Rapid detection of ratoon stunting disease; final report 2013/001

Berna, A
Sugar Research Australia

http://hdl.handle.net/11079/17018

Downloaded from Sugar Research Australia Ltd eLibrary
## SRA Research Project Final Report

### Rapid detection of ratoon stunting disease

<table>
<thead>
<tr>
<th>SRA Project Code</th>
<th>2013/001 (CES001)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Project Title</strong></td>
<td>Rapid detection of ratoon stunting disease</td>
</tr>
<tr>
<td><strong>Key Focus Area in SRA Strategic Plan</strong></td>
<td>Pest, disease and weed management</td>
</tr>
<tr>
<td><strong>Research Organisation(s)</strong></td>
<td>CSIRO</td>
</tr>
<tr>
<td><strong>Chief Investigator(s)</strong></td>
<td>Amalia Berna, Stephen Trowell, Barry Croft</td>
</tr>
</tbody>
</table>

### Project Objectives

This project’s objective was to develop a rapid 'sniff test' to diagnose ratoon stunting disease (RSD) using a new technology called 'Enose' or electronic nose. The aim was to be able to identify contaminated cane within minutes in the field, rather than wait for samples to be aggregated, shipped to, and tested at a central laboratory and with a procedure that takes more than a day to complete. Some species of pathogenic bacteria can be characterized by the volatile chemicals they produce and it is believed that *Leifsonia xyli* will be the same. The proposed test could be applied to a large number of sap samples or to freshly cut cane in a sampling chamber. The headspace would be drawn off the sample and analysed by Enose with minimal processing. The test could be automated and performed in a laboratory or, if desired, close to or in the field. The eNose test would improve RSD management and reduce costs to Productivity Service companies.

<table>
<thead>
<tr>
<th><strong>Milestone Number</strong></th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Milestone Due Date</strong></td>
<td>1 May 2016</td>
</tr>
<tr>
<td><strong>Date submitted</strong></td>
<td>12th May 2016</td>
</tr>
<tr>
<td><strong>Reason for delay</strong></td>
<td>The final report required to be approved by CSIRO reviewers before submission to SRA</td>
</tr>
<tr>
<td><strong>Milestone Title</strong></td>
<td>Final Report</td>
</tr>
<tr>
<td><strong>Success in achieving the objectives</strong></td>
<td>☐ Completely Achieved  ☒ Partially Achieved  ☐ Not Achieved</td>
</tr>
</tbody>
</table>
| SRA measures of success for Key Focus Area (from SRA Strategic Plan) | • Industry supported through effective pest, disease and weed diagnostic capabilities and awareness and training programs.  
• Capability to provide entomology, pathology and weed expertise to meet the pest, disease and weed diagnostic and management needs of the industry. |

© Sugar Research Australia Limited 2016

Copyright in this document is owned by Sugar Research Australia Limited (SRA) or by one or more other parties which have provided it to SRA, as indicated in the document. With the exception of any material protected by a trade mark, this document is licensed under a Creative Commons Attribution-NonCommercial 4.0 International licence (as described through this link). Any use of this publication, other than as authorised under this licence or copyright law, is prohibited.

http://creativecommons.org/licenses/by-nc/4.0/legalcode - This link takes you to the relevant licence conditions, including the full legal code.

In referencing this document, please use the citation identified in the document.

Disclaimer:
In this disclaimer a reference to “SRA” means Sugar Research Australia Ltd and its directors, officers, employees, contractors and agents.

This document has been prepared in good faith by the organisation or individual named in the document on the basis of information available to them at the date of publication without any independent verification. Although SRA does its best to present information that is correct and accurate, to the full extent permitted by law SRA makes no warranties, guarantees or representations about the suitability, reliability, currency or accuracy of the information in this document, for any purposes.

The information contained in this document (including tests, inspections and recommendations) is produced for general information only. It is not intended as professional advice on any particular matter. No person should act or fail to act on the basis of any information contained in this document without first conducting independent inquiries and obtaining specific and independent professional advice as appropriate.

To the full extent permitted by law, SRA expressly disclaims all and any liability to any persons in respect of anything done by any such person in reliance (whether in whole or in part) on any information contained in this document, including any loss, damage, cost or expense incurred by any such persons as a result of the use of, or reliance on, any information in this document.

The views expressed in this publication are not necessarily those of SRA.

Any copies made of this document or any part of it must incorporate this disclaimer.

# Table of Contents

Section 1: Executive Summary ................................................................. 4  
Section 2: Background ........................................................................... 5  
Section 3: Outputs and Achievement of Project Objectives ..................... 5  
  Materials and methods ......................................................................... 6  
  Results and discussion ......................................................................... 8  
  Conclusions ......................................................................................... 12  
Year 2: Diagnostic potential of Enose ..................................................... 12  
  Materials and methods ......................................................................... 12  
  Results and discussions: ...................................................................... 16  
  Agreed milestones additional to the original plan ................................. 18  
  Conclusions ......................................................................................... 20  
Year 3: Enose test in the field ................................................................. 21  
  Materials and methods: ...................................................................... 21  
  Results and discussions: ...................................................................... 22  
  Conclusions ......................................................................................... 25  
Overall Conclusions: ............................................................................. 25  
Section 4: Outputs and Outcomes ............................................................ 25  
Section 5: Intellectual Property (IP) and Confidentiality ........................ 26  
Section 6: Industry Communication and Adoption of Outputs ............... 26  
Section 7: Environmental Impact ............................................................ 27  
Section 8: Recommendations and Future Industry Needs .................... 27  
Section 9: Publications .......................................................................... 27  
References ............................................................................................. 29
Section 1: Executive Summary

Ratoon Stunting Disease (RSD) is hard to detect and control. Incidence of the disease depends on how strictly growers follow control measures aimed at excluding infected cane from the propagation cycle. RSD is caused by the bacterium Leifsonia xyli, which infects the xylem of sugarcane. RSD has no easily attributed symptoms but can cause yield losses of 5-60%, depending on the variety susceptibility and/or whether plants are stressed or otherwise.

The current RSD diagnostic assay is based on an antibody test (EB-ELISA), which requires the plates to be shipped to a central laboratory, which screens many samples, but this is slow and logistically complicated. DNA-based tests are highly sensitive but require complex logistics and not cost effective.

To address the above issues, we proposed to develop a rapid “sniff test” using diagnostic volatiles presumptively released from infected cane. Such a test could be applied either to a large number of sap samples or, directly, to freshly cut pieces of cane. In both cases, the headspace would be drawn off the sample and analysed by an electronic nose (Enose) without further processing. This could be automated and performed in a laboratory, or, if desired, close to or in the field. Our proposed method is non-destructive and could be used to screen sugar cane directly.

Main results of this project:
1. There are specific volatiles (principally styrene and ethyl benzene) that are diagnostic for Leifsonia xyli infection of sugar cane.
2. Using gas chromatography mass spectrometry (GC-MS) these specific volatiles allow diagnosis of Leifsonia xyli infection with ≥94% accuracy across years, in low and high infection samples and across cultivars.
3. The all or nothing nature of volatiles response (no association with level of infection) suggests that a systemic plant response generates these volatiles.
4. A small percentage (7.7%) of sugar cane sap samples not infected with Leifsonia xyli give atypically high levels of styrene and ethylbenzene presumably due to some other unidentified disease/s or physiological condition.
5. There were very few false negatives using GC-MS/GC-QToF-MS (0.7-2.1%).
6. Of the Enoses tested, DiagNose with k-nearest neighbours (KNN) gave the best classification rate (≥80%) on aged samples in the lab but the protocol was not significantly more convenient than current laboratory based methods.
7. The lab based Enose protocols were shortened for field testing and, under these conditions, the DiagNose did not discriminate infected from non-infected samples (~50% accuracy). It was found that the problem was due to the lower signal levels in fresh samples compared with samples that had been stored frozen.

Whilst we demonstrated that current commercial Enoses did not have the sensitivity nor selectivity to be used to diagnose RSD in the field, we were able to identify and validate robust volatile biomarkers. As improved Enose/biosensor technologies are developed they should be tested for their ability to diagnose RSD and possibly other plant diseases.

Outputs of this project:
Section 2: Background

There is an ongoing need for simple, sensitive, fast and accurate detection of microbes associated with plants and plant tissue. The requirement encompasses microbes that are serious plant pathogens as well as those that cause illness in animals or humans. Examples of the latter include *Aspergillus flavus* and *Listeria monocytogenes*. *Leifsonia xyli*, which causes RSD, is a pathogen of economic significance for the sugar industry. Control of RSD depends on maintenance of hygiene and exclusion of infected cane from the propagation cycle. The current EB-ELISA diagnostic tool used by the industry has been effective in helping Australian growers manage RSD but it is slow and cumbersome and costs a total of up to $0.5M per annum. Easier and faster methods of detecting *L. xyli* would be valuable since they would accelerate the decision-making process and reduce costs. Plant and animal pathogens emit, or trigger the plant to emit, characteristic volatile organic compounds (VOCs). The gold standard method for analysing VOCs is to collect a gas sample from the headspace above a plant or plant tissue and analyse it with a gas chromatograph coupled to a mass spectrometer (GC-MS). Unfortunately, although this equipment is very accurate and can usually identify VOCs that are diagnostic for disease, it is expensive and requires a well-equipped central laboratory with highly trained operators. Electronic nose (Enose) technology is simpler and cheaper and, although it is less accurate than GC-MS, offers the ability to make an on-the-spot diagnosis. Several papers have been published describing the application of Enose technology to detect plant pathogens or food contamination. Cultures of *L. xyli* have been reported to have a characteristic smell but infected sugarcane VOCs have not been tested. We therefore tested whether GC-MS and Enose can diagnose RSD.

Section 3: Outputs and Achievement of Project Objectives

Project objectives, methodology, results and discussion

**Project objectives:**

**Year 1: Volatiles associated to RSD**

During the project’s first year, we used gas chromatography mass spectrometry (GC-MS) to analyse ≥100 of samples which were infected and uninfected with RSD. We established protocols and a list of characteristic chemicals for each sample type. EB-ELISA was run in parallel. We compared the
infection levels with the levels of specific, potentially diagnostic, volatiles using multivariate analysis. The GC-MS data supported a successful classification of at least 90% of the samples and the project passed its first phase.

**Materials and methods**

*Plant samples and ELISA test*

Samples (n=146) of RSD-infected and disease-free cane were collected from the SRA Woodford Experiment Station. Samples came from three sites and nine varieties (Table 1). All plants tested in this study were 12 months old. A2 and D1 blocks, from which samples were taken, were on the same clay-loam soil type, and approximately 250 m apart at Woodford (GPS co-ordinates as follows S 26 55’ 42” E 152 46’ 42”). Kallangur propagation source is located approximately 40 km from Woodford and the soil type is a krasnozem (S27 13’ 24” E152 46’ 42”).

**Table 1. Sugar cane variety, sample size, sample source, and average ELISA results of collected samples.**

<table>
<thead>
<tr>
<th>Variety</th>
<th>Number of samples</th>
<th>Source (site)</th>
<th>ELISA (Mean ± SD*)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INFECTION SAMPLES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KQ228</td>
<td>5</td>
<td>RSD resistance trial (A2)</td>
<td>1.98 ± 0.30</td>
</tr>
<tr>
<td>Q110</td>
<td>8</td>
<td>RSD resistance trial (A2)</td>
<td>2.18 ± 0.64</td>
</tr>
<tr>
<td>Q124</td>
<td>9</td>
<td>RSD resistance trial (A2)</td>
<td>2.11 ± 0.68</td>
</tr>
<tr>
<td>Q138</td>
<td>10</td>
<td>RSD resistance trial (A2)</td>
<td>2.76 ± 0.33</td>
</tr>
<tr>
<td>Q183</td>
<td>5</td>
<td>RSD resistance trial (A2)</td>
<td>1.41 ± 0.50</td>
</tr>
<tr>
<td>Q232</td>
<td>8</td>
<td>RSD resistance trial (A2)</td>
<td>2.18 ± 0.64</td>
</tr>
<tr>
<td>Q240</td>
<td>10</td>
<td>RSD resistance trial (A2)</td>
<td>0.72 ± 0.24</td>
</tr>
<tr>
<td><strong>Total 55</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>HEALTHY SAMPLES</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KQ228</td>
<td>12</td>
<td>Woodford propagation (D1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Q110</td>
<td>9</td>
<td>Woodford propagation (D1)</td>
<td>0.04</td>
</tr>
<tr>
<td>Q124</td>
<td>13</td>
<td>Woodford propagation (D1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Q138</td>
<td>3</td>
<td>Woodford propagation (D1)</td>
<td>0.05</td>
</tr>
<tr>
<td>Q155</td>
<td>5</td>
<td>Woodford propagation (D1)</td>
<td>0.04</td>
</tr>
<tr>
<td>Q183</td>
<td>10</td>
<td>Woodford propagation (D1)</td>
<td>0.04</td>
</tr>
<tr>
<td>Q232</td>
<td>6</td>
<td>Woodford propagation (D1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Q240</td>
<td>15</td>
<td>Woodford propagation (D1)</td>
<td>0.04</td>
</tr>
<tr>
<td>Q90</td>
<td>14</td>
<td>Woodford propagation (D1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Q110</td>
<td>1</td>
<td>Kallangur propagation</td>
<td>0.02</td>
</tr>
<tr>
<td>Q155</td>
<td>3</td>
<td>Kallangur propagation</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Total 91</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*SD = standard deviation*
Stalk vascular extracts for ELISA and phase contrast microscopy were collected by applying positive air pressure to stalk pieces. The pieces were taken from near the stalk base (Figure 1). Extracts were collected in microcentrifuge tubes and frozen at −20 °C until required for analysis. An additional 1 ml of extract was collected for GC-MS analysis.

![Image](image.png)

**Figure 1:** Xylem vascular extract from sugar cane using positive pressure

The ELISA procedure has been described previously [1]. Absorbance of the colour reaction in the ELISA was read on a Biorad microplate reader at 405 nm. Samples with an absorbance reading of > 0.2 were considered positive. Samples with 0.05-0.2 ELISA reading were considered doubtful and re-examined by phase contrast microscopy (1000x magnification) for the presence of the characteristic bacteria. Doubtful samples were considered positive only if both the ELISA and microscopy were positive.

**GC-MS analysis**

For GC-MS analysis of the sugar sap, xylem extract (1 mL) was placed into 10 mL vials and incubated at 40°C with shaking (500 rpm) for 10 min. Headspace extraction was carried out for 40 min with a solid phase micro-extraction (SPME) fibre (Aldrich, Bellefonte, PA) composed of fused silica partially cross-linked with 65 μm polydimethylsiloxane/divinylbenzene (PDS/DVB) and the SPME analysis was carried out using an autosampler (CombiPAL, Switzerland). After absorption, headspace volatiles were transferred to the GC injection port, which was equipped with a 0.8 mm i.d. splitless glass liner, at 240°C. Desorbed volatile compounds were separated in an Agilent (ex-Varian) 3800 GC, equipped with a ZB-5MS (Phenomenex, California U.S.) fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness). The oven temperature was programmed to rise from 30°C (held for 2 min) to 80°C at 20°C/min (held for 1 min) then to 100°C at 20°C/min (held for 1 min) and finally to 230°C at 30°C/min (held for 2 min). The GC column output was fed into an Agilent (ex-Varian) 1200 mass selective detector. The GC-MS transfer line was heated at 250°C with He as the carrier gas (1 mL/min). Mass spectrometry was performed in electron impact mode at 70 eV scanning over the range m/z = 35-350 Da.

**GC-MS data processing**

The GC-MS data was processed in the following steps: (1) normalisation, (2) baseline correction, (3) peak detection, and (4) peak alignment. As the GC-MS sample analysis was performed over a period of several weeks, gradual changes in the technique’s sensitivity means the data collected on different days were subject to a “day of analysis” effect. Therefore, the data was normalised using
a blank sample measured on the day of analysis (i.e. analysis of an empty glass vial) to overcome this temporal effect. The baseline drift of each single ion chromatogram (SIC) was corrected using Asymmetric Least Square (ALS) [2]. We employed peak detection as described before [3]. For each peak detected, we calculated the area under the peak and aligned the peaks through a combination of piecewise method [4] and dynamic time warping, where the target peaks were chosen based on a dendrogram alignment scheme [5].

Feature selection
We used the mutual information (MI) approach [6] to find the optimal subset of features (peak areas) that gave the best classification performance. The feature subset was selected by maximising the mutual information \( I(Z, C) \) between the selected features \( Z'' = \{Z''_1, \ldots, Z''_n\} \) and class \( C \):

\[
I(Z, C) = \sum_{z,c} p(Z, C) \log \frac{p(C|Z)}{p(C)}
\]

We employed the Kraskov–Grassberger technique [7] (estimator 2) for estimating the mutual information. The code used for the estimation is publicly available as a Java Information Dynamics Toolkit [8].

Classification based on selected features
Two common classifiers, support vector machine (SVM) [9] and k-nearest neighbours (kNN) [10], were used to train a model and classify the test data. We performed the classification using the one-against-all (leave-one-out) cross-validation [6].

Results and discussion

ELISA reading and volatiles levels in sugar sap
The average absorbance measured by the ELISA assay for all infected plots in the trial ranged from 0.72 ± 0.24 and 2.76 ± 0.33 (Table 1). The cultivar with the highest infection levels was Q138 from plot A2.

In total 81 compounds were detected in the headspace of healthy and infected xylem extract. Alcohols, organic acids, aromatic compounds, aldehydes and ketones were among the chemicals found in the headspace. The majority of the compounds were identified by reference to the NIST library or standards.

Diagnostic RSD volatiles
Using the MI feature selection described above on the GC-MS data, seven ions corresponding to seven volatiles were shown to be diagnostic of RSD infection (Table 2). With the exception of 2-ethyl hexanoic acid, the diagnostic volatiles were present at higher concentrations in RSD infected samples compared to healthy ones.
Table 2: Diagnostic RSD volatiles and their characteristic ions

<table>
<thead>
<tr>
<th>Rt* (min)</th>
<th>Compound name</th>
<th>m/z</th>
<th>Relative abundance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.957</td>
<td>Dimethyl sulfide</td>
<td>62</td>
<td>i&gt;h</td>
</tr>
<tr>
<td>2.195</td>
<td>2-Methyl propanal</td>
<td>41</td>
<td>i&gt;h</td>
</tr>
<tr>
<td>5.296</td>
<td>Ethyl benzene</td>
<td>106</td>
<td>i&gt;h</td>
</tr>
<tr>
<td>5.681</td>
<td>Styrene</td>
<td>104</td>
<td>i&gt;h</td>
</tr>
<tr>
<td>7.564</td>
<td>2-Ethyl-4-methyl 1-pentanol</td>
<td>57</td>
<td>i&gt;h</td>
</tr>
<tr>
<td>7.634</td>
<td>2-Ethyl-1-hexanol</td>
<td>41</td>
<td>i&gt;h</td>
</tr>
<tr>
<td>8.649</td>
<td>2-Ethyl hexanoic acid</td>
<td>73</td>
<td>h&gt;i</td>
</tr>
</tbody>
</table>

*Rt=retention time, 'i'=infected and 'h'=healthy samples

Sulphur compounds like dimethyl disulfide are universal bacterial metabolites and may be present in higher concentration in infected samples due to the degradation of sulphur-containing amino acids such as methionine and cysteine, which has been previously reported for *Salmonella typhimurium* contamination of vegetables and meat [11, 12].

Styrene, an aromatic hydrocarbon, is known to be emitted from plants [13, 14]. Styrene sources of in the environment are of two types: anthropogenic and natural [15]. Anthropogenic sources are mainly due to petrochemical and polymer-processing industries or automobile exhaust fumes. Natural styrene formation has been observed as a trace metabolite in foods, in particular, cheeses [16]. Styrene is also known to be naturally synthesised by some plant species, including several trees in the *Styracaceae* family (including several *Styrax* species) [17]. Styrene is believed to be synthesized from excess L-phenylalanine, via decarboxylation of cinnamic acid involved in secondary metabolism of plants [17]. It is also known that microorganisms, such as *Penicillium* spp., *Aspergillus niger* and *Saccharomyces cerevisiae*, do promote the decarboxylation of cinnamic acid to form styrene [13]. Based on this, it is therefore plausible that styrene is naturally produced in healthy sugar sap and that infected plants could regulate styrene synthesis.

2-Ethyl-1-hexanol has been previously reported as a natural compound in grapes [18]. It is possible that 2-ethyl-1-hexanol could be metabolically related with 2-ethyl hexanoic acid since the latter can be produced by oxidation of the former. *Leifsonia xyli* may be able to reduce the organic acid and form the alcohol (Table 2).

The other compounds have not been reported to be associated with plants or bacteria. However, it is known that VOCs are used by microorganisms to give the producer an advantage in terms of colonisation by suppressing other bacteria growth [19]. Also, when a plant is subjected to abiotic or biotic stress, a set of responses is activated to combat the disease process. Plants under stress emit a much greater variety of volatiles and in greater quantities to serve mainly as indirect plant defence [19].

*Classification of samples using volatile profile*
Of the two classifiers tested and using all seven compounds, kNN gave slightly better results for sugar sap than SVM: 98.6% and 97.94% correct classification respectively. Styrene and ethyl benzene were the first two volatiles selected using mutual information that were able to discriminate healthy and infected samples. Ethyl benzene on its own could be used to correctly classify 97.9% of samples while styrene can be used to classify 98.6% of samples (Table 3).

If we use styrene only as a predictor of infection, two misclassified samples were found: one false negative and one false positive (Table 3).

Table 3: Confusion matrix for RSD diagnosis using a kNN classifier with ethyl benzene and styrene levels.

<table>
<thead>
<tr>
<th>Class</th>
<th>Predicted</th>
<th>Ethyl benzene&lt;sup&gt;A&lt;/sup&gt;</th>
<th>Styrene&lt;sup&gt;B&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>healthy</td>
<td>infected</td>
</tr>
<tr>
<td>True</td>
<td>healthy</td>
<td>89</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>infected</td>
<td>1</td>
<td>54</td>
</tr>
</tbody>
</table>

<sup>A</sup>Classification = 97.9 %  
<sup>B</sup>Classification = 98.6 %

As mentioned above, in general there were higher volatile responses for infected samples than for healthy samples, except in the case of seven anomalous samples, which were RSD free (Figure 2). It may be that these could have been infected by some other plant disease, or that there could be some unknown processes involved with these particular samples.

When the volatile levels were correlated with the ELISA reading we found that there was no simple dose-response relationship between diagnostic volatile levels and the infection levels (Figure 2). However the volatile levels were signaling plant infection and we were able to tentatively establish thresholds for RSD detection based on these levels. By visual inspection of Figures 2 three zones could be identified; for example, in the case of ethyl benzene, samples with very high levels (≥12 normalized peak area) of the compound indicate plant infection by other diseases or other physiological state (Figure 2(a)). Samples with intermediate levels of ethyl benzene (between 1.8 and 12 normalized peak area) indicated RSD infection while samples with levels ≤1.8 normalized peak area represented healthy samples. Based on these threshold values, there are three misclassified samples (shown by asterisks in Figure 2(a)): two false positive and one false negative, which correspond to the misclassified samples shown in Table 3. The ‘all or nothing’ nature of the volatiles response would suggest systemic plant generation of these volatiles.

The potential utility of these findings is that ‘intermediate’ levels of ethyl benzene would indicate RSD infection of the plant.
Figure 2: Normalized peak area for (a) ethyl benzene and (b) styrene for healthy (blue) and infected (red) samples, with samples sorted in order of increasing ELISA reading. Seven anomalous healthy samples with higher levels of ethylbenzene/styrene can be observed (large blue peaks). Black lines indicate RSD thresholds based on volatile levels and asterisk shows the misclassified samples.

**Varietal characterisation**

An unexpected finding was that the volatile profiles could be used for cultivar discrimination. For example, healthy stalks from cultivars KQ228 (n=12) and Q90 (n=14) coming from the same site (block D1) were discriminated on the basis of the peak area of volatiles found in the headspace of the samples (Figure 3). These results could potentially be used to confirm cultivar identity.

![Principal component analysis using GC-MS data of cultivars KQ228 and Q90. Both cultivars are healthy samples from a 12 month old plant and grown on the same site.](image-url)
Conclusions

Gas chromatography-mass spectrometry analysis of the headspace of xylem extract from infected and uninfected sugar cane identified specific alcohols, aromatic hydrocarbons and an aldehyde as signatures of RSD infection. In particular, styrene and ethyl benzene were the best candidates to signify RSD infection. Using these compounds, we obtained better than 98% correct classification demonstrating that they are useful biomarkers for RSD prediction. To test the robustness of this approach, we analysed samples from different sites, different varieties and from a larger range of infections in Year 2.

Year 2: Diagnostic potential of Enose

Two Enose devices were compared for their ability to detect RSD in sugarcane. One Enose uses metal oxide sensors and cyclically varies the operating temperature of the sensors to generate more discriminating signals, while the second uses a different sensing technology based on conducting polymers and has advantage of being faster compared to the first device. We used results obtained by GC-MS and EB-ELISA to train these two commercially available Enoses, using “leave one out” cross-validation and/or training and test set methodology. One device (DiagNose) achieved 80% accuracy in classifying infected and uninfected sugar sap indicating success with this phase of the project. The DiagNose undertook field testing in Year 3.

Materials and methods

Plant samples and ELISA test

In total 140 samples (RSD-infected and disease-free cane) were collected from the Woodford Experiment Station and Maryborough (-25.5130565, 152.7509417) both in Queensland, Australia. Samples came from three sites and six varieties (Table 4). All plants tested in this study were 8 to 12 months of age. Blocks E1 and E5 were 200 m apart, D1 is about 100 m from E1 and 200 m from E5. Approximately about 50% of the samples were RSD infected.
Table 4: Sugar cane variety, sample size, sample source, and average ELISA results of collected samples.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Number of samples</th>
<th>Source (site)</th>
<th>ELISA (Mean ±SD(^A))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Infected samples</td>
<td></td>
</tr>
<tr>
<td>Q110</td>
<td>15</td>
<td>Woodford (E1)</td>
<td>0.577 ±0.123</td>
</tr>
<tr>
<td>Q124</td>
<td>15</td>
<td>Woodford (E1)</td>
<td>0.440 ±0.148</td>
</tr>
<tr>
<td>QA01-5267</td>
<td>10</td>
<td>Woodford (E1)</td>
<td>0.520 ±0.116</td>
</tr>
<tr>
<td>Q90</td>
<td>10</td>
<td>Woodford (E1)</td>
<td>0.516 ±0.074</td>
</tr>
<tr>
<td>Q171</td>
<td>10</td>
<td>Woodford (E1)</td>
<td>0.259 ±0.060</td>
</tr>
<tr>
<td>Q138</td>
<td>9</td>
<td>Maryborough (Beaver Rock Rd)</td>
<td>0.351 ±0.105</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total number of samples : 69</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Healthy samples</td>
<td></td>
</tr>
<tr>
<td>Q110</td>
<td>15</td>
<td>Woodford (E5)</td>
<td>0.051 ±0.013</td>
</tr>
<tr>
<td>Q124</td>
<td>15</td>
<td>Woodford (E5)</td>
<td>0.048 ±0.014</td>
</tr>
<tr>
<td>QA01-5267</td>
<td>10</td>
<td>Woodford (F5)</td>
<td>0.085 ±0.051</td>
</tr>
<tr>
<td>Q90</td>
<td>10</td>
<td>Woodford (E5)</td>
<td>0.038 ±0.038</td>
</tr>
<tr>
<td>Q171</td>
<td>10</td>
<td>Woodford (F5)</td>
<td>0.056 ±0.018</td>
</tr>
<tr>
<td>Q138</td>
<td>11</td>
<td>Maryborough (Beaver Rock Rd)</td>
<td>-0.04 ±0.019</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total number of samples : 71</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disease (cultivar)</th>
<th>Healthy</th>
<th>Infected</th>
<th>Source (site)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorotic streak (RP193-67)</td>
<td>3</td>
<td>3</td>
<td>Woodford (F5)</td>
</tr>
<tr>
<td>Leaf scald (QC03-92)</td>
<td>1</td>
<td>1</td>
<td>Woodford (E5)</td>
</tr>
<tr>
<td>Leaf scald (Q63)</td>
<td>3</td>
<td>3</td>
<td>Woodford (D1)</td>
</tr>
</tbody>
</table>

\(^A\)SD = standard deviation

A small number of xylem extracts from sugarcane plants infected with Chlorotic streak and Leaf Scald were also collected (Table 4), this was done to test if the previously identified RSD diagnostic volatiles are also diagnostic for other sugar cane diseases. Chlorotic Streak and Leaf Scald are found in sugar cane growing areas and can cause serious yield losses, particularly in flood prone and waterlogged parts of the industry. Main symptoms of chlorotic streak are irregular creamy-white streaks with distinct margins and internal stalk reddening similar to those seen with ratoon stunting disease. Leaf Scald disease symptoms are irregular, ‘pencil line’ streaks and less reddening of internal stalks tissues [20].

Electronic noses (Enoses):

The following electronic noses were tested to identify RSD samples:
a. **DiagNose**

The DiagNose (The eNose Company - the Netherlands) contains an array of 12 metal oxide sensors, SnO$_2$/Pd/2 (triplicate), WO$_3$ (triplicate), SnO$_2$/Pt/2 (triplicate), SnO$_2$/Cu, SnO$_2$/Ag and one undisclosed type sensor. The metal-oxide sensors behave as semiconductors. When oxygen adsorbs and ionizes at the sensor surface the conductivity becomes low. Oxygen removal as a result of interaction with volatiles results in a measurable increase in conductivity. The DiagNose cyclically varies the operating sensor temperatures in order to generate more discriminating signals. The DiagNose was initially flushed with instrument air (purified atmospheric air containing less than 0.1ppm total hydrocarbons) at a flow rate of 320 ml/min for 1 hour prior to sampling to obtain a constant low baseline. The sample (1 ml in a 10ml glass vial) was placed in a block heater set at 27°C for 10 minutes before sampling. The block heater was agitated during incubation and sampling. The headspace in the vial was pumped to and from the DiagNose via tubing in a closed loop (Figure 4). The flow from the DiagNose’s internal pump was regulated to 40 ml/min using a needle valve. Polytetrafluoroethylene (25mm x 0.2 µm, Supelco, Bellefonte, PA) filters were placed at the inlets to minimise contamination of the DiagNose chamber with either moisture or particulates. The volatiles in the headspace were sampled for 5 minutes. The sensors were then flushed with instrument air for 30 min to desorb the volatiles and return the sensors to the baseline. The line sampling the sugar sap samples was also flushed with air for 5 min to remove sample carry over in the tubing before the next sample. The sampling order for each day’s analysis started with an empty vial, followed by a 1% sucrose standard. Sugar sap samples were alternated between healthy and infected samples. The analysis finished with another 1% sucrose standard.

![Figure 4. DiagNose used for sugar sap volatileanalysis.](image-url)

b. **Cyranose**

The volatiles in the headspace above sugar sap samples were also assessed using a Cyranose 320 Enose. The Cyranose contains 32 individual thin-film carbon-black polymer composite chemiresistor sensors in an array. The sensors are composed of a conductive thin film that is
deposited between electrodes on a ceramic substrate. Analytes from the sample vapour are absorbed onto the conductive film which increases the resistance across the sensor. When the analytes are removed, the polymer relaxes into its original state, restoring the conductivity. The sample (1 mL in a 10ml vial) was placed in a heating block at 27°C for 5 minutes. The heating block was agitated at a slow speed to increase the volatile releases into the headspace above the sample (Figure 5). Cyranose sensors were first purged with instrumental air for 50 sec on a medium pump speed to give a steady baseline. The headspace above the sugar sap sample was sampled for 40 secs using a medium pump speed, after which the sensors were flushed with instrument air for 20 secs at high pump speed to desorb any remaining analytes from the sensors. This method allowed a sample to be analysed every 5 min.

![Figure 5: Cyranose for volatile collection from sugar sap](image)

**Data processing, feature selection and classification methods**

The following data processing, feature selection and classification methods were applied to Enose data:

1. **DiagNose:**
   1.1. **Pre-processing:** data from DiagNose was z-scored, i.e. the variations standardised, to remove day of analysis effects.
   1.2. **Feature selection** was used to select a subset of features, $\nu \subseteq \{1, 2, 3K, m\}$, where $m$ is the total number of features, such that the resulting subset of features gives the best classification performance for the given size constraint $n$ on the number of features in $\nu$.
   1.3. **Classification:** two types of classifiers were run on the data:
      i. **Support Vector Machine (SVM)** (Coster and Vapnik, 1995): this method constructs a hyperplane to separate training data in different classes, i.e. it ‘divides’ the data into separate clusters. SVM classifies any new unseen data according to which side of the plane the new data lays.
      ii. **K Nearest Neighbours (kNN)** (Russell and Norvig, 1995): this method classifies a new unseen data based on the $k$ nearest (Euclidean distance) training data from the new data. The new data's class is determined to be that with the most data points in the neighborhood.
2. Cyranose

2.1. Pre-processing:
   i. Removal of sensor drift: drift of the signal was removed using an algorithm based on asymmetric least squares (ALS) smoothing. ALS estimates the baseline by penalising error values.
   ii. Baseline subtraction and division: for each sample there is period were clean air is run through the sensors and a baseline is the average of the last five measurements before the introduction of air, the calculated baseline value is then subtracted; the result is then divided by the baseline value.
   iii. Normalisation: the data was normalised by performing z-score (standardizing the variance).

2.2. Feature selection and classification: The same feature selection and classifications methods used for DiagNose were also used for Cyranose.

Results and discussions:

ELISA testing was performed for all samples (Table 4). The infected samples had generally lower ELISA readings compared to samples from year 1 of the project. The cultivars with the highest and lowest levels of infection were Q110 and Q171 respectively both from Woodford.

The Enose results presented below were from 140 samples and for the two electronic noses:

a. DiagNose

From the different normalizations tested, the optimal z-scoring method [21, 22] was found with using each sensor temperature. Feature selection shows that the DiagNose sensors can only just detect the difference between healthy and infected (response in blue, Figure 6(a)). A cluster of sensor-time features identified as the most informative sensors (red circle in Figure 6(a), were based on the sensor 11 response (type SnO$_2$/Ag) during the first minute of analysis. These features during these times suggested that the analysis and recovery times could be significantly reduced thus making the sample analysis much faster.
Figure 6(a) Heat map showing mean differences between healthy and infected samples. In red circle are the informative sensors. (b) Classification results for 140 samples, healthy vs. RSD infected, using support vector machine (SVM) and k-nearest neighbor (kNN). The black line shows the naïve classification results, and the grey line shows statistical significance of $p=0.05$.

The classification results indicate 86.4% correct classification (Figure 6B). There were 9 false positive and 11 false negative samples out of 140 samples (Table 5). The best classifier was kNN with 11 features providing >80% classification. The following samples were false negatives: 107, 92, 93, 95, 9, 15, 24 and 298, from cultivars Q90, Q110, Q124, Q138 and Q171 while false positives were identified with samples 228, 231, 232, 234, 184, 186, 222, 122, 135, 149 and 155 from cultivars QA01-5267, Q90, Q110, Q124 and Q171. The majority of the misclassifications came from cultivar Q171.

Table 5: Confusion matrix for RSD diagnosis using kNN classification with 11 DiagNose features.

<table>
<thead>
<tr>
<th>True class</th>
<th>Predicted class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>healthy</td>
</tr>
<tr>
<td>healthy</td>
<td>63</td>
</tr>
<tr>
<td>infected</td>
<td>11</td>
</tr>
</tbody>
</table>

1. Cyranose

The feature selection showed that the Cyranose sensors did not detect the difference between healthy and infected samples. A cluster of sensor-time features were identified as the most informative sensors (red circle in Figure 7a) which were based on the second and third sensors during the first minute of analysis. The classification rates were below statistical significance (i.e. <60%) using SVM and KNN classifiers.
Figure 7 (a): Heat map showing mean differences between healthy and infected samples for Cyranose. In red circle are the informative sensors. (b) Classification results for 140 samples, healthy vs. RSD infected, using support vector machine (SVM) and $k$-nearest neighbor (kNN). The black line shows the naive classification results, and the grey line indicates statistical significance at $p=0.05$.

**Agreed milestones additional to the original plan**

*RSD diagnostic volatiles using GC-QTOF-MS (gas chromatography quadrupole time-of-flight mass spectrometry)*

**GC-QTOF analysis.** CSIRO purchased a GC-QTOF-MS which is a more sensitive GC-MS capability. We used this equipment to analyse sugar sap with the objective of potentially finding more diagnostic compounds and validate our earlier findings.

For the sugar sap, the xylem extract (1 mL) was placed into a 10 mL vial and incubated at 40°C with shaking (500 rpm) for 10 min. Headspace extraction was carried out for 40 min with a solid phase micro-extraction SPME fibre (Aldrich, Bellefonte, PA) composed of fused silica partially cross-linked with 65 μm polydimethylsiloxane/divinylbenzene (PDS/DVB). The SPME analysis was carried out using an autosampler (Agilent Technologies GC Sampler 120, Switzerland). After absorption, headspace volatiles were transferred to the GC injection port, equipped with a 0.75 mm i.d. ultra inert straight glass liner, at 250°C. Desorbed volatile compounds were separated in an Agilent Technologies 7890B GC System (Figure 8), equipped with a ZB-5MS fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness). The oven temperature was programmed to rise from 30°C (held for 2 min) to 80°C at 20°C min$^{-1}$ (held for 1 min) then to 100°C at 20°C min$^{-1}$ (held for 1 min) and finally to 230°C at 30°C min$^{-1}$ (held for 2 min). The flow rate of the He carrier gas was set to 1.9 mL/min. The GC column output was fed via a Deans Switch into an Agilent Technologies 7200 Accurate-Mass Q-TOF GC/MS. The GC-QTOF transfer line was heated at 280°C with a He flow rate of 3.7 mL/min. Mass spectrometry was performed in electron impact mode with an emission current of 35.0 μA over the range 35-350 m/z. The temperature of the source and quadrupole were set at 230 and 150°C, respectively. The collision cell had a N$_2$ flow of 1.5ml/min.
Data was pre-processed and analysed using approaches described for the standard GC-MS previously.

Figure 8: Agilent GC-QTOF-MS used for the analysis of harvest year 2014 samples.

**Results.** Using feature selection, nine ions corresponding to nine different volatiles were shown to be diagnostic for RSD infection (the VOC identification is a work in progress). The majority of the compounds showed higher levels in infected samples compared to healthy ones. In year 2 (2014), the samples came from more diverse sites and RSD samples had lower infection levels. GC-QTOF-MS identified more than 180 VOCS making classification more challenging. The classification results for 141 training samples, healthy vs. RSD infected, are shown in Figure 9. The classification was consistently around 94% for number of features > 20.
Figure 9: GC-QTOF-MS classification results for 140 samples, healthy vs. RSD infected, using support vector machine (SVM) and k-nearest neighbor (kNN). The black line shows the naïve classification results, and the grey line shows statistical significance of $p=0.05$. For each size constraint (number of features, x-axis), classification is performed between the classes and the accuracy rate (y-axis) is shown.

If the first feature (a single compound) was used then the classification rate was around 80% using SVM classifier. If more than 20 features are used (9 compounds in total) then the classification success rate increase to 94% using the SVM classifier. These results suggest that even with more diverse samples and lower levels of infection the instrument can still discriminate samples with high accuracy.

There were 6 false positive and 3 false negative identifications from a total of 140 samples (Table 6). From a practical point of view it is important to minimize false negatives. The majority of misclassified samples came from cultivar Q110. The false negatives were identified as 228, 122, and 150 while the false positives were identified as: 3, 12, 13, 33, 298 and 83. Of the misclassified samples, three were identified as either false negative or positive by both the DiagNose and GC-QTOF-MS (ID 228, 298 and 122). Sample ID 298 (Q138 - M Sommerfeld Beaver Rock Rd Maryborough) came from a RSD positive plot and this single stalk was found healthy.

Table 6: Confusion matrix for RSD diagnosis using SVM classification with nine diagnostic VOCs (20 features).

<table>
<thead>
<tr>
<th>True class</th>
<th>Class</th>
<th>Predicted class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>healthy</td>
<td>infected</td>
</tr>
<tr>
<td>healthy</td>
<td>65</td>
<td>6</td>
</tr>
<tr>
<td>infected</td>
<td>3</td>
<td>66</td>
</tr>
</tbody>
</table>

RSD diagnostic set of volatiles is unique to this disease

Analysis of headspace and feature selection procedure showed that for CS and LD samples there were six and eight distinct volatiles respectively. Since the objective of this test was to find out if the features ($m/z$ vs retention time) were unique to RSD, we did not seek to identify the volatiles for CS and LS but we looked at common features between RSD features and the other diseases tested. There were no common features between CD and RSD, while for LS samples, only one compound was found in common to RSD.

ELISA test revealed that the Chlorotic Streak (CS) and Leaf Scald (LS) samples were free of RSD. Despite the limited numbers tested and the validation required in a larger sample size, the results are encouraging as it potentially shows unique combination of compounds for RSD detection.

Conclusions

The DiagNose was shown to be the most effective for detecting RSD infected sugar sap with >80% correct classification.
There is a need to reduce the sample size and analysis time as extracting 1 ml of xylem extract is time consuming and the present sample time of 35 min makes the test impractical for field deployment.

The validation of RSD diagnosis using GC-QTOF-MS was successfully conducted and the potential of this approach was confirmed since a classification rate of 94% was achieved using nine diagnostic volatile compounds.

**Year 3: Enose test in the field**
The DiagNose Enose device was leased/purchased and located at the premises of a Productivity Service Company or elsewhere in or close to a cane growing area. The Enose was trained with fresh samples of known status and Enose predictions were found to be less 50% accurate compared to the data obtained from ELISA analysis. It was found that the problem was due to the lower signal levels in fresh samples compared with samples that had been stored frozen.

**Materials and methods:**

*Sample:*
Two sample types were tested:

A. Xylem extract (1 mL) as originally done at CSIRO labs.
B. Xylem extract on filter membrane (150 µL of xylem used in A on nylon filter membranes)

*Cultivars:*
The collected cultivars and the sample number processed by the Enose are shown in Table 7. In total 10 cultivars were collected from Woodford Experimental Station in QLD.

Table 7: Sugar cane variety, sample size, sample source, and average ELISA results of collected samples for Enose field test.

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Number of samples</th>
<th>Source (site)</th>
<th>ELISA (Mean ±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Q124</td>
<td>15</td>
<td>Woodford (E1)</td>
<td>0.58 ±0.21</td>
</tr>
<tr>
<td>Q171</td>
<td>14</td>
<td>Woodford (E1)</td>
<td>0.35 ±0.14</td>
</tr>
<tr>
<td>Q200</td>
<td>10</td>
<td>Woodford (E1)</td>
<td>0.36±0.14</td>
</tr>
<tr>
<td>Q246</td>
<td>14</td>
<td>Woodford (E1)</td>
<td>0.28±0.09</td>
</tr>
<tr>
<td>Q110</td>
<td>10</td>
<td>Woodford (E1)</td>
<td>0.41±0.19</td>
</tr>
<tr>
<td>Q208*</td>
<td>8</td>
<td>Woodford (E1)</td>
<td>0.13±0.19</td>
</tr>
<tr>
<td>Q138*</td>
<td>8</td>
<td>Woodford (E1)</td>
<td>0.50±0.26</td>
</tr>
<tr>
<td>Q183*</td>
<td>9</td>
<td>Woodford (E1)</td>
<td>0.46±0.22</td>
</tr>
</tbody>
</table>
### Enose analysis:

The protocols used of Enose related data pre-processing and analysis were the same as used in Year 2. The main change in the protocol was a reduced Enose analysis time (from 35 min to 20 min) and samples were freshly collected and analysed immediately (no storage and freezing).

### Results and discussions:

A. Xylem extract

The classification rates for 240 samples (coming from 10 cultivars) were below 50% (Figure 11) which was disappointing since the preliminary results in Year 2 of the project showed 86% success rate.
Figure 11.- Enose classification results of 240 samples, healthy vs. RSD infected, using support vector machine (SVM), k-nearest neighbor (KNN), Bayesian networks (BN). The black line shows the naïve classification results, and the grey line shows statistical significance at $p=0.05$. For each size constraint (number of features, x-axis), the classification was performed between classes and the accuracy rate (y-axis) is shown. Sample volume was 1 ml.

A possible reason for the difference between the results obtained in Year 2 (in CSIRO labs) and Year 3 (in Woodford Experimental station) was that the Woodford samples were freshly collected and immediately analysed by the Enose, while the CSIRO samples (Year 2 of the project) had been stored at -80 °C for at least four months after collection prior to analysis with the Enose. It was hypothesised that the freezing process may have generated higher amount of the volatiles due to disruption of the small vascular tissues present in the sugar sap. This would have increased the Enose power to discriminate samples.

To test this, fresh samples vs stored samples (6 month at -80 °C) were analysed using GC-QTOF-MS and compared, particularly searching for the diagnostic volatiles. The results demonstrated that these compounds were either absent or significantly lower in concentration in fresh samples compared to those which had been stored (Figure 12). This suggest that freezing samples with storage below 0 °C and thawing at room temperature before analysis affects the headspace volatile composition. It is well known that freezing produces large ice crystals which can cause tissue disruption which in turn can release enzymes which can generate secondary volatiles [23].

In future work, shorter freezing and sample thaw times will be investigated to test whether lower abundances of volatile compounds result compared to longer storage times. This method could provide a solution for Enose analysis of the sugar sap samples.
Diagnostic compound 1

Cultivar Q246

Abundance a.u.

0.0E+00 5.0E+04 1.0E+05 1.5E+05 2.0E+05 2.5E+05 3.0E+05

Fresh Xylem Stored Xylem

Diagnostic compound 2

Cultivar Q246

Abundance a.u.

0.0E+00 1.0E+07 2.0E+07 3.0E+07 4.0E+07 5.0E+07

Fresh Xylem Stored Xylem

Figure 12. Ion abundance (arbitrary units, AU) for two diagnostic volatile compounds (1 and 2) present in fresh and stored xylem samples, measured using GC-QTOF-MS. Healthy samples are shown in blue while red indicates infected samples.

B. Xylem extract on filter membrane.

A sub set of the samples (Table 7) was used to test the ability of the Enose to detect RSD in a smaller xylem volume on a nylon filter. The results showed that a smaller volume decreased the Enose’s ability to sense the diagnostic volatiles with the classification rate around 0.5 and below statistical significance (Figure 13). It is likely that there was insufficient volatile compounds in the headspace for detection by the Enose.

Figure 13.- Enose classification results of 80 samples, healthy vs. RSD infected, using support vector machine (SVM), k-nearest neighbor (kNN), Bayesian networks (BN). The black line shows the naïve classification results, and the grey line shows the line of statistical significance, \( p=0.05 \). For each
size constraint (number of features, x-axis), classification was performed between the classes and the accuracy rate (y-axis) is shown. Samples are 150 µl of xylem on nylon filter membranes

Conclusions

The Enose was not able to predict RSD infection when fresh xylem samples are analysed using previously established protocols and particular type of chemical sensor used in the Enose. The information on the diagnostic volatiles associated with RSD is critical and could potentially attract the attention of chemical sensor developers and, in future, more sensitive, selective and cheap sensors could be offered to the sugar industry for detection of RSD in the field.

Overall Conclusions:

1. There are specific volatiles (principally styrene and ethyl benzene) that are diagnostic for *Leifsonia xyli* infection of sugar cane.
2. Using GC-MS these specific volatiles allow diagnosis of *Leifsonia xyli* infection with ≥94% accuracy across years, in low and high infection samples and across cultivars.
3. The all or nothing nature of the volatiles response (no association with level of infection) suggests that a systemic plant response generates these volatiles.
4. A small percentage (7.7%) of sugar cane sap samples not infected with *Leifsonia xyli* gave atypically high levels of styrene and ethylbenzene presumable due to another unidentified disease/s or physiological condition.
5. There were very few false negatives using GC-MS/GC-QTOF-MS (0.7-2.1%).
6. The DiagNose with kNN gave the best classification rate (≥80%) on aged samples in the lab but the protocol was not significantly more convenient than current methods.
7. The protocols were shortened for field deployment and, when tested in the field, the classification rate for the DiagNose was lower (~50% accuracy) compared to laboratory based trials. It was found that the problem was due to the lower signal levels in fresh samples compared to those which had been frozen at -80 °C for 4 months.

Whilst currently available commercial Enoses are not ready for deployment for RSD diagnosis in the field, this work has identified and validate robust volatile biomarkers for RSD detection. As improved Enose/biosensors technologies are developed, it would be cost-effective that these are tested for their ability for RSD diagnosis and possibly other plant diseases.

Section 4: Outputs and Outcomes

Outputs:

1. 37th Australian Society of Sugar Cane Technologists (ASSCT) Conference Proceedings, April 2015 (Appendix 1).
2. Article published in the CaneConnection newsletter winter edition that came out in July 2015 (Appendix 2).
3. Poster presentation accepted for Plant Physiology and Pathology conference (Dallas, 9-10 June 2016). Title: “Indirect detection of ratoon stunting disease in sugar cane” (Appendix 3).
4. Draft manuscript with full results of the diagnostic volatile compounds found in RSD infected sugar cane using GC-MS and across two harvest years is in preparation and for submission to Analytica Chimica Acta (impact factor: 4.513) (Appendix 4).
5. SOP for Electronic nose operation in the field (Appendix 5).

Outcomes:
1. GC-MS screening is viable as a research tool for RSD detection
2. Current generation of Enose fails due to lack of sensitivity and low throughput of samples
3. Based on knowledge of diagnostic volatiles new Enose technology can be applied.

Section 5: Intellectual Property (IP) and Confidentiality

We came to the view that specific results (use of the diagnostic volatile markers styrene and ethylbenzene) represent a patentable invention. However, CSIRO and SRA agreed that any license earnings from the small commercial market for a device based on detecting these volatiles is too small to outweigh the very substantial upfront costs (in time and money) of patenting. Patent protection was not sought and the results have been placed in the public domain.

Section 6: Industry Communication and Adoption of Outputs

Key messages:

Gas chromatography-mass spectrometry:
- Diagnostic volatiles allowed discrimination between RSD and healthy samples
- Classification is between 94%-98%
- There is not a simple linear relationship between the levels of the volatiles and levels of infection. Higher levels of the volatiles are likely to be a systemic response of the plant due to infection

Enose preliminary results in the lab:
- 86% correct classification

Enose preliminary results in the field:
- 50% correct classification when samples size was 150 ul xylem extract in nylon
- <50% correct classification when sample size 1ml xylem extract and 20 min analysis time
- There is an effect on the generation of the volatiles when samples are frozen, stored and thawed
Communication of these results were made available to Peter Sampson through the milestone reports. Relevant results were also communicated to collaborators and Peter Sampson in three annual meetings: the 31st of July 2014 in Woodford, the 20th April 2015 in Canberra and the 22nd January 2016 via TC.

a) What new information, if any, is available on the adoption of project outputs?
The new information available on project outputs is the list of volatiles associated to RSD. The results will be published and the list can be used to research and diagnose RSD in the laboratory and it will have value independent of the diagnostic instrument or methodology in use.

b) List any newsletters, fact sheets or any other media coverage.
An Enose article was published in the CaneConnection newsletter winter edition that came out in July 2015.

c) Identify any further opportunities to disseminate and promote project outputs at seminars, field days etc.
To disseminate the GC-MS results more widely we are preparing a manuscript with full results of the diagnostic volatile compounds found in RSD infected sugar cane using GC-MS and across two harvest years. Paper will be submitted to the Analytica Chimica Acta (impact factor: 4.513) which is one of the top journals in analytical chemistry and agriculture area. This paper will be the first paper showing RSD associated volatile compounds and their utility as potential indicators of the disease. The paper will potentially attract the attention of chemical sensor developers and in the future, more sensitive, selective and cheap sensors could be offered to the sugar industry to detect RSD quicker.

Section 7: Environmental Impact
The Enose or GC-MS technology, if adopted in the future, would decrease the use of ELISA tests and this will reduce the environmental hazard associated with the use and disposal of laboratory material used in ELISA.

Section 8: Recommendations and Future Industry Needs
As new, fast and more sensitive Enose/biosensor technologies are developed for other applications this can be tested against RSD diagnostic volatiles for use in the sugar industry.

Section 9: Publications
1. 37th Australian Society of Sugar Cane Technologists (ASSCT) Conference Proceedings, April 2015 (Appendix 1).
2. Article published in the CaneConnection newsletter winter edition that came out in July 2015 (Appendix 2).
3. Poster presentation accepted for Plant Physiology and Pathology conference (Dallas, 9-10 June 2016). Title: “Indirect detection of ratoon stunting disease in sugar cane” (Appendix 3).
4. Draft manuscript with full results of the diagnostic volatile compounds found in RSD infected sugar cane using GC-MS and across two harvest years is in preparation and for submission to Analytica Chimica Acta (impact factor: 4.513) (Appendix 4).
5. SOP for Electronic nose operation in the field (Appendix 5).
References