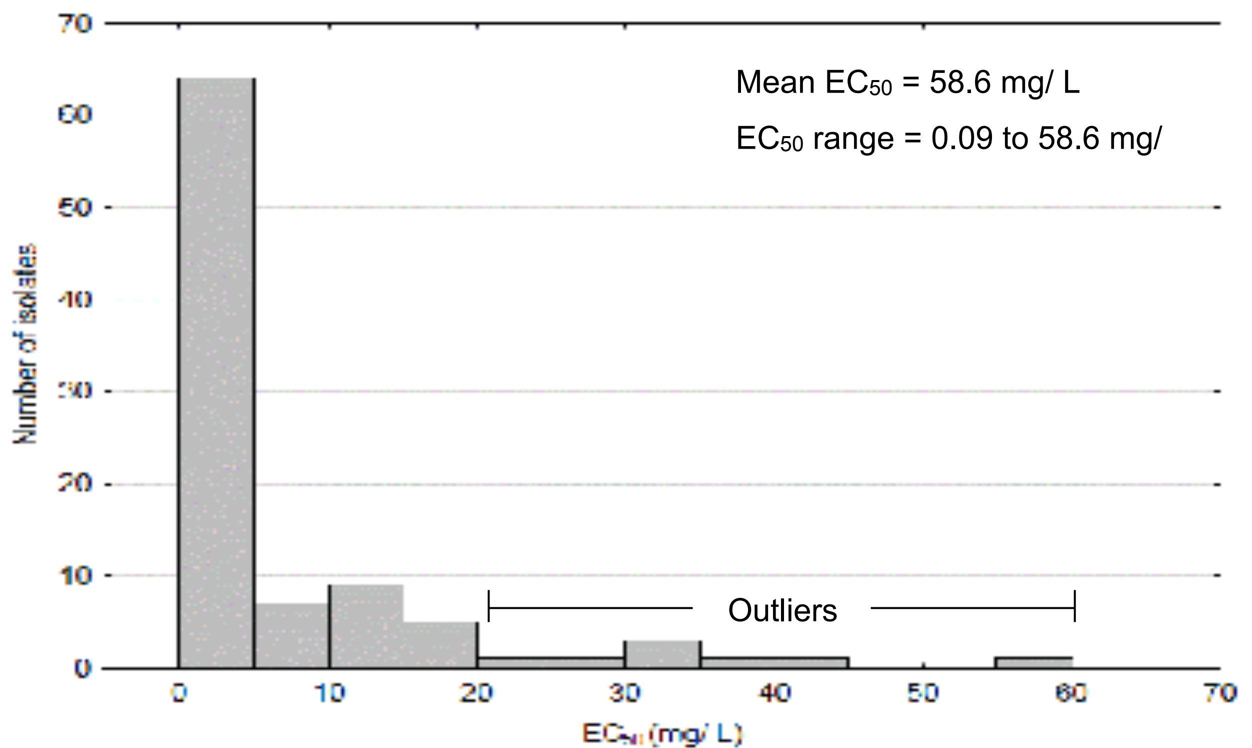

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Figure 3: Pooled EC_{50} distribution of pyrimethanil for the *in vitro* mycelium assay of an *N. alba* population from 'Cripps Pink' apple. The population was not exposed to the fungicide in the orchard or at harvest.

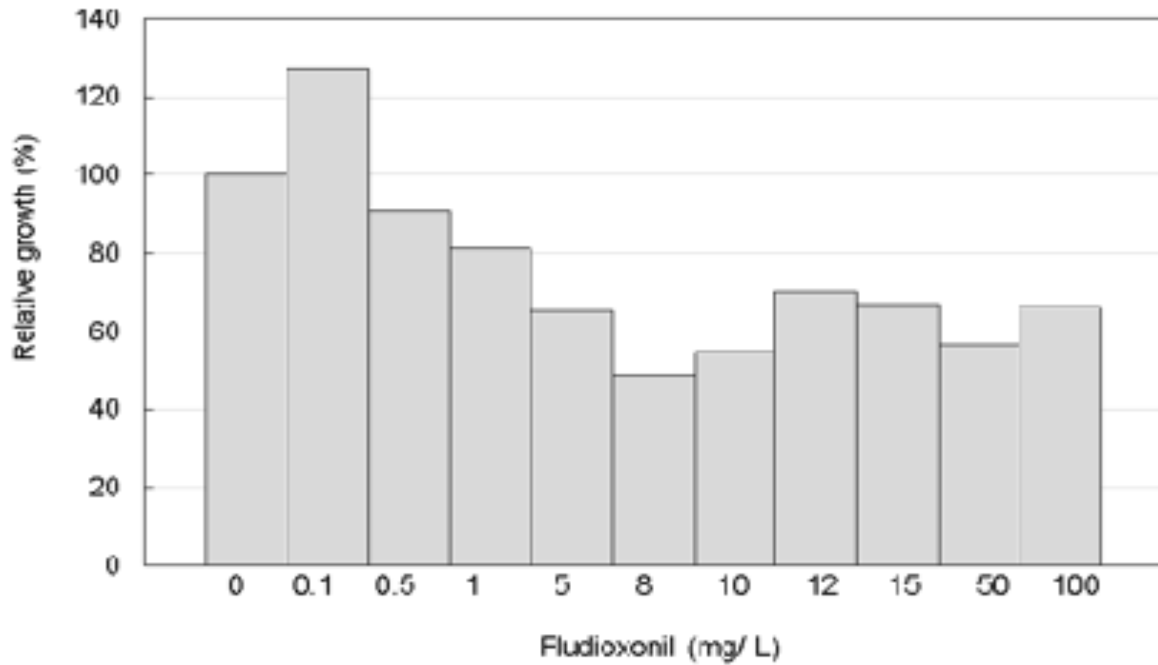

Figure 4: *In vitro* mycelial growth determined for a *N. alba* population from a commercial 'Cripps Pink' orchard in the Witzenberg Valley not exposed to the phenylpyrrole fungicide fludioxonil.

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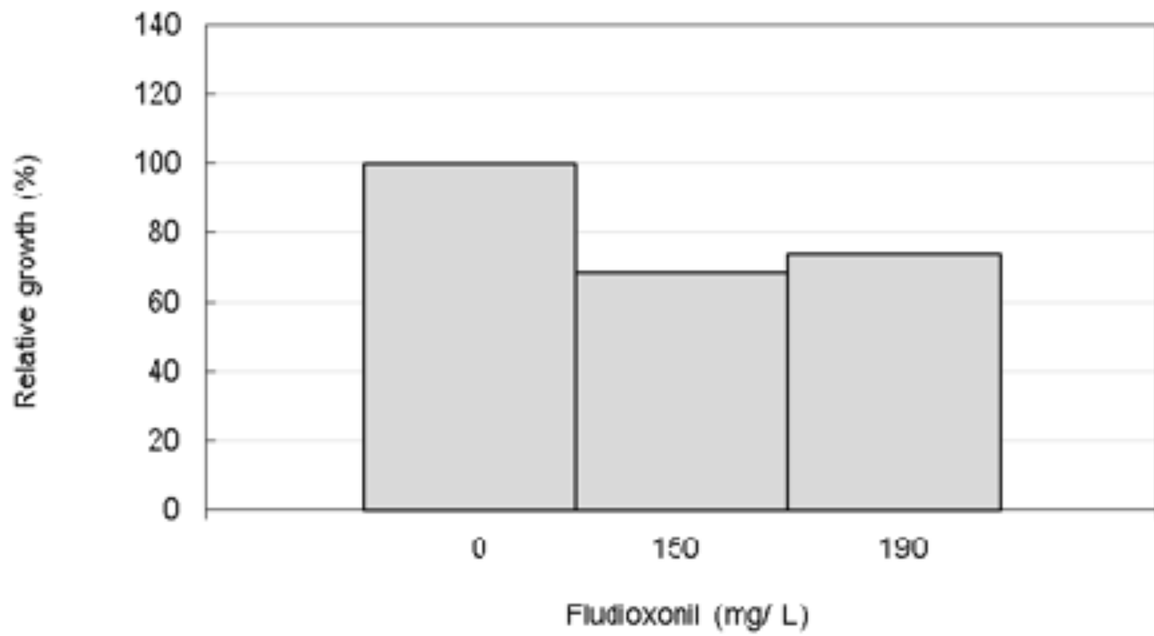

Figure 5: Mycelial growth determined for 37 *N. alba* isolates towards the highest soluble concentration of fludioxonil (190 mg/ L) in acetone.

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ACCUMULATED OUTPUTS

2011

Identification of *N. alba* present on preharvest and stored 'Cripps Pink' apple;

Development of rapid detection method, comprising of a spore removal wash-method and designed genus specific-primers.

2012

Determined the *in vitro* fungicide sensitivity of flusilazole on *N. alba.*

2013

Compilation of disease incidence and distribution data for BER in the Western Cape.

2014

Developments of double enzyme restriction digest to distinguish between the four BER species.

Determined the window period, or timing, of *N. alba* conidia on fruit surfaces during the growing season using PCR-RFLP.

2015

Identification of *N. alba* present on preharvest and stored 'Cripps Pink' apple

Identification of *N. alba* and *N. perennans* on 'Packham's Triumph' pear

Determined the *in vitro* fungicide efficacy of fludioxonil and pyrimethanil on *N. alba*

MsC Degree – Jessica Rochefort

a) TECHNOLOGY DEVELOPED, PRODUCTS AND PATENTS

1. Rapid detection method for South African *Neofabraea* spp. on the surface of apples.
2. Double enzyme restriction digests for differentiating between four *Neofabraea* species causing postharvest fruit rot.

b) SUGGESTIONS FOR TECHNOLOGY TRANSFER

1. Popular articles.
2. Verbal presentations at agricultural events.
3. Informal Q and A sessions with producers and packhouses.

c) HUMAN RESOURCES DEVELOPMENT/TRAINING

Student Name and Surname	Student Nationality	Degree (e.g. MSc Agric, MComm)	Level of studies in final year of project	Graduation date	Total cost to industry throughout the project
Honours students					
Masters Students					
Jessica Rochefort	South Africa	MSc	3	9/12/2015	130 000
PhD students					

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Postdocs					
Alana Den Breeyen	South Africa	PhD			100 000
Support Personnel (not a requirement for HORTGRO Science)					
Elveresha Davids	South Africa	Technician			
Michell Leibrandt	South Africa	Technician			

PERSONS PARTICIPATING IN THE PROJECT (Excluding students)

Initials & Surname	Highest Qualification	Degree/ Diploma registered for	Race (1)	Gender (2)	Institution & Department	Position (3)	Cost to Project R
C. L. Lennox	Doctorate		W	F	Stellenbosch University, Dept. Plant Pathology	PL	NA
A. Den Breeyen	Doctorate		W	F	Weeds Pathology Unit, ARC-PPRI	PF/Coll	100 000
Elveresha Davids	Grade 12		B	F	SU, PP	TA	75 000
Michelle Leibrandt	Grade 11		B	F	SU, PP	TA	75 000

[1]Race B = African, Coloured or Indian
W = White

[2]Gender F = Female
M = Male

[3]Position Co = Co-worker (other researcher at your institution)
Coll = Collaborator (participating researcher that does not receive funding for this project from industry)
PF = Post-doctoral fellow
PL = Project leader
RA = Research assistant
TA = Technical assistant/ technician

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

Den Breeyen A & Lennox CL 2012. Towards the development of a rapid detection method for bull's eye rot in apples. Integrated Plant Protection in Fruit Crops", Subgroup "Pome Fruit Diseases", IOBC-WPRS Bulletin 84: 5-8

e) PRESENTATIONS/PAPERS DELIVERED

Den Breeyen & Lennox 2011. Etiology of *Neofabraea* spp. on storage apples in the Western Cape. Presented at the Southern African Society of Plant Pathology Conference, 23rd – 27th January 2011, Kruger National Park, South Africa.

Den Breeyen & Lennox 2011. A study on bull's eye rots of apples in South Africa. Presented at the 9th International Congress on Postharvest Pathology, 11th – 14th April 2011, Spain;

Den Breeyen & Lennox 2011. Towards the development of a rapid detection method for bull's eye rot in apples. Presented at the 9th International IOBC/WPRS Workshop on Pome Fruit Diseases, 29th August – 2nd September 2011, Belgium.

Den Breeyen A & Lennox CL 2012. Distribution and Incidence of Bull's Eye Rot of Apples in South Africa. Extended abstract presented at the 2012 CIGR Section VII International Technical Symposium on "Innovating the Food Value Chain". Postharvest Technology and Agri-Food Processing, Stellenbosch, South Africa, 25-29 November, 2012.

Den Breeyen A & Lennox CL 2013. A review of *Neofabraea alba* on 'Cripp's Pink' apples in South Africa. Presented at the 48th Congress Of The Southern African Society For Plant Pathology, 20 – 24th January 2013, Bella Bella, South Africa.

Rochefort J, Den Breeyen A & Lennox CL 2014. Fungicide sensitivity of a South African *Neofabraea alba* population towards fungicides mancozeb, flusilazole, pyrimethanil and fludioxonil. Extended abstract presented at IOBC-WPRS Stellenbosch 2014 Workshop taking place at the Stellenbosch Institute for Advanced Study (STIAS), Stellenbosch, South Africa from 24 – 28 November 2014.

Rochefort J, Den Breeyen A & Lennox CL 2014. Orchard Detection, Disease Incidence and Distribution of Bull's Eye Rot on 'Cripp's Pink' apples in the Western Cape of South Africa. Presented at IOBC-WPRS Stellenbosch 2014 Workshop taking place at the Stellenbosch Institute for Advanced Study (STIAS), Stellenbosch, South Africa from 24 – 28 November 2014.

Rochefort J, Den Breeyen A & Lennox CL 2015. Disease incidence and distribution, and timing of bull's eye rot infection on 'Cripp's Pink apple in growing regions of the Western Cape. Presented at the 49th Congress Of The Southern African Society For Plant Pathology, 18 – 21th January 2015, Bloemfontein, South Africa.

Rochefort J, Den Breeyen A & Lennox CL 2015.Determining the sensitivity of a *Neofabraea alba* population from 'Cripp's Pink' apple towards fungicides pyrimethanil and fludioxonil. Presented at the 49th Congress Of The Southern African Society For Plant Pathology, 18 – 21th January 2015, Bloemfontein, South Africa.

BUDGET

TOTAL COST SUMMARY OF THE PROJECT

YEAR	CFPA	DFTS	Deciduous	SATI	Winetech	THRIP	OTHER	TOTAL
2011			105 000					105 000
2012			120 000					120 000
2013			150 000					150 000
2014			175 000					175 000

2015				175 000					175 000

EVALUATION BY INDUSTRY

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Project number	

Project name	

Name of Sub-Committee*	

Comments on project

Committee's recommendation (Review panel in the case of PHI)

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

Chairperson____ Date____________________

*SUB-COMMITTEES

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Version 2015

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

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

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