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| Project title**:** | Exploring whether redberry disease of blackberry is caused by a mite-transmitted virus |
| **Project number:** | SF 149 |
| **Project leader:** | Stuart MacFarlane, JHI |
| **Report:** | Final report - August 2015 |
| **Previous report:** | N/A |
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| **Location of project:** | JHI |
| **Industry Representative:** | Salih Hodzhov, WB Chambers & Son |
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| **Date project completed (or expected completion date):** | August 2015 |

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## AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

Stuart MacFarlane

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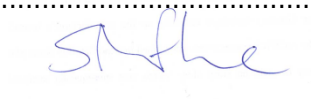
Signature ..... Date: 22.08.2015



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# GROWER SUMMARY

## Headline

- In this project, at least three new viruses have been discovered in blackberry, but their contribution to the condition called redberry is not yet known.

## Background and expected deliverables

Trials to date looking at acaricides for the control of the blackberry mite thought to be responsible for this condition have shown little control of redberry despite good mite control. This suggests either something other than the mite is causing the condition or that the mite is transmitting something which is systemic in the plant and causing significant damage with relatively low mite levels. This project has carried out in-depth sequencing of plant tissues affected by redberry to try to identify whether viruses are responsible for this condition and whether the blackberry mite is transmitting them. Finding out if the condition is indeed virus-associated will inform control measures and could lead on to screening different blackberry genotypes to look for tolerance or resistance to the virus.

## Summary of the project and main conclusions

The project has revealed that redberry-affected blackberry plants do carry viruses. None of these viruses is known to be transmitted by mites and so, at present, there is no evidence that links the blackberry mite and these viruses in the disease process. However, it is now possible to use the tests devised in this project to examine more closely whether any of these viruses actually are present in the blackberry mite and whether they have any association with the redberry disease.

## Financial benefits

A typical blackberry crop can be worth up to £10,000 per tonne and in 2015 in some plantations as much as 30% of fruit showed redberry symptoms. This project was designed as a foundation for further work on identifying the causal agent(s) of redberry disease in blackberry. No immediate financial benefits were predicted.

## Action points for growers

- The project was not designed to produce immediate recommendations for growers to follow.

# SCIENCE SECTION

## Introduction

Redberry disease is thought to be caused by the blackberry mite *Acalitus essigi* injecting toxic saliva into developing drupelets whilst feeding. However recent research (Cross et al. 2012 - HDC project SF116), has clearly demonstrated that although the blackberry mite can be effectively controlled by sprays of rapeseed triglycerides (Codacide Oil) and abamectin (Dynamec), this control did not result in prevention or reduction of the redberry symptoms. This suggests that the redberry condition is not just a result of direct mite feeding damage.

The James Hutton Institute has confirmed that the raspberry leaf and bud mite, which is in the same family as the blackberry mite (Eriophydae) and causes a leaf blotch and crumbly fruit symptoms in raspberry, is associated with a new virus: Raspberry Leaf Blotch Virus (RLBV). This demonstrates that new, mite-transmitted viruses that cause significant disease in fruit and other crops do remain to be discovered. Testing of, and understanding if, UK blackberry plants and mites do carry a virus that is the cause of redberry disease would inform future work on control of this condition and, if a virus is responsible, provides the potential to screen genotypes for virus resistance for incorporation into breeding programs.

## Materials and methods

### *Sampling*

In the summer of 2014 eight blackberry plantations in England of cv. Loch Ness, showing redberry symptoms were identified by ADAS fruit consultants. Sampling commenced on 9 July when fruit at the earliest sites started to ripen and finished on 24 August. Samples were taken from an individual but representative plant within each plantation and included five of the newest fully expanded leaves from the top of the floricane and five fruit displaying redberry symptoms. These were bagged separately and posted first class to JHI for analysis.

**Figure 1**. Redberry on blackberry in the field

**Figure 2**. Redberry in harvested fruit

### *Isolation of RNA from blackberry berries and leaves*

Twelve samples of leaves and fruits from different redberry affected blackberry plants were supplied by Janet Allen and Harriet Roberts (Table 1).

**Table 1**. Blackberry samples used for virus discovery

| ID number | Region | Variety | Sample number | Comments |
|---|---|---|---|---|
| BB1 | South East | Loch Ness | 1 | |
| BB2 | East Anglia | Loch Ness | 2 | |
| BB3a | South East | Loch Ness | 3a | 3a and 3 b same plantation different symptoms |
| BB3b | South East | Loch Ness | 3b | |
| BB4 | South East | Loch ness | 4 | |
| BB5 | East Anglia | Loch Ness | 5 | |
| BB6 | West Mids | Loch Ness | 6 | |
| BB7 | West Mids | Loch Ness | 7 | |
| BB8 | West Mids | Loch Ness | 8 | |
| BB9 | East Anglia | Loch Ness | 9 | |
| BB10 | East Anglia | Loch Ness | 10 | Fruit did not survive transport |
| BB11 | East Anglia | Chester | 11 | Fruit did not survive transport |
| BB12 | South East | Loch ness | 12 | |

Single leaves from the different samples were combined to give four groups: Group 1 (samples 1 and 2); Group 2 (samples 3 to 5); Group 3 (samples 6 to 8); Group 4 (samples 9 to 12). Group 5 was a bulk of the fruits from 10 of the samples combined. The leaves and fruits were frozen in liquid nitrogen and ground to a fine powder using a chilled mortar and pestle. Two methods for RNA extraction from these samples were tried. The first, a trizol extraction followed by sample clean-up using a Qiagen RNeasy column, was found not to produce high-quality RNA. The second method, a hot borate buffer extraction followed by sample clean-up using a Qiagen RNeasy column was more successful.

The hot buffer extraction method is based on the paper of Pang *et al*., 2011 (Pang, M., Stewart, J.D. and Zhan, J. 2011. A mini-scale hot borate method for the isolation of total RNA from a large number of cotton tissue samples. African Journal of Biotechnology 68, 15430-15437).

The details of the method are as follows:

To prepare 10mls Hot Borate Extraction (XT) Buffer: mix 0.0154 g 10 mM DTT, 0.1 mL Nonidet, 0.4 g Polyvinylpyrrolidone (PVP)-40, heat to 85 ºC and maintain the buffer at 85 ºC before use.

*Day 1*

Transfer frozen ground tissue (c. 0.25ml) to a 1.5 mL centrifuge tube. Add 800 µL of the hot XT Buffer (85 ºC), add 2.0 µL of Proteinase K (10 mg/mL). Mix by flicking or shaking so the sample is completely dispersed.

Incubate at 42 ºC for 1.5 hours, then add 90 µL 2M Potassium Chloride (KCl) to precipitate proteins from the extract. Vortex gently to mix the samples. Incubate on ice for 1 hour. Centrifuge at max rpm for 10 min at 4 ºC.

Transfer the supernatant to a new tube. Add 270 µL 8M Lithium Chloride (LiCl) so the final concentration is 2M LiCl. Incubate on ice overnight.

*Day 2*

Pre-cool the centrifuge to 4 ºC. Centrifuge the precipitated samples at max rpm for 10 min at 4 ºC. Discard the supernatant. Wash the RNA pellet in 500 µL of ice-cold 2M LiCl. Make sure the pellet is dispersed in the solution to minimize the retention of contaminants.

Centrifuge at max rpm for 3 min at 4 ºC. Discard the supernatant.

Repeat the washing/centrifugation twice more.

 

Suspend RNA pellet in 250 µL of 1X TE Buffer (pH 8.0) and gently vortex. Sample may be warmed to room temperature in a heating block for 5 min to facilitate diffusion. Centrifuge at max rpm for 5 min at 4 ºC to remove any insoluble material.

Save the supernatant by transferring to new a centrifuge tube. Add 1/10 volume (about 25 µL) of Potassium Acetate (KAc) pH 5.5. Incubate on ice for 15 min. This will remove positively charged polysaccharides, residual proteins, and other salt-insoluble material. Remove samples from ice. Centrifuge at max rpm for 8 min at 4 ºC.

Transfer supernatant to new a centrifuge tube and discard the pellet. Add 1/10 volume (about 25 µL) of 2M Sodium Acetate (NaAc) pH6.0. Add 2.5 x volumes (about 800 µL) of cold 100% ethanol. Store precipitated RNA samples at – 80 ºC. When required for use, collect RNA by centrifugation (max rpm for 10 min at 4 ºC), wash pellet with 70% ethanol, air dry then re-suspend in RNAse-free water (or TE buffer).

### *Rationale for using Next Generation Sequencing (NGS) for virus detection*

Identification of viruses in redberry-affected blackberry plants was done using so-called “next generation sequencing” (NGS). This is an extremely high-throughput chemical analysis in which the genetic material (RNA) from any viruses within the sampled plant is captured and stored as electronic sequence data. About 60 million separate pieces of sequence data were captured for each of three plant samples. Using computer programmes, overlapping parts of the different pieces of sequence data were identified, allowing longer assemblies (contigs) to be created. Then the assembled sequences were compared to the sequences of known viruses, stored in publicly available sequence databases.

Viruses among the blackberry-derived sequences could potentially be either identical or similar to sequences in the databases, thereby identifying either known or related viruses in the blackberry samples. Viruses always produce enzymes that are involved in their multiplication (replicases, RNA polymerases, DNA polymerases) which have very well-conserved protein subdomains. The virus-matching analysis searches for small pieces of sequence that contain these well-conserved domains, so that even new viruses that have fairly few matches in these domains will be identified. However, completely novel viruses that have none of these conserved domains will not be identified by this approach.

### *Several different issues affect the effectiveness of NGS identification of viruses:*

1. **Sample preparation.** The majority of plant viruses have RNA as their genetic material. Isolation of RNA from plant samples will capture the virus sequences but they will be only a small fraction of the total RNA, the majority of which will be host

plant RNA. Some plant viruses have genomes of DNA that will not be captured by the RNA isolation, however, these DNA viruses do produce some RNA during virus multiplication and this will be captured in the RNA sample. RNA viruses, generally, produce an amount of double-stranded (ds)RNA during their multiplication. dsRNA is produced in only low amounts by the host plant, so that isolation of dsRNA from infected plants will enrich for the capture of virus sequences. Potential problems are that not all viruses seem to produce appreciable amounts of dsRNA and the dsRNA isolation procedure requires extra laboratory work that increases costs and reduces workflow speed. The level of accumulation of the virus and the uniformity of its distribution in the infected plant will also affect the ease of its detection.

2. **Depth of read sequencing.** Depending on the amount of RNA sample submitted for sequencing and the volume of reaction applied to the sequencing machinery, it is possible to recover different “amounts” of sequence data from each sample. Obtaining the sequence of a very highly expressed RNA requires the analysis of less sample than does obtaining the sequence of a very rare RNA. Obtaining the same sequence multiple times (i.e. having a greater “read depth”) gives greater confidence that the sequence is real and is not an artefact of the sequencing or sequence assembly processes. However, sequence reads obtained only once or a few times may still be real but will have to be confirmed by other methods.

3. **Cost of sequencing.** The greater the volume of reaction mixture applied to the sequencer, the greater the cost of the sequencing procedure. In addition, RNA samples submitted for sequencing need to be enzymatically treated to convert them to DNA, cleave them into a preferred size-range, and add adaptors to their ends to allow the sequencing reaction to take place. Different samples can be tagged by bar-coding so that mixtures of samples can be analysed simultaneously, with the results being separated by computer using the sample-specific tags. This allows smaller volumes of reaction mixture to be applied to the sequencing machinery, thus, saving costs. However, this must be balanced by the cost of making different bar-coded libraries for each sample. For this project we decided to combine tissue samples from different red berry-affected plants into three bulks. Total RNA (comprising blackberry plant RNA and virus RNA) was isolated from these bulked samples, one from berries and two from leaves. Leaf samples one and two were derived from several different batches of leaves taken from different plants, with no overlap between the source leaves in each sample.

 

To compensate for this bulking of input material, a fairly deep sequencing reaction was done for each RNA sample (>60 million reads per sample).

### *Submission of RNA for Illumina sequencing*

After undergoing quality-control checks at JHI, three RNA samples (SMF10 [fruit], SMF11 [leaf group 1] and SMF12 [leaf group 2] were delivered to the University of Glasgow Polyomics facility for ribosomal RNA depletion and Illumina sequencing (75 cycles of paired-end sequencing using an Illumina NextSeq500 sequencer).

The output from the sequencer was: SMF10; 4,566,629,003 bases in 61,312,256 reads (4.6 billion bases, 61 million reads, or 30 million paired reads). SMF11; 5,044,129,501 bases in 67,724,078 reads (5.0 billion bases, 68 million reads, or 34 million paired reads). SMF12; 5,988,417,055 bases in 80,424,324 reads (6.0 billion bases, 80 million reads, or 40 million paired reads).

### *Sequencing data analysis*

The sequence datasets from Glasgow Polyomics were analysed for the presence of potential viral sequences using the VirFind pipeline (http://virfind.org/j/) which gives lists of sequences that have similarity to database nucleotide and protein sequences of known viruses.

#### RT-PCR confirmation of virus infection of blackberry samples

Primers were designed to amplify fragments of the viral sequences identified by the NGS analysis. New RNA samples were extracted from the powdered leaf and berry samples (that had been stored at -80ºC). Reverse transcription and polymerase chain reaction (PCR) amplification were done in a combined reaction using Illustra Ready To Go RT-PCR beads as recommended by the manufacturer (GE Healthcare). Amplification products were analysed by gel electrophoresis and DNA fragments of the expected size were isolated using a Millipore DNA Gel Extraction Kit, cloned into pGemTeasy and sequenced at the JHI sequencing facility.

## Results

Combining the results from all three RNA samples that were sent for analysis, 214 potential viral nucleotide sequences were identified in the data, and 1570 potential viral protein sequences were also identified. These were then examined individually to remove sequence with only low/partial similarity to database sequences.

The NGS technique is able to identify sequences that are present at very low levels in a sample. This means that it is very easy to detect contaminating sequences that were present in the laboratory environment at the time of sample preparation. For example, Tobacco rattle

virus and Tomato mosaic virus sequences were present in the berry fruit sequence dataset, however, these viruses are probably contaminants from other experiments being undertaken in the laboratory.

**Black raspberry necrosis virus (BRNV)** and **raspberry leaf mottle virus (RLMV)** were also detected. These viruses are a common presence in raspberry samples that we routinely analyse, however, they were present in all three sequence datasets which gives more confidence to them being actually present in the sampled blackberry plants.

Other virus sequences identified in the datasets are:

**Tobacco necrosis virus** (extensive coverage of the virus genome, present in all three datasets).

**Satellite tobacco necrosis virus** (two satellites found, present in all three datasets), these satellites depend on Tobacco necrosis virus to be able to multiply and spread.

A previously unknown **luteovirus** was found. Most partial sequences are in the berry dataset but an assembly of almost the complete virus genome sequence is present in leaf dataset SMF12.

Possible **ourmiavirus** sequences (with similarities to ourmia melon virus and Epirus cherry virus) are present in all three datasets. These sequences do have some similarity to viruses from fungi, and so their identification as plant virus sequences is not yet clear.

Possible **tymovirus** sequences (with similarities to switchgrass mosaic virus and others). No complete sequence is present in the datasets but multiple sequences (>25) are present, although these may not all be from the same, single virus.

RT-PCR testing was done of new RNA samples extracted from the same powdered leaf and berry samples that were subjected to NGS. Twenty seven different pairs of primers were designed to amplify the most likely viral sequences identified in the NGS data. However, at the time of writing this report not all of these primer pairs had been tested – this work is ongoing.

The initial experiments have confirmed (by sequence analysis of cloned RT-PCR fragments) that **black raspberry necrosis virus**, **raspberry leaf mottle virus**, a novel **luteovirus, a tymovirus and an ourmiavirus** are definitely present in the blackberry samples, although not all of the samples were shown to contain each of these viruses. Testing for the presence of Tobacco necrosis virus is ongoing and has not yet been unambiguously confirmed.

 

## Discussion

The NGS analysis of blackberry leaf and berry RNA has succeeded in showing that these plants were infected by several viruses. RT-PCR tests are already available to detect two of these viruses in berry fruit crops (BRNV and RLMV), and we have used the NGS data to designed additional RT-PCR tests to enable us to detect several of the other identified viruses in blackberry (and perhaps other crop plants). These RT-PCR tests should also be applicable to testing of the blackberry mite, thus, completing all the Scientific Objectives of this project.

However, to understand whether one (or a combination of) these viruses plays any role in redberry disease will require a much larger survey of affected and unaffected plants to test for their presence.

**The mite-transmitted virus** (RLBV) that was previously found to be associated with leaf blotch disease in raspberry belongs to the Emaravirus genus. No sequences similar to any emaravirus were found in these samples. Several other types of virus are known to be transmitted by mites, for example, viruses from the genus Trichovirus cause diseases in tree fruits such as apple, apricot, cherry, grapevine and peach. In addition, the other mite-transmitted viruses Blackcurrant reversion virus and Citrus leprosis virus are also associated with severe disease in fruit crops. However, the data produced by this study did not contain any sequences of viruses that are known currently to be mite-transmitted, although for many viruses information about transmission vectors is poor or even absent.

The **black raspberry necrosis virus** and **raspberry leaf mottle virus** are known to be transmitted by aphids. Because no recent surveys have been done to examine the range of virus infection in blackberry, it is not known how widespread these two viruses are in UK blackberry crops. Previous work demonstrated that blackberry was susceptible to these viruses (Jones & McGavin, 1998).

**Tobacco necrosis virus** is known to infect many plant species, including apple, raspberry and strawberry, although, we cannot find any reports of it being found in blackberry. This virus is known to be transmitted by a water- and soil-borne fungus (*Olpidium brassicae*).

All **luteoviruses** are so far known to be transmitted by aphids. The luteovirus we have discovered was previously unknown and we have no information on its plant host range and transmission characteristics.

**Tymoviruses**, which are a very broad group of viruses, are known to be transmitted by beetles and leafhoppers, although for some of these viruses, including grapevine-infecting viruses, the vector is not yet known. We have so far obtained only a small part of the tymovirus sequence which, however, is different to those previously reported. Further work is required

to obtain the full sequence of this virus and then to begin to understand the host range and transmission characteristics of the virus.

**Ourmiaviruses** are a very poorly understood group of plant viruses and, currently, no vector is known for any of the three viruses within this group. Similar to the blackberry tymovirus, it is necessary to obtain more information about the nature of this virus before we can be sure of its involvement (or not) in redberry disease.

**Aphids as vectors of disease in blackberry**. BRNV and RLMV are quite commonly-occurring viruses in UK raspberry crops, and are known to be transmitted by the large raspberry aphid (*Amphorophora idaei*). In experiments, some other aphid species (*Aulacorthum solani* and *Macrosiphum euphorbiae* for BRNV, and *M. euphorbiae* for RLMV) have transmitted these viruses. Currently in England the principle aphid species found on commercial blackberry is blackberry cereal aphid (*Sitobion fragariae*) and less often the permanent blackberry aphid (*Aphis ruborum*). The true involvement of these and other aphid species in virus transmission to blackberry is not well studied. However, there is currently only a limited range of pesticides available for aphid control in protected blackberry i.e. only pyrethrins (on label all edible protected and outdoor crops), thiacloprid (EAMUs for outdoor and protected crops and pymetrozine (Chess WG EAMU for protected and Plenum WG for outdoor crops), chlorpyrifos and pirimicarb use is restricted to outdoor blackberry. Investigation of potential involvement of aphids in blackberry disease in the UK may gain greater importance if the aphid-transmitted viruses identified in this project can be linked to redberry symptoms.

## Conclusions

- At least five viruses were detected in the redberry affected blackberry samples
- Two of these viruses (BRNV, RLMV) were already known to be able to infect blackberry but there have been no reported studies linking them to disease symptoms in blackberry
- Three new viruses were discovered – their effects on blackberry and their means of transmission are not known
- No viruses were found that are known to be mite-transmitted
- Diagnostic tests have been designed to detect these viruses
- These tests can be used to examine further blackberry samples and also blackberry mites to understand if any of these viruses is strongly associated with redberry disease

- Although only the cv. Loch Ness was assessed in this study all commercially grown cultivars are susceptible to this condition therefore further virus investigation would be very valuable to the industry

## Knowledge and Technology Transfer

N/A

## References

Jones, A.T. & McGavin, W.J. (1998). Infectibility and sensitivity of UK raspberry, blackberry and hybrid berry cultivars to *Rubus* viruses. *Annals of applied Biology* **132**, 239-251.

 