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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

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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.

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Grower Summary

Headline

- Methods for the detection of solanaceous viroids affecting tomato have been developed and validated.

Background and expected deliverables

In recent years, solanaceous viroids have been appearing frequently in tomato crops around the world, including the UK e.g. *Columnea latent viroid* in 2007. Others found include *Citrus exocortis viroid, Tomato apical stunt viroid* and *Tomato chlorotic dwarf viroid*. There are others too, which are yet to appear in Europe. These viroids have also been detected in a range of solanaceous and non-solanaceous ornamental species, in the UK, Europe and elsewhere in the world. While the origin of these viroid infections is unconfirmed, evidence exists to strongly suggest that seed-borne disease is one probable route of infection.

There are a number of viroids that can infect solanaceous crops. The most important of these belong to the genus *Pospiviroid*, of which *Potato spindle tuber viroid* (PSTVd) is a type member. In 2003 there was the first outbreak of this pathogen on tomatoes in the UK. In the last decade a number of pospiviroids have appeared in tomato crops around the world including *Columnea latent viroid* (CLVd), *Citrus exocortis viroid* (CEVd), *Tomato apical stunt viroid* (TASVd) and *Tomato chlorotic dwarf viroid* (TCDVd). There are other rare pospiviroids that can infect tomatoes, but these too are yet to appear in Europe.

In the UK in 2007, CLVd was identified for the first time, with three separate outbreaks in crops of tomato var. 'Santa'. The impact of this viroid on the crop was high - with between 20-60% of plants affected. The costs incurred were significant in terms of lost yield and control measures. Similar problems also occurred in France. In addition, these viroids have been detected in a range of solanaceous and non-solanaceous ornamental species, in the UK, Europe and beyond. In the UK, TCDVd has been identified in petunia, in addition to findings of PSTVd in ornamental *Solanum* and *Datura*. The origin of these viroid infections is unconfirmed. Ornamentals might be possible sources of infection. Much evidence exists to strongly suggest that seed-borne disease is one probable route of infection. For example with the CLVd outbreaks in England and France, the only common linking factor was one variety being supplied by one seed company. Seed transmission has been demonstrated for some viroids including PSTVd and TASVd in tomato. However, this work has never been done for CLVd.

Seed-borne infection is one of the most likely and hopefully the most easily eliminated source of disease. This project focuses on CLVd aiming to:

- detect and control seed-borne infection
- validate detection methods
- determine the type and frequency of seed-borne transmission
- investigate efficacy of seed treatments for viroid control
- detect latent infection in tomato seedlings

Successful realisation of these goals and implementation of their outcomes will benefit the British tomato industry by providing the means to help reduce the impact of solanaceous viroids, control or eliminate them from the seed, plant, fruit production and supply chains

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Summary of the project and main conclusions

- Year one of this project has been development and validation of real-time Polymerase Chain Reaction (RT PCR) assays for the detection of solanaceous viroids, primarily CLVd.

- A validation pack has been compiled for accreditation of the existing Fera CLVd TaqMan PCR assays. UKAS 17025 accreditation has been granted.

- Further assays have been developed to allow broad range detection of solanaceous viroids under the matching Defra/EUPHRESCO viroid research project. These assays are currently undergoing validation by ring-testing at laboratories across Europe to ascertain the range of detection.

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- Once this work is complete a screening package will be available to both the seed production and plant propagation industries.

Financial benefits

This work will lead to a better understanding of the risk of seed-borne infection. The validation of detection methods and the determination of the type and frequency of seed-borne transmission; the efficacy of seed treatments for viroid control and detection of latent infection in tomato seedlings will lead to improvements in management strategies for these diseases. The ultimate aim of this work is the provision of a cost effective screening service for each stage of the tomato production chain.

Action points for growers

- A new CLVd assay has been designed, validated and incorporated into a new testing serivce. This is now available to industry through Fera. The new testing service has been specifically focused on the detection of CLVd from seed stocks.

- This new testing service has achieved ISO 17025 accreditation; one of the highest quality standards for laboratory-based tests.

- Further action points will be apparent on the completion of year two of the project, when the risks of seed borne infection and strategies for management of seed borne infection will be quantified.

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Science Section

Introduction

This project has been investigating the detection of *Columnea latent viroid* from tomato seeds and growing plant material using RT-PCR.

CLVd is an important pathogen of tomato crops. Symptoms include stunting, leaf distortion, bronzing, and a 'crunchy' leaf symptom. CLVd is not an EU-listed pathogen, however, as it is closely related to PSTVd and has the potential to cause a very serious problem in tomato crops, statutory plant health action has been taken where outbreaks have occurred.

There is currently no EU protocol for the detection of CLVd. The work detailed below was undertaken to optimise, validate and gain UKAS accreditation for the detection of CLVd. The UKAS assay has been accredited to cover the detection of CLVd in tomato seed, symptomatic and asymptomatic tomato leaf material. The accreditation also covers symptomatic material form other plant species. The principles used to validate this assay are identical to those used for validation of the PSTVd assay.

Further assays have been developed for broad range detection of pospiviroids including specific assays for the detection of *Citrus exocortis viroid* (CEVd) and *Tomato apical stunt viroid* (TASVd). When combined with the existing specific assays for *Potato spindle tuber viroid* (PSTVd), *Columnea latent viroid* (CLVd), *Tomato chlorotic dwarf viroid* (TCDVd) and *Chrysanthemum stunt viroid* (CSVd) this will further enhance the suite of viroids for which Fera has specific assays available. Additionally a generic *pospiviroid* assay has also been designed to detect the six viroids listed above. This generic assay is currently undergoing a programme of ring testing against a broad range of viroid isolates, the results of this further ring testing for validation will be available before the end of this project.

Materials and methods

Validation of CLVd assay: RNA extraction

Two methods were compared for extracting RNA from tomato seed samples:

1. CTAB RNA extraction (adapted from Lodhi *et al* 1994).

2. Fera Kingfisher RNA extraction (in-house magnetic bead extraction method)

The method used a dilution series to ascertain the reliability of detection at three dilutions: 'neat', 1:10 and 1:100. A homogenate of infected seed was used for the 'neat' assays, this was then diluted in a homogenate of healthy/uninfected seed to achieve 1:10 and 1:100 dilutions. Three repeats were carried out for each dilution in the series.

Validation of CLVd assay: Sensitivity and limits of detection of the CLVd real-time PCR assay

The sensitivity of the assay was determined in three ways:

1. Leaf dilution: Infected leaf homogenate dilution series in healthy leaf homogenate (Sensitivity)
2. Seed dilution: Infected leaf homogenate dilution series in healthy leaf homogenate (Sensitivity)
3. Seed 'simulated sample' dilution: Infected seed placed in bulk of uninfected seed (Limits of detection)

The method used in the first two parts of this work were similar to that used in the RNA extraction validation, where homogenate of infected leaf or seed was diluted in known uninfected leaf or seed homogenate as appropriate to give dilutions down to parts per billion

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($1:1^9$). Each dilution was performed twice and for each of these the assay was carried out twice.

The final part of this aspect of the work was to simulate seed lot samples with low infection rates. Prior to homogenisation, one seed taken from known infected material was placed in 50 seeds from a known uninfected seed lot. This was tested twice. This 'dilution' was also duplicated. Using the same principle duplicate simulated samples were also generated for 1:100, 1:200, 1:300, 1:400, 1:500, 1:600 and 1:700.

Validation of CLVd assay: Specificity of assay

As part of the design process for the assay the TaqMan primers and probe were designed to a conserved region of the genome which had commonality across the sequence data available.

Determination of CLVd health status in seed samples intended for future work

Bulks of seed from known outbreak sites have been obtained to allow the seedling grow-out work early in year two of this project. Several kilograms of seed of both cv. Santa and cv. Sweet Million have been retained for this purpose.

Prior to further work being conducted on seed transmission rates, detection in asymptomatic seedlings and efficacy of seed treatments it has been necessary to demonstrate that the seed lots acquired for further work were infected with CLVd. To achieve this the following has been carried out:

1. A seed sub-sample has been tested using the CLVd assay.
2. A determination of seed infection rate has been made by testing a sample of each cultivar. This was done by testing sub-samples from each seed lot. 15 batches of 100 seeds were tested. A determination of infection rate was calculated using the ISTA (International Seed Testing Association) produced programme 'SeedCalc8'.

Results and Discussion

Validation of CLVd assays: RNA extraction

A comparison of RNA extraction using both CTAB and Kingfisher extraction methods was carried out to ascertain the most suitable method for routine use within the laboratory for the detection of CLVd (Table 1). Although CTAB extraction does give lower Ct values throughout the dilution series these differences are not statistically significant. CTAB extraction involves an overnight incubation step and each step in the process is labour intensive; and hence more expensive. Kingfisher extraction is more rapid and is also more amenable to automation. Due to the high numbers of seed sub-samples which would be tested from a screened seed lot Kingfisher is a more suitable method for RNA extraction and is now routinely in use for this assay.

Validation of CLVd assay: Sensitivity and limits of detection of the CLVd real-time PCR assay

The results from Table 2 indicate that the real-time PCR assay is capable of detecting a CLVd leaf positive down to $1:1^5$ (1:100,000). This dilution series was carried out using symptomatic material. The results from seed dilution series (Table 3) show that the TaqMan assay can detect CLVd down to $1:1^2$ (1:100). This may be due to the fact that there are more inhibitors present in seed and that this affects the efficiency of the reaction. Additionally, as the true concentration of CLVd in the reference samples was undetermined, it is likely that the titre of viroid in the growing plant was much higher than the titre of viroid in the seed sample. This could also help to account for the apparent differences in relative sensitivity between the seed and leaf dilution series.

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Table 1. Raw data for the comparison between CTAB and Kingfisher from seed material.

Sample Name	Method	Ct	Delta RN
Neat 1	Kingfisher	17	1.6
Neat 2	Kingfisher	17	1.6
Neat 3	Kingfisher	17	1.6
1:10 (1)	Kingfisher	24	1.6
1:10 (2)	Kingfisher	20	1.6
1:10 (3)	Kingfisher	20	1.6
1:100 (1)	Kingfisher	23	1.5
1:100 (2)	Kingfisher	23	1.5
1:100 (3)	Kingfisher	23	1.5
Neat 1	CTAB	14	1.6
Neat 2	CTAB	14	1.4
Neat 3	CTAB	14	1.4
1:10 (1)	CTAB	17	1.6
1:10 (2)	CTAB	21	1.4
1:10 (3)	CTAB	20	1.4
1:100 (1)	CTAB	20	1.4
1:100 (2)	CTAB	20	1.4
1:100 (3)	CTAB	19	1.6

Table 2. Leaf dilution series showing sensitivity of CLVd TaqMan assay (represented by a serial dilution of infected leaf material in healthy leaf material).

Dilution	Ct	Ct	Delta RN
Neat	14.00	13.94	2.39
Neat	14.23	13.69	2.39
$1{:}1^1$	17.76	17.49	1.46
$1{:}1^1$	17.01	17.79	2.10
$1{:}1^2$	21.33	21.38	1.78
$1{:}1^2$	21.08	21.13	1.84
$1{:}1^3$	24.71	24.60	1.00
$1{:}1^3$	24.33	24.98	0.95
$1{:}1^4$	28.55	28.71	0.18
$1{:}1^4$	28.17	27.52	0.32
$1{:}1^5$	31.23	30.87	0.08
$1{:}1^5$	24.98	35.74	0.03
$1{:}1^6$	Undetermined	Undetermined	0
$1{:}1^6$	Undetermined	Undetermined	0
$1{:}1^7$	Undetermined	Undetermined	0
$1{:}1^7$	Undetermined	Undetermined	0
$1{:}1^8$	Undetermined	Undetermined	0
$1{:}1^8$	Undetermined	Undetermined	0
$1{:}1^9$	Undetermined	Undetermined	0
$1{:}1^9$	Undetermined	Undetermined	0

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Table 3. Sensitivity of CLVd TaqMan assay (represented by a serial dilution of infected seed material in healthy seed material).

Dilution	Ct	Ct	Delta RN
Neat	21.47	20.90	1.60
Neat	22.45	22.35	1.30
$1:1^{1}$	31.52	30.33	0.12
$1:1^{1}$	26.61	25.76	0.80
$1:1^{2}$	31.21	31.43	0.15
$1:1^{2}$	23.60	24.23	1.30
$1:1^{3}$	Undetermined	Undetermined	0
$1:1^{3}$	Undetermined	Undetermined	0
$1:1^{4}$	Undetermined	Undetermined	0
$1:1^{4}$	Undetermined	Undetermined	0
$1:1^{5}$	Undetermined	Undetermined	0
$1:1^{5}$	Undetermined	Undetermined	0
$1:1^{6}$	Undetermined	Undetermined	0
$1:1^{6}$	Undetermined	Undetermined	0
$1:1^{7}$	Undetermined	Undetermined	0
$1:1^{7}$	Undetermined	Undetermined	0
$1:1^{8}$	Undetermined	Undetermined	0
$1:1^{8}$	Undetermined	Undetermined	0
$1:1^{9}$	Undetermined	Undetermined	0
$1:1^{9}$	Undetermined	Undetermined	0

Results of the determination of limits of detection are outlined in Table 4. Due to the design of the experiment, this gives a more realistic measure of the sensitivity of the assay for practical purposes. CLVd was detected in bulks of 700 seeds where only 1 seed was known to be infected. This potentially means that testing could be carried out in bulks of 700 seeds, however, the standard protocol applied under the UKAS 17025 accreditation limits bulks sizes to 100 seeds to give added confidence in this test.

Table 4. Limit of detection of the CLVd TaqMan assay for seed lot testing as determined by testing simulated seed samples.

Sample Name	Ct	Ct	Delta RN
Neat	17.41	17.36	2.51
1 CLVd seed in 50	21.58	22.10	2.08
1 in 50	21.54	21.32	2.26
1 in 100	25.29	25.29	1.39
1 in 100	22.07	22.00	1.87
1 in 200	22.48	22.89	1.50
1 in 200	22.53	22.31	1.89
1 in 300	24.96	25.07	1.40
1 in 300	24.30	24.51	1.65
1 in 400	27.35	27.44	1.02
1 in 400	23.96	23.96	1.57
1 in 500	26.67	26.78	0.66
1 in 500	24.56	24.80	1.47
1 in 600	25.08	25.16	1.41
1 in 600	26.28	26.47	1.28
1 in 700	25.72	26.07	1.23
1 in 700	24.52	24.81	1.32

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Validation of CLVd assay: Specificity of assay

The assay was designed to regions of the genome which were common to all 26 CLVd isolates where sequence data was available via public databases (i.e. GenBank). This desktop study indicates that the assay should detect all known isolates of CLVd. This *in silico* work has been supported by testing using the 3 known isolates of CLVd which were available from European outbreak sites. In contrast testing using isolates of other pospiviroids, including PSTVd, TASVd, TCDVd, CEVd and CSVd, failed to yield any positive results; thus demonstrating the specificity of the new assay for CLVd and CLVd alone.

These assays have also been used in a laboratory proficiency test, produced by FAPAS. The purpose of this proficiency test was to detect CLVd from blind samples containing the target, a related target (e.g. PSTVd) and an unrelated target (e.g. *Potato virus Y*). The work was assessed as 'satisfactory' meaning that the status of each sample was determined correctly.

Additionally, the assay has been used to detect CLVd in 184 sub samples taken from 24 seed lots. In each case the detection of CLVd was confirmed using PCR and subsequent genetic sequence comparison.

Determination of CLVd health status in seed samples intended for future work

A 100 seed sample of each variety was tested using the real-time PCR assay. In each case the seed lots were shown to contain CLVd (Table 5). Further to this, a determination of seed health was carried out to ascertain approximate percentage infection in each seed lot. For each variety, 12 out of 15 seed sub-samples tested was positive for CLVd. This gives a calculated mean infection rate of 1.6% (Table 6). As the results from both varieties were the same this indicates a very low rate of seed infection, which may not be variety dependant.

Table 5. Determination that seed lots for future study contain CLVd.

Variety	Ct	Delta RN
Santa	24	1.13
	24	1.08
Sweet Million	29	0.37
	29	0.37

Table 6. Determination of seed infection rate in acquired seed lots.

Variety	No. of Bulks	No. of seeds per Bulk	Total No. of seeds tested	No. Bulks Positive	Mean Infection rate	Lower 95% CL	Upper 95% CL
Santa	15	100	1500	12	1.60%	0.73%	3.09%
Sweet Million	15	100	1500	12	1.60%	0.73%	3.09%

Conclusions

During the course of this work reliable extraction of CLVd RNA from seed and growing plant material has been demonstrated. Whilst the CTAB method was slightly more efficient at extracting RNA, this was not significantly better than magnetic bead extraction. The benefits this method confers for speed of extraction and suitability for automation make this more suitable for the high throughput conditions, necessary for running an efficient seed screening service at a cost-effective price.

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The results listed above demonstrate that the Fera CLVd real-time PCR assay will reliably detect the target pathogen. This has been further demonstrated through the extensive validation procedures carried out to gain UKAS accreditation for testing tomato seed for CLVd using this assay. Further assay development work, carried out under DEP funding, is currently undergoing ring testing and these results will be included in the year 2 project report.

Seed has been obtained and has been shown to be infected with CLVd. Work is currently ongoing to demonstrate that this CLVd infected seed is infectious and seedling grow out work will begin shortly.

Technology transfer

Fera has a system in place for transfer of R&D assays to frontline diagnostic teams. This assay has been validated and UKAS ISO17025 accreditation for this procedure in now being offered the industry. The work conducted in the coming year will allow screening package for both seed and seedlings to be offered to the industry.

References

Lodhi MA, Ye GN, Weeden NF and Reisch BI (1994). A simple and efficient method for DNA extraction from grapevine cultivars and Vitis species. *Plant Mol. Biol. Reptr.* **12**: 6-13

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