Project Title:	Parsnip Yellow Fleck Virus: development of a disease management strategy
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Summary of work completed 2002/03 to date

Monitor vector migration using water traps

The network of water traps established in 2000and 2001 was developed further with sites utilised throughout the major carrot growing regions in England (Figure 1). Aphids caught weekly in the water traps were removed and sent to the ADAS laboratory for identification.

The timing of migration and abundance of aphids varied throughout the country. Migration began at the end of April in Suffolk and Nottinghamshire and by early May most sites had caught aphids. Aphids tended to be more abundant in the north of England (Figure 2), although there was distinct variation within regions.

Test for virus incidence in potential host plants

Two types of carrot sample were received for testing in 2002. The majority were from ADAS and collected without regard to symptoms from several sites and on three occasions. Other samples were collected because of the presence of symptoms either early in the season (by Howard Hinds) or on the occasion of the last meeting (July 16th) at Skelmersdale (by consortium members attending the meeting). All samples were tested by ELISA; a subset of these was tested by PCR as bulks of 5 samples. For random samples PCR tests were done on the samples collected on one date.

For ELISA the antiserum was that raised against the Wisbech isolate whilst for PCR six primer pairs designed to detect all *Anthriscus* isolates so far sequenced were used. A smaller subset of the samples was used to inoculate *Nicotiana benthamiana* and *N. occidentalis* in the glasshouse. Those used for inoculation included positives by other tests and samples with symptoms as well as some chosen at random.

In total 626 samples (not including the Skelmersdale samples) were tested by ELISA of which 4 were positive (0.6%). None of the four samples tested from H.H. gave a positive. Furthermore 201 samples (mainly as sets of 5) were tested by PCR; 1 random sample was positive. A further sample selected because of symptoms seen and 5 of 5 samples from H.H. were also positive by PCR; one of these was a parsnip. Thirty samples from the random collections were put to herbaceous hosts and one of these gave symptoms. All five samples from H.H. gave symptoms when put to plants (Table 1). For plants collected at Skelmersdale there was some correlation of symptoms in carrot with PCR positive and getting virus into herbaceous host (Table 2). Use of different PCR primers gave differentiation of viruses (Table 3). At least seven different genotypes of virus in 10 tested. Numbers were too small to draw conclusions about correlation of virus types with symptoms (Table 4).

The results indicated that the very low levels of virus present in 2002 (at least as detectable by methods used here) were insufficient to look at trend in season (Table 5). PCR detects some isolates that were not detected by anti-Wisbech strain antiserum and there was some correlation of infection shown by PCR with symptoms at Skelmersdale. Furthermore PCR diagnosis indicates that the virus appears very variable (correlates with earlier sequencing results).

Acquisition and transmission of AYV and PYFV

Further studies were undertaken to determine the effect of temperature and inoculation time on the transmission of AYV. Results from experiments conducted at 10ºC and 20ºC were supplemented with data from studies carried out at 15ºC and four inoculation access periods (IAP), 2, 10, 30 minutes and 24 hours. Five alate viruliferous *C. aegopodii* were transferred to each of 30 *Anthriscus cerefolium* seedlings for each IAP. Aphids were sprayed off with insecticide after each IAP, the seedlings were returned to a glasshouse cubicle and maintained at 18ºC. After a further three weeks, all seedlings were extracted for RNA using the CTAB extraction method and tested for virus using the AYV/COX multiplex TaqMan® assay. Results indicated that AYV transmission efficiency at 15ºC was between values recorded previously at 10ºC and 20ºC for all IAP (Figure 3).

Pesticide efficacy against aphid vectors

Bioassays of pesticide efficacy against alate carrot willow aphids were undertaken. Leaf discs of parsnip were dipped into solutions of known pesticide concentration (200, 100, 50, 25, 20, 10, 5, 2.5, 1 and 0.5% recommended field rate) or deionised water (control) and five aphids were caged onto the plant material. After 24 and 48 hours aphid mortality was assessed. To date nine concentrations (200, 100, 50, 25, 20, 10, 2.5, 1 and 0.5% recommended field rate) have been used with Aphox (pirimicarb), four concentrations (10, 5, 2, 1%) with Hallmark (λ-cyhalothrin), and four concentrations (200, 100, 50, 25%) with Dovetail (pirimicarb, λ-cyhalothrin mixture).

All products were highly successful in controlling carrot willow aphids. Probit analysis indicated that concentrations as low as 10 and 2% recommended field rate of Aphox and Hallmark respectively, could reduce aphid numbers by 90% within 24 hours (Table 6). Further studies are underway to provide data to complement these preliminary results.

A second bioassay was undertaken to investigate the period of effective insecticide residue. Leaf discs were dipped into a solution of pesticide at recommended field rate (treatment) or deionised water (control) and aphid caged onto the plant material sequentially each day. Aphid mortality was assessed 24 hours after insects had been caged onto the leaf discs. To date investigations have focused on Dovetail (pirimicarb, λ-cyhalothrin mixture). Results showed that the product provided excellent aphid control up to nine days post application (Figure 4). Further studies are underway to provide data to complement these preliminary results.

Develop multiplex PCR to detect PYFV and AYV in aphids

Previous work has seen the development of virus specific assays for *Parsnip yellow fleck virus* (PYFV) and *Anthriscus yellows virus* (AYV). Primers for both gel based RT-PCR and also primers and probes for TaqMan PCR have been designed to sequence within the polymerase gene region. In addition TaqMan assays have been developed for use as internal controls for both the cytochrome oxidase gene from plants and also the actin gene from *Cavariella aegopodii.* The TaqMan assays have

been labelled and optimisations been carried out in such a way as either virus can be multiplexed with either of the internal controls, giving robust detection of either PYFV or AYV in either plants or aphids. Or that AYV and PYFV can be multiplexed together to give detection of either virus at the same time. However, since the sequence available (the polymerase gene) for PYFV is very variable the range of isolates detected by the TaqMan assay developed is poor (Table 7) and its utility is limited to that of a lab tool, to aid in discriminating infected and healthy plants in the transmission studies.

To resolve the problem of variation an alternative format for real time PCR has been investigated, using degenerate primers and incorporation of SYBR green into the products. This should allow increased sensitivity and sample throughput compared with a gel-based assay whilst allowing a much larger range of isolates to be detected by using degenerate primers. A number of primer sets (11 in total) have been used in the SYBR green assay. We have shown that although primer dimer amplification is a problem with each set of primers, (giving an amplification plot in each case); it is possible to distinguish between the production of primer dimers and the virus specific product by looking at the melting profile of the products (Figure 5). Although the assay needs further optimisation and testing, it is likely that it will allow the detection of many different isolates of PYFV in a high-throughput fashion. Preliminary data also suggests the detection of products through the incorporation of SYBR green in this way is more sensitive than observing PCR products on a gel.

A number of aphid samples from trap catches for each of the years of the project so far have been tested for the presence of RNA. No RNA has been detected in any of the trapped aphids. Follow on studies in the lab have shown that RNA viability is extremely low in aphids stored in water, the data shows that aphids caught in water would not be suitable for virus testing. A number of other solutions have been investigated which may preserve the RNA within the aphid; among the solutions tested those based on methanol have been shown to effectively preserve the RNA for long periods of time.

Investigate molecular variability of PYFV and AYV

Alignments were made from the peptide sequences. Any areas of sequence similarity identified were then aligned using the nucleotide sequence. Areas of nucleotide sequence homology were identified within the polymerase gene, between the following viruses - *Parsnip yellow fleck* (Parsnip strain), *Rice tungro spherical virus, Maize chlorotic dwarf virus* and the Comovirus *Cowpea severe mosaic virus,* which was found to be similar to members of the *Sequiviridae* within the polymerase region.

Following alignment of the polymerase region of all the characterised members of the *Sequiviridae* regions of sequence homology were identified and degenerate PCR primers were designed. Using one of the primer sets 310nt of the polymerase gene of a number of isolates from carrot, cow parsley and celery was amplified by RT-PCR. In addition an AYV isolate (isolated on Chervil) was also amplified. The PCR products were sequenced, aligned and a cladogram of the putative translation products developed (Figure 6).

The isolates from cow parsley and carrot weakly clustered together (amino acid identities 80-100%) and isolates from celery and parsnip weakly clustered together (86-100%). The amino acid identity between these two clusters was 86-100%. AYV clustered with the other waikaviruses (78% and 68% identity with RTSV and MCDV, respectively). The sequences show that the polymerase gene of PYFV is very variable.

Concluding Points

- Yellow water traps were used successfully to monitor micro-migration of aphids. Vector migration was variable within and between years and regions.
- Virus incidence was relatively low in 2002 and absent in some regions. PCR tools detected some isolates that were not detected by anti-Wisbech strain antiserum. Furthermore PCR diagnosis indicated that the virus appeared to be very variable.
- Inoculation periods as low as several minutes were necessary for successful transmission of virus. Transmission efficiency was very high, close to 100%, when conditions were favourable.
- Laboratory bioassays indicated that insecticides were effective at controlling aphids, and residue tests showed that coverage could last up to nine days.
- Existing molecular diagnostics tools have been refined further to allow the detection of many different isolates of PYFV in a high-throughput fashion.
- Molecular analysis of virus sequences indicated that the polymerase gene of PYFV is very variable, and that isolates from cow parsley and carrot weakly clustered together (amino acid identities 80-100%) while isolates from celery and parsnip weakly clustered together (86-100%).

Tables

Table 1. Carrot samples 2002 - regular collection; not selected for symptoms

Source	**ELISA**		**PCR**		**To Plants**	
	Nos	+ve	Nos	+ve	Nos	+ve
A. Rickwood	330	3	100	0	3	0
Gleadthorpe	120	0	40	0	5	1
HuntaPac						
Lancs	60	1	40	1	6	0
Yorks	10	0				
Cheshire	11	0				
Stockbridge TC	30	0	10	0	3	0
WCF Farm Produce (Lancs)	20	0	10	0	3	0
Total	581	4 (0.7%)	200	1 (0.5%)	20	1

Table 2. Skelmersdale Plants with symptoms: ELISA, PCR and inoculation to plants.

Symptoms on carrot	Nos plants	ELISA	PCR	Inoculation (Symptoms)
Green/pale/necrosis	4	0/4*	0/1	0/2
Yellowing/necrosis	**5**	**0/5**	**1/1**	**2/2**
Bright yellow/stiff	*3*	*0/3*	*0/1(ns)*	*1/2*
Yellow/red/stiff	**4**	**0/4**	**2/2**	**2/2**
Intense red/yellow	1	0/1		0/1
Deep bronze/purple	*6*	*0/6*	*0/1(ns)*	*1/2*
Mixed	1	0/1		0/1

Bold = where symptoms give PCR positive/symptoms in plants
Italics= herbaceous plant with no symptoms negative by PCR but plant with symptoms yet to be tested.

Table 3. Differentiation amongst three virus isolates by PCR (Skelmersdale)

Primer pairs		2aF/1R	2F/2R	2aF/3R	3F/3R	4F/4R	Uni
Yellowing/necrosis		+w	+	+	+	-	+
Yellow/red/stiff	1	-	-	+	+w	-	+
	2	+w	-	+w	-	+	+

+ = amplification
+w = weak amplification
- = no amplification

Table 4. Early season samples submitted with symptoms.

Number plants	=	5
+ve by ELISA	=	0 (parsnip not tested)
+ve by PCR	=	5
+ve by plant inoculation	=	5

N.B. one sample was a parsnip.

Table 5. Distribution of positives in non-selected carrot samples by week number

Week Nos		25	26	30	34
ELISA					
	Tested	200	161	210	50
	+ve	1	0	3*	0
PCR					
	Tested			200	
	+ve			1*	

* These samples not from same site.

Table 6. Probit analysis (LD_{90}) of Aphox (pirimicarb), Hallmark (λ-cyhalothrin) and Dovetail (pirimicarb, λ-cyhalothrin mixture) against alate carrot willow aphids

	LD_{90}	
Product	24hours	48 Hours
Aphox	10.0	4.2
Hallmark	2.0	-
Dovetail	-	-

Table 7. Table illustrating number of nucleotide mismatches between primers/probes developed and the isolates sequenced and detection of PYFV isolates using TaqMan assay.

ISOLATE	F PRIMER	3' END	PROBE	5' END	R PRIMER	3'end	TOTAL
489 C	1	0	2	0	3	0	6
486 B1	1	0	2	0	3	0	6
489 e2*	1	0	1	0	3	0	5
519 E	1	0	4	0	3	0	8
519 G	1	0	2	0	2	0	5
486 B4*	0	0	0	0	3	0	3
489 H	0	0	0	0	2	0	2
V2005207**	2	0	3	0	2	0	7
527 F	2	0	4	0	4	0	10
wis-C*	1	0	2	0	3	0	6
V2003614**	0	0	2	0	4	0	6
CV065	2	0	5	0	3	0	10
508	3	0	5	0	3	0	11
P121	3	0	5	0	3	0	11
CV 506	1	0	9	0	4	0	14
513 C	3	1	5	0	5	0	13
513 B	3	1	5	1	5	0	13
A421	3	0	6	0	4	0	13
516 B	0	0	4	0	4	0	8
515 A	0	0	6	0	2	0	8
4266**							
4242							
518 A							

Strong +ves * Not tested

Weak +ves ** - ve COx

Figures

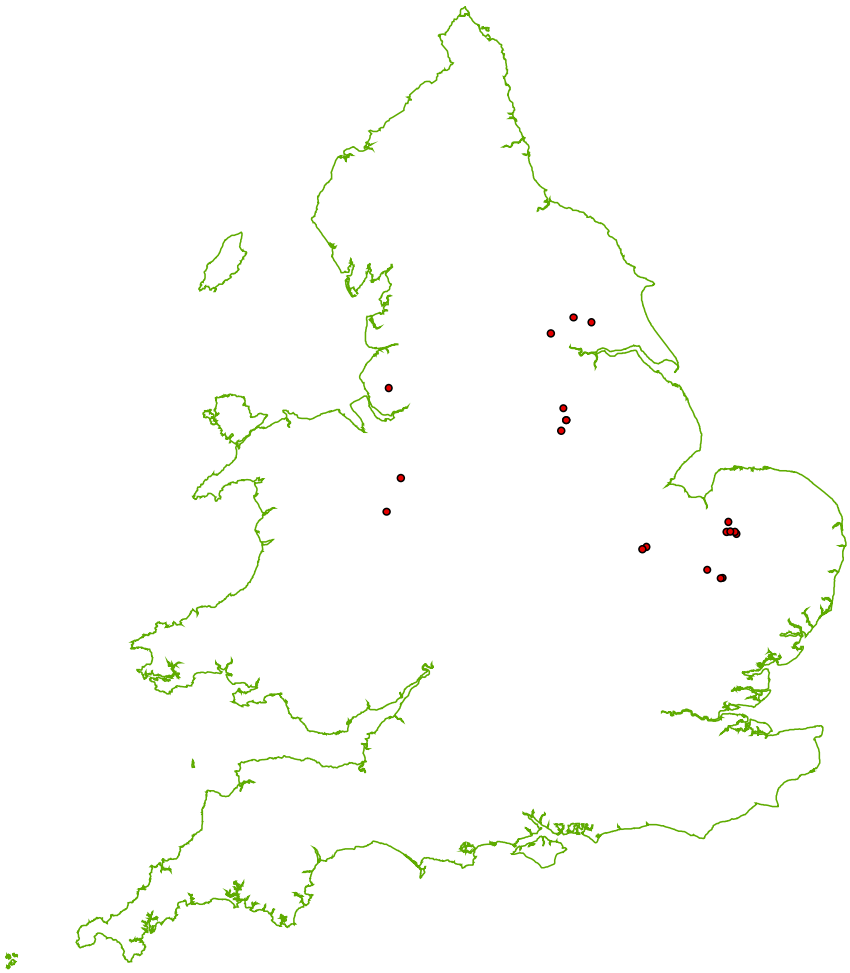


Figure 1. Locations of water traps, 2002

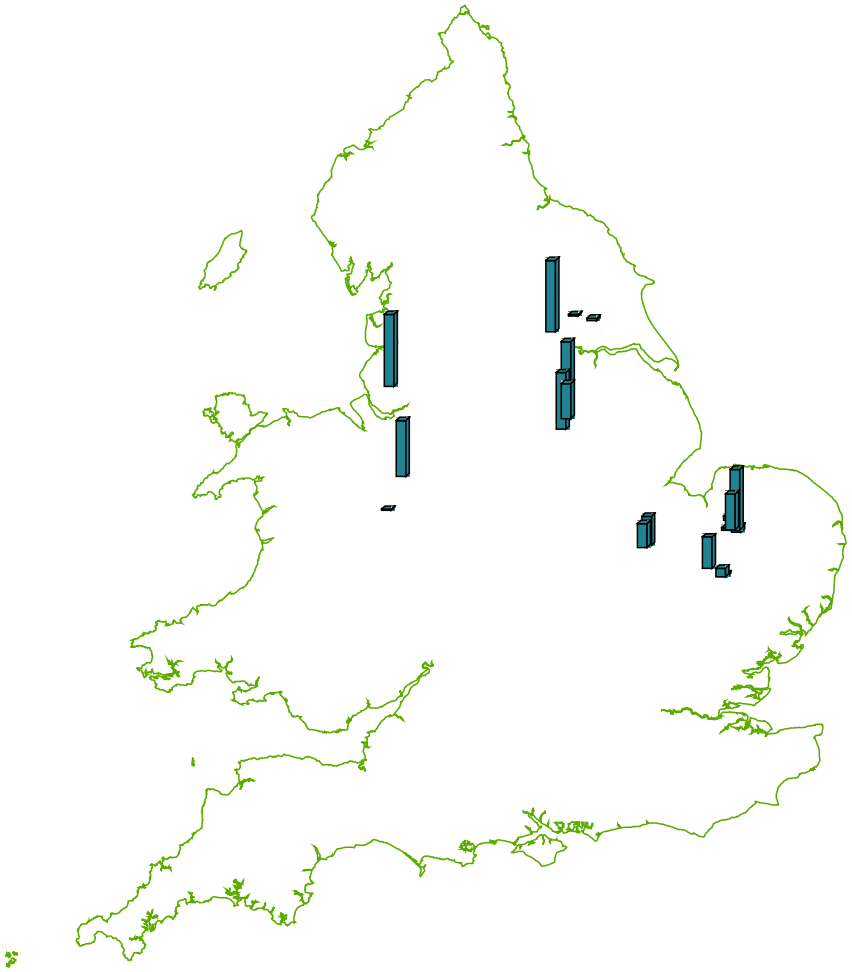


Figure 2. Accumulated number of aphids caught at each water traps location, 2002

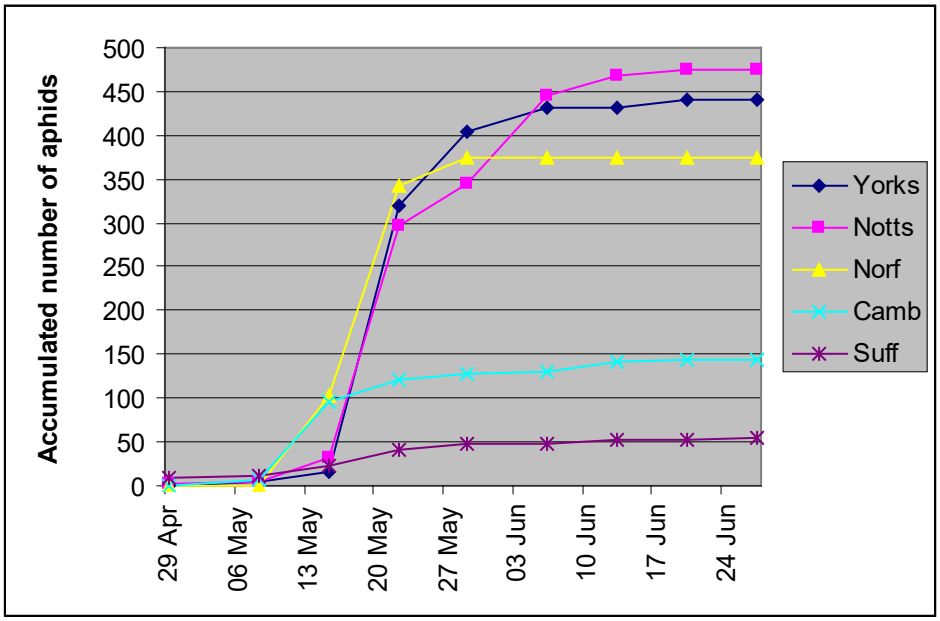


Figure 2. Accumulated regional water trap catches, 2002

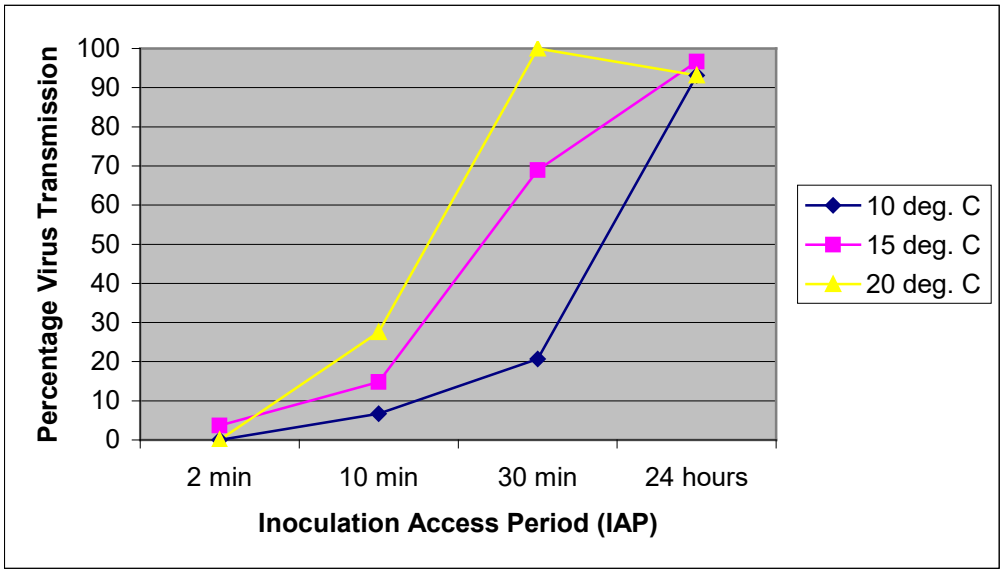


Figure 3. Effect of temperature and inoculation time on transmission of AYV

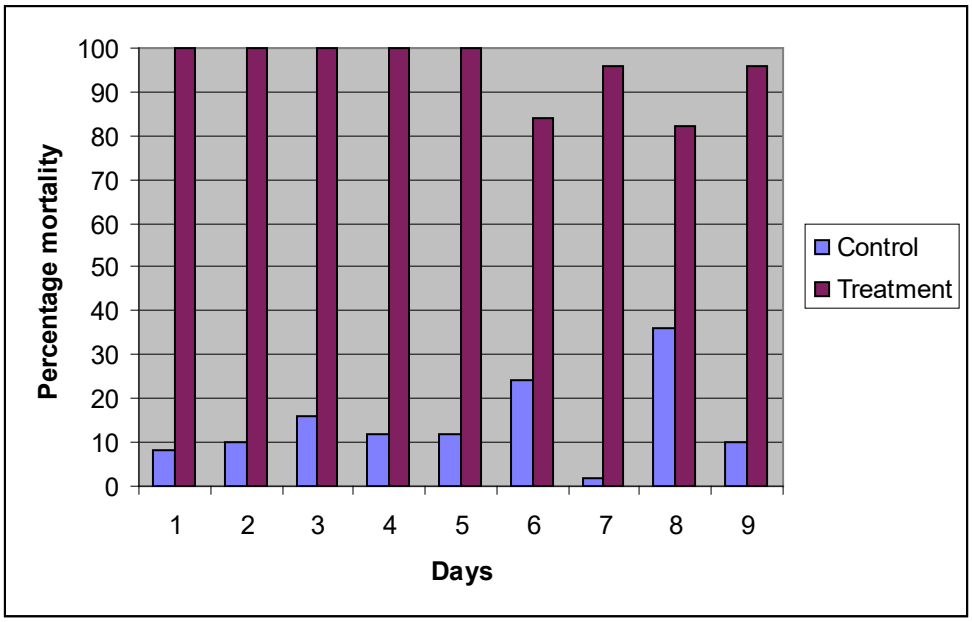
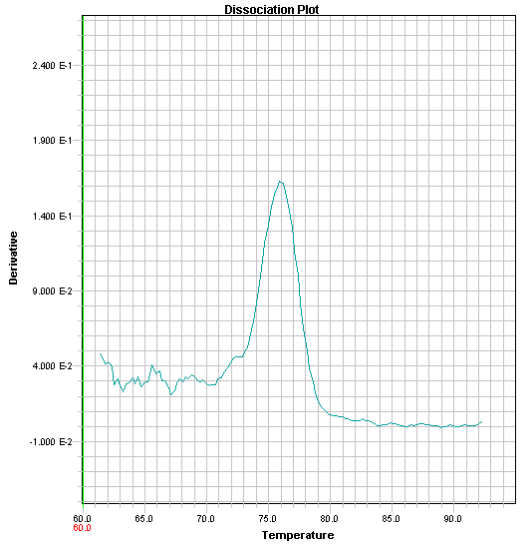


Figure 4. Residue efficacy of Dovetail against alate carrot willow aphids

A



B

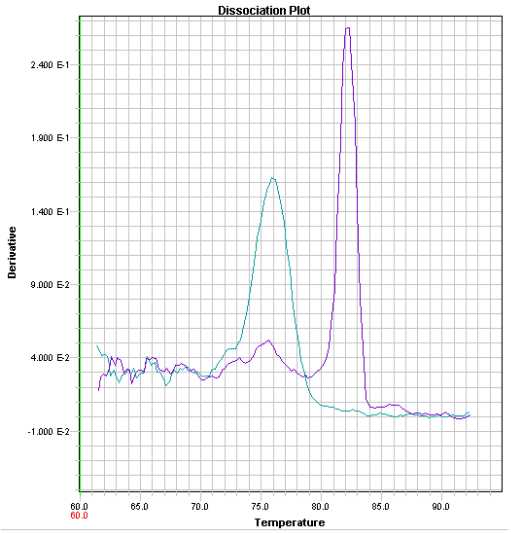


Figure 5. Dissociation plots for (A) water control and (B) PYFV infected (purple) and healthy (green) samples following amplification in the presence of SYBR green

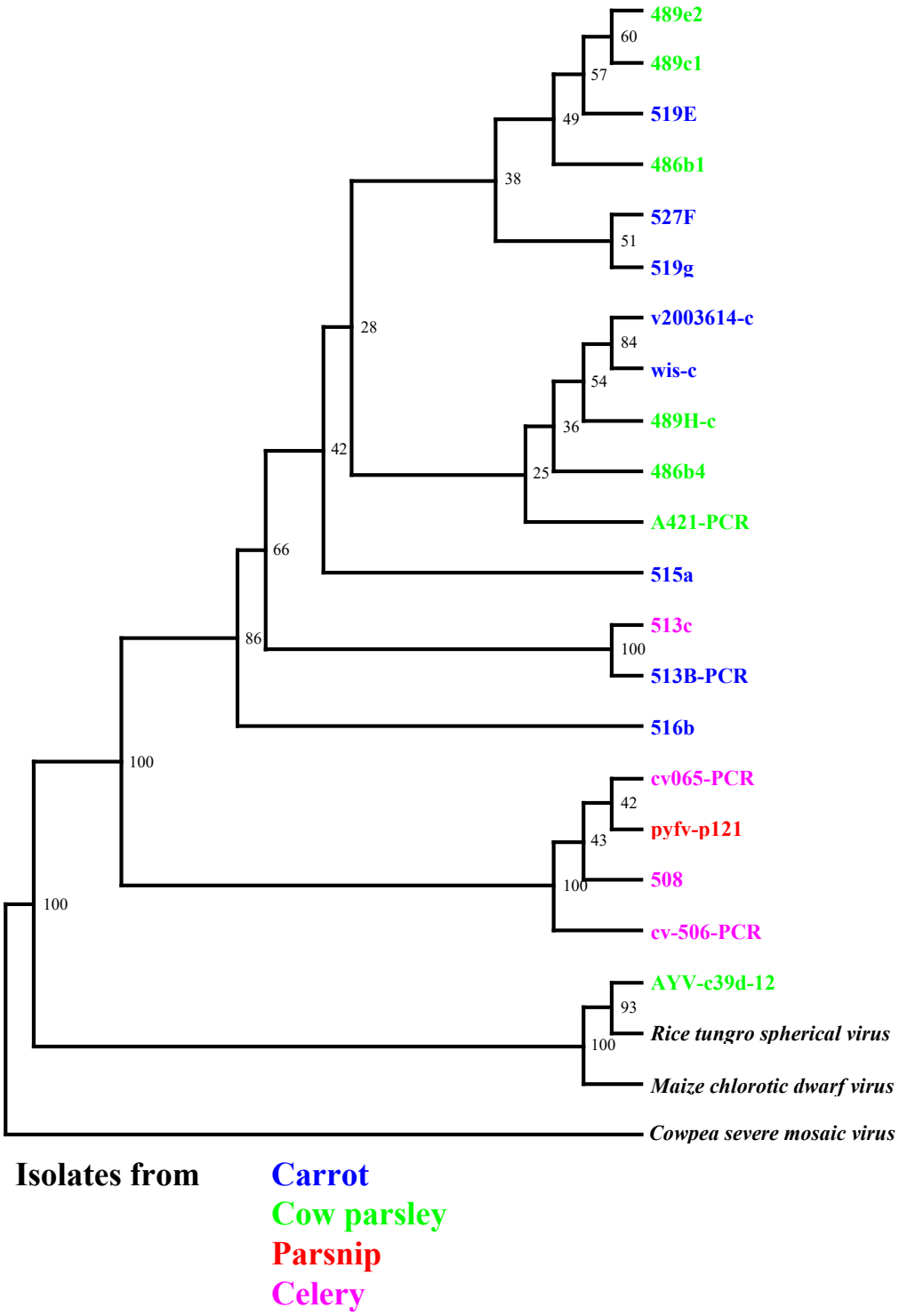


Figure 6. Cladogram showing the clustering of the translation products for a number of isolates of PYFV, and isolate of AYV and the other members of the *Sequiviridae.*

Numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred out of 100 trees, following bootstrap re-sampling.