Project title:	Detection and elimination of solanaceous viroids in tomato seeds and seedlings.
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Project leader:	Mr Adrian Fox, Fera
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Key staff:	Dr Wendy Monger
Location of project:	Fera, Sand Hutton, York
Project coordinator:	Mr John Overvoorde, Defland Nurseries
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The results and conclusions in this report are based on an investigation conducted over a two-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.

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

Headlines

- Methods for the detection of solanaceous viroids affecting tomato have been developed and validated.
- Transmission of CLVd from seed lots known to carry inoculum remains unproven.

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* (CLVd) in 2007. Others found include *Citrus exocortis viroid* (CEVd), *Tomato apical stunt viroid* (TASVd) and *Tomato chlorotic dwarf viroid* (TCDVd). Others, such as *Tomato macho planta viroid*, have yet to appear in Europe. They 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.

Since seed-borne infection is the one of the most likely and hopefully the most easily eliminated sources of disease, this project has focused on:

- the detection and control seed-borne infection
- the validation of detection methods
- determination of the type and frequency of seed-borne transmission
- efficacy of seed treatments for viroid control
- detection of 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 and fruit production and supply chains.

There are a number of viroids which can infect solanaceous crops. The most important of these belong to the genus *Pospiviroid*, of which *Potato spindle tuber viroid* (PSTVd) is the 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 CLVd, CEVd, TASVd and TCDVd. There are other rare pospiviroids that can infect tomatoes but which 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 (cv. 'Santa'). The impact of this viroid on the outbreak crops was high (with between 20-60% of plants affected) and the costs were significant in terms of lost yield and control measures (see Figure 1).

Figure 1. Tomato mother plants (cv. Santa) artificially inoculated with CLVd, showing leaf curling, yellowing and necrosis and fruit deformation

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. While the origin of these viroid infections is unconfirmed, and ornamentals might also 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, TASVd and TCDVd in tomato. However this work has not, as yet, been carried out for CLVd.

Summary of the project and main conclusions

- Year one of this project was concerned with development and validation of real-time 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.

- Both the generic and specific viroid assay suites were successfully tested under the EUPHRESCO DEP project by 10 laboratories across Europe.

- Validation work is currently being carried out on the TASVd and TCDVd specific real-time PCR assays to provide reliable, cost effective solutions for detection of these viroids from seed.

- 25 500 seeds were grown from stocks known to contain natural infection with CLVd. No evidence of seed transmission was recorded.

- Artificial inoculation of tomato with CLVd can result in low numbers of seed with low viability.

- 200 seeds were grown from mother plants experimentally infected with CLVd. No evidence of seed transmission was recorded.

Financial benefits

Since seed-borne infection is the one of the most likely and hopefully the most easily eliminated sources of disease, 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 and 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. 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 and fruit production and supply chains, with the ultimate aim of this work being 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 service. 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.

- Validation of the new TASVd and TCDVd assays against seed borne are undergoing final validation and will shortly be available to the industry through Fera. Both of these viroids have been detected from recent European tomato viroid outbreaks.

- A full suite of specific assays for the detection of tomato affecting pospiviroids has been completed. These are validated against known symptomatic leaf material. Validation against seed will be carried out as material becomes available.

- A ‘generic’ pospiviroid assay has been designed for broad range detection of tomato affecting pospiviroids.

- Following a comprehensive grow out of more than 25 000 seedlings originating from seed from an outbreak site known to contain a low incidence of inoculum has failed to demonstrate seed borne transmission of CLVd.

- Seed produced from artificially inoculated plants has similarly failed to demonstrate seed borne transmission of CLVd. In many cases inoculated plants failed to set fruit and where inoculation was delayed until fruit set, many plants either produced low numbers of seeds or failed to produce viable seed.

- Although seed borne transmission of CLVd remains unproven similar closely related viroids have been shown to be seed transmitted although the precise conditions for this remain elusive.

Science Section

Introduction

This project has been investigating the detection of *Columnea latent viroid* (CLVd) from tomato seeds and growing plant material using real-time 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 from 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. The generic and specific assays were tested by 9 institutes as part of the Euphresco DEP project. This 'ring testing' was carried out as an inter-laboratory proficiency test, where different extraction methods and testing regimes were followed depending upon the availability of equipment and extraction reagents used by each participating laboratory. This methodology was deemed to be the best process to ensure robust validation of the probe and primer sets under evaluation. The results of this 'ring testing' are presented.

Seed transmission has been demonstrated for some viroids including PSTVd, TASVd and TCDVd in tomato (Singh, 1970; Antignus *et a*l, 2007; Singh and Dilworth, 2009). However, the transmission of CLVd has not previously been investigated. Following outbreaks of CLVd in the UK in 2007 for which there was strong circumstantial evidence of seed being

implicated as the source of these outbreaks and as a consequence of a further outbreak of this viroid in 2009, the second part of this project focused on attempting to quantify the risk posed by inoculum (infected) seed in tomato propagation. This was investigated using seed obtained from known outbreak sources, which had been shown to contain low levels of inoculum seed. This was to 'simulate' a commercial seed situation. Due to the erratic and often elusive nature of seed transmission of viroids a simultaneous experiment was carried out to produce seed from artificially inoculated mother plants. This, in theory, would produce high incidence inoculum seed and the methodology for this was adapted from investigations into TCDVd seed transmission (Singh and Dilworth, 2009). Further work investigating symptomology of infected seedlings, detection of latent infection and seed treatments would depend upon confirmation of seed transmission.

Detection Methods

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 seed homogenate dilution series in healthy seed 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 ($1:1^9$). 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.

DEP 'ring' test of detection methods

Under the EUPHRESCO DEP (Detection and Epidemiology of Pospiviroids) project, Fera coordinated an interlaboratory validation test of real-time PCR assays to assess new molecular tools for the detection and diagnosis of pospiviroids. To this end assays were developed at Fera using generic primers for 6 pospivoroids (PSTVd, TCDVd, CEVd, CLVd, TASVd, CSVd) to allow testing for a suite of the most commonly intercepted pospiviroids in a

'single' assay. In addition to this work, assay primer sets were also developed for the specific detection of TASVd (3 assay primer sets were designed to this viroid) and CEVd (2 assay primer sets). The Fera CLVd assay was also included within the suite of assays tested within this project.

Across Europe 8 partner laboratories, including Fera; the French and Dutch plant protection services; as well as several university research laboratories, participated in the ring-test for the real-time PCR assays. In addition the official plant health laboratory in New Zealand also participated in the validation testing. It was noted that the process followed did not completely correspond to a ring-test as laboratories could use their own reagents and extraction method. This allowed a thorough interlaboratory validation of the assays themselves as laboratories were extracting viroid RNA using their own preferred methods. Each participating laboratory received a number of 'blind' samples of leaf material infected with the following: PSTVd, TASVd, TCDVd, CEVd and CSVd. In addition known control samples of positive PSTVD RNA extract, a 'healthy' control and water 'blank' were also provided.

All assays under test were challenged against all samples within the validation testing. Hence each assay was tested against its intended target as well as the other tomato affecting viroids to test specificity of each assay.

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.

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	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	2.1
$1:1^{2}$	21.33	21.38	1.78
$1:1^{2}$	21.08	21.13	1.84
$1:1^{3}$	24.71	24.6	1.0
$1:1^{3}$	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

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	20.9	1.6
Neat	22.45	22.35	1.3
$1:1^{1}$	31.52	30.33	0.12
$1:1^{1}$	26.61	25.76	0.8
$1:1^{2}$	31.21	31.43	0.15
$1:1^{2}$	23.6	24.23	1.3
$1:1^{3}$	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	22.1	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	1.87
1 in 200	22.48	22.89	1.5
1 in 200	22.53	22.31	1.89
1 in 300	24.96	25.07	1.4
1 in 300	24.3	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	24.8	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

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.

DEP ‘ring’ test of detection methods

These results were presented at an ad hoc EPPO working group meeting on tomato affecting viroids in Ljubljana, Slovenia (October 2009).

For the specific primers 100 % agreement between laboratories was recorded for the viroids concerned. However, whilst in all cases the target viroid was detected by the specific assay, in some cases these specific assays cross reacted with other target viroids. These were as follows:

- TASVd assay 2: One laboratory detected a TCDVd positive.
- TASVd assay 3: Three laboratories detected a positive from the PSTVd sample and one a positive from the TCDVd.
- CEVd assay 2: One laboratory detected TCDVd, one detected TASVd, one detected CSVd.
- CLVd assay: One laboratory found the healthy sample to be ‘positive’.

In the case of CLVd it is not thought that this is a true cross reaction, but rather contamination in the assay sample possibly during the RNA extraction phase. This conclusion is based upon the batch production method used for the healthy control material and the fact that no other laboratory detected this finding. It is also possible, given that other laboratories did not detect these cross reactions that they were also due to contamination in the RNA extraction stage. On the basis of these results the TASVd specific assays are currently undergoing further validation work at Fera to ensure they are both robust and sensitive for use in seed screening programmes.

For the generic pospiviroid test, the percentage of agreement varied between 78% and 100% depending on the viroid:

- One laboratory obtained negative results for samples which should have tested positive for PSTVd, TCDVd, CSVd.
- One laboratory obtained a negative result for a sample which should have tested positive for CSVd.
- One laboratory obtained a negative result for a sample which should have tested positive for CEVd.

Seed transmission studies

Material and methods

Determination of CLVd health status in samples for seed transmission studies

Bulks of seed from known outbreak sites were 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 was necessary to demonstrate that the seed lots acquired for further work were infected with CLVd. To achieve this, the following testing was carried out:

1. A seed sub-sample was tested using the CLVd assay.
2. A determination of seed infection rate was 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'.

CLVd Seed grow out from commercial seed linked to UK outbreak

The seed bulk identified for use in the seed transmission work was subdivided by weight into seed lots, each containing 50 seeds. Each of these lots was broadcast sown into a seed tray containing potting compost (John Innes No. 1), initially thirty seed lots per batch were sown per repetition of the grow out experiment, giving a total of 1500 seeds per repetition. These lots were grown under controlled environment conditions at a constant temperature of 24ºC and day length of 16 hours.

Following germination and grow out, all plants were harvested from each tray and tested using the ISO 17025 accredited test as described above to detect the presence of CLVd in seedlings. This testing was conducted after 6 weeks growth, with further testing carried out after 10 weeks growth.

In later repetitions of the experiment larger batches of 3000, 6000 and finally 7500 seeds were sown, grown out and tested. In total 25, 500 seeds were grown and tested in this experiment.

Production and grow out of artificially inoculated seed

Plants of cv. Santa were produced and artificially inoculated with CLVd inoculum which had been collected and stored since the 2007 UK outbreaks. The method for production of plants and collection of seeds was adapted from Singh and Dilworth (2009). Plants were inoculated in batches of six plants at a time, and grown under controlled environment conditions for fruit production at 24ºC/16hr day length until senescence. There were two main deviations from the published method. (a) timing of inoculation and (b) seed treatment following harvest. In each case infected plants were tested using real-time PCR to ensure that inoculation had been successful and symptoms were clearly observed (See Figure 1).

Figure 1. Tomato mother plants (cv. Santa) artificially inoculated with CLVd, showing leaf curling, yellowing and necrosis and fruit deformation

Timing of inoculation had to be altered as initial experiments prevented fruit set resulting in very few fruit containing few seed. Delaying inoculation for the second repetition of this work gave higher numbers of fruit, but again there were low numbers of seeds which were often deformed and had low viability. For the third repetition inoculation was delayed until fruit had started to set on the second truss, this yielded a greater quantity of seed and greater viability. With around 200 viable seeds resulting from the final six inoculated plants.

An acid wash treatment (adapted from Thyr *et al.* (1973)) was applied to remove the suspicion that any transmission observed could be arising from infected pulp or sap on the seed coat. Seeds were grown out in 2 batches of 100 seeds in a controlled environment room at 24ºC. Following germination and grow out, all plants were harvested from each tray and tested using the ISO 17025 accredited test as described above to detect the presence of CLVd in seedlings. This testing was conducted after 6 weeks growth, with further testing carried out after 10 weeks growth.

Results and discussion

Determination of CLVd health status in seed samples from known outbreak sources

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	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%

CLVd Seed grow out from commercial seed linked to UK outbreak

Levels of seed transmission reported for experimentally infected seeds of the viroid PSTVd indicate that between 7-11% transmission could be expected from '100%' infected seed. However, this data was from artificial inoculation of tomato mother plants and the true level of seed transmission from and 'outbreak' seed lot for PSTVd has not been investigated. In the 2007 UK outbreaks of CLVd, where seed transmission was strongly suspected to have been the infection source, a much lower incidence of initial transmission was observed. During one of these outbreaks only 3 plants were initially observed with symptoms from 56 000 planted (approximately 1 infected plant in 19 000). Thus any meaningful work on an outbreak seed lot would involve growing out tens of thousands of seeds. In total over 25 000 seedlings were grown and tested using real-time PCR. No viroid symptoms were observed in growing seedlings and no viroid was detected from any plants (See Table 7).

Table 7. Tomato seedling grow-out results showing the number of batches grown and tested and the outcome of the testing

Batch number	Seedlings per Batch	CLVd Result
1	1500	Negative
2	1500	Negative
3	1500	Negative
4	1500	Negative
5 &6	3000	Negative
7	3000	Negative
8	6000	Negative
9	7500	Negative
Total	**25 500**	**Negative**

Production and grow out of artificially inoculated seed

Artificially infected mother plants clearly exhibited symptoms of viroid infection during fruiting, with both foliar symptoms and deformation of fruit (Figure 1). From the 200 seeds obtained from artificially infected mother plants no viroid was detected nor were there any observable symptoms in progeny plants. Thus neither grow out experiment has successfully demonstrated seed transmission of CLVd in tomato.

Singh (2009) used untreated seed from infected mother plants and obtained high proportions of viroid transmission. However, using this method seed coat contamination could contribute to an erroneously high transmission rate in the published data. The results from this work indicate that seed transmission levels for CLVd are lower than would be expected from previously published work on other viroids, if it occurs.

Table 8. Results from growing out seeds produced from artificially infected mother plants.

Batch number	Seedlings per Batch	CLVd Result
1	100	Negative
2	100	Negative

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.

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. The species specific viroid assays developed and ring-tested under the EUPHRESCO DEP project all detect the targets they were designed against. Starting with the TASVd assay, further validation work will now be conducted to allow screening of seed lots using this assay. Further validation is required to ensure the assay is both robust and sensitive enough for use in testing seed samples where viroid concentrations may be

extremely low. The possible cross reactions from some of these assays do not present any major issues within the diagnostic context as these assays would not be used as 'stand alone' tests. Current diagnostic protocols for PSTVd, TCDVd and CLVd all include a second confirmatory test using DNA sequence comparison techniques. The DEP generic assay requires further development due to concerns over the robustness and sensitivity of this test when using the assay against seed borne viroid infections. An unfortunate consequence of increasing the detection range of the primer set has been a decrease in the sensitivity of the assay.

Seed was obtained from seed lots known to be associated with commercial outbreaks of CLVd. Subsequently this material was shown to be infected with CLVd at a low concentration (C. 1.5% seed infection). Further studies of seed lots where viroid contamination has been detected show this to be a typical level of viroid inoculum in a contaminated seed lot (data not presented). The batch grow-out experiment, testing over 25 000 seedlings, failed to demonstrate seed transmission of CLVd from this seed. This does highlight that not all infected seed will result in infected plants and there may also be other, as yet unidentified, factors influencing the levels of seed transmission.

Having based further artificial inoculation work on that carried out by Singh (2009), where successful transmission of TCDVd was demonstrated, the first point to note was that several attempts had to be made to produce viable seed from infected mother plants. In the first repetitions of the artificial inoculum experiment plants failed to produce fruit if inoculated early, or fruit yielded very low numbers of seeds (often <10 seed per fruit). These seeds were often deformed and on attempting to grow them out were found to be non-viable. Infecting plants later, around the second truss stage, did allow viable seed to be produced, although numbers of seeds per fruit were still low. Eventually 6 artificially inoculated plants yielded 200 seeds. These results indicate that even on discovering CLVd infection in a seed production system the chances of infection in daughter plants will be low due to the low numbers of viable seeds arising from inoculum plants. Further inferences on the detection of latent seedling infection cannot be made due to the absence of seedling infection arising from either naturally or artificially infected seeds.

Further work is currently underway at Fera to investigate the link between seed inoculum and CLVd infection. Due to the lack of evidence for true seed borne infection, experiments are being carried out into transmission of CLVd following events of seed coat contamination.

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 to the industry.

The work currently being conducted on the TASVd assays will allow a screening package for both seed and seedlings to be offered to the UK tomato industry. As time and availability of source material allows further assays will be validated for this purpose.

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