‘Wash and Grow’: the development of non-destructive tomato seed testing

PC 229

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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

- A rapid, new non-destructive testing method has been developed which is capable of detecting the presence of *Pepino mosaic virus* (both original tomato and new US2 strains)*, Potato spindle tuber viroid* (and potentially other tomato-infecting viroids) and *Tomato mosaic virus* with no loss of viability.

Background and expected deliverables

In the UK, the three most important viral pathogens of tomato (*Pepino mosaic virus,* PepMV*; Potato spindle tuber viroid,* PSTVd*; Tomato mosaic virus,* ToMV) can all be transmitted via infected seed. As all of these pathogens are readily spread by mechanical means, even a single infected plant originating from seed transmission, is enough to start an outbreak. The cost of such outbreaks can be substantial (in excess of £100k), once loss of production and necessary hygiene measures are taken into account.

Unfortunately, it is often impossible to guarantee that commercial seed is free of these pathogens and as a result British growers run a high risk of introducing these pathogens into their crops by using infected/contaminated seed. This risk can be minimised by testing seed prior to planting. However, given that large numbers of seeds need to be tested (typically several hundred) and that often the unit cost of each seed is high (e.g. over 50p per seed), the overall cost of this testing is often prohibitive, as current methods require that the seed is destroyed (destructive seed testing).

As a result, the development of methods that permit the reliable detection of the target pathogens, without compromising the performance of the seed (e.g. germination rate, genetic stability etc) could provide a cost-effective means for growers to ensure the health status of their seed stocks.

Specific deliverables:

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- A non-destructive seed testing method has been developed that allows the reliable and sensitive detection of the three key pathogens:
    1. *Pepino mosaic virus* (both tomato and new US2 strains)
    2. *Potato spindle tuber viroid* (and other tomato-infecting viroids)
    3. *Tomato mosaic virus*
- This will form part of a rapid and cost-effective testing service, which will be made readily available to the British tomato industry, including tomato growers and plant propagators.

Summary of the project and main conclusions

- A method for non-destructive testing of tomato seeds was successfully developed. The method was able to detect a single seed infected with both PepMV or PSTVd within a batch of 600 uninfected seeds.
- The method is rapid allowing testing to be turned around within 48 hours.
- The method has been extensively validated by comparative testing with the existing destructive method, using batches of commercial seed. Overall the sensitivity of both methods was found to similar. However, different performances were observed for different viruses, with non-destructive testing proving less sensitive for PepMV detection but more sensitivive for ToMV detection.

Financial benefits

- One of the major pathways by which virus/viroid infections are introduced into tomato crops is via seed. By testing seed this risk can be virtually eliminated and hence the cost implications of an outbreak via this route are removed.
- Destructive testing can be used, but has the disadvantage of being expensive due to the large numbers of seed destroyed during testing and the high unit cost of each seed. In many cases the cost of testing in seed alone could typically be £50-250 (for a 500 seed test).

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- TaqMan is an established technology, which has proven cost effective for routine use in diagnostic services. As the method is rapid, it can easily be used within the tight deadlines used by propagators and growers.
- Propagators will be able to have seeds tested prior to sowing. This will allow decisions to be made regarding whether batches should be used or not, or whether some type of seed treatment should be employed.
- Improved confidence in health status of tomato plants supplied by British propagators.
- There is the potential to use this research as a starting point for future work on other seed-borne pathogens of tomato and other protected crops, thus fast-tracking any future R&D effort.
- The technology will be made available through an existing, well-established diagnostic service. This is cost effective as issues such as quality assurance (including ISO 9001 and 17025) and availability of appropriate licenses (legally required for using PCR technology) are already in place, hence no need for additional expenditure.

Action points for growers

1. Pepino mosaic virus: for many years now it has been assumed that PepMV has been spread via infected/contaminated seed. The sudden arrival and rapid spread of the new US2 strain in Europe and the USA, associated with findings on seed, has highlighted the potential risks of spreading PepMV via this pathway. Many now regard commercial seed as posing a serious risk and as a result are looking to take routine action. Seed testing for this pathogen is obviously one action that growers should consider.

2. Tomato mosaic virus: while ToMV has been effectively controlled for many years now by the use of resistance, the introduction of some varieties which lack this (e.g. Sante), has resulted in the reappearance of ToMV related problems in the UK. For this reason, the testing of seed prior to sowing for non-resistant varieties is highly recommended.

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3. Viroid diseases: PSTVd and other tomato-infecting viroids have become a real risk to the British tomato industry in recent years. In addition to the first UK PSTVd outbreak in tomato in 2003, outbreaks of this and other viroids have been occurring regularly in Europe over the last few years. For this reason, new sensitive real-time PCR assays have been developed that can detect not only PSTVd but also the other tomato viroids. These can now be used for seed testing. While routine testing of all seed is obviously recommended, special attention should be paid to seed coming in from outside of Europe. For further advice see HDC Factsheet 09/06.

4. Sampling rates: the amount of seeds required to perform a satisfactory test has always been a major issue and has often resulted in a compromise between testing enough seed to ensure reliability, off-set against the cost of destroying seed during testing. Using well-established seed testing statistics, it can be predicted how many seeds are required to detect a pathogen to a particular level with a certain confidence. For example, to ensure a 95% confidence of detecting at least a 1% infection, a sample of 300 seeds is required. Further examples are given in Table 1.

Table 1: Number of seeds that should be tested in order to ensure detection of infection at various levels ('x' %) with a 95% confidence level

Sample size	'x' % infection
3000	0.1
1000	0.3
600	0.5
500	0.6
400	0.75
300	1.0
150	2.0
100	3.0

Based on these figures, we are recommending at least a 1000 seed test. The current International Seed Federation PepMV protocol recommends 3000 seeds. However it should be noted that this is a protocol recommended for seed companies, where obviously the cost of seed is less of an issue. For

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every 1000 seeds, these will be tested as two sub-batches of 500 seeds. For smaller seed batches e.g. trials or specialist varieties, we would recommend discussion with CSL diagnostics.

5. Seed testing: for further details of seed testing services, please contact CSL diagnostics at diagnosis@csl.gov.uk or (01904) 462324.

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Science Section

Introduction

Preliminary data generated by CSL (unpublished) showed that a non-destructive approach to the detection of viral pathogens in tomato seed could work in principle. This work also established a basic methodology. Following that proof-of-concept, the purpose of the studies described here was to provide further evidence of the efficiency and reliability of such an approach. This was intended to form the basis of a rapid cost-effective test which could then be made available to the British tomato industry.

Real-time PCR assays

A key component of this project has been the use of reliable real-time PCR assays for the detection of all the target pathogens. These have all been developed previously under Defra Plant Health Division funding. All the assays have been validated against a wide range of target isolates (at least 10 in all cases, but actually many more in some cases), as well as a range of non-target isolates including related viruses or viroids (e.g. in the case of ToMV this has been other tobamoviruses including *Tobacco mosaic virus* etc; for PepMV this has been other potexviruses including *Potato virus X*)

1. Effect of various lysis buffers on tomato seed germination

Materials and Methods

Single replicates of 50 uninfected seed from cv. Moneymaker (Kings) were immersed in 2.5 ml of the following solutions: CTAB buffer, Tris-HCl buffer pH 8; Guanidine hydrochloride (GHCl); and guanidine isothiocyanate (GITC). A water control was tested in parallel. Each tube was mixed on an end-over-end shaker for 20 minutes. The tomato seeds were then washed 3 times in 30 ml sterile distilled water (SDW), before the germination was assessed at 5, 7, 9, 12 and 14 days post treatment, using the method detailed below. In order to assess possible longer-term effects, seedlings (10) from treatments not showing signs of inhibition following germination were removed and grown on in a glasshouse.

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Germination assessment

Seed germination assessments were based on International Seed Testing Association (ISTA) guidelines. Seeds were placed on damp filter paper within a 90 mm petri dish with a hole drilled in the lid to allow gaseous exchange. A damp filter paper was placed on top of the seed and a wick run to a water source to ensure the seedlings remained moist. Plates were placed in duel cycling incubator with supplemented lighting. Temperature was maintained at 30°C for 8 hours with lighting and at 20°C for 16 hours without lighting. Germination was assessed periodically depending on the experiment.

2. Comparison of wash step and extraction protocol

Replicates (3) of 45 uninfected seed were added to 5 PepMV infected seed and immersed in 2.5 ml of either water or GITC buffer in a 6 ml tube. Each tube was left to agitate on an end-over-end shaker for 20 minutes. Seeds were then washed 3 times in 30 ml SDW and assessed for germination as previously described. The wash solution was vortexed and aliquotted into 4 x 0.5 ml samples. Random duplicates were processed using one of the following 2 methods:

Lithium chloride extraction

An equal volume of water (500µl) was added prior to the addition of 1 ml 4 M LiCl. The tube was mixed and placed at 4°C overnight. The entire sample was spun at 13000 g at 4°C for 25 mins to pellet the RNA. The pellet was then washed with 70% EtOH, re-spun for 10 mins and then air-dried. Finally the pellet was resuspended in 100µl molecular grade water.

Kingfisher extraction

An equal volume of GITC buffer (500µl) was added prior to the addition of 50 µl Magnesil beads. The standard plant RNA Kingfisher method was then followed with wash buffer in well 2, 70% ethanol in wells 3 and 4 and 200 ul of molecular grade water in well 5.

Real-time PCR

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All RNA extracts were tested using the real-time RT-PCR assays designed previously at CSL. PepMV testing was completed using both the current assay, designed to detect the 'older' tomato strain and a new assay, designed to detect the US2 strain.

3. Optimisation of drying method

The development of a suitable drying protocol is crucial for subsequent storage and successful germination. Moneymaker seed were divided into 300 seed batches, soaked in water for 1 hour and then towel dried. Triplicate batches were then subjected to one of the following drying treatments:

1. Placed in an airtight box containing silica gel and left at room temperature for 48 hours.
2. As (1) but placed at 28°C
3. Placed directly at 28° in the absence of silica gel.

After the drying treatment, each batch was placed at 110°C for 48 hours, the % moisture calculated and the calculations compared to those obtained for commercial seed prior to soaking.

4. Post-treatment storage

The proposed treatment of washing seed in water followed by drying in the presence of silica get at room temperature was tested for the effect on germination using different post treatment storage conditions. Replicates (3) of 50 seed cv. Moneymaker were treated and dried before being stored for 14 days at either room temperature or 4°C. Replicate samples from untreated seed were also incubated for 14 days at room temperature and 4°C to act as controls. Seed were then soaked in water for 1 hour and a germination test completed as described previously. Germination was assessed periodically for 17 days.

5. Sensitivity determination

An experiment was conducted to determine the sensitivity of the non-destructive method for the detection of PepMV, ToMV and PSTVd. Batches of 300 or 600 seed of cv. Moneymaker were mixed with acid washed seed

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from fruit infected with PepMV, TMV, or PSTVd. Duplicate samples of each virus were prepared as follows:

1. 1 infected seed in 600 Money maker seeds (0.16% infection)
2. 1 infected seed in 300 Money maker seeds (0.32% infection)
3. 3 infected seeds in 300 Money maker seeds (1% infection)
4. 30 infected seeds in 300 Money maker seeds (10% infection)

A control was prepared using Moneymaker seed in the absence of any additional known infected seed. In addition, a control was prepared containing no seed to act as a buffer control. All samples were tested for the presence of each virus using specific real-time RT-PCR assays.

6. Comparative testing of non-destructive vs destructive seed testing

Seed from three commercial seed batches were tested using the optimised non-destructive method and the current destructive method for testing seed. Each seed batch was split into batches of 2 x 300 seeds for non-destructive testing and 3 x 50 seeds for destructive testing. Extracts were tested for the presence of ToMV and PepMV using real-time PCR.

Further parallel testing was carried out using nine batches of commercial seed, which had all previously tested positive for PepMV US2 strain. Each batch was re-tested using both methods with sample sizes ranging from 10-200 of uncoated seed.

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Results and Discussion

1. Effect of various lysis buffers on tomato seed germination

The buffer with the highest germination rate was GITC. All the other buffers tested adversely affected the germination of tomato seeds suggesting they are not suitable for use in a non-destructive method (Table 2). Of 10 seedlings taken for the growing on test, 8 were reared successfully for both the GITC treatment and the water control suggesting the GITC had no adverse effect on plant production.

Table 2: Percentage germination (of 50 seed) after a 20 minute treatment with a range of different buffers. A water control was tested in parallel.

Treatment	Number of days post treatment			
	5	7	9	12
CTAB	0	0	0	10
Tris HCl	4	10	20	26
GHCl	0	6	30	52
GITC	2	26	50	68
Water	4	42	58	66

Subsequent germination test comparisons involving just GITC and water (with 3 replicates of 50 seeds each), showed there was no difference in germination rate between either treatment: GITC (79%; Stdev 11.7; n=3) and water (79%; Stdev 6.1; n=3).

2. Comparison of wash step and extraction protocol

PepMV was successfully detected in washings from all the samples tested. The overall the use of GITC was marginally better for the detection of PepMV than simply washing seeds in water (Table 3). This is probably due to the fact that this buffer will offfer better protection to the viral RNA, against degradation by RNase activity. In addition, levels of PepMV were comparable between samples extracted using the lithium chloride and the Kingfisher method (Table 3). Therefore the combination of washing tomato seeds in

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GITC buffer followed by a simple lithium chloride precipitation was adopted as the method of choice.

Table 3: Real-time RT-PCR results showing the efficiency of PepMV detection using combinations of extraction method and wash solution. A lower threshold cycle (C_T) indicates a higher concentration of target in the sample.

Extraction method	Wash solution	PepMV C_T	Standard deviation (of 3 samples)
Kingfisher	GITC	32.43	1.19
	Water	35.65	0.69
Lithium chloride	GITC	31.56	0.72
	Water	32.02	0.25

3. Optimisation of drying method

Both drying treatments performed at 28°C reduced the % moisture of the seed to a level below that found in unsoaked seed (data not shown). The drying treatment performed at room temperature in the presence of silica gel produced seed with almost identical % moisture content as the unsoaked seed with respective values of 8.62% for the dried versus 8.41% for the unsoaked seed. Therefore sealing the seed in a box containing silica and incubating at room temperature for 48 hours is a suitable method for drying seed after the washing treatment.

4. Post-treatment storage

Similar germination profiles were recorded for seeds from both controls, suggesting temperature had no effect on germination in the absence of the initial soaking and drying treatment. Germination after 17 days of treated seed stored at room temperature was significantly lower than treated seed stored at 4°C when tested at the 10% significance level ($p=0.051$). The results suggest treated seed should be stored at 4°C prior to planting (Fig. 1).

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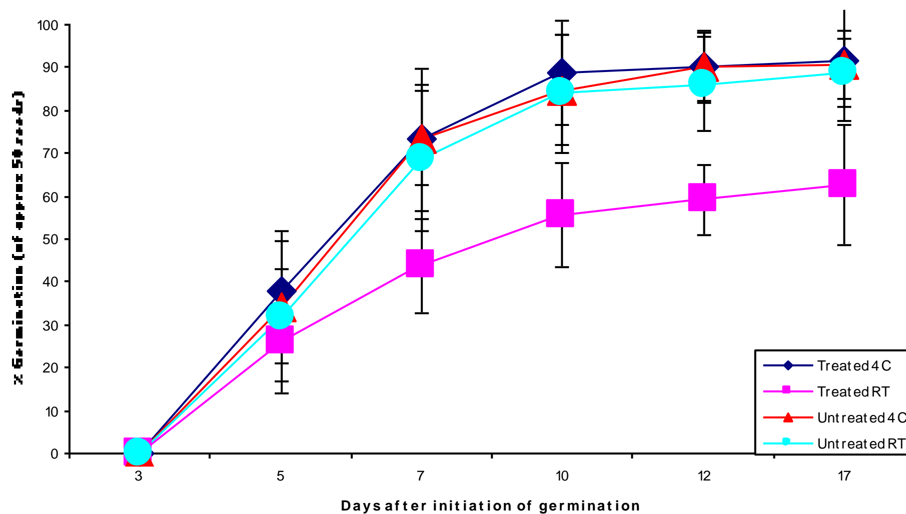

Figure 1: Percentage germination for treated and non-treated batches of 50 seeds (cv. moneymaker) after 14 days of storage at either room temperature or 4°C. Standard deviations are presented from three 3 replicate batches.

5. Sensitivity determination

All three viruses Pep MV, PSTV and ToMV, were readily detected in all the seed dilutions tested from 10% to 0.16% infection (Fig. 2). All buffer controls tested negative for all three viruses suggesting there was no virus contamination in the buffer. However, the control prepared from the commercially-bought Moneymaker seed tested strongly positive for ToMV (negative for PepMV and PSTVd), suggesting this seed stock was infected/contaminated with ToMV, when supplied.

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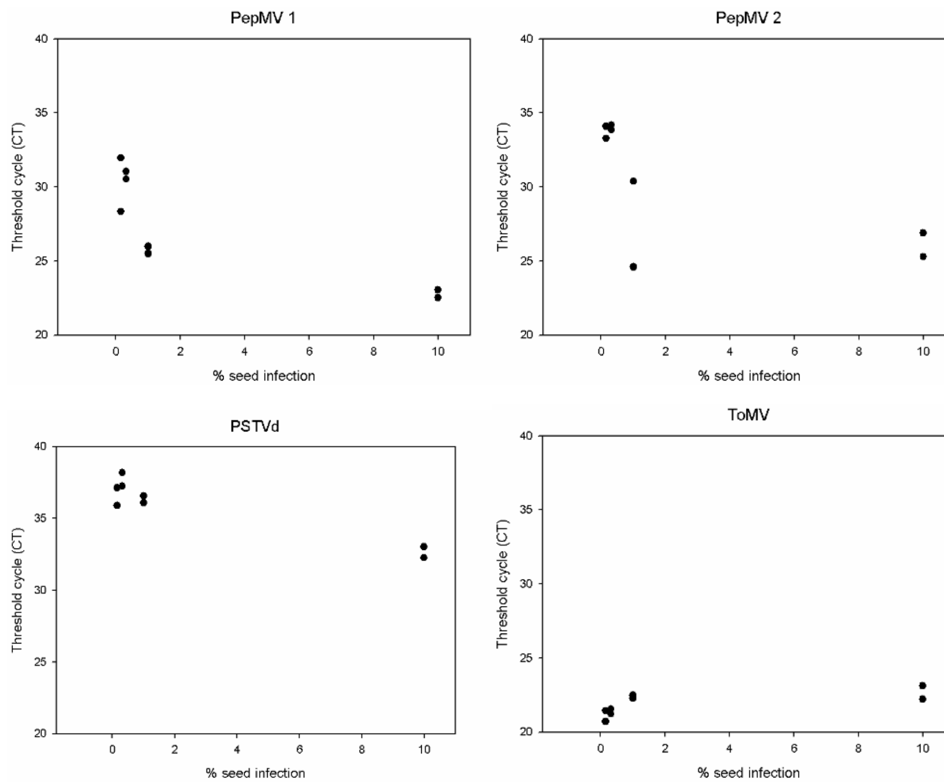

Figure 2: Detection of PepMV, PSTVd and ToMV in seed artificially amended with 0.16%, 0.32%, 1% and 10% virus infected seed using real-time RT-PCR. Duplicate samples were processed for each dilution. Each sample consisted of 300 seeds, except 0.16% infection, where batches of 600 seed were tested. (CT: A lower threshold cycle indicates a higher concentration of target in the sample).

6. Comparative testing of non-destructive vs destructive seed testing

The destructive method proved slightly more sensitive than the non-destructive method for the detection of PepMV (Table 4). In contrast, the non-destructive method was notably more efficient at detecting ToMV than the destructive method (Table 4). Overall these results would indicate that while the non-destructive method is around 10 times less sensitive for PepMV detection, it is around 10-fold more sensitive for ToMV detection. These results are certainly interesting and it is unclear why the performance of the non-destructive method should be so consistently different for two different viruses. The extremely low levels of viral RNA being detected for the PepMV contaminated seed could be seen as one factor. However, earlier studies carried out with PepMV-contaminated seeds produced from infected fruit grown at CSL, and cleaned using non-commercial methods (and hence more

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heavily contaminated), showed similar increases in Ct (around 3-4 cycles); again indicating a reduction in sensitivity of around 10 times.

Table 4: Real-time RT-PCR results comparing non-destructive (ND; 2 x 300 seeds) and destructive (D; 3 x 50 seeds) RNA extraction methods for the detection of PepMV and ToMV in seed stocks. Each sample was tested in duplicate wells; the number of wells testing positive are presented in parenthesis. A lower threshold cycle (C_T) indicates a higher concentration of target in the sample. A C_T of 40 indicates a negative result.

Seed batch	Replicate	PepMV C_T		ToMV C_T	
		D	ND	D	ND
A	1	31.54 (2)	34.97 (1)	28.86 (2)	23.09 (2)
	2	31.68 (2)	35.83 (1)	29.66 (2)	22.86 (2)
	3	40.00 (0)	-	29.98 (2)	-
B	1	32.29 (1)	37.89 (1)	30.76 (2)	23.80 (2)
	2	35.18 (1)	40.00 (0)	29.87 (2)	23.64 (2)
	3	32.08 (2)	-	29.96 (2)	-
C	1	40.00 (0)	37.33 (1)	32.79 (2)	24.87 (2)
	2	35.67 (2)	37.96 (2)	32.52 (2)	24.86 (2)
	3	32.64 (2)	-	33.54 (1)	-

Similar results were obtained for the comparative testing carried out on nine commercial seed batches, contaminated with PepMV. While overall the non-destructive method gave increased Ct values (hence detected less viral RNA), all stocks did test positive by both methods. Like the results shown above, there was a degree of variability, but again this can be explained by the low levels of target present on the seed and the small samples being tested in this case (i.e. 20 –200 seeds).

However, while there does indeed appear to be some difference in sensitivity between destructive and non-destructive methods, it is clear that the overall reliability of both methods is very similar, with both methods still being able to detect the presence of viral RNA. It is worth noting that the amounts of PepMV RNA being detected on some of the seed batches being tested is extremely low (towards the limit of detection) and could potentially be contamination occurring during seed cleaning. For seed batches genuinely infected with virus, it is extremely likely that the amount of virus being

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detected would be greater and hence readily detected by either method. It should also be noted that much of this work has been carried out using relatively small batches of seed and is therefore susceptible to variability. The use of larger seed samples, as suggested for the final testing service, would give more consistent results. This is of course a major benefit of the non-destructive method. While it is not directly better than destructive testing, in terms of straight-line sensitivity etc, it does overcome the issue of seed numbers being limiting due to cost and hence allows an increase in sensitivity/reliability by encouraging more seeds to be tested.

Conclusions

- A novel method was developed for the non-destructive testing of tomato seeds for three different pathogens: PepMV, ToMV and PSTVd.
- The method was sensitive, capable of detecting a single seed infected with PepMV and PSTVd in a batch of 600 healthy seeds. Sensitivity for ToMV is likely at least the same, although could not be demonstrated due to the test seeds being contaminated with virus at source.
- The non-destructive seed test proved highly comparable to the destructive method currently used by CSL, in terms of overall detection of viral RNA on seeds.
- Testing showed that the method developed has no adverse effects on seed viability (germination) or plants grown from those seeds.
- Although it is likely that seeds will be processed soon after non-destructive testing, storage is possible, provided it is done chilled (at 4°C). It is recommended that seed tested with the non-destructive method should not be left no more than 2 weeks before sowing.

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Technology transfer

1. An article ('Sowing the seeds of no doubt') was written and submitted for publication in HDC News. It has been published in the September 2006 issue.
2. Rick Mumford gave a presentation at the annual British Tomato Growers Association conference in Coventry on 28th September 2006. This presentation focused on recent developments in tomato virology including presenting data related to non-destructive seed testing.

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