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# Grower Summary

## Background

Outbreaks of *Parsnip yellow fleck virus* (PYFV) have become common in carrots with crop losses suffered by growers throughout the UK. Infections are distributed randomly in fields with first symptoms appearing in late May and early June resulting in severely stunted plants and the death of many individual plants. Later in the season, larger plants develop mottled foliage that is discoloured with yellow flecks. Plants infected with virus may develop secondary and/or misshapen roots and throughout the season, the tops of infected plants can develop die back and rot.

PYFV is detected in all stages of the crop and from carrots in storage. The virus has also been detected in symptomless plants and has been detected in cow parsley. Although outbreaks of the virus might appear sporadic, results from the Netherlands suggests that they can be frequent often resurging following years of little or no apparent virus incidence.

PYFV is transmitted by the willow-carrot aphid *Cavariella aegopodii* but vectors can only successfully transmit PYFV to carrots after acquiring a helper-virus, *Anthriscus yellows waikavirus* (AYV). With the widespread incidence of PYFV, it has been suggested that pesticides might have limited success in controlling the spread of vectors and virus as the plant protection products available are not sufficiently fast acting to prevent the relatively short periods of aphid feeding required for virus transmission. However, the effect of different pesticide groups on PYFV transmission is unknown and without a clear understanding of the viruses and their vectors, pesticide use to prevent damage could be indiscriminate leading to excessive insurance sprays.

## Objectives

1. Determine the phenology, migration and behaviour of aphids which can transmit PYFV in carrots
2. Identify virus reservoirs and determine the acquisition, transmission, and molecular variability of PYFV and AYV
3. Develop a prototype strategy that will allow growers to implement sustainable management of PYFV and its vectors

## Summary of Results

- A network of aphid traps was established (over four years) in all major carrot-growing areas in the UK and utilised to monitor vector migration. Results from the network revealed significant regional and annual variation in vector migration.
- A mathematical model has been developed that predicts the migration of aphid vectors into carrot crops. Based on artificial neural network techniques the system has proven successful at predicting the first aphid flights

- The molecular variability of PYFV and its helper virus AYV has been investigated using PCR. It is reported in the scientific literature that two fairly homogenous 'serotypes' of PYFV exist but results revealed that the Anthriscus-strain of PYFV is highly variable and many distinct isolates are present in wild and cultivated hosts
- The incidence of virus in wild and cultivated host plants was investigated using ELISA and PCR techniques. Results indicated that PYFV in hogweed forms a distinct clade (family) to that in cow parsley/carrot, so hogweed cannot be the source of virus for carrot crops. Cow parsley is likely to be the main source of PYFV and AYV
- The initial PCR primer sets have been sent to diagnostic laboratories and diagnostic assays are currently available to growers, providing a much more robust method for testing for PYFV than was available prior to the start of this project
- Bioassays of insecticide efficacy against alate vectors were undertaken with the most commonly used products employed by the carrot industry in the UK with the addition of two new neonicotinoids developed by Bayer CropScience. These were successful in controlling aphids to varying degrees. Additional bioassays indicated that effective insecticide residue periods of up to twenty-one days post application are possible.

**New Crop Management Strategy**

The advances made within this project have enabled the development of a new management strategy for the control of PYFV in carrots. The proposed new strategy is as follows:

1. Using the ANN derived phenology model, the consortium could provide regional predictions (e.g. via the internet/press release etc…), at the beginning of February, March, April and May, of the first flight of the willow-carrot aphid based on environmental data from the major carrot growing regions.
2. Using the regional prediction date as a guide, growers should set up field specific water traps in their crops a week or two before the predicted date. The contents should be analysed weekly to ascertain the actual date of the beginning of the annual migration of the willow-carrot aphid into the growers crops.
3. Using the results from their field specific water traps, the grower should begin their spray programme once the first willow-carrot aphids have been trapped.
4. Growers should utilise products that have been shown to be effective both topically and residually e.g. the new neonicotinoid products or Dovetail (bearing in mind the requirements for carrot root fly control)
5. Where possible, growers should continue to use the water traps to monitor the incoming populations of willow-carrot aphids and potentially amend their spray programme once the end of the aphid flight has occurred (e.g. following two consecutive trap samples with no carrot-willow aphid)

The use of this new management strategy will provide a rational approach to the problem of PYFV, leading to better targeting of insecticides and, in low risk years, a reduction in insecticide use. This will benefit both the grower (reduced virus due to better targeting; reduced input costs due to lower insecticide use) and the environment (fewer chemicals in the environment and lesser effects on non-target organisms due to reduced insecticide use).

This new management strategy is relatively cost effective, but will require funding from the industry to put it in place. The development of the predictions will require the acquisition of the environmental data and some resource to cover the cost of reparamatising the ANN and producing the predictions. The use of field specific trapping requires a third party to sort and identify the willow-carrot aphids out from the rest of the trap catch. This cost could be borne either by individual growers or by the industry as a whole. Fortunately, there is a service already in place for a similar scheme in seed potato crops, so negotiations with the service provider are recommended. Currently, the neonicotinoid products are still in development, so until they are marketed other insecticides will need to be used.

**A Diagnostic Service for Growers and Consultants**

The development and implementation of practical diagnostic tools for detection of PYFV within plants will help consultants and farmers to identify and quantify the extent of virus incidence within their fields and surrounding vegetation. Sophisticated diagnostic tools to detect PYFV in plants and aphids provide growers/consultants with the means of assessing virus levels and thus, support informed management decisions.

# Executive Summary


## Background

*Parsnip yellow fleck virus* (PYFV) is a perennial problem for the carrot growing industry, which in bad years can lead to losses of up to 20% in some crops and in 1998, was estimated at a total of around nearly £5 million. This latest epidemic highlighted the need for a better understanding of the epidemiology of the virus complex (PYFV and the required helper virus, *Anthriscus Yellows Virus*, AYV), the biology of its vector, the willow-carrot aphid (*Cavariella aegopodii*) and the requirement for a new management strategy.

## Objectives

1. Determine the phenology, migration and behaviour of aphids which can transmit PYFV in carrots
2. Identify virus reservoirs and determine the acquisition, transmission, and molecular variability of PYFV and AYV
3. Develop a prototype strategy that will allow growers to implement sustainable management of PYFV and its vectors

## Methods

The biology and phenology of the principal virus vector and the biology and epidemiology of the virus complex that leads to disease transmission were investigated using a combination of

- Field based trapping and non-crop plant searching (for the vector) and sampling (of potential crop and non-crop virus hosts)
- Laboratory based molecular investigations for the development of diagnostic tools, enabling the analysis of field collected and experimentally generated plant samples
- Laboratory investigations of the ability of the vector to acquire and transmit the helper virus, AYV
- Lab based efficacy studies on the topical and residual effect of insecticides
- Artificial Neural Network (ANN) and statistical techniques using environmental and vector data (from the Rothamsted Suction Trap Network) to develop models that were able to predict the first flight of the vector (i.e. when the first aphids are likely to arrive into the field)

## Results

- There is considerable variation, particularly in the number of willow-carrot aphids, but also in the timing of first flight and peak, between fields both within and between regions.

- It is possible that crops sown with a reduced seed rate attract a greater number of aphids. In the only year where investigation into this was possible (2003), the sites with a lower cropping density had a significantly higher catch of the willow-carrot aphid.
- PYFV was found to be highly variable, making it difficult to develop diagnostic tools that are guaranteed to detect all strains of the virus
- There are two separate clades of PYFV, one that infects carrots and cow parsley (Anthriscus strain) and another that infects parsnip and hogweed (Parsnip strain) and it is therefore unlikely that hogweed acts as a source plant for PYFV in carrots.
- New TaqMan® assays for the detection of both AYV and PYFV in plant material were developed and proved invaluable for laboratory testing of plant material.
- The helper virus can be acquired and transmitted by the willow-carrot aphid after only two minute feeding periods at temperatures as low as 15ºC, but efficiency is low
- Maximum virus acquisition efficiency was reached by 24 hours acquisition access period (AAP) at temperatures equal to or above 15ºC. Maximum virus transmission efficiency was reached by 24 hours (at 10-15ºC) or 30 minutes (20ºC)
- This speed of acquisition may indicate that AYV is not confined to the vascular regions but is distributed throughout the leaf, like PYFV.
- Serial transmission of these semi-persistent viruses is possible, with some willow carrot aphids able to transmit the virus up to 4 days after acquisition of the AYV/PYFV complex. The potential for serial transmission combined with the temperature effects on transmission time and the general principle that increasing temperature increases the probability of aphid movement between plants (Walters & Dixon, 1984) suggest that the risk of virus spread is increased in warmer weather due to greater within-crop movement by viruliferous aphids.
- Preliminary trials showed that the three main insecticides commonly used in umbelliferous crops (Aphox (pirimicarb), Hallmark (λ-cyhalothrin) and Dovetail (pirimicarb and λ-cyhalothrin)) and two new neonicotinoid products were all very effective against winged willow-carrot aphids.
- The residual effect of the most effective products (a new neonicotinoid (YRC+D OD) and Dovetail) is enough to provide good long-term protection against aphids, with swift and considerable residual activity occurring up to a week after application and slower activity up to three weeks after application. (This is in laboratory conditions, so environmental effects such as solarisation and rainfall will likely reduce the activity of these residues)
- The ANN predictive model has been validated successfully against data observed in carrot crops at several sites in the UK. Five models were developed using environmental data from January to May and the models based on ANNs performed better than those developed using previously established multivariate techniques.

**New Crop Management Strategy**

The advances made within this project have enabled the development of a new management strategy for the control of PYFV in carrots. The proposed new strategy is as follows:

1. Using the ANN derived phenology model, provide regional predictions (e.g. via the internet/press release etc…), at the beginning of February, March, April and May, of the first flight of the willow-carrot aphid based on environmental data from the major carrot growing regions.
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The use of this new management strategy will provide a rational approach to the problem of PYFV, leading to better targeting of insecticides and, in low risk years, a reduction in insecticide use. This will benefit both the grower (reduced virus due to better targeting; reduced input costs due to lower insecticide use) and the environment (fewer chemicals in the environment and lesser effects on non-target organisms due to reduced insecticide use).

This new management strategy is relatively cost effective, but will require funding from the industry to put it in place. The development of the predictions will require the acquisition of the environmental data and some resource to cover the cost of reparamatising the ANN and producing the predictions. The use of field specific trapping requires a third party to sort and identify the willow-carrot aphids out from the rest of the trap catch. This cost could be borne either by individual growers or by the industry as a whole. Fortunately, there is a commercial service already in place for a similar scheme in Seed Potato crops, so negotiations with the service provider are recommended. Currently, the neonicotinoid products are still in development, so until they are marketed other insecticides will need to be used.

# Science Section

## Background

*Parsnip yellow fleck virus* (PYFV), although sporadic, is a perennial problem for carrot growers in the UK and other carrot growing regions on mainland Europe. Occasional major outbreaks of the disease, the most recent being the epidemic of 1998, have resulted in significant crop losses. This latest epidemic highlighted the need for a better understanding of the epidemiology of the virus and biology of its vector. A consortium, including scientists and representatives from the carrot-growing industry, was formed to research and develop a disease management strategy for the PYFV system.

PYFV, type member of the genus *Sequiviridae*, was first reported on parsnip (*Pastinaca sativa* L.) in the UK by Murant and Goold (1968). The willow-carrot aphid (*Cavariella aegopodii* Scopoli) is the primary vector, transmitting the virus semi-persistently, but successful transmission can only occur after the acquisition of the *waikavirus* helper, *Anthriscus yellows virus* (AYV), also a semi-persistent virus.

*PYFV*

There are many isolates of PYFV, which are derived from either of two serotypes: the parsnip strain (PYFV-P), from parsnip, transmissible to parsnips and celery (*Apium graveolens* L.); or the *Anthriscus* strain (PYFV-A), from cow parsley (*Anthriscus sylvestris* (L.) Hoffm.), transmissible to carrots. The two serotypes also differ from each other in host range (Hemida & Murant, 1989); they both display a restricted range of host plants, with all natural hosts occurring in the family Umbelliferae. Natural infections from both strains occur in cultivated crops of celery, chervil (*Anthriscus cerefolium* (L.) Hoffm.) and coriander (*Coriandrum sativum* L.), in carrot (*Daucus carota* L.) from PYFV-A and in parsnip from PYFV-P. Natural infections of PYFV in wild hosts have been recorded in cow parsley, wild carrot (*D. carota*) and hogweed (*Heracleum sphondylium* L.), the latter with PYFV-P only (Elnagar & Murant, 1974).

Symptoms vary from host to host (Murant & Goold, 1968) with leaf yellowing and dieback recorded in Chervil, coriander and carrot, yellow flecks, leaf mosaic and chlorotic vein banding in parsnip and hogweed and no conspicuous symptoms in cow parsley or celery. Diagnostically susceptible hosts (i.e. indicator plants used under laboratory conditions) include species outside of the Umbelliferae family such as spinach (*Spinacia oleracea*), tobacco (*Nicotiana benthamiana* Domin.) and *Chenopodium* spp.

Some growers and consultants believe that PYFV is also the primary agent responsible for a root disorder found in carrots which is similar in appearance to crown rot (Figure 1). The roots of these carrots, showing PYFV symptoms in their foliage, assume a cigar shape, and when dissected, reveal brown, sometimes circular patches.

While natural transmission of PYFV in carrot is primarily by the willow-carrot aphid studies in the Netherlands by Van Dijk and Bos (1989) indicate that other aphid species can also transmit PYFV, including *C. theobaldi* Gillette and Braag, *C.*

*pastinacae* L. and the pea aphid, *Acyrthosiphon pisum* Harris. Efficiency of transmission by these other aphid species is low and appears to be strain specific, which, in turn is governed by host range.


Figure 1. Comparison of healthy (A) and suspected PYFV-infected carrot roots (B). Symptoms include misshapen and cigar-shaped roots. Photographs courtesy of Howard Hinds (Plantsystems).

PYFV cannot be vectored directly from plants that are immune to AYV, such as carrot or parsnip (Murant & Goold, 1968). As with all semi-persistently transmitted viruses, it is lost by the vectors on moulting and is not transmitted to its progeny, nor is it transmitted by seed or pollen (Murant & Goold, 1968; Brunt *et al*., 1996a).

*AYV*

As described above, AYV is essential for the successful acquisition and therefore transmission of PYFV. Van Dijk and Bos (1985) cited AYV as playing a vital role in the ecology of viral dieback of carrots in the Netherlands, the causal agent being PYFV-A. Van Dijk and Bos (1989) also describe the isolation of AYV using *C. theobaldi* and *A. pisum*, previously unreported as vectors of this virus.

Unlike PYFV, AYV appears to be confined solely to species of the Umbelliferae family, with some species, such as carrot and parsnip, showing immunity to the virus. Symptoms are described by Van Dijk and Bos (1989) as distinct leaf rolling, yellowing and reddening, which were recorded in chervil, coriander, rough chervil (*Chaerophyllum temulum* L.), *Physocaulis nodosus* and upright hedge parsley, (*Torilis japonica* (Houtt.) DC.). Elnagar and Murant (1976a) also describe similar symptoms (at that time considered typical) in chervil. While AYV has been isolated from naturally infected cow parsley (Murant & Goold, 1968; Van Dijk & Bos, 1989), infection has not been correlated with specific symptoms in this species.

*Willow-carrot aphid*

Willow-carrot aphid is an important, widespread polyphagous pest of a number of umbelliferous crops, including carrots, parsnips, celery and parsley causing significant yield losses in early and mid season crops (Anon, 1976). Other cultivated umbellifers, such as chervil and fennel may also be attacked. As well as causing direct damage to these crops (Dunn, 1965), willow-carrot aphid is also the primary vector of *Carrot motley dwarf virus* (CMDV).

Willow-carrot aphid is essentially heteroecious, surviving alternately on primary (woody) and secondary (herbaceous) hosts throughout its life history. Willows (*Salix* spp.), in particular the goat willow (*S. caprea* L.), the crack willow (*S. fragilis* L.) and the white willow (*S. alba* L.) (Dunn, 1965; Anon. 1976; Anon, 1982) are the primary host species, although the aphid can over-winter anholocyclically on its secondary umbelliferous hosts (Anon, 1982). The aphid's over-wintering phase occurs on willows in the form of physically resistant shiny black eggs. They are laid around the bud axils in October and November (Dunn, 1965) when they enter diapause (Kennedy & Stroyan, 1959). Egg hatch occurs in February and March, depending on temperature (Dunn, 1965). If this occurs before leaf break, the young fundatrices feed through the bark of young shoots before moving to the foliage and catkins, where the colony passes through either one or two generations of apterous fundatrigeniae (Dunn, 1965; Kundu & Dixon, 1995). Parthenogenetic adult fundatrices first appear in early April, while some winged forms of fundatrigeniae develop in May (Dunn, 1965). These alatae, migrating over a period of five to six weeks, may return to the primary willow host species *or* colonise their secondary umbelliferous hosts, especially carrot crops.

During the summer months, willow-carrot aphid reproduces parthenogenetically, giving rise to all-female generations (Moran, 1992). Peak numbers of migrants are seen in carrots from late May to mid-June (Dunn, 1965; Tyler, 1998), followed by a population decline. This shift in population dynamics is largely caused by the development of alatae from the second and third alienicolae generations, which migrate from the early-sown carrots to later-sown carrots, hedgerow umbellifers and willows (Anon, 1976). Wild umbelliferous species flower from May to late autumn (Table 1), providing willow-carrot aphid, which inclines to colonise the flower heads feeding on the short stalks and calyces, a succession of hosts throughout the growing season (Dunn & Kirkley, 1966). When the umbel on which the aphid is feeding develops seeds and desiccates the alienicolae disperse to another appropriate flowering host. Dunn and Kirkley (1966) observed willow-carrot aphid on early flowering umbellifers, such as cow parsley, when carrots were being colonised by the aphid, then on the subsequently flowering goutweed (*Aegopodium podagraria* L.), pignut (*Conopodium majus* (Gouan) Loret) and *C. temulum.* While many wild umbellifers (such as cow parsley) die down completely before winter, or form rosettes too late for recolonising willow-carrot aphid, others such as hogweed may produce enough growth to support an over-wintering population, especially when mown down sufficiently early to allow for regrowth (Dunn & Kirkley, 1966). Umbelliferous crops left in the ground over winter (e.g. carrots) may also act as hosts for viviparous forms of willow-carrot aphid, with winged adults from these crops developing earlier than those on the primary willow hosts.

Table 1. Flowering times of secondary host plants (after Kundu & Dixon, 1995)

| GROUP | HOST COMMON NAME | HOST BOTANICAL NAME | FLOWERING PERIOD |
|---|---|---|---|
| Early flowering (late spring to early summer) | Cow parsley | *Anthriscus sylvestris* (L.) Hoffm | April to early June |
| | Alexanders | *Smyrnium olusatrum* L. | April to June |
| | Pignut | *Conopodium majus* (Gouan) Loret | May to June |
| | Ground elder (goutweed) | *Aegopodium podagraria* L. | May to June |
| | Rough Chervil | *Chaerophyllum temulum* L. | Late May to early June |
| | Hogweed (cow parsnip) | *Heracleum sphondylium* L. | April to September |
| Late flowering (summer to early autumn) | Hemlock* | *Conium maculatum* L. | June to July |
| | Greater burnet-saxifrage | *Pimpinella major* (L.) Hudson | June to August |
| | Wild carrot | *Daucus carota* L. | June to August |
| | Fine-leaved Water-Dropwort | *Oenanthe aquatica* | June to September |
| | Fool's parsley | *Aethusa cynapium* L. | June to October |
| | Wild parsnip | *Pastinaca sativa* L. | July to August |
| | Upright hedge-parsley | *Torilis japonica* (Houtt.) DC. | July to September |
| | Wild angelica | *Angelica sylvestris* L. | July to October |
| | Fennel | *Foeniculum vulgare* Miller | July to October |

*In field trials, Dunn and Kirkley (1966) found few willow-carrot aphid alatae and little colonisation of this species

While cow parsley is unlikely to support over-wintering colonies of willow-carrot aphid, alatae can be produced on this host before the migration from willow to carrots is complete (Dunn & Kirkley, 1966). These aphids may be viruliferous and as such, early flowering umbellifers could be important virus reservoirs for aphids transmitting PYFV to carrots.

Studies by Murant and Goold (1968), Bem and Murant (1979) and later Van Dijk and Bos (1985) indicated that PYFV-A only occurs in cow parsley and that PYFV-P may occasionally occur in cow parsley, but is mainly isolated from hogweed. Whilst other Umbelliferae are susceptible to PYFV, they react with dieback (Van Dijk & Bos, 1985) and hence appear to be less significant as sources of virus spread. Infection in cow parsley is latent and this, coupled with the perennial nature of the species, suggests that it may be considered the main source of infection for PYFV-A. As willow-carrot aphid is the only *Cavariella* species to feed on both cow parsley and carrots (Van Dijk & Bos, 1985) it may be regarded as the primary vector of PYFV.

Although apterous willow-carrot aphids are more efficient vectors than alatae under controlled laboratory conditions (Elnagar & Murant, 1976a), the more mobile alatae actively spread PYFV in the field. Early vector transmission investigations (Elnagar & Murant, 1976a) indicate that willow-carrot aphid may acquire AYV or the AYV-PYFV complex in a minimum acquisition access period (AAP) of 10-15 minutes,

inoculating the viruses to test plants in a minimum inoculation access period (IAP) of two minutes. Additionally, AYV-infected aphids may acquire PYFV in a minimum AAP of two minutes, acquiring and inoculating PYFV in a minimum total time of 12 minutes.

Natural infection of PYFV occurs after willow-carrot aphid migrate in May and June. Migrating alatae may feed on cow parsley before alighting on newly uncovered early carrot crops and other Umbelliferae (Dunn, 1965; Dunn & Kirkley, 1966). As there is a high incidence of PYFV and AYV in cow parsley, it is regarded as an important virus reservoir, with the spread of the virus from cow parsley continuing at low levels when the aphids migrate for the second time in June and July (Van Dijk & Bos, 1985). As carrots are immune to AYV, the spread of virus within crops is limited to random plants, aphids retaining the virus complex for maximum of four days (Elnagar & Murant, 1976a).

*Pesticide usage*

Information on the use of insecticides specifically for the control of PYFV and its vectors is not available (D. Garthwaite pers. com.) and the effect of insecticide applications on virus transmission efficiency of PYFV vectors has yet to be investigated. Furthermore, considering Elnagar and Murants' findings (1976a) that the virus complex can be acquired and transmitted within 12 minutes, pesticide applications may not always provide effective disease and vector control on their own. Studies undertaken by Van Dijk and Bos (1985), in which they recorded limited effect of systemic insecticide treatment on the percentage of carrot seed plants with PYFV, perhaps bear out this view. However, without a clear understanding of the viruses and their vectors, pesticide use to prevent damage could be indiscriminate leading to excessive prophylactic sprays.

*Current PYFV control strategy*

At present growers rely on regular insecticide sprays to control willow carrot aphid and combat PYFV. Rothamsted Insect Survey data, combined with crop monitoring and grower experience can be used to time the start of this spray programme. Alternatively with early crops, sprays start as soon as the polythene is removed, usually in early May, when the plants are around the 5-6 true leaf stage. In main crops, sprays also start in early May once they are at the expanded cotyledon to one true leaf stage. Sprays are usually fortnightly and continue until the end of June.

Sprays can be of the aphid specific carbamate pirimicarb (various products) alone or alternatively a tank mix of pirimicarb and a pyrethroid usually deltamethrin (Decis) or λ-cyhalothrin (Hallmark). The advantage of including a pyrethroid is that it will deter aphid feeding and therefore virus transmission. Also, if the flight period for carrot fly coincides with that of aphids, a pyrethroid will give control of both pests.

Reliance on calendar dates to time spray programmes can lead to unnecessary treatments as aphid migration will be primarily determined by temperature. On occasions growers have been caught out by early aphid migration and no control measures were applied. Reliance on Rothamsted Insect Survey data could also

lead to inaccurate timing of insecticide sprays, as the data is only available at least a week after the aphids have been trapped and suction sampling at 40 feet above the ground may not necessarily be representative of what is happening at crop level. The current project aims to provide a much better understanding of parsnip yellow fleck virus and its vectors, willow carrot aphid, which will in turn improve risk assessment and rationalise control strategies.

**Aim**

To develop diagnostic assays for PYFV, to identify its aphid vectors, to quantify the interaction between PYFV and its helper virus and to identify environmental conditions that lead to virus spread in order to support the development of a management strategy for the disease in UK carrot crops.

**Objectives**

1. *To determine the phenology, migration and behaviour of aphids that can transmit PYFV in carrots*
2. *To identify virus reservoirs and determine the acquisition, transmission and molecular variability of the PYFV complex*
3. *To develop a prototype strategy that will allow growers to implement sustainable management of PYFV and its vectors*

At the end of project year 2002/03 the outstanding objectives were revised and the efforts in the final year of the project were targeted towards three new objectives (below). These are described in detail, following the details of work conducted for the three original objectives (above).

*1R. Correlate disease symptoms with virus presence*

*2R. Investigate the relationship between PYFV in cultivated and weedy hosts*

*3R. Control of PYFV using rational vector management*

Summary of work conducted to meet each objective:

*Objective 1. Determine the phenology, migration and behaviour of aphids that can transmit PYFV in carrots*

Identify aphids from existing on-going trap networks

A network of water traps was established in each project year at commercial field sites in England. During years one and two at each site, circular yellow water traps (30cm diameter, 15cm deep) were supported on fibreglass canes in the centre of carrot crops at crop height. Traps were sampled by industrial partners as often as possible and all insects collected were sent to the ADAS laboratory. All aphids were identified to species and results were relayed back to the industrial partners within 48 hours of receiving the samples.

In years three and four, a rectangular yellow water trap (400mm x 300mm) was laid on the soil surface on a south or southwest headland approximately 5m into the crop. Traps were sampled twice weekly for 10 weeks. The trap collecting fluid was strained though a muslin sheet and any insects caught were collected and returned to the ADAS laboratory for identification. The trap catches were sorted using a binocular microscope and aphids of *Cavariella* spp. isolated and identified to species level. Other aphid species were collected and stored in 70% alcohol for identification later.

Several species of aphids were caught in the water traps in carrot crops. Aphid species *C. aegopodii*, *C. theobaldi* and *C. pastinacae*, were caught frequently although willow-carrot aphid is the only species known to colonise carrot crops (Van Dijk & Bos, 1985; Blackman & Eastop, 2000). Other species were also abundant, including the bird-cherry oat aphid, *Rhopalosiphum padi*, potato aphid, *Macrosiphum euphorbiae*, black bean aphid, *Aphis fabae*, cabbage aphid, *Brevicoryne brassicae*, grain aphid, *Sitobion avenae*, rose aphid, *Macrosiphum rosae*, vetch aphid, *Megoura viciae*, and the nettle aphid, *Macrosiphum evansi*.

Monitor the phenology and migration of aphid vectors into carrot crops

*Year 2000 growing season*

In 2000, field sites (Figure 2) were selected based on a history either of PYFV or of known infestations of willow-carrot aphid. The methods used in the previous section were followed to establish and service traps.

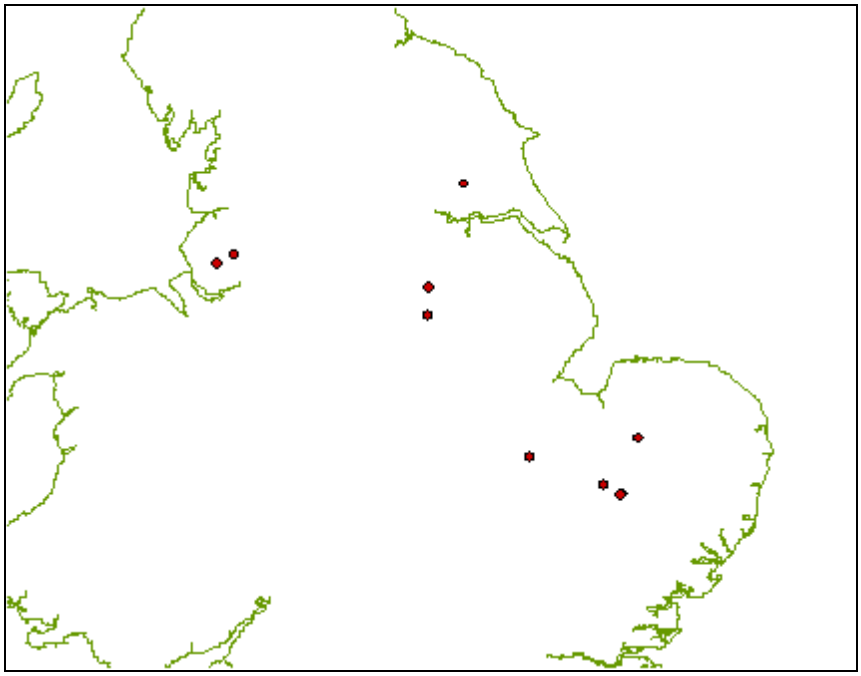


Figure 2. Aphid monitoring sites, 2000

Thirteen field sites were established around England in the major growing regions of umbelliferous crops (Table 2).

Table 2. Location of field sites and crops grown, 2000

| Region | Site (code) | Trap type | Crop | Grid ref |
|---|---|---|---|---|
| Cambridgeshire | Barway (Cambs) | Rectangular | Celery | TL 572798 |
| Lancashire | Lathom (Lancs 1) | Rectangular | Carrots | |
| | Tarleton (Lancs 3) | Rectangular | Cerery | |
| | Skelmersdale (Lancs 3) | Circular | Carrots | |
| Norfolk | Attleborough (Norfolk) | Rectangular | Parsnips | TM 004905 |
| Nottinghamshire | Edwinstowe (Notts 1) | Rectangular | Carrots | SK 636668 |
| | Harby (Notts 2) | Rectangular | Parsnips | SK 884724 |
| | SV (Notts 3) | Circular | Carrots | |
| Shropshire | Maddock (Shrops) | Circular | Carrots | |
| Suffolk | Herringswell 1 | Rectangular | Carrots | TL 723706 |
| | Herringswell 2 | Rectangular | Carrots | TL 725715 |
| Yorkshire | Cawood (Yorks 1) | Rectangular | Carrots | SE 550360 |
| | Market Weighton (Yorks 2) | Circular | Carrots | |

*Results, 2000*

At most sites, the total number of non-*Cavariella* species was greater than that of *Cavariella* species, however, of this species, willow-carrot aphid was most abundant all sites described in Table 2, with the exception of sites ‘Notts 3’ and ‘Yorks 2’, where *C. pastinacae* was the most abundant. Regional variation in willow-carrot aphid numbers was evident (Figures 3a-c), although during a ten- to eleven-week monitoring period, overall catches of willow-carrot aphid were low. Catches were particularly sparse in the northwest and East Anglian regions, while most catches of this species were collected from the Nottinghamshire sites. Due to the low number of catches in northwest and East Anglian sites, migration peaks of willow-carrot aphid are not obvious (Figures 3b & c), but in Nottinghamshire, it was most abundant from the end of May to the second week in June (Figure 3a).

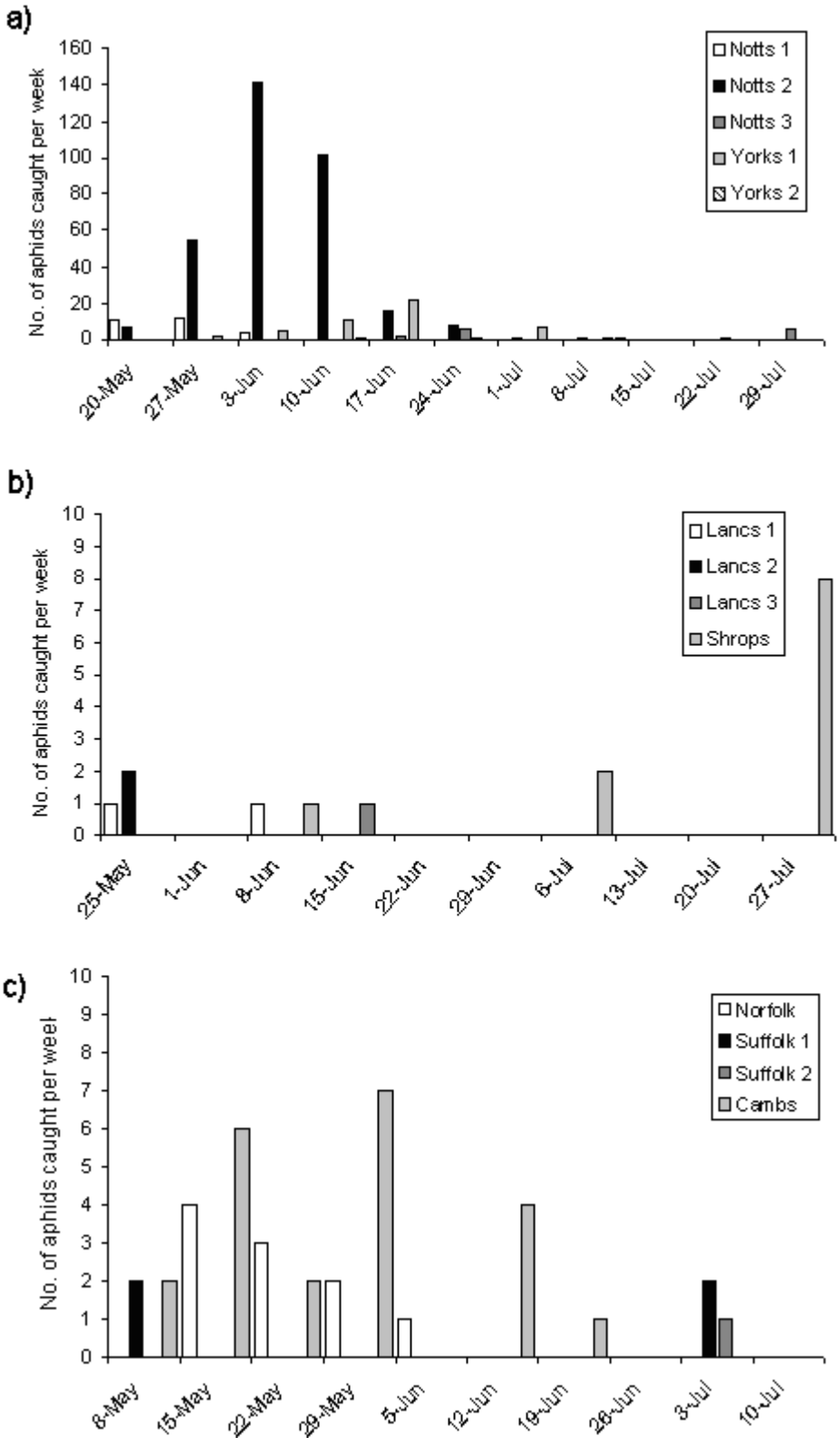



Figure 3. Weekly water trap catches of willow-carrot aphid from northeast England (a), northwest England (b) and East Anglia (c), 2000

*Year 2001 growing season*

In 2001 the network of traps established in 2000 was expanded to 20 sites. These were again located throughout the major carrot growing regions of England (Table 3). All sites were located in carrot fields.

Table 3. Carrot crops monitored for willow carrot aphid, 2001

| Region | Site | Grid reference |
|---|---|---|
| Cambridgeshire | Isleham | TL 628753 |
| | Yaxley | TL 184915 |
| Lancashire | Burscough | |
| Merseyside | Rainford | |
| Norfolk | Beachamwell | TF 745053 |
| | Cockley Cley | TF 825059 |
| | Gooderstone | TF 778027 |
| | Great Cressingham | TL 888991 |
| | Hillborough | TF 834025 |
| | Ickburgh | TL 795958 |
| | South Pickenham | TF 853038 |
| | Swaffham | TF 781087 |
| Nottinghamshire | Blyth | SK 645859 |
| | Budby | SK 617700 |
| | Thoresby | SK 645711 |
| Shropshire | Whitchurch | |
| Yorkshire | Cawood | SE 550360 |
| | Elvington | |
| | Thorpe Le Street | |

*Results 2001*

Aphid incidence was extremely low in 2001 and only a single willow-carrot aphid was caught throughout the 10-week monitoring period. Therefore trapping was continued for two or more weeks but no more willow carrot aphids were recorded. This result is difficult to explain and it is possible that the migration was early and before traps were set. However, data from the Rothamsted Insect Survey also indicated a very low incidence of willow carrot aphid in comparison with 2000, 2002 and 2003 (Table 4). Therefore, it is possible that water trap catches reflect the true level of aphid migration in 2001.

Table 4. Suction trap catches of willow-carrot aphid caught at four Rothamsted sites (Courtesy of Dr. R. Harrington, Rothamsted Research).

| Site | Year | | | |
|---|---|---|---|---|
| | **2000** | **2001** | **2002** | **2003** |
| Askham Bryan, North Yorkshire | 205 | 11 | | 245 |
| Preston, Lancashire | 323 | 9 | | 175 |
| Kirton, Lincolnshire | 470 | 150 | | 157 |
| Brooms Barn, Suffolk | 943 | 44 | | 137 |

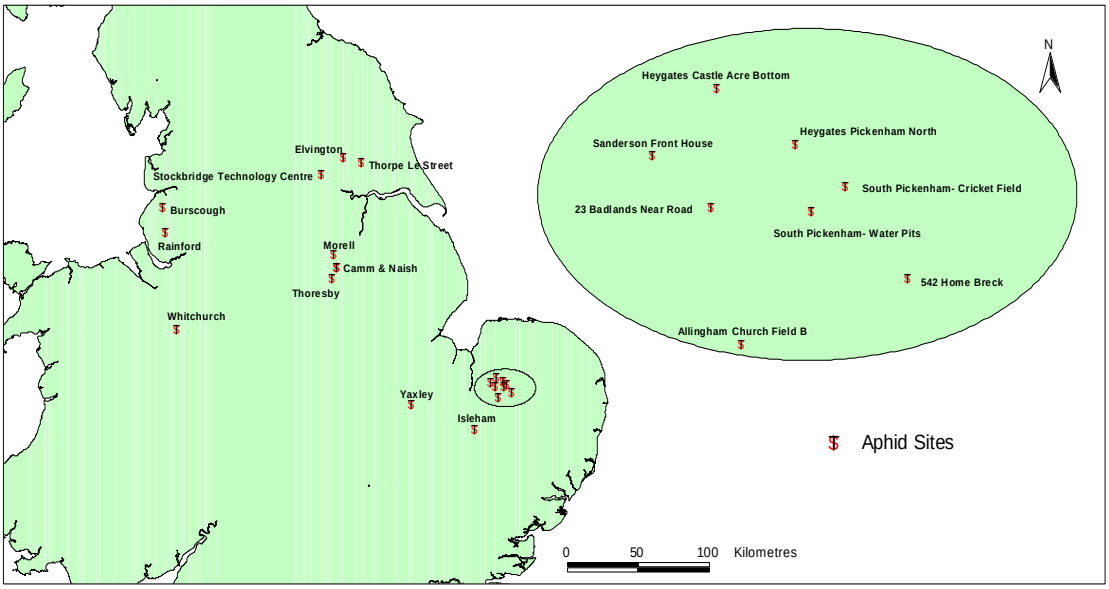



Figure 4. Aphid monitoring sites, 2001

*Year 2002 growing season*

In 2002, the network of water traps established in 2000 and 2001 was increased to twenty sites throughout the major carrot growing regions in England (Figure 5 & Table 5). Aphids caught weekly in the water traps were removed and sent to ADAS for identification.

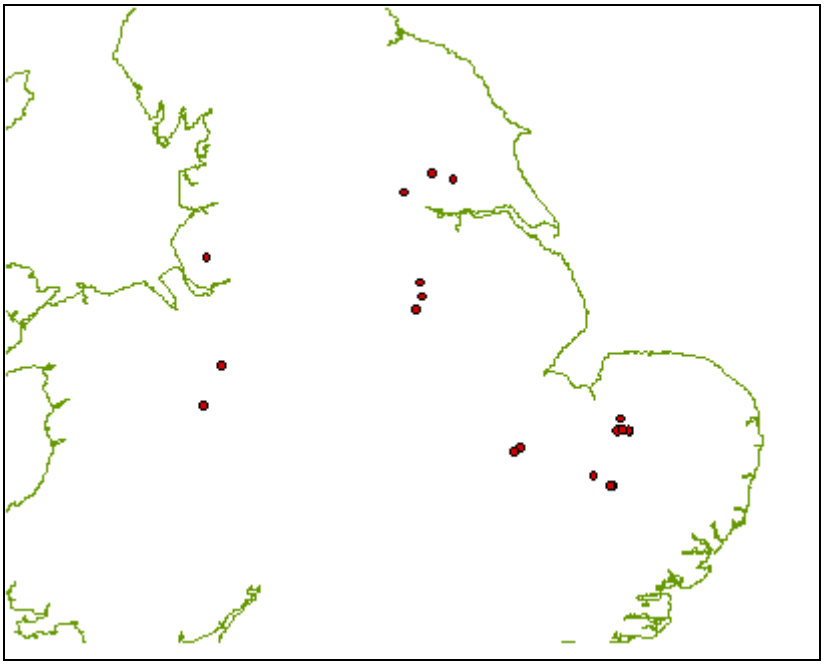


Figure 5. Aphid monitoring sites, 2002

Table 5. Location of field sites and carrot crops grown, 2002

| Region | Site (Code) | Grid reference |
|---|---|---|
| Cambridgeshire | Isleham 1(Cambs 1) | TL 207922 |
| | Isleham 2 (Cambs 2) | TL 178907 |
| | Isleham 3 (Cambs 3) | TL 618767 |
| Cheshire | Thornleigh Park (Cheshire) | SJ 367668 |
| Lancashire | Rainford (Lancs) | |
| Norfolk | Hilborough 1 (Norfolk A) | TF 815012 |
| | Hilborough 2 (Norfolk B) | TF 805025 |
| | Narborough 1 (Norfolk C) | TF 748024 |
| | Narborough 2 (Norfolk D) | TF 760093 |
| | Gooderstone (Norfolk E) | TF 774027 |
| Nottinghamshire | Blyth (Notts A) | SK 645859 |
| | Budby (Notts B) | SK 617700 |
| | Thoresby (Notts C) | SK 645711 |
| | Babworth (Notts D) | SK 662778 |
| North Yorkshire | Cawood (Yorks A) | SE 550360 |
| | Thorpe Le Street (Yorks B) | SE 821432 |
| | Elvington (Yorks C) | SE 711480 |
| Shropshire | Tibberton (Shrops) | SJ 6820 |
| Suffolk | Terringwell (Suffolk A) | TL 722712 |
| | Upton (Suffolk B) | TL 709707 |

*Results, 2002*

The timing of migration and abundance of willow-carrot aphid varied throughout the regions. Migration began at the end of April in Suffolk and Nottinghamshire and by early May most sites had caught this species (Figures 6a-f). Willow-carrot aphid was most abundant in the north of England (Figures 6a & b), with lowest numbers caught in the northwest regions and Suffolk (Figures 6e & f). Migration peaks, were evident at most sites and mainly occurred in mid-May.

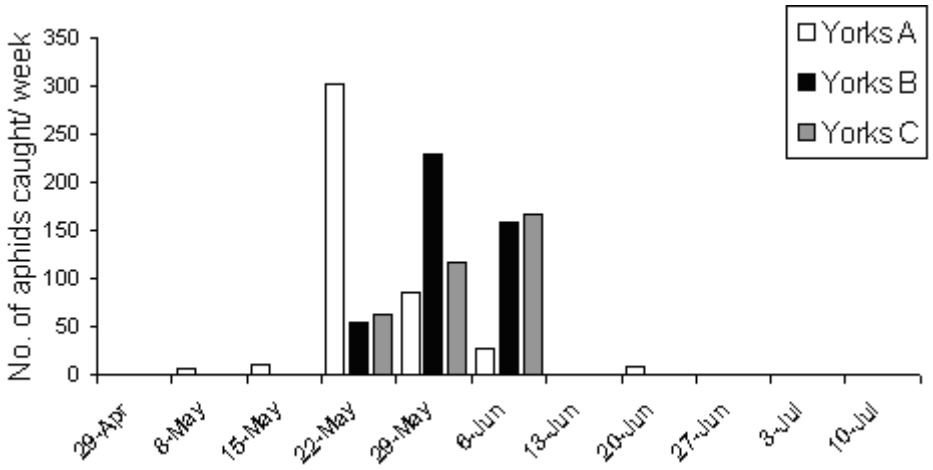



Figure 6a. Weekly water trap catches of willow-carrot aphid, North Yorkshire, 2002

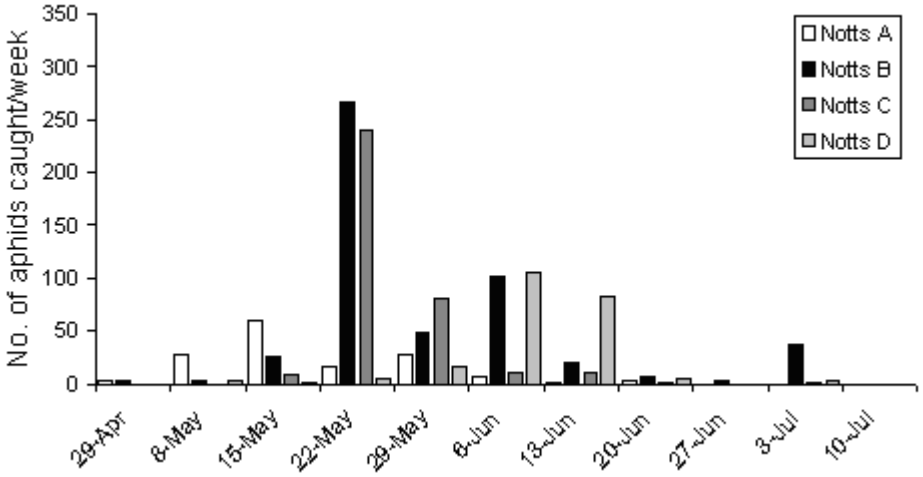



Figure 6b. Water trap catches of willow-carrot aphid, Nottinghamshire, 2002

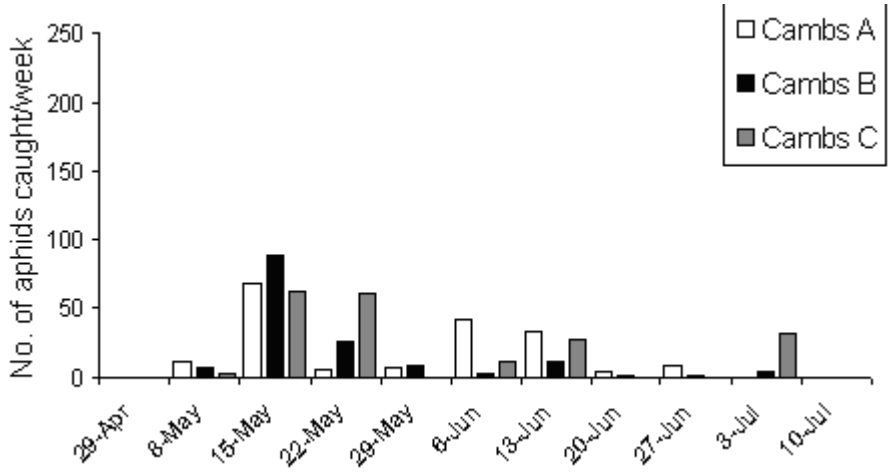



Figure 6c. Weekly water catches of willow-carrot aphid, Cambridgeshire, 2002

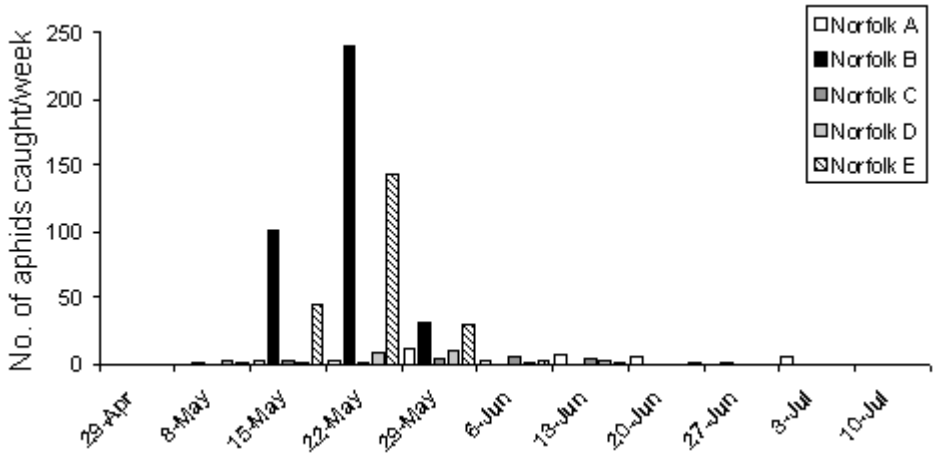



Figure 6d. Weekly water catches of willow-carrot aphid, Norfolk, 2002

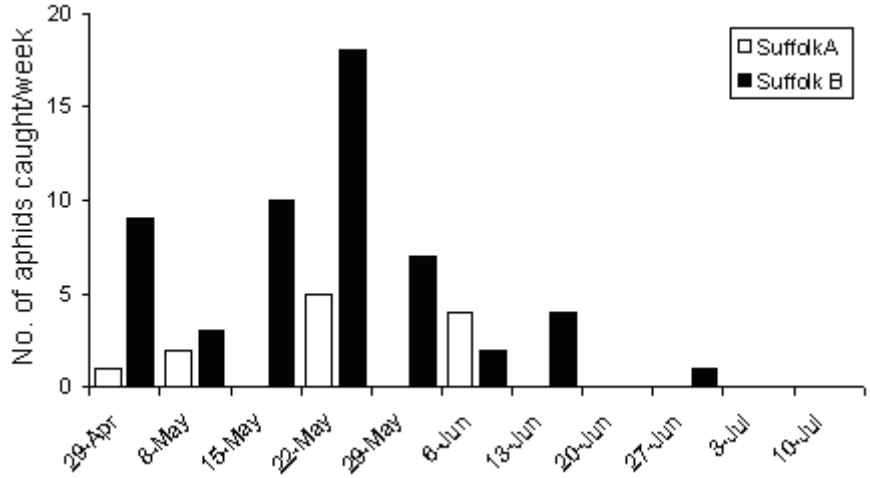



Figure 6e. Weekly water catches of willow-carrot aphid, Suffolk, 2002

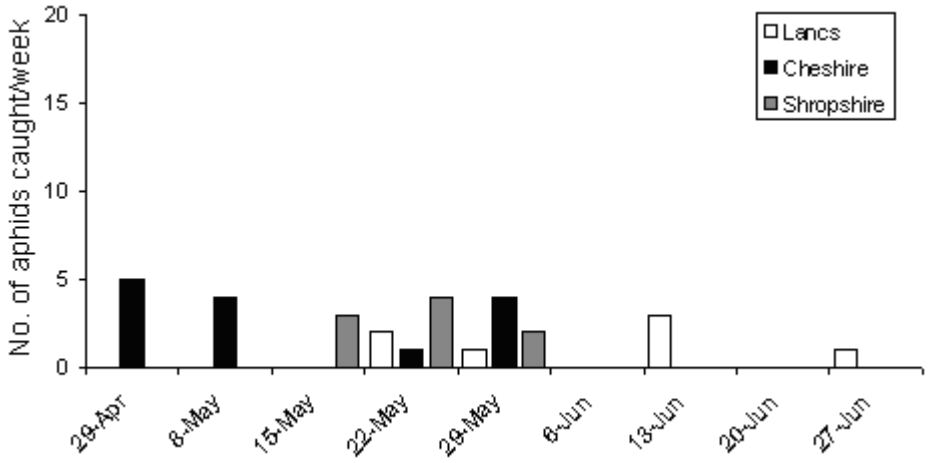



Figure 6f. Water trap catches of willow-carrot aphid, northwest England, 2002

*Year 2003 growing season*

A monitoring programme was undertaken in 2003 to provide further information on the timing and duration of willow-carrot aphid migration into carrot crops to support subsequent development of novel forecasting tools. Three areas of England, Lancashire, North Yorkshire and Suffolk/Cambridgeshire were used that had experienced problems with parsnip yellow fleck virus in the past. As before yellow water traps were used to monitor aphid activity.

Water traps were sited in carrot fields in the three geographical regions, with six sites in each region (Table 6). At Burscough (sites A and B), two sites were used during the monitoring programme and grid references are given for both. At Burscough A, traps were originally sited in an overwintered carrot crop that had been strawed up. Once these carrots were harvested, the traps were moved (on 22.5.03) to a new crop. Burscough B was a very large field and only half of this was cropped with carrots. Traps were initially located in the half of the field not cropped with carrots and were later moved (on 22.5.03) once it became clear where carrots were to be drilled. At two sites in each region, there was an area of carrots that was untreated with insecticides, including granular nematicides such as Temik applied at drilling. Each untreated area was a minimum size of 6 m x 50 m and adjoined the field margin. Untreated areas were used to sample plants for analysis to determine the presence of parsnip yellow fleck virus.

With the exception of Burscough B, two water traps were placed in each field, week beginning 17 March 2003. If carrot crops were not present, the traps were placed in the field margins so that they were not disturbed by any field cultivations. Traps were in position for approximately 16 weeks and were visited and emptied on a weekly basis. Traps consisted of a yellow cat litter tray (44 cm long, 32 cm wide, 9.5 cm deep) and were three-quarters filled with water. A drop of detergent was added to break the water surface tension, ensuring any insects that were caught drowned. In addition, a Campden tablet was crushed and sprinkled into each trap to help preserve the catch. The trap was covered with chicken wire to prevent birds taking the catch. A small hole was drilled into the side of each trap approximately 1 cm from the top so that they did not flood and overflow during heavy rainfall.

Table 6. Sites monitored for willow carrot aphid, 2003

| Region | Site | Grid reference |
|---|---|---|
| Lancashire | Asmall | SD 396083 |
| | Burscough A | SD451137/ 453136 |
| | Burscough B | SD447141/ 447149 |
| | Burscough C | SD446128 |
| | Hoscar Moss | SD462120 |
| | Ince Blundell | SD335039 |
| North Yorkshire | Buttercrambe | SE712568 |
| | Filey | SE068798 |
| | Holme on Spalding Moor | SE789370 |
| | Riccall | SE631361 |
| | Sand Hutton | SE688576 |
| | Sherburn | SE966777 |
| Suffolk/Cambridgeshire | Castle Acre, Suffolk | TF828152 |
| | Cranwich, Suffolk | TL772963 |
| | Fordham, Cambridgeshire | TZ620719 |
| | Freckenham, Cambridgeshire | TZ675728 |
| | Higham, Cambridgeshire | TZ753663 |
| | Narborough, Suffolk | TF780127 |

Traps were emptied by tipping the contents into a plastic kitchen sieve lined with muslin. The muslin was stored in a collection tube and returned to the laboratory. Catches were examined within five days of capture and all *Cavariella* spp. were identified and counted. Catches of *Cavariella* spp. were retained in tubes of 70% alcohol and labelled with the site and date of capture. The field margins of all eighteen fields used for water trapping were examined for the presence of wild umbelliferous plants when extension growth started in March/April. A random sample of 50 cow parsley plants was taken at each site and examined in the laboratory for overwintering aphids. Where fifty plants could not be sampled, as many as possible were collected. If cow parsley was not present the most abundant umbelliferous species, such as hogweed or hedge parsley, was collected. Samples were examined for aphids using an alcohol rinsing technique.

*Results, 2003*

The general trend in trap catches of willow carrot aphid was similar at all sites (Figures 7a-c). First aphids were caught late April/early May with peak numbers recorded around late May/early June. In general, catches then declined, with the exception of Castle Acre, Suffolk, and very few aphids were trapped in July. The high catch of aphids at Castle Acre between 12 and 18 June is difficult to explain.

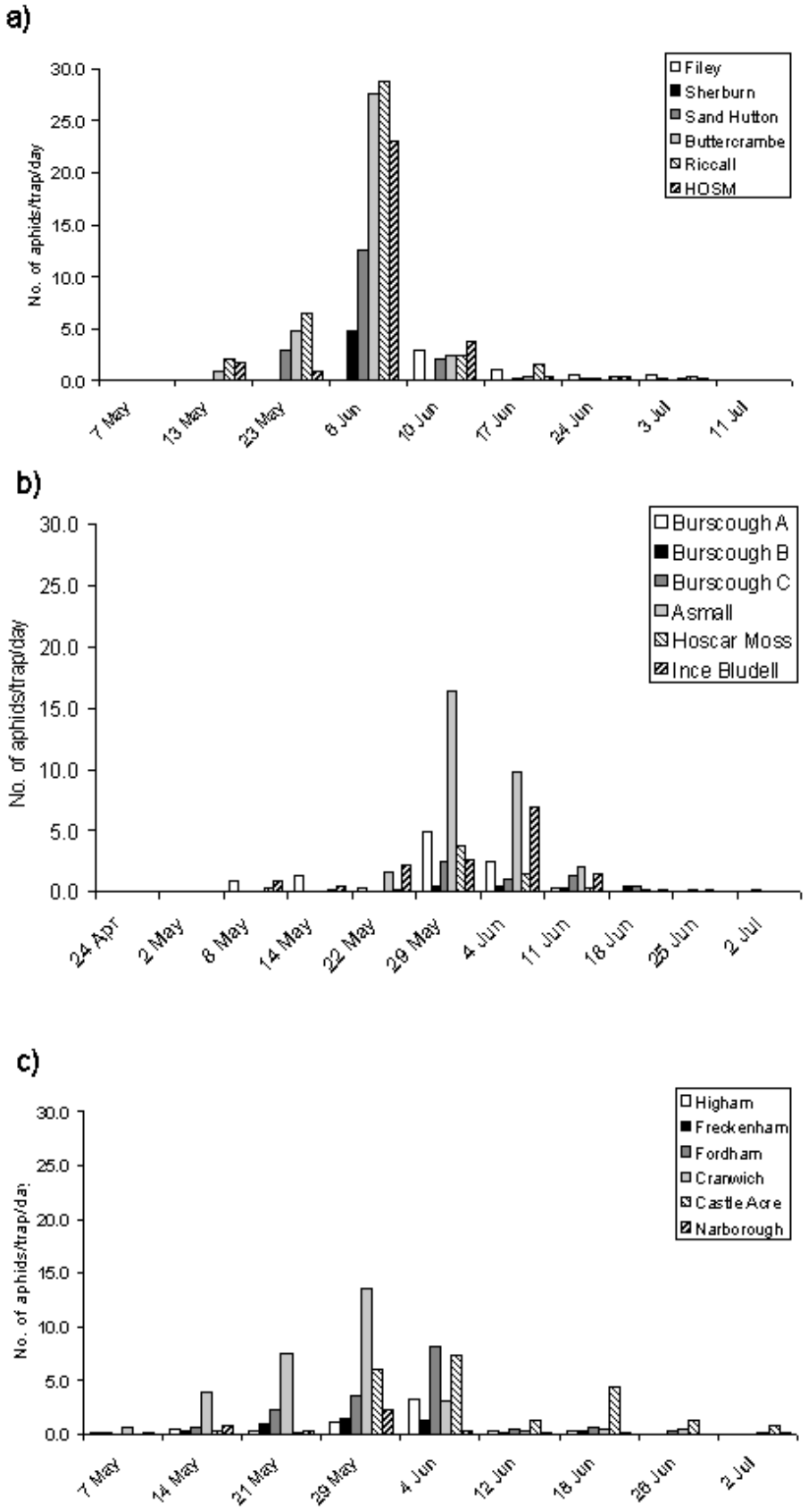



Figure7. Water trap catches of willow-carrot aphid in N. Yorks (a), Cambridgeshire (b) and Lancashire (c), 2003.

Peak catches were recorded in Yorkshire. Traps at Buttercrambe, Holme on Spalding Moor and Riccall all caught more than 20 willow carrot aphid/trap/day in the period 23 May – 2 June. The highest catch was at Riccall where 28.2 aphids/trap/day were caught. In Cambridgeshire/Suffolk, Cranwich had the highest aphid catch at 13 aphids/trap/day between 29 May and 6 June. In Lancashire, most aphids (16/trap/day) were found at Asmall between 29 May and 4 June.

It was interesting that the highest aphid catches in Yorkshire corresponded with sites (Buttercrambe, Holme on Spalding Moor, Riccall) that had been sown at lower than normal seed rates in order to encourage the growth of larger carrots for processing. A conventional crop for packing is sown at 2,100,000 seeds/ha whereas a low-density crop for processing is sown at about 800,000 seeds/ha. The data for the low-density crops were compared with catches at the other three Yorkshire sites (Filey, Sand Hutton, Sherburn, sown at normal seed rates) to determine if there was any correlation between low-density crops and aphid catches. The analysis assumed a split plot design with crop density as the main plot factor and trapping date as the sub-plot factor. This is not strictly correct as trapping date cannot be allocated randomly and there is likely to be a correlation between aphid catches at a particular site on different trapping occasions. Consequently, AREPMEASURES, a Genstat procedure, was used to undertake the analysis. This combats the problems associated with trapping date by calculating a constant called the Greenhouse Geisser epsilon. This has a value of between zero and one and gives a measure of the likely correlation between trapping dates. If there is no correlation, the value is one. In this case, the value was 0.24, which indicates a relatively high level of correlation. The degrees of freedom in the sub-plot stratum of the analysis of variance are multiplied by the Greenhouse Geisser epsilon to give revised values when testing for significance. The degrees of freedom in the sub-plot stratum for trapping date, crop density and their interaction were 8, 8 and 32 respectively. Multiplying by the Greenhouse Geisser epsilon gives 2, 2 and 8 and these values were used to test for significance in “F” tables.

There was significant difference between crop densities ($P < 0.05$, Table 7) and trapping dates ($P < 0.001$) but the interaction between these variates was non-significant. A significant effect of trapping date is as expected as aphids initially migrate into plots at low numbers, ultimately reach a peak and then decline. A significant effect of crop density suggests that aphid catches are greater where crops are grown at a low crop density. Where cereals were grown through a permanent understorey of clover, numbers of cereal aphids were reduced in comparison with a conventionally drilled crop (Clements & Donaldson, 1997). One possible reason is that aphids rely on the contrast between cereal seedlings and a bare soil background to be able to detect the crop. The clover crop provided a green background and camouflaged the emerging cereals. Where carrots are drilled at reduced seed rates and wider row spacings than normal, there would be a greater contrast between carrot seedlings and bare soil than in a conventionally grown crop. This might be more attractive to willow-carrot aphid. However, care should be taken when interpreting these results as comparison of seed rates was not possible within the same sites. Consequently, there could be confounding factors influencing trap catches at Buttercrambe, Holme on Spalding Moor and Riccall, other than seed rate alone. However, the influence of crop density on aphid immigration into crops is worthy of further investigation.

Table 7. The effect of sampling date and crop density on numbers of willow carrot aphid (number/trap/day) in Yorkshire

| | Sample date | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Crop density** | **07/05** | **13/05** | **23/05** | **02/06** | **10/06** | **17/06** | **24/06** | **03/07** | **11/07** | **Mean** |
| High density | 0 | 0.01 | 0.20 | 0.63 | 0.36 | 0.15 | 0.14 | 0.10 | 0.01 | 0.18 |
| Low density | 0.02 | 0.40 | 0.64 | 1.44 | 0.59 | 0.23 | 0.11 | 0.10 | 0.03 | 0.39 |
| Mean | 0.01 | 0.20 | 0.42 | 1.03 | 0.47 | 0.19 | 0.12 | 0.10 | 0.02 | |

SED (4 df) for cropping density = 0.059; SED (8 df) for trapping date = 0.116; SED (8 df) for interaction = 0.165

Freckenham, Cambridgeshire, was the only site at which willow-carrot aphids were found on umbelliferous plants in the field margin. At this site, three willow-carrot aphids were found on cow parsley. A number of other aphid species were found but these were not identified. Details of the plant species sampled at each site are given in Table 8. No umbelliferous plants were collected from the Lancashire sites.

Table 8. Number of willow-carrot and other aphids recovered from umbelliferous plants in field margins (sampled March/April at start of extension growth)

| **Site** | **Plant species** | **Number of samples** | **Number of aphids** | |
|---|---|---|---|---|
| | | | Willow-carrot aphid | Other aphids |
| **Cambridgeshire** | | | | |
| Higham | Cow parsley | 33 | 0 | 3 |
| | Hemlock | 3 | 0 | 8 |
| | Hog weed | 14 | 0 | 0 |
| Freckenham | Cow parsley | 40 | 3 | 18 |
| Fordham | Cow parsley | 50 | 0 | 0 |
| **North Yorkshire** | | | | |
| Buttercrambe | Cow parsley | 50 | 0 | 0 |
| Filey | Cow parsley | 11 | 0 | 57 |
| | Hemlock | 36 | 0 | 0 |
| Holme on Spalding Moor | Cow parsley | 41 | 0 | 42 |
| Riccall | Cow parsley | 50 | 0 | 111 |
| Sand Hutton | Cow parsley | 20 | 0 | 0 |
| | Hogweed | 5 | 0 | 0 |
| Sherburn | Bur chervil seedling | 4 | 0 | 16 |
| | Bur chervil plants | 14 | 0 | 0 |
| **Suffolk** | | | | |
| Cranwich | Bur chervil | 64 | 0 | 0 |
| | Cow parsley | 10 | 0 | I |
| | Rough chervil | 26 | 0 | 5 |
| Castle Acre | Cow parsley | 50 | 0 | 0 |
| Narborough | Bur chervil | 4 | 0 | 2 |
| | Hemlock | 50 | 0 | 3 |

*Conclusions & Discussion*

With the exception of four sites in 2000, willow carrot aphid was the most numerous *Cavariella* spp trapped throughout four years of monitoring. Numbers caught varied between years and between regions. Catches in 2000 were relatively low with no more than 11 willow carrot aphids/trap/day. In 2001 only one aphid was caught. Although it is possible that some migration occurred before traps were set, it is likely that 2001 was a low risk year for PYFV. Catches of willow carrot aphid increased again in 2002 with just over 20/trap/day being caught at the peak of migration in the third week of May.

Willow carrot aphids were trapped any time between late April and late July with peak catches tending to occur around mid May to early June. Nottinghamshire and Yorkshire were the regions to catch most willow carrot aphid with the highest catch occurring in 2003 at Riccall with 28.2 aphids/trap/day. The variability in timing and numbers of willow carrot aphid caught demonstrate the value of water traps for timing control measures against the pest.

Highest catches of willow carrot aphid were in low-density carrot crops in Yorkshire. Numbers of aphids were significantly higher than in conventional crops in the same region. However, care must be taken in interpreting this result as conventional and low density crops were not compared at the same site. Throughout the monitoring programme, numbers of willow carrot aphids in umbelliferous plants around field margins was very low.

*Objective 2. Identify virus reservoirs and determine the acquisition, transmission, and molecular variability of the PYFV complex*

<u>Collect potential weed hosts from the margins of carrot crops and test them for virus incidence using ELISA</u>

*Year 2000 growing season*

Samples of umbelliferous crops and potential weed hosts in fields with water traps were taken weekly during the aphid migration period in 2000. Plant samples were selected at random and all were tested for PYFV infection using ELISA. As cow parsley is considered the primary source of PYFV (Murant & Goold, 1968; Bem & Murant,1979; Van Dijk & Bos,1985) samples of this species were also tested for the presence of AYV using PCR. In addition, the industrial partners within the consortium were encouraged to collect samples of crops they suspected to be infected with PYFV and their virus status was confirmed.

A botanical survey of potential weed hosts was conducted at three sites. Field margins and hedgerows surrounding fields were sampled for the presence of umbelliferous weeds and the relative frequency/cover of plant species was assessed using the DAFOR (Dominant, Abundant, Frequent, Occasional, Rare) scheme for measuring abundance.

Seventy-nine samples of umbelliferous weeds and crops were received for PYFV detection. PYFV was detected in eighteen samples of carrot, celery, parsnip and cow parsley but was not found in hogweed or other weeds (Table 9). Isolates were stored and used for subsequent molecular characterisation studies.

Table 9. Virus incidence in potential hosts collected from margins of carrot crops tested by ELISA, 2000

| Host | No. of plants tested | No. of plants PYFV positive | % of plants PYFV positive |
|---|---|---|---|
| Carrot | 34 | 6 | 18 |
| Celery | 12 | 2 | 17 |
| Parsnip | 10 | 3 | 30 |
| Cow parsley | 17 | 7 | 41 |
| Hog weed | 4 | 0 | 0 |
| Other weeds | 2 | 0 | 0 |
| **TOTAL** | **79** | **18** | **23** |

*Year 2001 growing season*

Samples of umbelliferous crops and potential weed hosts in fields with water traps were taken weekly during the aphid migration period in 2001. A total of 15 to 20 plant samples were taken per week with five or six leaves taken from an individual host. Samples were collected by ADAS and sent to HRI-Wellesbourne.

Following problems of significant serological activity against uninfected herbaceous test plants in 2000 when using a plate-trapped-antigen (PTA) ELISA, two new antisera were raised in rabbits. The first, raised using PYFV extracted from *N. benthamiana*, had only very weak serological activity. The second, raised against PYFV from spinach had good activity and, unlike the first antiserum, worked well in double-antibody-sandwich (DAS) ELISA. As DAS ELISA is normally more specific than PTA ELISA, the former was used in 2001.

*Results*

A total of 172 samples were tested, 26% of which tested positive for PYFV by ELISA (Table 10). Infection was detected in three hosts species of eight sampled. Infection rates were highest early in the season for all three hosts and declined erratically but steadily through the season (Table 11). PYFV was not detected in other weed hosts except for rough chervil where almost half of the samples tested were infected. In general, weeds were tested later in the season and an early PYFV infection may have been missed. Virus incidence varied with geographical location. Infected carrots were detected in samples from Nottinghamshire and Cambridgeshire sites only, while PYFV infected cow parsley was detected in samples from all regions except Yorkshire. Infection in rough chervil was only found in samples from a site in Cambridgeshire.

A subset of fifty-four samples from those tested above were inoculated to herbaceous hosts and to test the validity of the ELISA results, these included samples which had tested negative for PYFV by ELISA as well as those which were positive. A total of 12 samples inoculated to herbaceous hosts produced symptoms, but when further tested by ELISA, all were negative for PYFV (Table 12).

Table 10. Virus incidence in potential hosts collected from margins of carrot crops tested by ELISA, 2001

| Host | No. of plants tested | No. of plants PYFV positive | % of plants PYFV positive |
|---|---|---|---|
| Carrot | 54 | 17 | 31 |
| Cow parsley | 54 | 20 | 37 |
| Rough chervil | 15 | 7 | 47 |
| Celery | 1 | 0 | 0 |
| Upright hedge parsley | 24 | 0 | 0 |
| Hemlock | 1 | 0 | 0 |
| Hog weed | 21 | 0 | 0 |
| Wild Parsnip | 2 | 0 | 0 |
| **TOTAL** | **172** | **44** | **26** |

Table 11. Temporal distribution of PYFV incidence in carrots and wild hosts, 2001

| Host plant | Week No. | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **21*** | **22** | **23** | **24** | **25** | **26** | **27** | **28** | **29•** | **33∞** |
| Carrots | | | 7/9 | 5/12 | | 3/13 | 1/7 | 1/4 | 0/5 | 0/4 |
| Cow parsley | 6/8 | 2/5 | 3/4 | 3/11 | 4/13 | 2/6 | 0/3 | | 0/1 | 0/3 |
| Rough chervil | | | | | 5/7 | | 1/3 | | 1/5 | |
| Celery/other weeds | | | | 0/1 | | 0/16 | 0/17 | 0/6 | 0/9 | |

* = Wk. Beginning 21/05/01; • = Wk. Beginning 16/07/01; ∞ = Wk. Beginning 13/08/01

Table 12. Inoculation of herbaceous hosts and subsequent ELISA testing

| Sample description | No. of plants inoculated | No. of plants with symptoms | No. of plants PYFV +ve (ELISA) |
|---|---|---|---|
| Carrot (PYFV +ve) | 7 | 1 | 0 |
| Cow parsley (PYFV +ve) | 8 | 0 | 0 |
| Rough chervil (PYFV +ve) | 1 | 0 | 0 |
| Carrot (PYFV +ve) | 13 | 2 | 0 |
| Cow parsley (PYFV +ve) | 14 | 4 | 0 |
| Rough chervil (PYFV +ve) | 1 | 0 | 0 |
| Hogweed (PYFV +ve) | 4 | 3 | 0 |
| Upright hedge parsley (PYFV +ve) | 4 | 2 | 0 |
| Wild parsnip (PYFV +ve) | 2 | 0 | 0 |

As there was no clear association between ELISA results and infection of herbaceous hosts in either 2000 or 2001, an alternative diagnostic test was investigated. PYFV group specific primers were developed at HRI and tested successfully on herbaceous hosts. RNA extracts were prepared from all hosts and stored at –80°C. When these primers were optimised, the RNA samples were tested for PYFV. Primers designed at CSL to AYV were also used to test the RNA samples, alongside carrot red leaf or general luteovirus primers.

*Year 2002 growing season*

Two types of carrot sample were received by HRI Wellesbourne for testing in 2002. The majority were sent by 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 or during the meeting of July 16th at Skelmersdale (by consortium members attending the meeting). All samples were tested by ELISA using antiserum raised against the Wisbech isolate of PYFV. A subset was also tested by PCR using six primer pairs (designed to detect all *Anthriscus* strain isolates sequenced to date).

A subset of the above samples was also used to inoculate *Nicotiana benthamiana* and *N. occidentalis* under glasshouse conditions. Those used for inoculation included samples selected at random, some that tested positive by ELISA or PCR and others with symptoms.

*Results*

In total 581 samples collected at random were tested by ELISA, four of which were positive (0.6%) (Table 13). Of 200 random samples tested by PCR, PYFV was detected in one. A total of 30 samples from the random collections were inoculated to herbaceous hosts and resulted in one plant expressing symptoms.

Table 13. Results of carrot samples tested for PYFV, selected in regular random collections (not selected by symptoms), 2002

| Source | ELISA | | PCR | | Inoculated 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** | **200** | **1** | **20** | **1** |

Five seedlings, each showing virus symptoms and die back, were collected early in the season and tested for PYFV by ELISA, PCR and inoculation to herbaceous hosts. One sample was parsnip (not tested by ELISA) and the remainder were carrots. All five samples tested positive for PYFV by PCR; ELISA detected no virus and all inoculated hosts expressed virus symptoms, indicating a good correlation with symptoms and PCR results (Table 14).

From the samples collected later in the season at Skelmersdale, some correlation of symptoms in carrot with PCR positive results and successful inoculation of virus to herbaceous hosts was evident, but not as conclusive as those tested earlier in the season (Table 15). The three PYFV-positive samples (detected by PCR) were tested further using a range of six different primer sets, the results of which illustrate the differentiation between isolates of the *Anthriscus* strain (Table 16).

Table 14. Testing for PYFV of early season samples submitted with symptoms, 2002

| Diagnostic test | No. +ve | No. –ve | No. with symptoms |
|---|---|---|---|
| ELISA | 0* | 4 | - |
| PCR | 5 | 0 | - |
| Inoculation to hosts | - | - | 5 |

*Parsnip sample was not tested

Table 15. Detection of PYFV in Skelmersdale carrots with symptoms: ELISA, PCR and inoculation to herbaceous plants.

| Symptoms on carrot | No. of Plants | ELISA | PCR | Inoculations |
|---|---|---|---|---|
| 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* | *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* | *1/2* |
| Mixed | 1 | 0/1 | | 0/1 |

**Bold =** where symptoms give PCR positive/symptoms in plants
*Italics*= Carrot which gave negative result by PCR did not give symptoms in herbaceous host; herbaceous hosts with symptoms were inoculated from carrots not tested for PYFV by PCR.

Table 16. Differentiation between three PYFV isolates (Skelmersdale) by PCR, 2002

| Expressed symptoms | Primer sets | | | | | |
|---|---|---|---|---|---|---|
| | 2aF/1R | 2F/2R | 2aF/3R | 3F/3R | 4F/4R | Universal |
| Yellowing/necrosis | +w | + | + | + | - | + |
| Yellow/red/stiff (sample1) | - | - | + | +w | - | + |
| Yellow/red/stiff (sample2) | +w | - | +w | - | + | + |

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

The very low levels of virus present in 2002 (at least as detectable by methods used here) were insufficient to consider in-season trends. PCR detected 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 indicated that the PYFV *Anthriscus* strain appears to be highly variable (which correlates with earlier sequencing results).

ELISA for routine detection of PYFV was further modified and developed using antibodies produced in HDC project FV 228 “Carrots: diagnosis of PYFV”. Small quantities of antisera prepared against the parsnip strain of PYFV (P121) and the Anthriscus strain of PYFV (A421), which were produced in the early 1980s, were obtained from SCRI for comparison. Freeze-dried leaf material infected with virus strains A121 and P421 was also obtained and established in culture by mechanical inoculation to *Nicotiana* spp. Samples from these cultures were used as reference strains for subsequent serological and molecular studies. Purification of PYFV was continued to underpin further monoclonal antibody production.

*Summary of Results (2000-2002)*

With each year of the project, improved methods of virus detection were developed and were utilised in subsequent sampling years. In addition, enhanced sampling techniques were implemented during each season, resulting in random samples and those selected for symptoms being separated for testing by the 2002 sampling season. While knowledge and techniques improved dramatically during the course of the project, comparison between years is not appropriate, due to the slightly variable testing and sampling methods used from year to year.

Develop multiplex PCR to detect PYFV and AYV in aphids

*Sequencing of PYFV and AYV*

One sample of cow parsley, identified as being PYFV positive, was found to be infested with willow-carrot aphid and a programme of serial acquisition and transmission experiments was undertaken to extract and isolate both PYFV and AYV. The programme involved approximately 1000 plants, using individual willow-carrot aphids and was successful in isolating AYV from PYFV. Cultures of each virus were established in suitable indicator plants.

DNA alignments were made from the peptide sequences of all the characterised members of the *Sequiviridae*. 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 virus* (parsnip strain), *Rice tungro spherical virus* (RTSV), *Maize chlorotic dwarf virus* (MCDV) and the cowpea severe mosaic *Comovirus* (CPSMV), which was found to be similar to members of the Sequiviridae within the polymerase region.

Following alignment of the polymerase region, areas of sequence homology were identified and eight degenerate PCR primers were designed (four forward and four reverse). The primers designed could be used in combination to amplify between 122 and 527 nucleotides of the polymerase gene (Figure 8; Table 17).

All PCR primer combinations used amplified DNA of the expected size following RT-PCR carried out on total RNA preps from PYFV (*Anthriscus* strain) infected *N. benthamiana* (Figure 9).

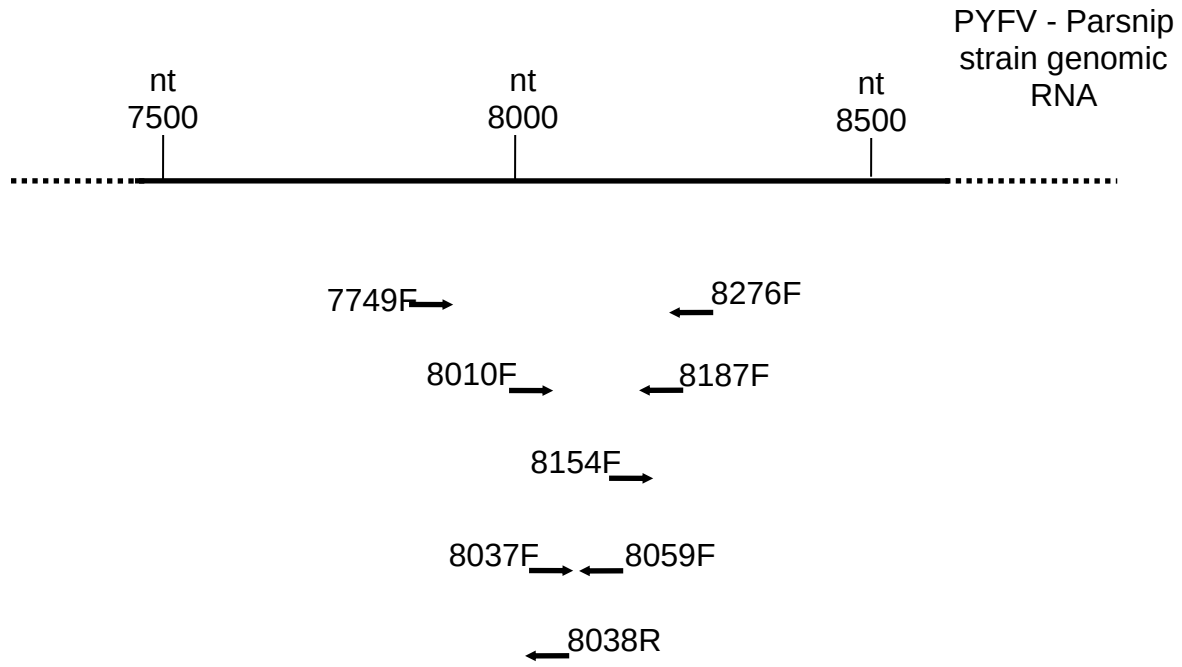



Figure 8. Schematic to illustrate approximate positions of degenerate primers tested for PCR detection of PYFV

Table 17. Primer combinations used to attempt to amplify sections of the PYFV polymerase gene

| Primer set | Forward | Reverse | Size (nt) |
|---|---|---|---|
| A | 7749F | 8276R | 537 |
| B | 7749F | 8187R | 438 |
| C | 7749F | 8038R | 289 |
| D | 7749F | 8059R | 310 |
| E | 8010F | 8276R | 266 |
| F | 8010F | 8187R | 177 |
| G | 8037F | 8276 | 239 |
| H | 8037F | 8187 | 150 |
| I | 8154F | 8276 | 122 |

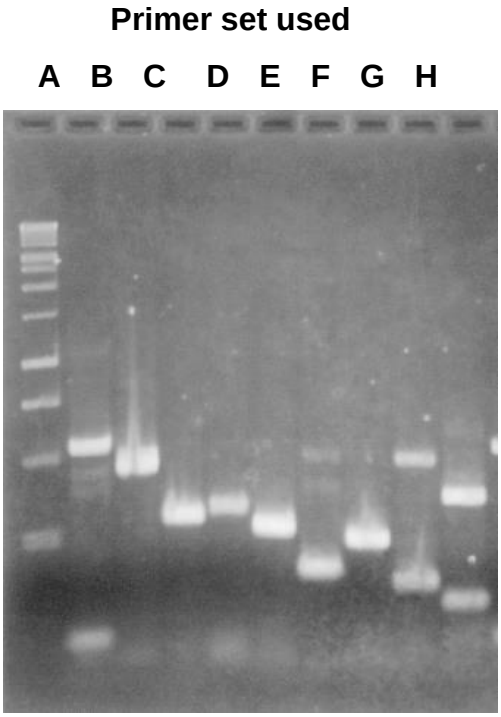



Figure 9. PCR primers combinations used on total RNA preps from PYFV (Anthriscus strain) infected *N. benthamiana*

*Primer and probe design for PYFV and AYV*

Primers for both gel based RT-PCR and also primers and probes for TaqMan PCR were designed to sequence within the polymerase gene for both viruses using Primer Express™ Version 1.0 software (Applied Biosystems, Foster City, CA, USA).

The PYFV primers and probe were designed to the sequence of the isolate 489-H within the most conserved region of the genome, identified from a multiple sequence alignment of twenty PYFV isolates (Table 18). The AYV primers and probe were designed to the sequence of the isolate separated from a mixed infection in cow parsley (C39-12). For both assays, the probes were covalently labelled at the 5´ terminal nucleotide with the 6-carboxyfluorescein (FAM) reporter dye and at the 3´ terminal nucleotide with the tetra-methylcarboxyrhodamine (TAMRA) quencher dye. The sequences of the primers and probes used for virus detection are given in Table 19.

Table 18. Panel of *Parsnip yellow fleck virus* isolates used in the design of primers and probe for the PYFV TaqMan assay

| Isolate | Original host | Isolate | Original host |
|---|---|---|---|
| 489 H-c | Anthriscus | wis-C | Carrot |
| 486 B1 | Anthriscus | V2003614 | Carrot |
| 489 e2 | Anthriscus | 513B | Carrot |
| 486 B4 | Anthriscus | 516b | Carrot |
| 489C1 | Anthriscus | 515a | Carrot |
| A421 | Anthriscus | cv-506 | Celery |
| 527 F | Carrot | 513c | Celery |
| 519 E | Carrot | CV065 | Celery |
| 519 G | Carrot | 508 | Celery |
| V2005207 | Carrot | P121 | Parsnip |

Table 19. Sequence of primers and probes used for the detection of PYFV and AYV.

| PRIMER/PROBE | SEQUENCE 5'-3' | PRIMER LENGTH (BP) |
|---|---|---|
| PYFV-489-H-399F | TGT GAG AGC TTG TGT CTA TGG WGA | 24 |
| PYFV-489-H-485R | TTC GAC ACA GTC TGT AGG TTG TACC | 25 |
| PYFV-489-H-427T* | AAC ATA GTG GCC ATC AAG CAG GAG GTC TT | 29 |
| AYV-C39-12-77F | AGA GAG GGT TTG CCG GAG AT | 20 |
| AYV-C39-12-158R | TTA ACG ACA TTA ACA ATG CTA TGA TAA ATC | 30 |
| AYV-C39-12* | ATG CTA AGT TTG ATG GCA TAG GCA GTG CTG | 30 |

Indicates dual-labelled fluorogenic TaqMan® probe in each set

In addition, TaqMan assays were developed for use as internal controls for both the cytochrome oxidase subunit one (COX 1) gene from plants and the actin gene from

willow-carrot aphid. When the internal controls are used alongside the PYFV and AYV assays it eliminates possible errors caused by false negative results. Both the PYFV and AYV TaqMan assays were optimised to ensure primers were working at their optimum capacity.

*Multiplex and singleplex TaqMan assays*

Following optimisation and limitation, either virus assay can be multiplexed with either of the internal controls, giving robust detection of either PYFV or AYV in either plants or aphids. Alternatively, the AYV and PYFV assays can be multiplexed together to give detection of either virus at the same time.

After primer optimisation, the PYFV assay was tested for specificity against a collection of twenty known PYFV isolates; these included a subset of the isolates used in primer and probe design with the addition of three different PYFV isolates (Table 20). The sequenced region of the PYFV genome was evaluated to determine whether it was conserved across all the known PYFV isolates. Results from this evaluation indicated that the assay could only be used to detect the isolate to which it was originally designed (PYFV 489-H), and a limited number of others, for use under laboratory conditions. It was evident that detection of field isolates was not possible because of the high molecular variability between isolates of the *Anthriscus* strain of PYFV.

Table 20. Results of the PYFV singleplex specificity trial against a range of known isolates, listed in order of reaction.

| ISOLATE | PYFV +ve -/+ | COX 1 +ve -/+ |
|---|---|---|
| 489-H | + | + |
| 519g | + | + |
| 486b1 | + | + |
| 513c | + | + |
| cv-506 | + | + |
| A421 | + | + |
| P121 | - | + |
| 519E | - | + |
| 515a | - | + |
| 527F | - | + |
| 516b | - | + |
| 508 | - | + |
| 489c1 | - | + |
| 513b | - | + |
| cv065 | - | + |
| 4242** | - | + |
| 518A** | - | + |
| V2005207* | - | - |
| v2003614* | - | - |
| 4266* | - | - |

Dark grey and light grey cells signify isolates that were strongly and weakly positive to the PYFV assay respectively. Uncoloured cells signify isolates that were undetected by the PYFV assay. * indicate those isolates that were tested for PYFV but were negative to the internal cytochrome oxidase control; ** indicate PYFV isolates that at the time of testing had not been sequenced, but were undetected by the PYFV assay.

Extended analysis of the specificity trial results was undertaken, utilising an alignment of the known nucleotide sequences of the PYFV isolates above, to determine whether the number of oligonucleotide base pair mismatches correlated with the specificity of the PYFV assay (Table 21). For example, would an isolate with a low number of mismatches correlate with a positive result and conversely, would an isolate with a high number of mismatches correlate with a negative result?

Table 21. Summary of the base pair mismatches of the range of tested PYFV isolates (excluding those that tested negative to the internal control).

| ISOLATE | F PRIMER | | PROBE | R PRIMER | | TOTAL No. OF MISMATCHES |
|---|---|---|---|---|---|---|
| | ENTIRE PRIMER | 3'END | ENTIRE PROBE | ENTIRE PRIMER | 3' END | |
| 489-H | 0 | 0 | 0 | 0 | 0 | 0 |
| 519g | 1 | 0 | 0 | 2 | 1 | 3 |
| 486b1 | 1 | 0 | 0 | 3 | 2 | 4 |
| 513c | 4 | 1 | 5 | 7 | 2 | 16 |

| | | | | | | |
|---|---|---|---|---|---|---|
| A421 | 6 | 2 | 5 | 5 | 2 | 16 |
| cv-506 | 3 | 1 | 9 | 5 | 3 | 17 |
| | | | | | | |
| 489c1 | 1 | 0 | 2 | 3 | 2 | 6 |
| 519E | 1 | 0 | 4 | 1 | 0 | 6 |
| 515a | 0 | 0 | 6 | 4 | 2 | 10 |
| 527F | 3 | 2 | 4 | 4 | 0 | 11 |
| 516b | 3 | 1 | 4 | 4 | 0 | 11 |
| 508 | 5 | 1 | 5 | 5 | 3 | 15 |
| P121 | 5 | 1 | 5 | 5 | 3 | 15 |
| 513B | 4 | 1 | 5 | 7 | 2 | 16 |
| cv065 | 5 | 1 | 9 | 5 | 3 | 19 |

Shaded cells indicate isolates detected by the PYFV assay. The data signify the number of oligonucleotide base pair mismatches compared to the PYFV primer and probe sequences, revealed by the alignment of nucleotide sequences of PYFV isolates.

Both 489-H and 519g isolates were strongly detected by the PYFV assay. No mismatches were found in the primer and probe sequences of PYFV 489-H as they were specifically designed to this isolate and only three mismatches were identified in those of 519g. Isolate 486b1 was weakly detected, but also had a low number of mismatches, while the remaining three isolates, also weakly detected, all had a high number of mismatches. In contrast, some isolates with a few mismatches were not detected at all. Other than the most strongly detected isolates, 489-H and 519g, there was no obvious correlation in the number of mismatches and positive detection of virus; indeed, three of those isolates detected had some of the highest number of mismatches. It has therefore not been possible to apply a fixed set of rules to the explanation for undetected sequences that are closely related.

*AYV and COX 1 multiplex assay*

As AYV in plant material was regularly available throughout the project, the AYV assay was multiplexed with the COX 1 internal control. Each assay was subjected to a primer optimisation matrix and the optimum concentration selected in each case. The COX 1 assay was also subjected to primer limitation to allow multiplexing with the AYV assay. Comparisons were then made with the AYV/COX 1 assay to ensure that they performed equally well in multiplex as well as in simplex formats. The resulting multiplex assay was used to detect virus in all subsequent acquisition and transmission experiments.

After the AYV and COX 1 assays were multiplexed, reagent and labour costs were reduced to a minimum, as just two rather than three reactions were required to detect both viruses and the internal control. Thus far, TaqMan technology does not allow multiplexing of more than two assays due to the limited availability of reporter dyes. However, with further optimisation alternative assays may be developed by multiplexing the AYV and PYFV assays to detect mixed infections or by multiplexing the PYFV assay with the COX 1 internal control when only PYFV detection is required.

*PYFV singleplex assay*

The fact that very few other isolates were detected demonstrated that the PYFV region sequenced thus far is highly variable. This limitation did not restrict the use of the assay in the laboratory as the primers and probes were specifically designed to the sequence of the key isolate in use. Furthermore, this assay could be the foundation for further developments in PYFV detection, whereby additional sequencing of the PYFV genome may reveal a more conserved region to which new primers and probes may be designed, widening the scope of the assay and increasing the range of isolates detected. Alternatively, using a combined approach, multiple assays may be developed for the detection of all the isolates sequenced for use in a single 'cocktail' assay.

*Sybr Green assay*

Further investigations into the potential for increasing the range of PYFV isolates detected, using the intercalating dye 'SYBR® Green' were undertaken, as a possible alternative to the TaqMan® chemistry. The fluorescent nature of the dye negates the need for expensive probes. While primer dimer (primers annealing to themselves) detection can be a problem with this method, using melting curve analysis enables amplified products to be distinguished by their melting characteristics (Figure 10).

Investigations were halted after validation trials produced anomalous results, with uninfected plant RNA extracts producing peaks normally associated with PYFV-infected material.

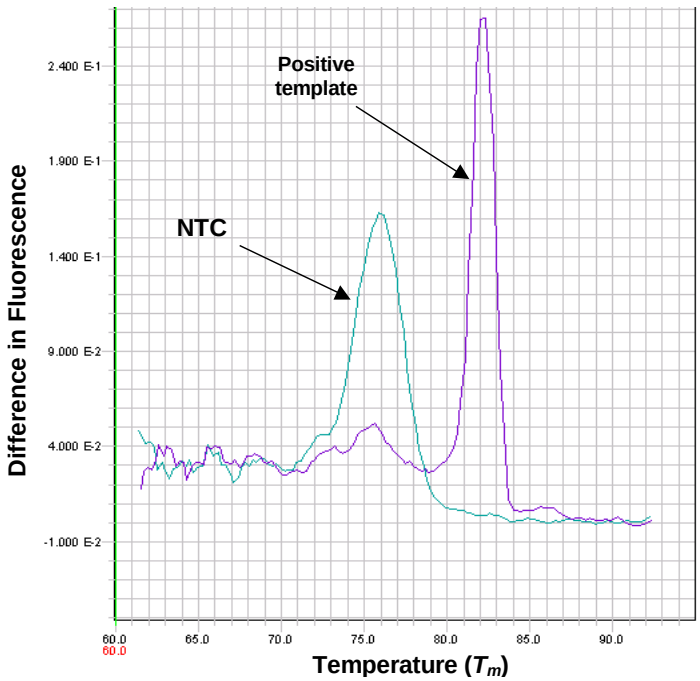



Figure 10. Melting curve analysis for a positive template and NTC (blank) reaction in a SYBR® Green assay. A single, low temperature peak (blue) indicates detection

of primer dimers produced in the NTC reaction. Double peaks, one at low and one at high $T_m$ (purple) indicate detection of primer dimers and product in a positive template reaction.

*Detection of virus in individual aphids*

The sensitivity of the two virus assays (using the actin internal control) was especially highlighted by experiments in which both AYV and PYFV were detected in individual viruliferous aphids (data not shown).

Aphids caught in water traps in 2001 and 2002 were stored at CSL. Sub-samples of the aphids were chosen randomly from both years, with the aim of detecting AYV and PYFV utilising TaqMan PCR. Initial analysis focused on the detection of RNA, as an indicator of the presence of virus, however, RNA could not be identified in any of the samples tested.

An extended laboratory study was undertaken to determine the effect of storage solution on RNA extraction. Aphids were stored in five solutions, distilled water, 70 and 100% ethanol, 50% methanol and RNA Later, for one, two or six weeks and the presence of RNA determined using TaqMan PCR. Results indicated that either 70 or 100% ethanol were the best storage solutions with RNA extracted from all vectors tested (Figure 11). RNA Later and 50% methanol were less effective, and no RNA was detected from vectors stored in distilled water.

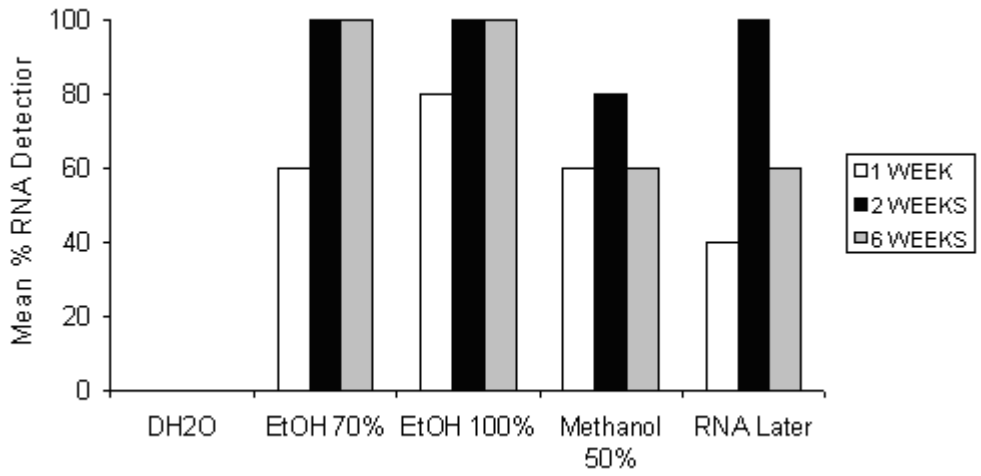



Figure 11. Effect of storage solution and duration on RNA extract from viruliferous aphids

*Conclusions*

The development of new TaqMan assays for the detection of both AYV and PYFV in plant material proved invaluable for laboratory testing of plant material. Confirmation of infection in plant material with either virus was obtained conclusively within a short time prior to using source plants for vector transmission

experiments, even when virus titre was very low. Although the assays were not designed for quantitative analyses, it was possible to extend analyses of results to determine the 'best' plants for use in these experiments where source plants with a high virus titre were desirable.

SYBR® Green has clear uses as an alternative to PCR and TaqMan® detection assays, confirming the efficacy of specific primer sets and negating the need for labour intensive post-PCR manipulations and expensive probes (Shu *et al*., 2003). However, the complexity of PYFV with its high variability between isolates, and resource (time) constraints within the project, prevented further SYBR® Green method development for the detection of PYFV.

The AYV and PYFV TaqMan assays are sensitive enough to detect virus in individual aphids but samples must be stored in a suitable preservative to ensure against RNA degradation.

Investigate the molecular variability of PYFV and AYV strains

Small sections of the helicase domain (B = 179nt) and the polymerase gene (A = 450nt) of the PYFV genome were amplified (in 2000 and 2001, respectively) using PCR and degenerate primers designed to closely related viruses (Figure 12). In each case, the PCR product was cloned and sequenced. Using primers designed within the polymerase gene and the helicase gene, in 2002, long range RT-PCR was used to amplify the region between these two conserved domains (C = 3500nt). The resulting PCR products were cloned and sequence was generated for the clones, giving approximately 4000nt of sequence in total.

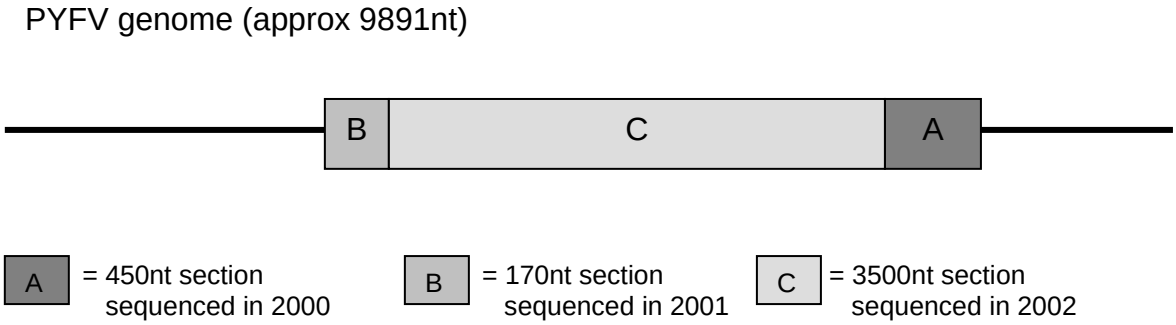



Figure 12. Diagrammatic representation of the PYFV genome and regions sequenced (boxed)

For AYV, further primers were designed within the region sequenced in 2000, and a technique known as 3 ′ RACE (Rapid Amplification of cDNA Ends) was employed to amplify the region between the poly-A tail (present in AYV but not PYFV) and the polymerase region already sequenced. The resulting PCR product was cloned and sequenced, resulting in the elucidation of a further 1673nt of the AYV genome, giving approximately 2000nt of sequence in total (Figure 13).

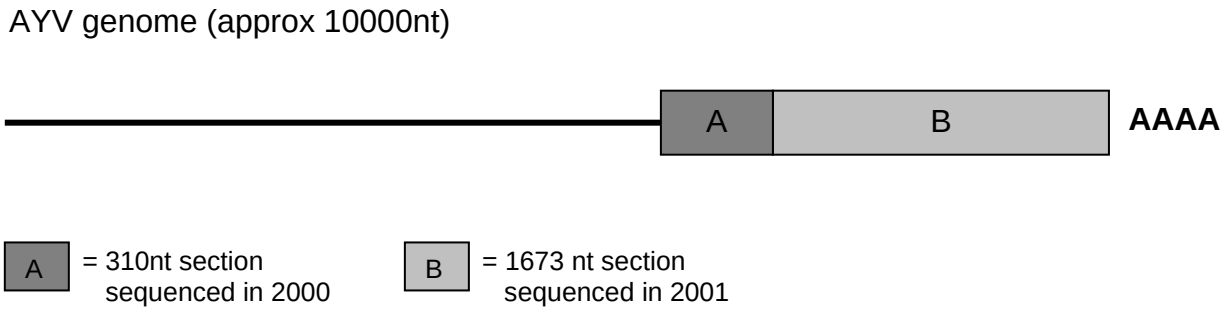



Figure 13. Diagrammatic representation of the AYV genome and regions sequenced (boxed)

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, the AYV isolate (isolated on Chervil) was amplified. The PCR products were sequenced, aligned and a cladogram of the putative translation products developed (Figure 14).

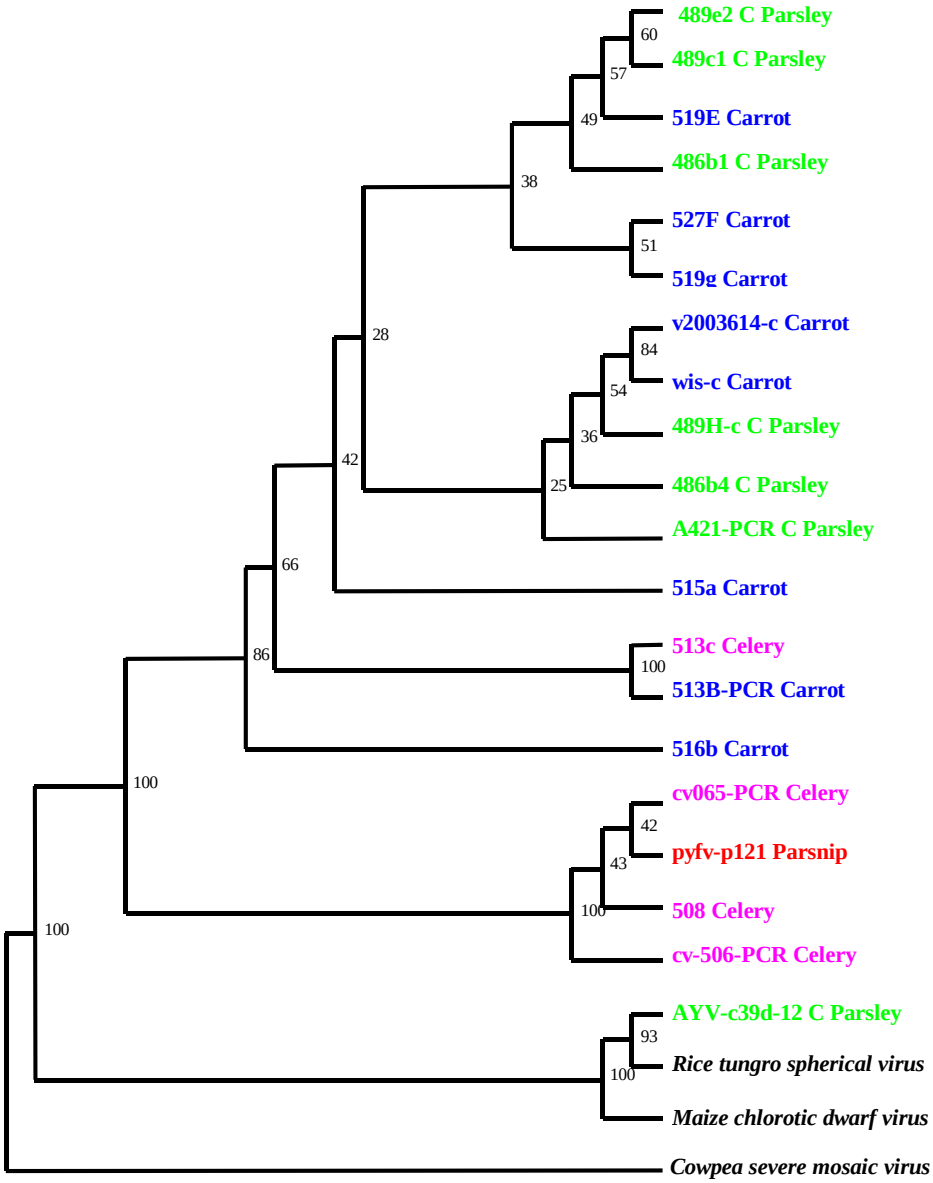



Figure 14. Cladogram showing the clustering of the translation products for a number of isolates of PYFV, and isolate of AYV and the other members of the *Sequiviridae.* Numbers at the forks indicate the number of times the group consisting of the species which are to the right of that fork occurred out of 100 trees, following bootstrap re-sampling.

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%). The amino acid identity between these two clusters was between 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 highly variable.

Determine virus acquisition and transmission efficiency by aphid vectors under controlled conditions

*Establish cultures of key aphid species*

While there is evidence that other aphid species, such as *C. pastinacae*, *C. theobaldi* and *A. pisum* are able to transmit PYFV and AYV (Van Dijk & Bos, 1989), their transmission efficiency is low. In addition, as they do not feed on carrot or cow parsley in the field (Börner, 1952; Van Dijk & Bos, 1985), they are not regarded as significant vectors of PYFV in carrot crops (Van Dijk & Bos, 1985). Acquisition and transmission studies on the PYFV system used willow-carrot aphid as the primary vector.

Non-viruliferous willow-carrot aphids were supplied by the Institute of Virology and Environmental Mycology (IVEM), Centre of Ecology and Hydrology, Oxford. They were maintained in culture on wild carrot in a glasshouse at 17ºC ± 2ºC with a 16-hour photoperiod, supplemented by 400-watt daylight bulbs in Holophane® lamps when necessary. Fresh host plants grown to the three-to-four true leaf growth stage were added every two to three weeks, at which time dead plants were removed and discarded. Adult alatae were used for all transmission studies, while mixed life stages were used in virus maintenance. Cultures were maintained at high density to promote the development of alatae.

*Allow aphids to feed on infected plants for varying times under different temperature regimes and determine minimum acquisition time*

Elnagar and Murants' (1976a) extensive experiments made important advances in the understanding of the virus-vector interaction in the PYFV system. The developments in molecular diagnostic tool design achieved in this study facilitated further development of the findings of Elnagar and Murants' (1976a) study of the virus-vector relationship, in particular the effect of temperature on transmission frequency. Thus, as AYV is pivotal to the transmission of PYFV, this component of the PYFV system was investigated in preliminary studies.

Laboratory investigations were undertaken to assess the efficiency of acquisition of AYV by willow-carrot aphid, using four different acquisition times (two min., 10 min., 30 min., and 24 hours) under three different temperature regimes (10ºC, 15ºC and 20ºC).

For each AAP exposure, test aphids were confined on AYV-infected source plants at the selected test temperature and timed for the selected AAP. Immediately after exposure to virus at the test AAP, viruliferous aphids were transferred to chervil test seedlings, five per seedling, caged with an aphid-proof Perspex tube and maintained at 18ºC ± 2ºC in a glasshouse for a standard 48 hours IAP. Following this standard IAP, the Perspex tubes were removed and the seedlings were sprayed with a systemic insecticide ('Rapid', a.i. $1gl^{-1}$ pirimicarb, in aerosol form, Syngenta) to remove all aphids. Treated seedlings were maintained at 18ºC ± 2ºC in a glasshouse for approximately three weeks post transmission until they were sampled for testing.

Approximately 0.1g of leaf material was collected (from at least three different parts of each plant to allow for any variation in virus dispersal) for extraction. Following the CTAB RNA extraction method adapted from Chang *et al*. (1993), the resulting

RNA preparations were tested for AYV using the AYV/COX 1 multiplex TaqMan® assay. This procedure was repeated for each test AAP exposure at each test temperature.

*Results*

The interaction between AAP and temperature was significant in its effect on aphid acquisition efficiency, indicated by the proportion of AYV-infected chervil test seedlings (d.f. = 6, 35, f = 3.04, $p < 0.01$). The length of time test aphids were allowed to feed on infected source plants (AAP) and the temperature at feeding were found to be independently highly significant in their effect on the efficiency of test aphids to acquire virus (d.f = 3, 35, f = 25.53, $p < 0.001$ and d.f = 2, 35, f = 10.78, $p < 0.001$ respectively).

At 10ºC, acquisition efficiency of willow-carrot aphid is low, irrespective of AAP (Figure 15). At this temperature, aphids required more than two minutes feeding time on AYV source plants to acquire virus, as indicated by the low proportion of AYV-infected chervil test plants. There was little difference in acquisition efficiency between 10-minute, 30-minute and 24-hour AAP, but it was significantly higher following a 24-hour AAP than after two minutes. After 24 hours AAP, transmission efficiency was recorded at 40%.

At 15ºC, acquisition feeding times of two, 10 and 30 minutes resulted in a similar percentage of seedlings becoming AYV-infected (<30% virus transmission), indicating that acquisition efficiency was low. However there was a significant difference after 24 hours when 94% virus transmission was recorded. At 20ºC, acquisition efficiency remained low when aphids were given two and 10 minute AAPs, but after 30 minutes and 24 hours, efficiency significantly increased with the percentage of AYV-infected chervil seedlings, recorded at 65 and 100% respectively.

Figure 16 highlights differences between AAPs, irrespective of the effects of temperature. Aphid acquisition efficiency increased with longer feeding periods on virus source plants, but was little affected by short AAPs. Aphid virus acquisition efficiency was less than 15% when given two or 10 minute AAPs, but increased significantly after acquisition feeds of 30 minutes and 24 hours with 36% and 81% seedlings infected respectively.

While an increase in temperature at acquisition feeding increases the efficiency of AYV acquisition by willow-carrot aphid, the relationship is not linear (Figure 17). The percentage of AYV-infected seedlings was significantly lower at a feeding temperature of 10ºC (17%), than at 15 or 20ºC, but a significant difference was not detected between the latter two temperatures, with 36% and 45% of the seedlings infected respectively.

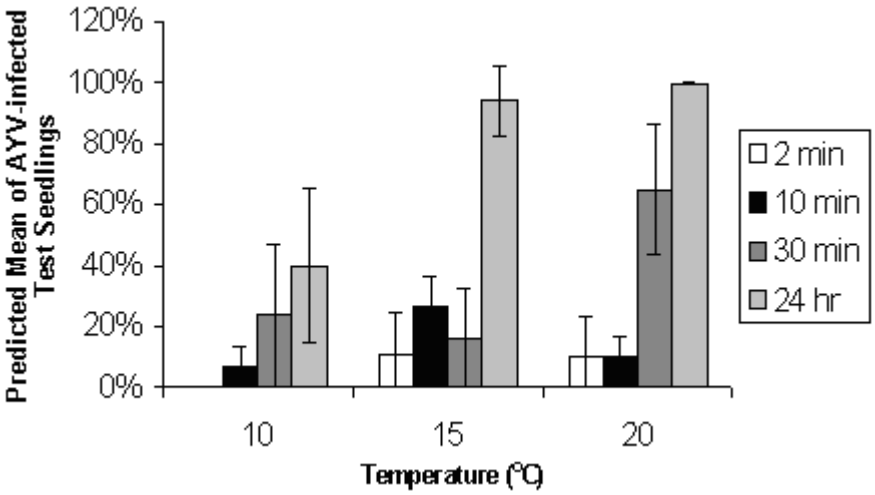



Figure 15. The efficiency of AYV acquisition by willow-carrot aphid alatae at different temperatures and acquisition access periods (AAP). Bars represent ± 2 SE at 95% confidence levels.

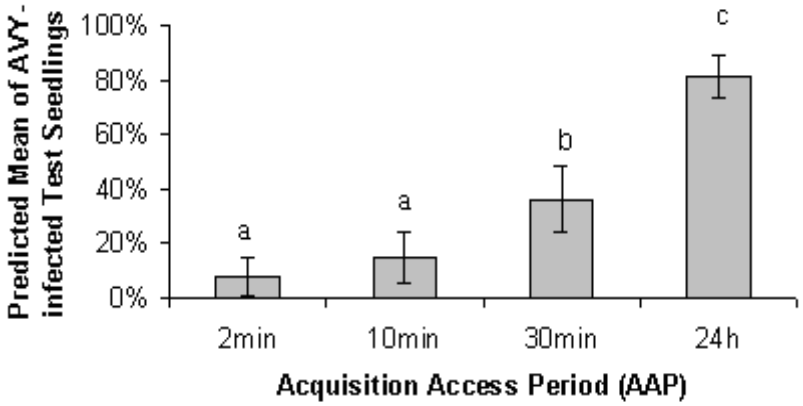



Figure 16. The effect of AAP on aphid acquisition efficiency, regardless of the effect of temperature. Means represented by bars with the same superscript letter do not differ significantly. Bars represent ± 2 SE at 95% confidence levels.

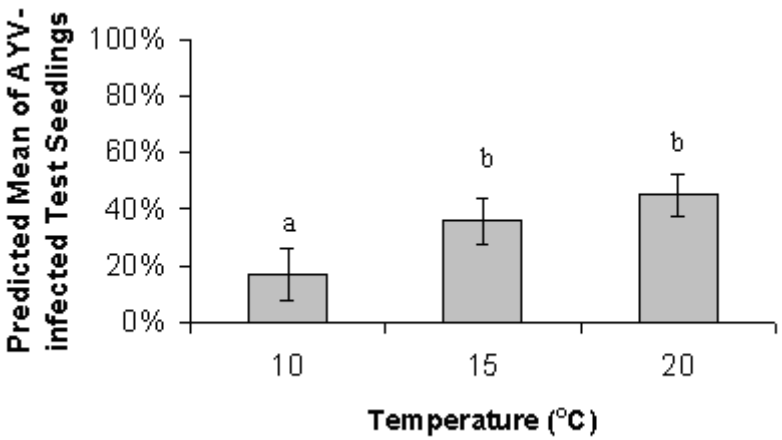



Figure 17. The effect of temperature at the time of the acquisition feed on aphid efficiency to acquire AYV, irrespective of the effect of AAP. Means represented by bars with the same superscript letter do not differ significantly. Bars represent ± 2 SE at 95% confidence levels.

*Allow aphids to feed on infected plants for varying times under different temperature regimes and determine minimum acquisition time*

Laboratory investigations were undertaken to assess the efficiency of transmission of AYV by willow-carrot aphid, using four different transmission times (two min., 10 min., 30 min., and 24 hours) under three different temperature regimes (10ºC, 15ºC and 20ºC).

In all transmission experiments, test aphids were confined on AYV-infected source plants for a standard 24 hours AAP at 18ºC. Immediately after exposure to virus at the standard AAP, viruliferous aphids were transferred to individual chervil test seedlings, five per seedling, caged with an aphid-proof Perspex tube for the selected IAP and maintained at the selected temperature regime. Following the selected IAP, the Perspex tubes were removed and the seedlings were sprayed with a systemic insecticide ('Rapid', a.i. $1gl^{-1}$ pirimicarb, in aerosol form, Syngenta) to remove all aphids. Treated seedlings were maintained at 18ºC ± 2ºC in a glasshouse for approximately three weeks post transmission until they were sampled for testing, as above.

*Results*

The interaction between IAP and temperature had a significant effect on aphid transmission efficiency (d.f. = 6, 35, f = 3.51, $p < 0.01$). The length of time viruliferous test aphids were allowed to feed on healthy chervil seedlings (IAP) and the temperature at feeding, both independently exerted highly significant effects on the efficiency of aphids to transmit AYV (d.f = 3, 35, f = 74.94, $p < 0.001$ and d.f = 2, 35, f = 14.04, $p < 0.001$ respectively).

At lower transmission feeding temperatures, a smaller percentage of test seedlings were infected with AYV (Figure 18) following an IAP of 2-30 minutes, indicating low transmission efficiency in the test aphids. However, after 24 hours IAP, plant infection was very similar at all three test temperatures, with the percentage of

infected seedlings reaching or exceeding 90%. Overall, the percentage of seedlings infected with AYV and therefore aphid transmission efficiency increases with temperature and IAP, with optimal transmission at 20ºC after transmission feeding times of 30 minutes to 24 hours (percentage of seedlings infected ≥ 90%).

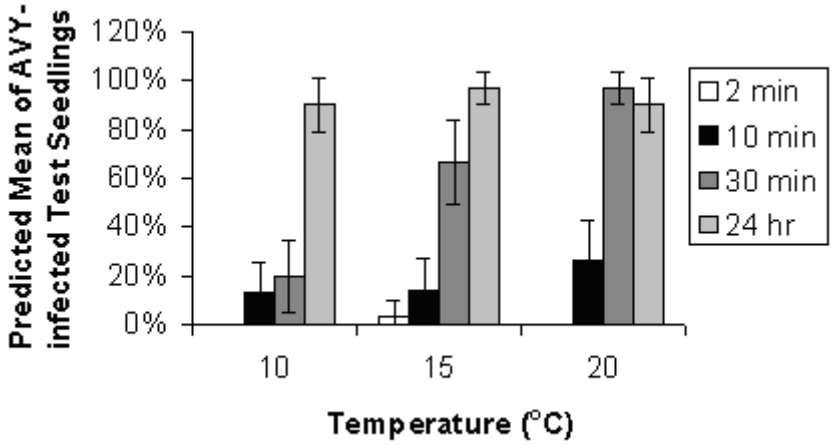



Figure 18. The efficiency of AYV transmission by willow-carrot aphid alatae at different temperatures and inoculation access periods (IAP). Bars represent ± 2 SE at 95% confidence levels.

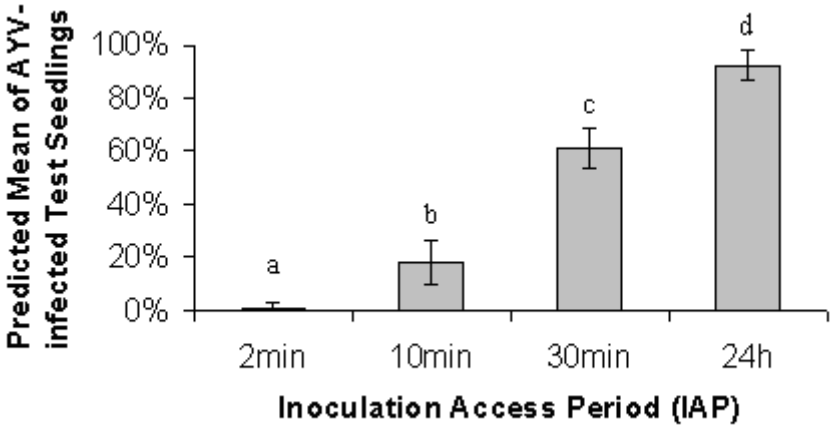



Figure 19. The effect of IAP on aphid transmission efficiency, regardless of the effect of temperature. Means represented by bars with the same superscript letter do not differ significantly. Bars represent ± 2 SE at 95% confidence levels.

Disregarding the effect of temperature, the IAP exerted a highly significant effect on aphid transmission efficiency, with the percentage of AYV-infected seedlings increasing significantly with each IAP, from 1% after 2 minutes to 92% after 24 hours (Figure 19).

While aphid transmission efficiency increases with temperature (Figure 20), this factor is less influential than IAP. The predicted mean percentage of infected

seedlings was significantly lower at 10ºC than at 15ºC or 20ºC with little difference in the effect of transmission efficiency recorded between the latter two temperatures.

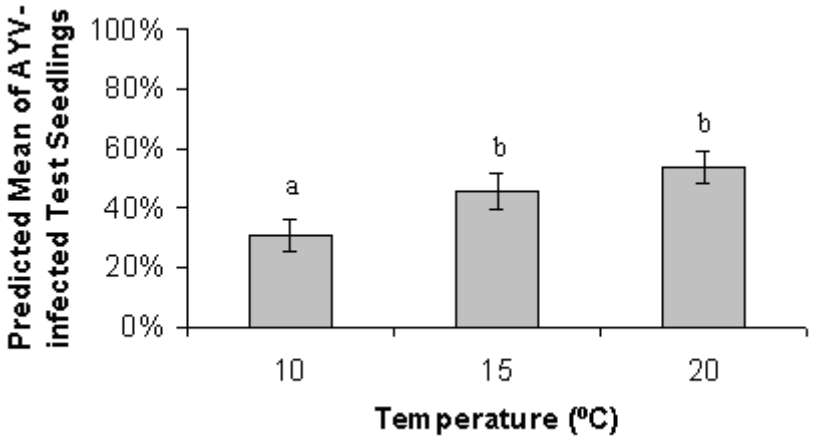



Figure 20. The effect of temperature on aphid transmission efficiency, regardless of the effect of IAP. Means represented by bars with the same superscript letter do not differ significantly. Bars represent ± 2 SE at 95% confidence levels.

*Discussion and conclusions*

The data from these preliminary experiments illustrate that the helper virus can be acquired and transmitted by willow-carrot aphid alatae after only two minute feeding periods at temperatures as low as 15ºC, but efficiency is low. Maximum virus acquisition efficiency was reached by 24 hours AAP at temperatures equal to or above 15ºC. Maximum virus transmission efficiency was reached by 24 hours (at 10-15ºC) or 30 minutes (20ºC).

Aphid feeding periods for both virus acquisition and transmission appear to be more influential than temperature. AAPs of 30 minutes or less yielded estimated percentages of infected test seedlings of 36% or less but aphid acquisition efficiency increased significantly by 24 hours (>80%). Again, a similar pattern was observed with differing IAPs. Short IAPs of 10 minutes and under yielded less than 20% estimated percentages of infected test seedlings, with significant increases observed from 30 minutes to 24 hours (61-92%).

These data partly support and extend the work of Elnagar and Murant (1976a) who concluded that the frequency of transmission increased with increasing AAP and that the percentage of test plants infected with AYV increased with longer IAP. During their investigations, they found the minimum AAP required for willow-carrot aphid to acquire AYV was 15 minutes (instead of two minutes as observed above), and the minimum IAP was as short as two minutes (mirrored by the above results). Conclusions drawn from this earlier virus transmission study indicated that the resulting minimum AAP data correlated with distribution of the virus in leaf tissue, described in subsequent work in which they used ultra-violet irradiation experiments to determine that AYV is confined to deep-lying plant tissue (Elnagar & Murant, 1976b). The longer feeding periods required to acquire AYV in Elnagar and

Murants' study indicated that aphids may need longer to probe for the deeper-lying phloem tissues. While the results from the above AYV *transmission* experiments corroborate the findings of Elnagar and Murant (1976a), those from the AYV *acquisition* experiments differ. Acquisition of AYV occurred after short acquisition feeds of two and ten minutes. Thus, contrary to the conclusion of Elnagar and Murant (1976a), that AYV is concentrated in deep-lying tissue and PYFV is distributed throughout the leaf, this study may indicate that AYV is not confined to the vascular regions but is also distributed throughout the leaf, like PYFV.

For both the AYV acquisition and transmission studies reported above, all test seedlings were infested with five aphids per seedling and the resulting data indicate the efficiency of acquisition or transmission of AYV by alate willow-carrot aphids. To determine the *probability* of transmission of AYV by *individual* aphids, further studies would be necessary, repeating the general method above, but giving *single* viruliferous aphids the opportunity to transmit virus to test seedlings.

If the results from the current study were extrapolated to the field situation, it would appear that the greatest risk of virus spread would be posed by aphids that spend up to 24 hours on their acquisition feeds and subsequently more than 30 minutes on their transmission feeds. However, use of data from controlled laboratory experiments to measure transmission efficiency of aphid vectors flying under field conditions should be treated with caution. As groups of five aphids were used in both the controlled laboratory acquisition and transmission experiments to increase the probability of infection, the resulting data may result in an artificially high vector efficiency. During the experimental acquisition and transmission feeds, especially the shorter periods, not all aphids were feeding or probing continuously. Conversely, individuals that probed or fed for longer periods were unnaturally interrupted when the timed feeding periods were terminated. Therefore, as well as using laboratory data to estimate vector efficiency, further trials should be undertaken to establish vector *propensity* (the probability of a vector to inoculate a plant with a virus under field conditions (Irwin & Ruesink, 1986; Perez *et al.*, 1995)) of virus in open-field surveys.

Time constraints and limited availability of both willow-carrot aphid alatae and PYFV source plants prevented further investigations in similar comparative experiments with PYFV. Indeed, sample numbers differed slightly between the acquisition and transmission trials above due to restricted supplies of alatae. During the summer months, willow-carrot aphid cultures inexplicably failed and the production of alatae was radically reduced, and re-establishment of thriving cultures occurred over an extended period.

The maintenance of PYFV in laboratory cultures was consistently problematic, with stocks of PYFV alone or PYFV and AYV in complex periodically failing, a phenomenon experienced in other contemporary laboratories. Repeatedly, stock plants infected with both viruses and subsequently used as source plants for vector transmissions in virus maintenance, yielded source plants with AYV only. This occurrence is not exclusive to the PYFV system. It has been observed elsewhere that when laboratory plants were infected with PYFV and Italian carnation mottle and after repeated passages, PYFV was eventually lost, although symptom expression remained unchanged. In each case, the most virulent virus flourished. Watson *et al*., (1964) observed attenuation of motley dwarf in field isolates that

caused severe stunting in carrots but became less virulent after several months of glasshouse sub-culturing.

It appears that for naturally occurring PYFV to survive, infections of PYFV/AYV in host plants must maintain a balance, such that PYFV never totally disappears from a local area, a theory perhaps supported by Elnagar and Murant (1976a) who suggest that infection with either virus is unaffected by the presence of the other. While the experience of this study is that in chervil AYV out-competes PYFV in mixed infections in individual plants, the two viruses have evolved to co-exist naturally in cow parsley and other biennial or perennial non-crop hosts. Experience in maintaining laboratory cultures of AYV (singly) and AYV/PYFV in complex suggests that AYV replicates much more efficiently than PYFV. Following this assumption, and considering that PYFV relies exclusively upon AYV for successful transmission, the loss of PYFV in laboratory cultures may have been caused by the accumulative affect of AYV being vectored far more efficiently that PYFV. After several vector transmissions, with fewer PYFV particles on each occasion, a smaller reservoir of virus would be available, thus resulting in the eventual total loss of PYFV but high titre of AYV.

While the maintenance of laboratory isolates of PYFV was problematic, field isolates were occasionally made available through the submission of samples from carrot growers, and these were subsequently cultured in laboratory test plants. However, difficulties arose in the method of virus detection. The TaqMan® assays were designed specifically to the isolates used within laboratory experiments. Due to the unusually high variability discovered between isolates, these assays were inappropriate for use in testing field isolates. The only alternative method of detection was by conventional PCR, which is highly labour intensive, more open to risk of contamination and not suited to the high sample throughput encountered after acquisition and transmission trials. Investigations focused upon the helper virus, with a view to extending trials to PYFV should an adequate source become available.

In conclusion, as the transmission of PYFV may only take place *after* the acquisition of AYV, and because carrot crops have immunity to AYV, reservoirs of the helper virus in the field are predominantly restricted to the non-crop hosts found in headlands and hedgerows. It appears that while a greater risk of virus spread will come from alatae feeding for periods of up to 24 hours at relatively high temperatures (e.g. 20ºC), aphids exposed to shorter feeding times and lower temperatures still have the ability to acquire and transmit AYV, thus maintaining at least a low level of virus pressure. Data from both the AYV acquisition and transmission experiments clearly show that the acquisition and transmission efficiency of willow-carrot aphid alatae is highly influenced by temperature and AAP/IAP.

*Objective 3. Develop a prototype strategy that will allow growers to implement sustainable management of PYFV and its vectors*

<u>Undertake laboratory assays on the effects of some pesticide groups on vector behaviour and virus spread.</u>

*Pesticide efficacy against aphid vectors*

In order to develop a rational management strategy for this disease, it is necessary to understand the effects of the main pesticides used against the willow-carrot aphid. Quantification of the topical and residual efficacy of the commonly used products will provide growers with further useful information when considering which insecticide is best for their disease management strategy.

A pilot study was undertaken in which three insecticides, selected from the Defra Pesticide Usage Survey (Garthwaite *et al*, 2001) were identified as the most commonly used in umbelliferous crops: Aphox (pirimicarb), Hallmark (λ-cyhalothrin) and Dovetail (pirimicarb and λ-cyhalothrin) and were used in preliminary bioassays to determine dose response of alate willow-carrot aphids.

Leaf discs of parsnip were dipped into solutions of known pesticide concentration (a range from 200 to 0.5% of the recommended field rate) or deionised water (control) and five aphids were caged onto each treated leaf disc. Aphid mortality was assessed after 24 and 48 hours

Dilutions were expressed as a percentage of the recommended field rate. Concentrations of 200, 100, 50, 25, 20, 10, 2.5, 1 and 0.5% were used with Aphox; concentrations of 200, 100, 40, 20, 10, 5, 2, 1% were used with Hallmark and concentrations of 200, 100, 50, 25% with Dovetail.

All products were highly successful in controlling willow-carrot aphids. Results indicated that when using Aphox at 25% the recommended field rate, aphid mortality was recorded at 82% after 24 hours exposure to treated leaf material, increasing to 100% after 48 hours. Similarly, when using Dovetail at 25% of the recommended field rate, aphid mortality was recorded at 100% after 24 hours. Hallmark was particularly effective, causing 100% aphid mortality at just 10% of the recommended field rate.

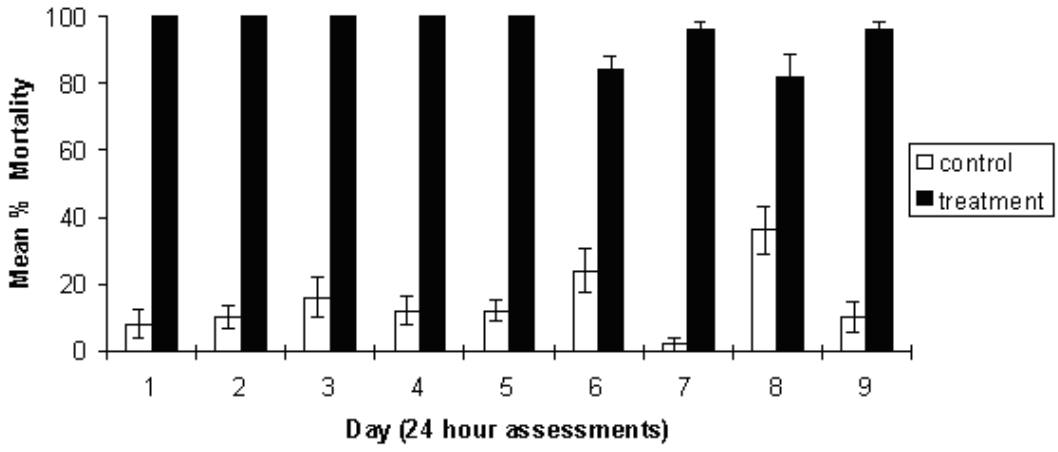



Figure 21. Residual efficacy of Dovetail against alate willow-carrot aphid alatae

During these early studies, a second bioassay was undertaken to investigate the temporal effect on insecticide residue, using alate willow-carrot aphids. Leaf discs were dipped into a solution of insecticide diluted to the recommended field rate or deionised water (control) and divided into subsets, each allowed to age from 0-9 days. Five aphids were caged onto treated leaf discs sequentially for a period of

nine days. Aphid mortality was assessed 24 hours after they had been caged onto the leaf discs. Results showed that the product provided excellent aphid control up to nine days post application (Figure 21).

A follow up to this pilot study took place under revised milestone 3R (see below).

Revisions to milestones (end 2002/2003)

At the end of project year 2002/03 the outstanding objectives were revised and the efforts in the final year of the project were targeted towards the following new objectives:

*1R. Correlate disease symptoms with virus presence*

*2R. Investigate the relationship between PYFV in cultivated crops and weedy hosts*

*3R. Control PYFV utilising rational vector management*

*Objective 1R. Correlate disease symptoms with virus presence;*

*Objective 2R. Investigate the relationship between PYFV in cultivated and weedy hosts*

*Introduction*

Objectives 1R and 2R are interconnected and were addressed together. Although the focus remained upon PYFV, where appropriate, samples were also tested for the three components of the carrot motley dwarf complex and for AYV the helper virus for PYFV.

*Materials and methods*

All testing was by PCR. Primers used at HRI are listed in Table 22. The same primers were used for testing at CSL except for carrot red leaf virus. Plant samples were used fresh or stored frozen at –70°C until required. RNA extractions used commercial procedures (at HRI Qiagen Rneasy; at CSL an automated system based on magnetic beads was used). RNA extracts in Rnase free water were stored frozen at –70°C until tested. Reverse transcription and PCR testing used standard procedures and reactions analysed by electrophoresis on agarose gels. Bands in ethidium bromide stained gels were normally scored as clearly positive or negative; some samples gave very weak bands and were scored as possibly positive.

All samples of cow parsley were tested for PYFV, *Carrot red leaf virus* (CRLV) and AYV. All carrot samples were also tested for CRLV and PYFV but as AYV does not infect this host, these samples were not tested for this virus. Carrot samples positive for CRLV were also tested for *Carrot mottle virus* (CmoV) and carrot redleaf associated RNA (CRLaRNA), which are dependent on CRLV for aphid transmission. To determine whether the latter two viruses were transmitted in the wild without transmission of CRLV, a proportion of samples apparently free of CRLV were also tested for these two viruses.

The majority of positive reactions were further analysed by sequencing complete amplicons (without cloning) using standard procedures (Big Dye; ABI) and automated sequencers. Sequences were aligned and compared using the DNAstar suite of programmes.

Table 22. Primers used during PCR testing

| Virus | Primer | Sequence 5'-3' |
|---|---|---|
| CRLV[1] | F(807) | GAG GTG AGA AAT CGC YTG AC |
| | R (808) | MGG CGC CAC ART GAT AGG |
| Annealing temperature used 52°C; Mg concentration 1.5 Mm; product 211 bp | | |
| CmoV[1] | F (896) | TGG WGT ICA CAA CAA CTC |
| | R (898) | AAG GCT TTG TAC AAC ATT GG |
| Annealing temperature used 52°C; Mg concentration 3 mM; product 408 bp | | |
| CRLaRNA | F (893) | TCT AGT TTC TCT CAA GTT CCA |
| | R (895) | CCT CAC CTR CCA ATT ATG G |
| Annealing temperature used 52°C; Mg concentration 1.5 mM; product 486 bp | | |
| PYFV[3] | F (856) | GCI AAY TTY GAY GGI ATG TTY CAY CC |
| | R (857) | TCM GCR TAI ACR CAI GCY CTI ACA |
| Annealing temperature 56°C; Mg concentration 1.5 mM; product 280 bp | | |
| AYV[2] | F (774) | TGA ATA GTT TTC TGA AGA AAG GAA AGA G |
| | R (775) | ATG GCA AAT CCT GAT GGC AT |

[1]Primers taken from Vercruysse *et al.,* (2000), Journal of Virological Methods, 88, 153-161.
[2]Designed at HRI.

Carrots were sampled from the untreated areas to determine presence or absence of virus. At one site in each region (Asmall Farm, Lancs, Higham, Suffolk and Sand Hutton, North Yorkshire), two hundred and fifty carrots were taken randomly from each untreated area. In addition, samples were also taken of any plants (up to twenty) that appeared to have virus symptoms, e.g. stunting, distortion, foliar discolouration or mottling. At the remaining three untreated areas, one hundred carrots were randomly sampled together with up to twenty plants that were showing virus symptoms. All samples were sent to HRI Wellesbourne for analysis for the presence of virus.

During the course of the project, there were reports of virus symptoms at two sites in North Yorkshire, Dunstall (Riccall) and Menagerie (Escrick) Farms, which were not being used for aphid monitoring. Additional plant samples (approximately 50 plants) were taken from these sites and comprised small stunted plants believed to have been infected at an early stage of development, intermediate sized plants infected at a later stage of development and healthy carrots.

Six sites were selected through the grower/consultant members of the consortium. Two sites were in East Anglia (Suffolk H and Norfolk CA), two in Yorkshire (WS and HSM) and two were in Lancashire (A and NH). Three of these sites were visited early in the season (H – 9/6/03; A – 11/6/03; WS – 16/6/03) and 50 carrot samples collected at three pace intervals. These were all young plants and no symptoms were seen on any of them. At least seventy cow parsley plants were then collected

from a number of sites graded in distance from immediately adjacent to the field up to approximately 2 km away. The precise positions of all collection sites (carrot and cow parsley) were fixed using GPS and the distances between them calculated. The outline plan had been to collect along a single transect away from the fields but cow parsley obviously occurs in scattered patches mainly in hedgerows and so a pragmatic approach to collecting was adopted; plants were collected from up to seven sites at increasing distances from each field but these did not form clear single transects.

Spring and early summer were very hot in 2003 and unfortunately, many of the cow parsley plants had gone to seed earlier than expected. This meant that not only was not all the tissue in ideal condition for testing but it was also difficult to distinguish what may have been viral symptoms from leaf reddening due to senescence; leaf colouration was recorded but once the results of the virus testing were available it was clear that there was no correlation of leaf colour with viruses (data not shown). Although no systematic search was made, significant numbers of aphids were only seen on a small proportion of the plants (mainly those remaining relatively non-senescent).

These three carrot fields and the other three sites were visited again later in the year by ADAS staff (samples received at HRI 15/08 –19/08) and variable numbers of samples collected from the now more mature carrots. Some of these plants showed symptoms reminiscent of those reported for the carrot motley dwarf complex (leaf yellowing or reddening and 'stiffening' of leaves). No symptoms suggestive of PYFV were seen. These plants were tested either as individuals where symptoms were present or as bulks of five plants where no symptoms were seen.

A seventh site of two individual fields (Dunstall (D) & Menagerie (M)) was identified during the year due the presence of necrosis and stunting symptoms reminiscent of those thought to be caused by PYFV. Samples were supplied to HRI (received 17/7/03) divided into three categories according to perceived severity of symptoms (i) PYFV (ii) possible PYFV and (iii) uninfected. This site was visited again in August (15/8/03).

*Results*

In order to obtain a broader assessment of the rate of occurrence and variation within viruses, further carrot samples were also obtained from 17 growers. None of these samples showed any symptoms and were sub-sampled by taking 20 plants at random from those supplied and these were tested individually (with several different leaves being sampled and these sub-samples pooled for each plant). These plants were all well grown and had *ca*. 30-40 cm of leaf growth. Supply dates ranged from mid-June to mid August (Table 23). These samples were tested for PYFV, CRLV, CMoV and CRLaRNA (but not AYV as this does not infect carrots).

Table 23. Location of randomly sampled field sites in 2003 and sampling dates.

| Site Number | Location | Date | Site Number | Location | Date |
|---|---|---|---|---|---|
| 1 | Suffolk | 18/6/03 | 10 | North Yorkshire | 11/07/03 |
| 2 | Norfolk | 18/6/03 | 11 | North Yorkshire | 11/07/03 |

| | | | | | |
|---|---|---|---|---|---|
| 3 | Cambridgeshire | 26/6/03 | 12 | North Yorkshire | 11/07/03 |
| 4 | Suffolk | 02/07/03 | 13 | North Yorkshire | 11/07/03 |
| 5 | Norfolk | 09/07/03 | 14 | East Yorkshire | 11/07/03 |
| 6 | Lancashire | 09/07/03 | 15 | North Yorkshire | 11/07/03 |
| 7 | Lancashire | 09/07/03 | 16 | Lancashire | 14/08/03 |
| 8 | Lancashire | 09/07/03 | 17 | Lancashire | 14/08/03 |
| 9 | Lancashire | 09/07/03 | | | |

Table 24. Average root weights (g) for the three symptom categories for two Yorkshire fields

| Class | 'Uninfected' | 'Possible' | 'PYFV Infected' |
|---|---|---|---|
| Field 1 (D) | 41.0 | 15.1 | 2.4 |
| Field 2 (M) | 59.6 | 17.9 | 4.3 |

Plants were initially assigned to symptom classes by the supplier

Table 25. Association of PYFV and CRLV with randomly selected carrot plants from three symptoms classes from two Yorkshire fields

| Site | Symptom class | PYFV[1] | CRLV[1] |
|---|---|---|---|
| Field 1 (D) | 'PYFV infected' | 15/16 | 0/16 |
| | 'Possible PYFV' | 5/16 | 0/16 |
| | 'Uninfected' | 0/10 | 0/10 |
| Field 2 (M) | 'PYFV infected' | 18/19 | 0/19 |
| | 'Possible PYFV' | 3/13 | 1/13 |
| | 'Uninfected' | 0/9 | 0/9 |

Plants were initially assigned to symptom classes by the supplier
[1]number of plants positive by PCR / number of plants tested

A number of parameters (e.g. root weight, plant length) were measured for the July samples from the seventh site (fields D & M) and it was clear that there was a strong correlation of perceived infection with stunting (Table 24). These plants had been assigned to three symptom categories by the supplier; approximately 10 – 20 randomly selected plants from each category for each field were tested for viruses. CRLV was detected in only one of these plants and neither CMoV or CRLaRNA were associated with this infection or with two very weak (probably artefactual) CLRV positive reactions (Table 25). There was a clear association of PYFV infection with the severest symptoms. There was only a weak association of PYFV with the moderately affected class (Table 25).

When Field 2 was visited again in August (15/8/03), the symptoms reminiscent of PYFV had disappeared but many plants were showing symptoms, suggesting

CRLV infection (leaf yellowing /reddening, stiffening and fine division). A further 34 plants were collected and tested but little virus was detected (Table 26).

Table 26. Virus detected in plants with or without 'CLRV-type' symptoms, Field 2(M) Yorkshire

| | PYFV | CRLV | CMoV | CRLaRNA |
|---|---|---|---|---|
| No Symptoms | 0/10 | 0/10 | -[1] | -[1] |
| Symptoms | 0/24 | 1/24 | 1/11[2] | 0/11 |

Plants collected 15/8/03
(Number positive / number tested)
[1] not tested;
[2]the positive plant is the same as that infected with CRLV.

At the Lancashire A site, cow parsley samples were collected from four sites in an arc from northeast through north to southwest of the carrot field (1 – adjacent to the field; 2 at 450 m; 3 at 750 m and 4 at 1450 m). Around the Suffolk site cow parsley was generally very patchily distributed and collection were made from only three sites from west to north–west of the field (1 – adjacent to the field; 2 at 500m; 3 at 1700 m). At the Yorkshire WS site the scattered patches of cow parsley were small and collections were made from 6 places, the first four forming a transect to the northwest (at 100, 400, 760 and 1030 m) whilst the fifth and sixth were to the northeast (560 m) and the southeast (2250 m). (This last carrot field had willows of various sizes beside a ditch adjacent to the carrot field, potentially providing an aphid over-wintering site very close to the field). At all sites, the carrots were small (Lancs A emergence unknown but *ca* 5-6 true leaves; Suffolk emerged *ca* 1/04; Yorks WS emerged mid May).

Overall, the three sites formed a triangle with the Yorks/Lancs sites *ca* 140 km apart and the Suffolk site 220 and 270 km away from these. The other three sites sampled in August were in the same areas as these first three.

No symptoms were seen on any of the early carrot samples and it was difficult to distinguish possible viral symptoms from senescence in the cow parsley.

Further details of the relative positions of main sites and the specific collection points are available on request.

No PYFV was found in any of the 150 early-collected carrots (three sites) or in any of the 237 late-collected samples from six sites. Many of the latter plants had symptoms of leaf reddening/yellowing and stiffening but no necrosis. Neither was PYFV detected in a small sample of carrots from Lancashire.

Despite the generally poor, senescent state of the plant material, PYFV was detected in some cow parsley plants at all sites (Table 27). As was expected (the hypothesis being that PYFV spreads from cow parsley to carrots but not in the opposite direction) there was no correlation of rate of infection with proximity to the carrot fields.

Table 27. PYFV infection detected in cow parsley at three sites with up to six collection points for the cow parsley[1], 2003.

| | Collection point | | | | | | |
|---|---|---|---|---|---|---|---|
| **Site** | **1** | **2** | **3** | **4** | **5** | **6** | **Total** |
| Suffolk | 3/20 | 5/25 | 1/25 | | | | 9/70(12.9%) |
| Lancs A | 0/25 | 0/5 | 0/20 | 0/10 | 2/10 | | 2/70 (2.8%) |
| Yorks WS | 2/9 | 0/11 | 0/10 | 3/15 | 0/10 | 0/21 | 5/76 (6.6%) |

[1]Note that the distance from the carrots to the cow parsley is generally in the order of collection (Number of plants positive by PCR / number of plants tested)

The majority of the amplicons were sequenced and presented as a combined cladogram with the results from two Yorkshire fields with symptoms. The small number of virus isolates encountered limited the degree of analysis possible but it appeared that the samples from Suffolk were slightly more likely to resemble each other than those from the other sites and isolates from the Lancs/Yorks sites were more like those from the two fields where PYFV was found in association with carrots with symptoms (Figure 22). Also included are sequences from five isolates from hogweed detected and sequenced earlier in the project; these formed a clade (family) very distinct from the cow parsley and carrot isolates. These are assumed to represent the previously reported ‘parsnip serotype’ whilst all others represent the ‘carrot serotype’. There were too few isolates to determine whether there was a consistent variation between collection points.

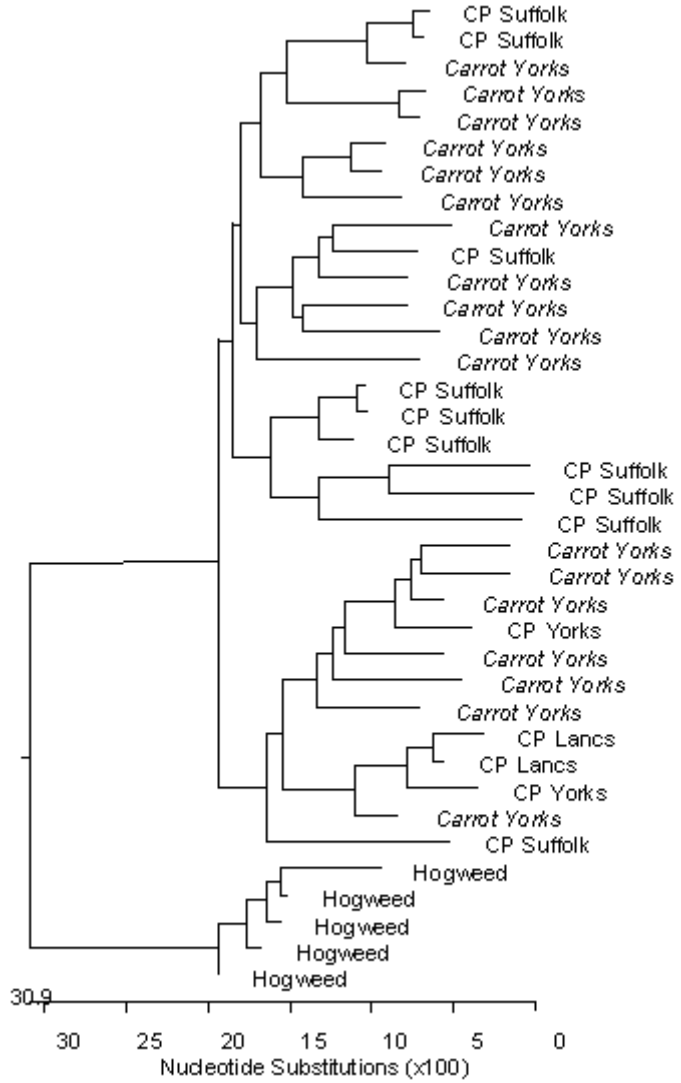



Figure 22. Cladogram of sequences of amplicons from PYFV from carrot, cow parsley (CP) and hogweed. Carrot isolates are all from the two fields in Yorkshire identified based on necrotic symptoms. Cow parsley isolates come from the three sites sampled in early June (Suffolk, Lancashire A and Yorkshire WS). Hogweed samples came from a variety of locations in previous years; all other samples are 2003.

AYV (which acts as a helper virus for PYFV) does not occur in carrots, therefore only the cow parsley samples were tested for this virus. Higher levels of AYV infection than PYFV were found at all sites (Table 28) and the low level of PYFV does not seem to be due to the absence of the helper virus in the areas tested. As expected, PYFV infection was mainly found where there were AYV infections (PYFV alone, 4 plants; both viruses 12 plants; AYV alone 47 plants). Not all amplicons were sequenced but in those that were, there was a generally low level of variability. There was some tendency for the isolates from within collection points to be more similar and for there to be a slight gradient of variation across the country. Two isolates were apparently particularly divergent but it is not clear whether these are due to errors in sequencing or genuine variations (Figure 23).

Table 28. AYV infection detected in cow parsley at three sites with up to six collection points for the cow parsley[1], 2003.

| | Collection point | | | | | | |
|---|---|---|---|---|---|---|---|
| **Site** | **1** | **2** | **3** | **4** | **5** | **6** | **Total** |
| Suffolk | 11/20[1] | 7/25 | 8/25 | | | | 26/70 (37.1%) |
| Lancs A | 6/25 | 0/5 | 6/20 | 4/10 | 6/10 | | 21/70 (30%) |
| Yorks WS | 0/9 | 1/11 | 1/10 | 9/15 | 0/10 | 1/21 | 12/76 (15.8%) |

[1]Note that the distance from the carrots to the cow parsley is generally in the order of collection. (Number of plants positive by PCR / number of plants tested)

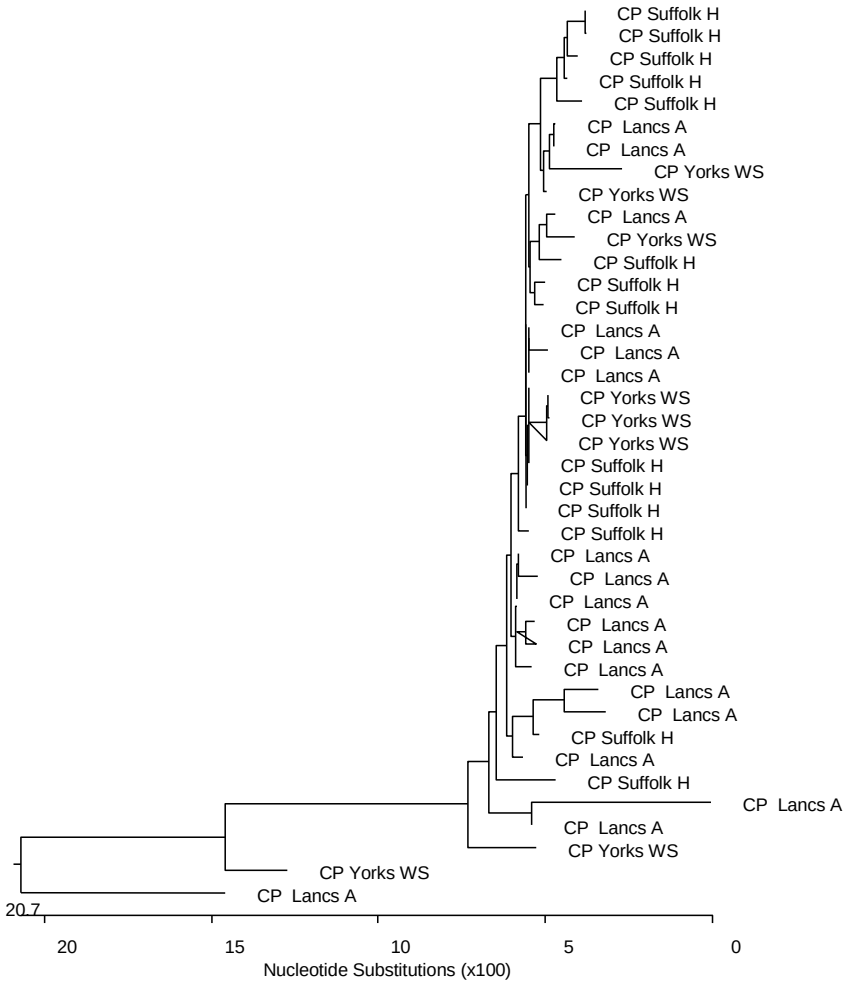



Figure 23. Cladogram of sequences of amplicons from AYV isolates in 2003. All isolates are from cow parsley and from the three sites sampled in early June 2003. Despite the young age of the carrots tested in June, quite high levels of CRLV were already present in one crop despite a complete lack of symptoms. CRLV was also present in the cow parsley samples (Table 29).

CRLV was also present in the crops tested in August but there was only a poor to moderate correlation with symptoms, which was quite variable by site (Table 30).

(Note that as most of these late sampled plants were deliberately selected for presence or absence of symptoms no overall level of occurrence can be given).

Table 29. CRLV infection detected in June 2003 in carrot and cow parsley at three sites with up to six collection points for the cow parsley[1].

| | | Collection point | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Site** | **Sample type** | **1** | **2** | **3** | **4** | **5** | **6** | **Total** |
| Suffolk | Carrot | | | | | | | 15/50 (30%) |
| | C. P. | 11/20 | 6/25 | 9/25 | | | | 26/70 (37.1%) |
| Lancs A | Carrot | | | | | | | 0/50 (0%) |
| | C. P. | 18/25 | 5/5 | 13/20 | 3/10 | 1/10 | | 40/70 (57.1%) |
| Yorks WS | Carrot | | | | | | | 2/50 (4%) |
| | C. P. | 2/9 | 2/11 | 1/10 | 4/15 | 2/10 | 4/21 | 15/76 (19.7%) |

[1]Note that the distance from the carrots to the cow parsley is generally in the order of collection.
(Number of plants positive by PCR / number of plants tested)

Table 30. Presence of CRLV in August, 2003 collected carrots at six sites in plants with and some without symptoms suggestive of 'carrot redleaf disease'.

| **Site** | **No Symptoms** | **Symptoms** |
|---|---|---|
| Suffolk | 5/20 (25%) | 17/25 (68%) |
| Norfolk | -[2] | 14/28 (50%) |
| Lancs A | 6/80[3] (7.5%) | 7/24 (29.2%) |
| Lancs NH | - | 1/20 (5%) |
| Yorks WS | - | 10/20 (50%) |
| Yorks HSM | - | 4/20 (20%) |

(Number of plants positive by PCR / number of plants tested)
[2] = none available or tested; [3] = tested as 16 bulks of five plants, by most probable number equivalent to 6/80 as given.

Sequence analysis of a selection of selected amplicons (Figure 24) clearly showed that the carrot virus isolates with two exceptions (out of twenty-eight when samples from previous years are included) were quite distinct from the cow parsley sequences. This strongly implies that the majority (over 90%) of infections by CRLV in carrots do not come from cow parsley. None of the thirty-seven cow parsley isolates was like the majority of carrot isolates. Within the cow parsley isolates there was a strong tendency for the Yorks/Lancs samples to resemble each other more than they did the Suffolk samples. Four of the fourteen cow parsley isolates from Suffolk formed a distinct sub-clade.

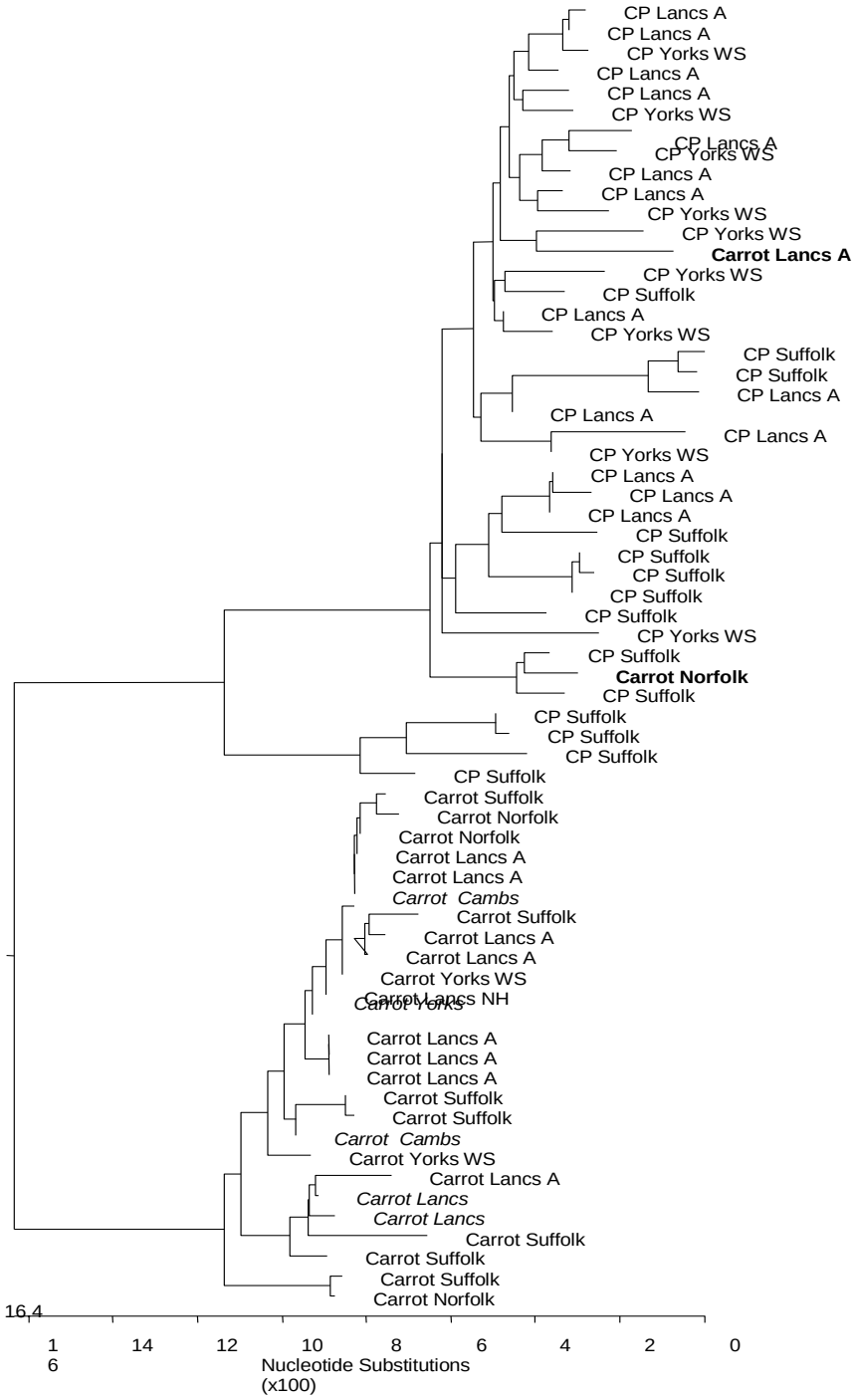



Figure 24. Cladogram of sequences of amplicons from CRLV from cow parsley (CP) or carrot. Cow parsley samples came from three sites (Suffolk H, Lancashire A and Yorkshire WS) collected in early June 2003; carrot samples come from these three sites and three others (Norfolk CA, Lancashire NH and Yorkshire HSM), collected in either June or August, 2003. Samples in italics were carrots (from counties as indicated) collected in 2002. Note that only two carrot samples (in bold) fall into the predominantly cow parsley clade. Samples from CSL testing of 17 sites not included as these used different primers and were not comparable (but the level of nucleotide diversity suggested one of nine samples was quite distinct and possibly equivalent to the 'cow parsley clade' above).

Carrot mottle virus is dependent on CRLV for transmission (and encapsidation) as part of the 'carrot motley dwarf' complex of viruses. Both carrot and cow parsley samples were mainly tested for the presence of this virus when CRLV was present although some CRLV negative plants were also tested to determine whether infections occurred without the helper virus (infections which might give symptoms but which would be non-transmissible 'dead-ends').

Infection of both carrots (Table 31) and cow parsley (Table 32) by CRLV were frequently associated with the presence of CMoV. Out of nineteen plants in which CRLV was not detected, only one appeared to be infected by CMoV alone. Where comparisons were possible, the presence of CMoV did not seem to affect the expression of symptoms.

Table 31. Occurrence of CMoV with CRLV in carrots from seven sites, 2003

| Site: | Crop | No Symptoms | Symptoms |
|---|---|---|---|
| Suffolk | Early Carrots | 12/15 (80%) | -[1] |
| | Late Carrots | 3/5 (60%) | 12/16 (75%) |
| Norfolk | Late Carrots | - | 14/28 (50%) |
| Lancs A | Early Carrots | 0/1 | - |
| | Late Carrots | 6/6 (100%) | 5/8 (62.5%) |
| Lancs NH | Late Carrots | 4/9 (44.4%) | 1/1 (100%) |
| Yorks WS | Early Carrots | 1/2 | - |
| | Late Carrots | - | 5/8 (62.5%) |
| Yorks HSM | Late Carrots | - | 3/4 (75%) |
| Yorks Field 2 | Carrots | - | 1/1 (100%) |

(Number of plants positive by PCR / number of plants tested)
[1] = none available or tested

Table 32. Occurrence of CMoV with CRLV in cow parsley from three sites, 2003

| | | Collection point | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Site | Sample type | 1 | 2 | 3 | 4 | 5 | 6 | Total |
| Suffolk | Cow Parsley | 0/11 | 0/6 | 0/9 | | | | 0/26 |
| Lancs A | Cow Parsley | 16/18 | 3/4 | 4/9 | 2/3 | 0/1 | | 25/35 (71.5%) |
| Yorks WS | Cow Parsley | 1/1 | 1/2 | 0/1 | 1/5 | 1/2 | 0/4 | 4/15 (26.7%) |

(Number of plants positive by PCR / number of plants tested)

Sequencing of the amplicons, again, clearly separated the majority of carrot isolates (30 of 32 of all sequences available) from the cow parsley isolates (18 sequences) and a single, previously published sequence of CMoV from parsley in Belgium (Figure 25). The cow parsley samples from Lancashire and Yorkshire were divided into 2 sub-clades but were not clearly distinguishable from each other. The

previously published parsley isolate from Belgium was very distinct from all the other isolates. Other than the two isolates that fell with the cow parsley isolates, there was no clear differentiation among the carrot isolates. This again supports the view that the majority (greater than 90%) of carrot isolates do not come from cow parsley.

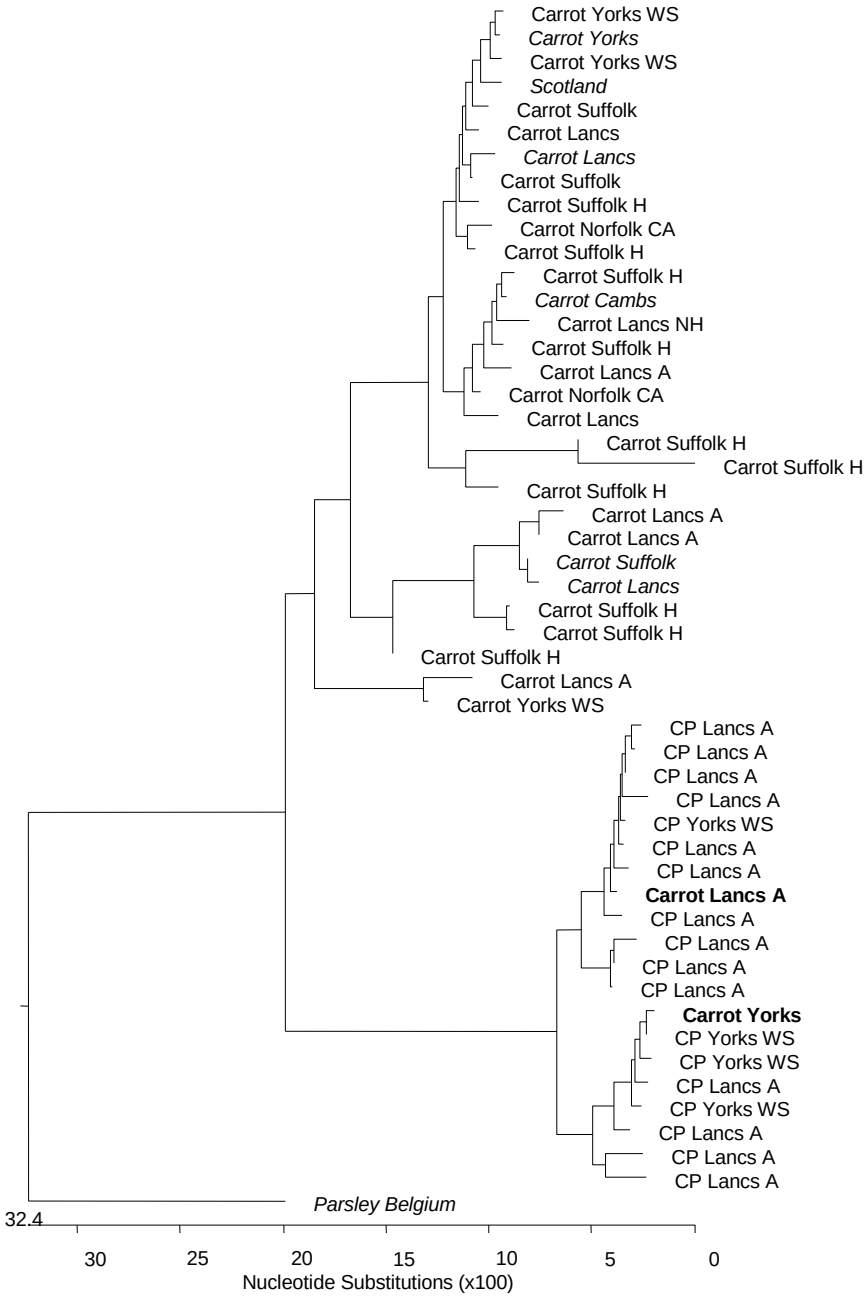



Figure 25. Cladogram of sequences of amplicons from CMoV isolates from cow parsley (CP) or carrot. Cow parsley samples came from three sites (Lancashire A, Yorkshire WS and Suffolk H (although no infection found at the last site)) collected in early June 2003; carrot samples come from these three sites and three others (Norfolk CA, Lancashire NH and Yorkshire HSM) and were collected in either June or August, 2003. Samples in italics were carrots (from counties as indicated) collected in 2002 except for that from Scotland and that from parsley in Belgium,

which were from published sources. Samples without letter suffixes were mainly tested at CSL except for the Yorkshire carrot sample falling into the mainly CP clade which came from the site showing PYFV symptoms in July. Note that only two carrot samples (in bold) fall into the predominantly cow parsley clade.

Carrot redleaf virus associated RNA (CRLVaRNA) is also dependent on CRLV for transmission (and encapsidation) as part of the 'carrot motley dwarf' complex of viruses. As with CMoV, in the main only CRLV positive samples were tested for CRLVaRNA. In carrots, CRLV was moderately frequently associated with the presence of CRLVaRNA but there was no clear association between the presence of CRLVaRNA and symptoms (Table 33). In plants without CRLV, CRLVaRNA was only found in one out of twenty-one plants tested.

Table 33. Occurrence of CRLVaRNA with CRLV in carrots from seven sites, 2003

| Site: | Crop | No Symptoms | Symptoms |
|---|---|---|---|
| Suffolk | Early Carrots | 5/15 (33.3%) | -[1] |
| | Late Carrots | 0/5 | 6/17 (35.3%) |
| Norfolk | Late Carrots | - | 0/9 |
| Lancs A | Early Carrots | 0/1 | - |
| | Late Carrots | 5/6 (83.3%) | 4/7 (57.2%) |
| Lancs NH | Late Carrots | - | 1/1 (100%) |
| Yorks WS | Early Carrots | 1/2 | - |
| | Late Carrots | - | 1/8 (12.5%) |
| Yorks HSM | Late Carrots | - | 0/4 |
| Yorks Field 2 (M) | Carrots | 0/1 | 1/1 (100%) |
| **Total No. of positive carrots = 24/77 (31.2%)** | | | |

(Number of plants positive by PCR / number of plants tested)
[1] = none available or tested

No infections were found in cow parsley (Table 34). The complete lack of CRLaRNA in 76 cow parsley, all of which were infected with CRLV, suggests that this species is not a host of this umbravirus and underlines the fact that the majority of CRLV infections in carrot do not come from cow parsley.

Table 34. Occurrence of CRLVaRNA with CRLV in cow parsley from three sites, 2003

| Site | Sample type | +ve plants/ No. tested |
|---|---|---|
| Suffolk | Cow Parsley | 0/26 |

| | | |
|---|---|---|
| Lancs A | Cow Parsley | 0/35 |
| Yorks WS | Cow Parsley | 0/15 |
| **Total No. of positive cow parsley = 0/76 (0%)** | | |

Sequencing of the amplicons again showed a slight tendency for isolates from specific sites to be more similar to each other than to those from other sites (Figure 26) and that a few isolates were quite divergent. It was already known from published sources that the two California isolates were quite distinct from each other and whilst the majority of carrot isolates from the UK were very similar to a25 (the predominant sequence type in California) a few isolates were either similar to a8 or were intermediate between isolates a25 and a8.

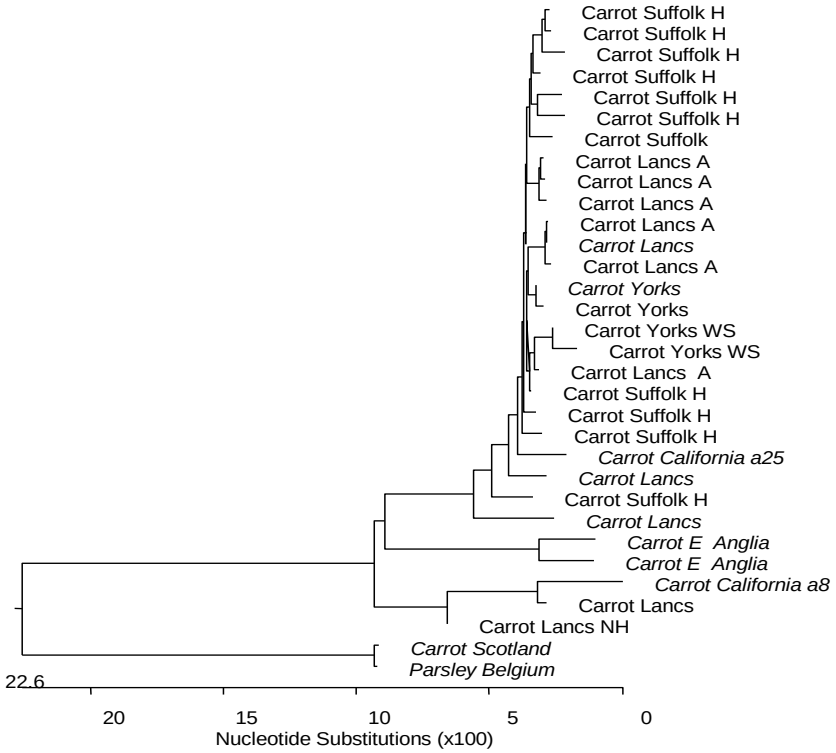



Figure 26. Cladogram of sequences of amplicons from CRLaRNA from carrot and one isolate from parsley in Belgium. Samples collected in early June, 2003, (Lancashire A, Yorkshire WS and Suffolk H) or August, 2003 (Norfolk CA, Lancashire NH and Yorkshire HSM) or on the dates given in table 2 (17 sites indicated by county but no suffix). Samples in italics were carrots (from counties as indicated) collected in 2002 except for those from Scotland and California and parsley in Belgium for which the sequences were taken from published sources.

The carrots collected from seventeen sites across England (Table 34) were tested for four viruses. In these tests at CSL, RT-PCR was also carried out for the mRNA from a 'housekeeping' gene consistently expressed in all growing plants (cytochrome oxidase) to determine the quality of the RNA extracts; where this mRNA could not be detected it was assumed that either inhibitors were present or extraction had failed (or was poor) for some reason.

The viruses detected are highlighted in Table 35. Virus was detected in samples from just five of the seventeen test sites. The samples from sites where no viral positives were detected were largely positive to COX 1, indicating that the RNA preparations were of good quality. Samples from site 9 were all negative to the COX 1 internal control but the reason for this complete failure at this one site is not known.

Table 35. Detection of PYFV, CRLV, CmoV and CRLV virus using RT-PCR in randomly sampled carrots from field sites in 2003; 20 samples were taken from each site.

| **Site** | **Number of positive samples** | | | | |
|---|---|---|---|---|---|
| | **Internal Control (COX 1)** | **PYFV** | **CRLV** | **CmoV** | **CRLVaRNA** |
| 1 | 19 | 0 | 0 | 0 | 0 |
| 2 | 20 | 0 | 0 | 0 | 0 |
| 3 | 20 | 0 | 0 | 0 | 0 |
| **4** | **19** | **0** | **5** | **4** | **3** |
| 5 | 20 | 0 | 0 | 0 | 0 |
| 6 | 20 | 0 | 0 | 0 | 0 |
| **7** | **20** | **0** | **3** | **2** | **2** |
| 8 | 20 | 0 | 0 | 0 | 0 |
| 9 | 0 | 0 | 0 | 0 | 0 |
| 10 | 19 | 0 | 0 | 0 | 0 |
| **11** | **20** | **0** | **2** | **0** | **0** |
| 12 | 20 | 0 | 0 | 0 | 0 |
| 13 | 18 | 0 | 0 | 0 | 0 |
| **14** | **20** | **0** | **1** | **0** | **0** |
| 15 | 20 | 0 | 0 | 0 | 0 |
| **16** | **20** | **0** | **2** | **0** | **0** |
| 17 | 20 | 0 | 0 | 0 | 0 |

No PYFV was detected in any of the 315 samples for which good quality RNA was prepared representing, sixteen sites spread across England from Suffolk to North Yorkshire and Lancashire.

The three viruses in the carrot red leaf complex were detected at five sites at an overall rate of between 1.6 and 4.1% of the 315 samples with good quality RNA. Surprisingly, two of the CMoV infections occurred in the apparent absence of the helper virus CRLV or one third of all the CMoV infections detected. The amplicons from all isolates detected were also sequenced and examined for evidence of

regional variation but the numbers were too low to be definitive on their own and where appropriate, the data has been incorporated in previous figures. As the CRLV detection at CSL used different primers these sequences could not be compared with those from the other sites but note that one of the CRLV isolates here was quite distinct from the other nine and may indicate a third 'cow parsley clade' isolate, giving a total of three out of 37 sequenced.

These results confirm that all the viruses, and especially PYFV, were infrequent or absent from carrots without symptoms in 2003.

*Discussion*

A recurring problem in this project has been the sporadic nature of the outbreaks of its primary focus, PYFV. This was again evident in the samples tested this season when PYFV was clearly rare in carrots, being detected in carrots only at one site but it was noteworthy that this site had been identified on the basis of the necrotic leaf symptoms and stunting thought to be associated with PYFV. This was the clearest single outbreak of this disease during the entire span of the project.

Considering only the most severely affected and least affected categories (as determined by the supplier) there was a very clear association between PYFV and the disease (94% detectable virus versus 0% for those without symptoms). For the intermediate category there was a much lower level of infection (28% detectable infection) but the cause of this poorer association is not known. It may that these plants are all infected but with lower levels of virus for some reason (e.g. more recent infections or less inoculum) or it may be that only some are infected and the remainder have similar symptoms/stunting for other reasons (other pathogens or non-disease effects). There was very little CRLV present in these plants. When this field was revisited a few weeks later, there was no evidence of necrotic plants and no PYFV could be detected in a sample of the remaining plants. It seems most likely that the heavily affected plants had died in the interim, removing infection from the field.

Why there should have been an outbreak just in this field is not known. Potential inoculum in the form of PYFV in cow parsley was present at the other sites but no infection was detected. This point will be discussed further below. The main conclusion of the testing in the 2003 season is that the only very severe outbreak of the viral necrosis disease seen in this project was closely associated with the presence of PYFV.

The cause of the leaf reddening and similar symptoms seen later in the season is less clear. Where direct comparisons were made there were higher proportions of plants apparently infected with one or more components of the carrot redleaf complex (by 3 – 4 fold) in those plants showing symptoms than in those not. However, the apparent proportion of plants with symptoms in which virus could be detected varied across sites from 5% to 68%. The cause of this is not known but may be due to fluctuations in the levels of virus (caused either by time or local environmental factors) or it may be that many of these symptoms are not viral in origin or due to some other virus. This was not investigated.

It is clear from the sequencing data (and the absence of CLRVaRNA in cow parsley) that the majority (>90%) of CRLV complex infections do not come from cow parsley. Possible alternate sources were not investigated here. There was

some evidence of a gradient of virus types in both the carrot and cow parsley populations across the country but not within the sites investigated. The data will be studied more intensively for large scale variation for a scientific publication but is not relevant to the project and will not be discussed further here.

The levels of PYFV found in cow parsley were too low to test the hypothesis that PYFV populations vary at the microscale of a few kilometres relevant to local spread. At the Suffolk site, the one sample from almost 2 km away from the others (in turn 500 m apart) was somewhat distinct but little can be inferred from a single sample. There was some evidence of differentiation of both PYFV and AYV populations at the national scale but again this is not relevant to this project.

The major conundrum of 2003 was the high level of PYFV in two closely spaced Yorkshire fields but almost complete lack of detectable virus in carrots at the other sites. The infected sites were for processing and therefore planted at lower densities than at the other sites, which may be relevant to aphid behaviour. It may also be that these crops were planted earlier than the main study sites. However, the difference in occurrence seems too great to be accounted for by these factors alone.

Another possibility is that infection did occur at other sites but they were tested too early (virus only just arrived) or too late (PYFV was not detected in late samples at the disease site, possibly because infected plants had died or been out-competed by uninfected plants). This seems less likely as there were no reports of disease in mid-season at the other sites. Potential inoculum was found at all three sites as low to moderate (2.8% to 12.9%) levels of PYFV were present in cow parsley at all the sites tested (as was the helper virus, AYV, at higher levels, 15.8% to 37.1%)

The more radical possibility, that cow parsley is in fact not the source of PYFV, must be considered. It is quite clear from the sequence data that most infections of carrots by the CRLV complex do not come from cow parsley. The spring of 2003 was hot and by the time of collection cow parsley, which is normally still quite green at this time (as in 2004), had finished flowering and was well into senescence with much mature seed seen. These plants did not seem to be in a good state to act as hosts for aphids and hence virus reservoirs. It may be that an alternative host served as the main reservoir for PYFV infection at the disease site. The nature of this possible alternative host is not known. Hogweed is another widespread umbellifer that follows cow parsley and known to be infected in the wild. However, earlier sequence data (and published serological data) tends to rule out the majority of PYFV in hogweed (if not all as only five sequences are available but these form a very distinct clade). However, it may be that ‘carrot serotype PYFV’ may occur in limited numbers of hogweed or that another host with a distinct distribution was involved in 2003. It is not clear at this stage whether we should be looking for a possible alternative host growing earlier or later than cow parsley. (An alternative PYFV host which normally grows earlier than cow parsley might only give infection in carrots in unusual years when weed growth is delayed and it overlaps with the carrots; on the other hand, PYFV may only be a problem at most sites in years when the hypothetical alternative host is actively growing in early enough to overlap with susceptible young carrots. The outbreak of ‘necrotic disease’ in the two Yorkshire fields may have occurred because at this site the ‘alternative host overlapped with the carrots due to particular local conditions.) Furthermore, the precise interplay of virus populations in one or more than one non-carrot hosts of

PYFV with the carrot crop may be dependent on the timing of aphid movements (and hence be environmentally driven). In certain years (like 2003) one host may give infections at particular site, whilst in other years a different host may give infections at other sites or more generally, depending on when aphid vectors move and between which plants. The lack of differentiation between the carrot and cow parsley PYFV populations (such as seen for CRLV and CMoV) suggests that there is a flow of infection between the two hosts; however, this flow may be via a third host which acts as the main reservoir for infection in carrots.

*3R. Control of PYFV utilising rational vector management*

Undertake bioassays to determine the impact of insecticides on PYFV transmission

Bioassay protocols were designed and agreed to investigate the effective period of insecticidal activity and impact on the transmission efficiency of willow-carrot aphid. However, the maintenance of PYFV in laboratory cultures was consistently problematic, with stocks of PYFV alone or PYFV and AYV in complex periodically failing, a trend experienced in other laboratories. Repeatedly, stock plants infected with both viruses and subsequently used as source plants for vector transmissions in virus maintenance, yielded source plants with AYV only. This experience suggests that AYV replicates much more efficiently than PYFV. With the agreement of David Cole and consortium members, additional bioassays were designed and implemented to evaluate the efficacy of a selected range of insecticides from three pesticide groups (carbamate, pyrethroid and neonicotinoid) against willow-carrot aphid alatae. Two laboratory bioassays were undertaken to evaluate: (a) the immediate effect of selected insecticides applied topically to willow-carrot aphid alatae using a Potter spraying tower, (b) the residual effect of selected insecticides against willow-carrot aphid using a laboratory booth sprayer.

*a) Bioassay to evaluate the immediate effect of insecticides applied topically to willow-carrot aphid.*

The range of insecticides for use was selected from the 1999 Defra Pesticide Usage Survey (Garthwaite *et al*., 2001) and with industry consultation. These insecticides were pirimicarb as 'Aphox' (carbamate), lambda-cyhalothrin as 'Hallmark' (pyrethroid), lambda-cyhalothrin and pirimicarb as 'Dovetail' (carbamate and pyrethroid), thiacloprid as 'YRC OD' (neonicotinoid) and thiacloprid and deltamethrin as 'YRC+D OD' (neonicotinoid). Chlorpyriphos as Dursban WG (organophosphate) was used as the toxic standard and distilled water ($DH_2O$) was used as the solvent and control. Aphox, Hallmark and Dovetail were supplied by Syngenta Crop Protection UK Ltd., 'YRC OD' and 'YRC+D OD' by Bayer CropScience Ltd. And Dursban WG by Dow Agrosciences Ltd. Each insecticide was applied to live willow-carrot aphid alatae at the recommended field rates.

The Potter tower was fitted with a medium spray nozzle and pressure was set at 10 psi to spray 2mg $cm^{-2}$ for Dovetail, Aphox and YRC OD and 3mg $cm^{-2}$ for Hallmark and YRC+D OD. Five test aphids were briefly immobilised with $CO_2$, transferred to a Petri dish base lined with a filter paper disc (the target), positioned on the Potter tower spray platter and sprayed. Immediately after spraying, the target was removed from the tower and treated aphids were transferred to the chervil leaflets (five aphids per leaflet) and caged. Assessments of mortality were made six, 10, 15 and 30 minutes then one, two and 24 hours post treatment. Aphids were scored as

alive or “dead” (i.e. when no movement was observed following mechanical stimulation).

The first bioassay was conducted to evaluate the immediate effect of the selected range of insecticides applied topically to adult willow-carrot aphid alatae. No mortality amongst control aphids was recorded throughout the seven assessment intervals. Mortality following exposure to the toxic standard was not recorded until after 15 minutes, but reached 100% after 1 hour (Figure 27). A highly significant difference in effect on percentage aphid mortality between treatments at each assessment period from six minutes to two hours ($p < 0.001$) was recorded.

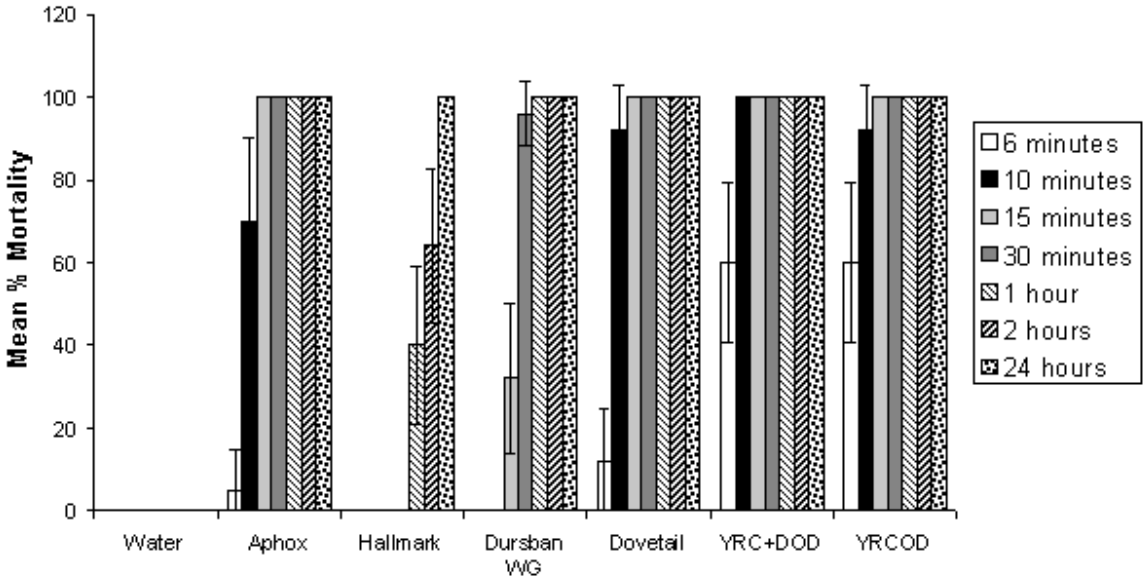



Figure 27. Topical application to willow-carrot aphid alatae – comparison of response to each treatment over time. Bars represent ± 2 SE at 95% confidence levels.

Aphid mortality was recorded after six minutes exposure to Aphox, Dovetail, YRC+D OD and YRC OD. Aphox and Dovetail both resulted in less than 15% mortality after this period of exposure, but efficacy of both the neonicotinoids was significantly higher, with 60% aphid mortality recorded for both products. Efficacy of neonicotinoid products increased after exposure of 10 minutes, resulting in more than 70% aphid mortality (YRC+D OD was 100% effective after 10 minutes). Hallmark toxicity was not recorded until after a one hour exposure (40% mortality). Longer exposure to all the products under trial resulted in higher aphid mortality. All products were 100% effective against willow-carrot aphid alatae after 24 hours exposure.

The two neonicotinoids were the fastest acting and most effective products in the topical application bioassay. After 10 minutes, there was no significant difference in efficacy recorded between these products and Aphox and Dovetail. Hallmark was the least effective product after short exposure periods.

*b) Bioassay to evaluate the immediate and residual effect of selected insecticides against willow-carrot aphid alatae using a laboratory Booth sprayer.*

A subset of the insecticides used in the bioassay 'a' was selected after consultation with consortium members. Whole parsnip (*Pastinacae sativa*) plants were sprayed in a laboratory booth sprayer at the recommended field rate for each product. Leaf discs were cut from treated plants and infested with willow-carrot aphid alatae at intervals of zero, one, two, three, seven, 14 and 21 days post treatment. Aphids were assessed for mortality after 15 minutes, one, two, 24 and 48 hours exposure to dry insecticide residue.

Seven test plants per replicate (five replicates) for each test product and control were sprayed in the calibrated laboratory booth sprayer. When dry, treated plants were selected and two 30mm diameter leaf discs cut from two discreet leaves from each plant. Leaf discs were mounted abaxial side up onto agar test arenas, two discs per arena, and five arenas per treatment. Treated plants that were not required for aphid infestation at the first post-treatment interval were transferred to a glasshouse and arranged on the benching in the same order in which they were sprayed.

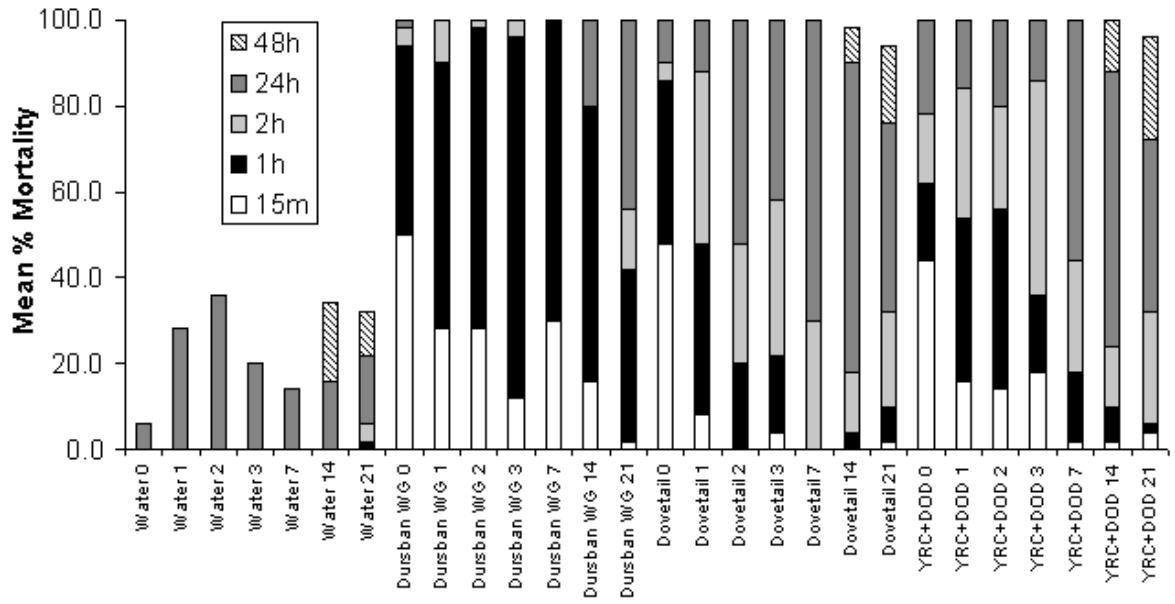



Figure 28. Residual effect on willow-carrot aphid alatae to test products from the day of spraying up to three weeks post application

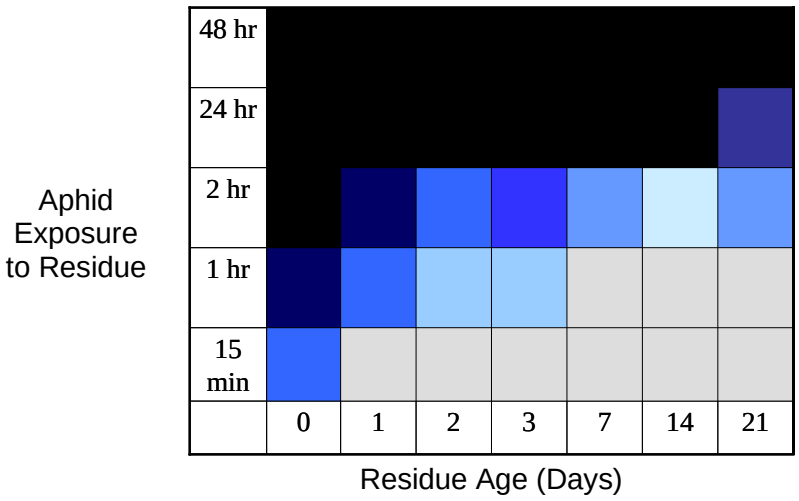
A subset of the products tested in the topical application bioassay was selected for use in the extended residual bioassay, based on the above results: Dovetail and YRC+D OD, with Dursban WG as the toxic standard and distilled water as the neutral control. Control aphids were largely unaffected by the distilled water treatment. However, while unusually high mortality was recorded in control aphids after 24 hours and 48 hours exposure to water-treated leaves, it was significantly lower than mortality recorded after exposure to leaves treated with the test products (Figure 28). Mortality following exposure to the toxic standard steadily increased with longer exposure to treated leaves, reaching 100% after 24 hours exposure for each treatment interval, up to 21 days post spraying.

Aphid mortality after 15 minutes exposure to Dovetail and YRC+D OD residues at each interval post treatment was recorded from 0-50%. The effect of these residues on aphid mortality was more noticeable after one-hour exposure, with efficacy decreasing significantly with the age of residue for both test products. Efficacy of Dovetail and YRC+D OD increased after 2 hours exposure of aphids to treated leaves, with maximum mortality recorded at 90% for Dovetail and 86% for YRC+DOD.

After exposure of 24 hours to product residues, aphid mortality was recorded at 100% for up to seven days post application, decreasing to 76% and 72% for Dovetail and YRC+D OD respectively after 21 days. Three weeks post treatment, aphid mortality was recorded over 90% when aphids were exposed to product residues for 48 hours.

Therefore, it would appear that after exposure to product residues, aphid mortality was recorded up to three weeks post application but that maximum mortality was achieved when aphids were exposed to residues for more than 24 hours. After shorter exposure periods, aphid mortality decreased with an increase in the age of residue for both Dovetail (Table 36) and YRC+D OD (Table 37) residues.

Table 36. Effect of Dovetail residue on mortality of willow-carrot aphid adult alatae from the day of application to 21 days post application




Percentage aphid mortality Key:

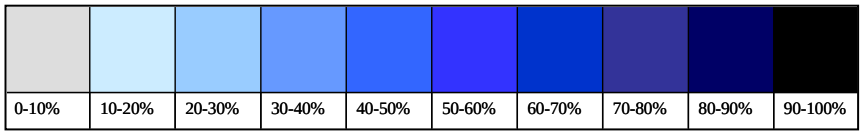
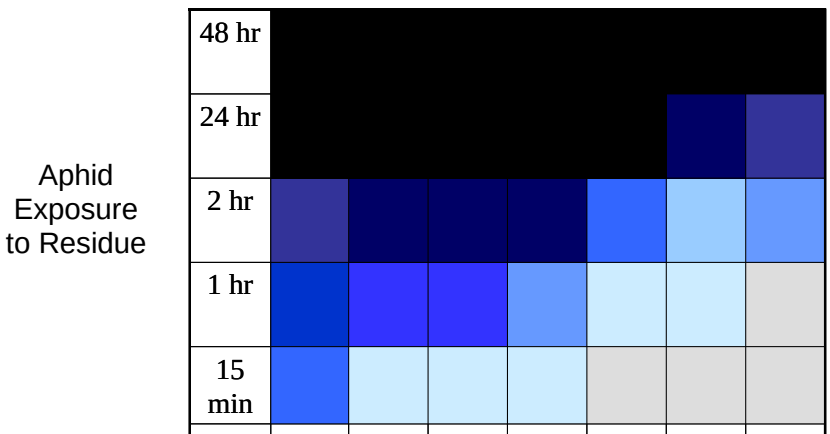
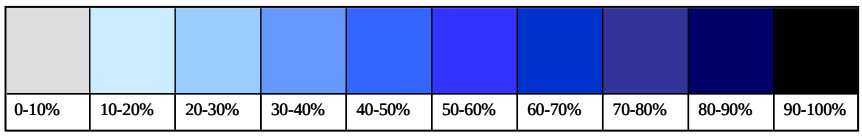



Table 37. Effect of YRC+D OD residue on mortality of willow-carrot aphid adult alatae from the day of application to 21 days post application



Residue Age (Days)

Percentage aphid mortality Key:




Investigate the potential for serial transmission of PYFV by aphid vectors

Preliminary experiment protocols were designed and implemented to investigate the potential of willow-carrot aphid adult alatae to serially transmit PYFV to carrot seedlings, under controlled laboratory conditions. Individual viruliferous aphids were sequentially allowed one, three and 24 hour IAPs on young carrot seedlings, for a maximum of four transfers. Three weeks post inoculation, each carrot seedling was sampled, extracted for RNA and tested for PYFV, using TaqMan® RT-PCR.

*Materials and Methods*

*Pre-test:*

Prior to the start of serial transmission experiments, chervil seedlings were infected with AYV and PYFV using willow-carrot aphid as the vector. Approximately three weeks post transmission, a subset of the inoculated chervil plants were tested using TaqMan® RT-PCR for AYV and PYFV. Plants with the highest virus titre were chosen by selecting the corresponding TaqMan® amplification curves showing the lowest $C_T$ and highest $\Delta R_n$ values.

Carrot seedlings were grown to the first true leaf stage, fifteen seedlings per half seed tray, two trays for each IAP. Willow-carrot aphid alatae were collected from the main aphid cultures 24 hours before the start of the experiment then transferred to the AYV/PYFV infected chervil source plant and allowed a standard 24 hours AAP at 20°C ±2°C.

*Test procedure:*

For each IAP (i.e. one, three and 24 hours) following the 24-hour AAP, individual viruliferous adult alatae were transferred from the virus source plant to single healthy carrot seedlings. They were contained by aphid-proof Perspex cages, maintained at 20°C ±2°C in a Sanyo CE cabinet and timed with a stop clock for the appropriate IAP. Immediately after the selected IAP, the individual aphids were transferred to fresh carrot seedlings. This process was repeated for each of four transfers (and each IAP), but after the final transfer, the cages were removed and the seedlings were sprayed with a systemic insecticide ('Rapid', a.i. $1gl^{-1}$ pirimicarb,

in aerosol form, Syngenta). Post inoculation, test seedlings were maintained in a Sanyo CE cabinet at 20°C ±2°C, until tested for virus.

Three weeks post inoculation, approximately 0.1g of leaf material was collected (from at least three different parts of each plant to allow for any variation in virus dispersal) for extraction. Following the CTAB RNA extraction method (adapted from Chang *et al*. (1993)), the resulting RNA preparations were tested for PYFV and COX 1 using TaqMan® RT-PCR.

*Results and Discussion*

Each IAP trial used 120 carrot seedlings (360 in total). Five seedlings from the 360 inoculated carrots had perished by the time of sampling. The remaining 355 seedlings were tested for PYFV and COX 1. In total, 346 carrot seedlings tested positive to the internal control, but none were positive to PYFV.

The failure of PYFV transmission to carrot seedlings, even after a 24hr IAP, would indicate that the test aphids were not viruliferous. However, the selected source plant tested positive for both viruses prior to the start of the experiment. AAPs of 24 hours or more have been used in other experiments as standard, with success, resulting in maximum transmission efficiency. Throughout the serial transmissions, some aphids produced progeny, indicating that they were also feeding and therefore able to transmit PYFV. Since this trial was conducted, it has become apparent that maintaining laboratory stocks of PYFV is difficult, with PYFV titre steadily decreasing with consecutive vector transmissions.

Results from experiments conducted at the beginning of this project, to separate AYV from a mixed infection of AYV and PYFV, indicate that willow-carrot aphid can transmit virus for several days. During one experiment, aphids were allowed to feed on a chervil source plant, infected with AYV and PYFV for 24 hours AAP. Groups of these aphids were then transferred to three healthy chervil seedlings for 24 hours IAP. This was repeated twice, but on the fourth day of transmission, surviving aphids were transferred individually to fresh chervil seedlings (39 in total) and allowed 72 hours IAP. Four weeks post inoculation, chervil seedlings were graded according to visible symptoms, which ranged from apical necrosis to chlorosis and reddening of the older leaves, to no symptoms at all. Twenty-six plants, inoculated by aphids on the fourth transmission date expressed virus symptoms, eighteen of which exhibited symptoms associated with AYV and PYFV in complex, the remainder exhibited symptoms associated with AYV only. While results from this experiment were not verified by PCR, the implication is that willow-carrot aphid does have the ability to transmit AYV and/or PYFV serially for at least four days.

<u>Development of a predictive forecast of vector phenology</u>

*Introduction*

The development of a successful prediction system for the population dynamics of willow-carrot aphid would be a significant step in the formation of a rational management strategy for this pest and associated viruses. However, the nonlinear

and complex nature of the population dynamics of insects makes this prediction very difficult (Lankin *et al.,* 2001). Due to the nature of PYFV epidemiology, it is alate willow-carrot aphid arriving into the crops that are likely to be the main vectors. Hence, predictions of the timing of aphid migrations from their overwintering sites will enable growers to make economically and ecologically beneficial decisions on the use of insecticides.

Weather conditions influence aphid population dynamics either directly by affecting development and survival, or indirectly by affecting natural enemies or the aphid host plant (McVean *et al.,* 1999; Worner *et al.,* 2002). It has been shown that for certain aphid species, the date of migration is linearly related to the mean temperature during the previous winter (Parker, 1997; Thomas *et al.,* 1993;Worner *et al.,* 1995, 2002). Various techniques have been employed in the past to predict both the timing and population dynamics of various aphid species, including simple linear regression models (Harrington *et al.,* 1990; Parker, 1997; Turl, 1980; Walters & Dewar, 1986) and multiple linear regression models (A'Brook, 1983; Basky, 2003; Harrington *et al.,* 1987). However, simple linear models do not account for a significant amount of the total variance causing a lack of predictive ability. With the multiple regression models there was a lack of understanding, due to the fact that the weather variables are highly correlated with each other.

However, there are two methods that have potential when considering the dynamics of a prediction system:

1. Howling *et al*. (1993) created a multiple regression model without the problems of correlation in the weather variables. By using principal components analysis (PCA) the correlations are removed allowing stepwise regression to be performed to give a multivariate linear regression model with high predictive power, although the model was only tested on the peach-potato aphid.
2. An artificial neural network (ANN) is an artificial learning mechanism that is suitable for investigations where the input and output values are known but the relationship is unknown. The `BlackBox' nature of ANNs have been their downfall, preventing the model's acceptance spreading into all fields. However, in areas where the interpretation of the model is not as important as its predictive capacity, ANN's ability to account for both nonlinear input/output relationships and the interactions between inputs is seen as highly valuable (Chaloulakou, 2003; Gardner & Dorling, 1999).

ANNs have been applied and tested in many different areas. Concentrating on the environmental applications of neural networks, Colasanti (1991) found similarities between ANN's and ecosystems and recommended the utilization of this tool in ecological modelling. They have been used for modelling the spatial dynamics of fish and predicting phytoplankton production (Giske *et al.,* 1998; Recknagel *et al.,* 1997; Scardi, 1996). Seigner *et al*. (1994) used ANNs to model the greenhouse effect. McRoberts *et al*. (1998) developed a descriptive model of crop quality in crop production, and found that ANNs worked considerably better than standard multivariate techniques. DeWolf and Francl (1997) found that ANNs worked in a similar way to multivariate nonlinear regression analysis, however, ANNs always seemed to provide more significant results.

By looking at the population dynamics of the forest pest pine needle gall midge, Chon *et al*. (2000) found that although the prediction of the number of pests would be considerably lower than the observed number, the timing of the population peaks matched almost perfectly. Lankin *et al*. (2001) found similar results with aphids, the timings of the predicted results nearly always coincided with the actual results, although the magnitude of aphid numbers would be very different. This method was improved by Worner *et al*. (2002) by using a sequential temporal cascading correlation algorithm.

In these and many other studies it has been shown that ANNs outperform their classical counterparts. Both types of model were developed in this project to predict the Julian date of willow-carrot aphid migration and their performance was compared to establish which is most applicable.

*Materials and Methods*

Aphid data from the network of traps described above and the Rothamsted Insect Survey (RIS) suction trap network was used to derive a forecasting system for willow-carrot aphid. The Julian date of first flight was calculated, whereby the first flight is defined as either the first occurrence of more than one aphid, or two consecutive sightings of an aphid in the trap. This method was used to prevent stochastic early fliers being mistaken for the start of the insect migration. Preliminary analysis showed this to be more highly correlated to the weather data than the first occurrence of the aphid species, as suggested by Harrington *et al*. (1990) and Howling *et al.* (1993).

Weather data was obtained from the UK Meteorological Office Land Surface Stations. The nearest Meteorological Station to each aphid trap was calculated by finding the shortest Euclidean distance between the trap site and every station. The weather variables are monthly averages of the standard weather variables. However, there were fewer sites with soil temperature more than 10cm deep, so only one soil temperature variable was included (Table 38).

If relatively few missing values (< 5%) were detected in the weather data they were estimated by calculating the maximum likelihood estimator of the unknown values using the data that was available (Pena & Tiao, 1991). However, for some years at certain sites, entire variables were missing, in these cases the records were completely ignored, thereby removing the chance of including additional errors caused by estimation.

Table 38. Weather variables used in this study

| Variable number | Description |
|---|---|
| 1 | Monthly mean screen temperature |
| 2 | Monthly mean screen minimum temperature |
| 3 | Monthly mean grass minimum temperature |
| 4 | Monthly mean accumulated day degrees below zero |
| 5 | Monthly mean accumulated day degrees above zero |
| 6 | Monthly mean 10 cm soil temperature |
| 7 | Monthly mean rainfall |

| | |
|---|---|
| 8 | Monthly mean sunshine duration |
| 9 | Monthly minimum screen minimum temperature |
| 10 | Monthly minimum grass minimum temperature |

There were 12 complete years of aphid trap and associated weather data. This historic data was separated into two sections: the training set and the testing set. The training set consisted of the first 10 years of data, the final two years of data formed the testing set.

Five different models were produced using both methods. These five models were trained using inputs from different times of the year. The first model used the weather data for January, the second used the weather data for both January and February, and so on until the fifth, which used weather data for January, February, March, April, and May.

*Multivariate Analysis.*

It is often found that there is a high correlation between weather variables (Howling *et al.,* 1993). One of the major assumptions of multivariate stepwise regression is that the variables are uncorrelated. If the variables are correlated the order in which the variables are presented to the stepwise regression becomes influential. Therefore PCA was performed on the 10 weather variables to remove any correlations. In the PCA the correlation matrix was used rather than the covariance matrix, so that the variables were standardized to counter the effect that differences of scale might have had in the analysis (Chatfield & Collins, 1980).

Stepwise regression was then performed by regressing the first flight data on the principal components with an eigenvalue greater than one. The F values were set to a level such that the variables were included or excluded if they gave at least a 5% increase to the significance of the model.

The five models (Jan, Jan-Feb, Jan-Mar, Jan-Apr, Jan-May) were calibrated using the training data. Predicted values were obtained by finding the PC scores for the test set and running them through the models and the 95% confidence intervals were calculated using the techniques suggested by Montgomery & Peck (1982).

*b) Neural Networks.*

There are various different types of neural networks and the main division is between those that are supervised and those that are unsupervised. The difference is that for a supervised network the user must say if the network output is right or wrong. A supervised network was used here, as the actual date of the first flight for the training set is known. The type of neural network used was a multilayer feed-forward network, which has a number of consecutive layers with a set amount of nodes (or neurons) in each layer. The first layer contains the input nodes and the final layer contains the output nodes, with the layers between known as hidden layers. Every node is connected to every other node in the next layer and these connections have a weight, which was randomly set between zero and one when the network was initialized (Figure 35).

There are a number of different algorithms to implement the learning process. The backpropagation algorithm was chosen because it can learn from examples (Lek & Gu'egan, 1999), and it can accept non-binary input values (Elizondo *et al.,* 1994; Venuri, 1988). The training occurs by iteratively changing these weights such that the error (the difference between the actual results and the predicted results) is minimized.

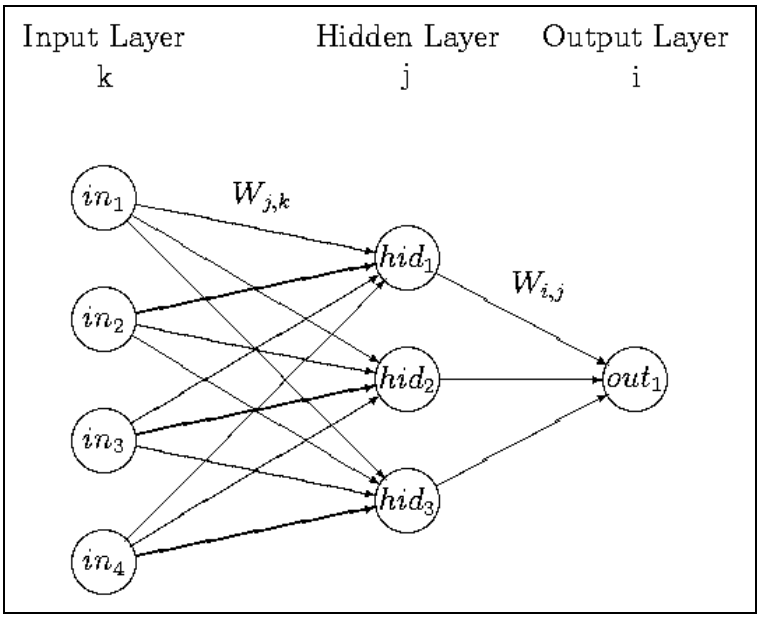



Figure 35. A Multi-layer feed forward neural network

This network has a nonlinear error surface due to the output for each of the hidden layers and the final output layer being passed through a sigmoid function (Equation 1)

$$f(x) = \frac{1}{1+\exp(-x)} \qquad \text{Equation 1}$$

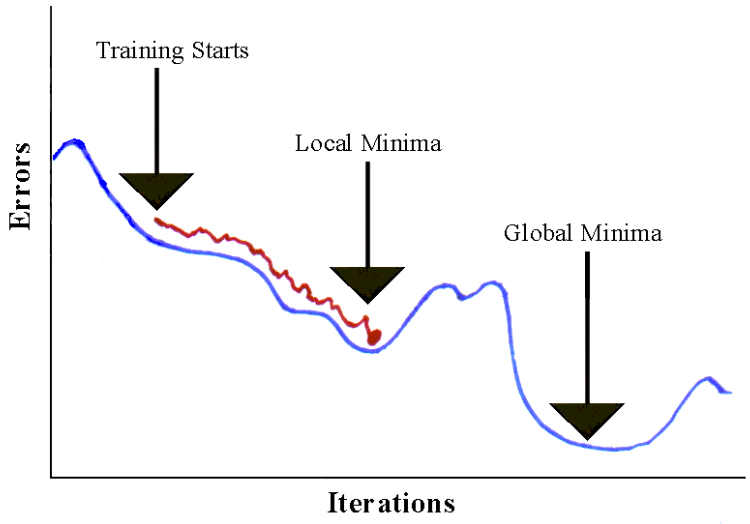



Figure 36. Errors plotted against iterations

The greater the number of hidden layers in the network the more ridges there will be on the error surface, so that any continuous (or discontinuous) function can be represented (Russell & Norvig, 2003). However, this approach is disadvantaged as, due to the nonlinearity of the network, it is possible that training will finish at a local minima (Figure 36), so the network may never attain the global minimum in the training process (Rogers, 1997).

The back-propagation algorithm (Box 1) has two processes occurring, the first is the feed-forward process, where the inputs are 'fed' forward through the network to produce an output. The process described here is for a single hidden layer network, as shown in Figure 35, although it can easily be extended to have more layers. The inputs to the nodes in the hidden layer are the weighted sum of the inputs (Equation 2).

$$Hid_j = \sum_k W_{j,k} In_k \qquad \text{Equation 2}$$

Where *j* is the node in the hidden layer; *k* the node in the input layer; $W_{j.k}$ the weight of the connection between *j* and *k*; and $In_k$ the value of the input node *k.*

The output for the nodes in the hidden layer ($HidOut_j$) is the resulting sigmoid (equation 1) of equation 2 (i.e. where $Hid_j$ is *x* in equation 1) . Similarly the nodes in the output layer can be calculated as the weighted sum of the outputs from the hidden layer.

$$Out_i = \sum_j W_{i,j} HidOut_j \qquad \text{Equation 3}$$

Here, the final results from the output layer is the resulting sigmoid of equation 3.

1. Initialise the network, including number of hidden layers and number of nodes in each layer.
2. Initialise the stopping condition (minimum mean square error, or maximum iterations), the learning rate (μ) and the momentum term (α). Randomise all weights so they have a value between 0 and 1.
3. For every sample in the training set repeat steps 47.

Box 1: Back propagation algorithm

The second process of the backpropagation algorithm is the backpropagation of errors (the differences between the actual and the predicted results), whereby the errors are calculated and back propagated through the network. The updating rule (also known as the delta rule) is found from the derivation of the error gradient. The weights between the hidden layer and the output layer are updated utilizing a learning rate and a momentum term. The weights between the input layer and the hidden layer are updated similarly.

This procedure continues for every sample in the training set until some stopping criteria is met. The criterion for deciding when a network is fully trained changes from implementation to implementation, however there are three standard stopping conditions (Russell & Norvig, 2003), being;

1. The number of iterations is above a certain threshold.
2. The error between the actual and the predicted results, or the average error for all samples in the training set, or the mean square error, is below a certain threshold.
3. For every sample in the training set the error is below a certain threshold.

The stopping criterion used in this study was the third type. Following recommended practices, all input variables were standardized to have a mean of zero and a standard deviation of 1 (Swingler, 1996). The actual Julian days were divided by 365, so they were transformed to be on a linear scale between zero and one, as if the inputs were left in their original state, equation 1 would make them so small the computer would round the inputs to zero. For the output variables equation 1 returns values between zero and one, so when multiplied by 365 will give the predicted Julian date.

The architecture of the neural network is a description of the number of hidden layers and the number of nodes contained in every layer. It is important that the architecture is chosen in such a way that the network represents the actual

process. However, this is very rarely known in advance (Russell & Norvig, 2003) and as a result the architecture must be estimated. Using the evolutionary optimization algorithm in “Pythia’’, (software designed for ANN development, see http://www.runtime.org/pythia) the three most appropriate architectures for all five models were found. For each of these three architectures five learning rates (0.2-0.8) and five momentum terms (0.3-0.7) were chosen. This gave 15 predictions for every model in the test set and the resulting 15 predictions were averaged to give an overall prediction. The purpose of the repeated sampling was to counteract the randomization of weights upon initialization.

*Model Comparison*

The models were first evaluated by looking at the predicted values and the actual values and calculating the coefficient of determination ($R^2$). Models were also compared by determining how many of the actual values lay outside of the 95% confidence interval range, or more importantly, how many of the actual values were lower than the predicted values. This procedure was selected as if the predicted value is higher than the actual value then the crop may already have become infested with aphids by the time sprays are applied. However, if the converse is true, the insecticide will have been applied by the time migration occurs.

*Multivariate Analysis Results*

The best results were found using the weather data for Jan – Apr (Figure 37), and Jan – May ($F = 5.274$; $df = 19,19$; $P < 0.007$; $R^2=0.709$ and $F = 5.788$; $df = 19,19$; $P < 0.007$; $R^2=0.769$ respectively). Both results are highly significant, however the majority of first catches occurred at the beginning of May. This means that if predictions cannot be given until the end of April then a high proportion of migrations may have already occurred by the time insecticides are used. Of the earlier data sets, the weather data for January gave the best result ($F = 2.306$; $df = 19,19$; $P > 0.05$; $R^2=0.639$), although this is not significant. The results for all five models are shown in Table 39.

Table 39. Results from both multivariate analysis and artificial neural network

| Multivariate statistics | | | | Neural networks | | | |
|---|---|---|---|---|---|---|---|
| model | R | F | P | model | R | F | P |
| Jan | 0.639 | 2.306 | 0.1 | Jan | 0.765 | 5.888 | 0.004 |
| Jan-Feb | 0.579 | 9.646 | 0.001 | Jan-Feb | 0.776 | 10.385 | 0 |
| Jan-Mar | 0.331 | 7.579 | 0.002 | Jan-Mar | 0.657 | 13.39 | 0 |
| Jan-Apr | 0.709 | 5.274 | 0.007 | Jan-Apr | 0.856 | 14.93 | 0 |
| Jan-May | 0.769 | 5.788 | 0.005 | Jan-May | 0.79 | 9.708 | 0.001 |

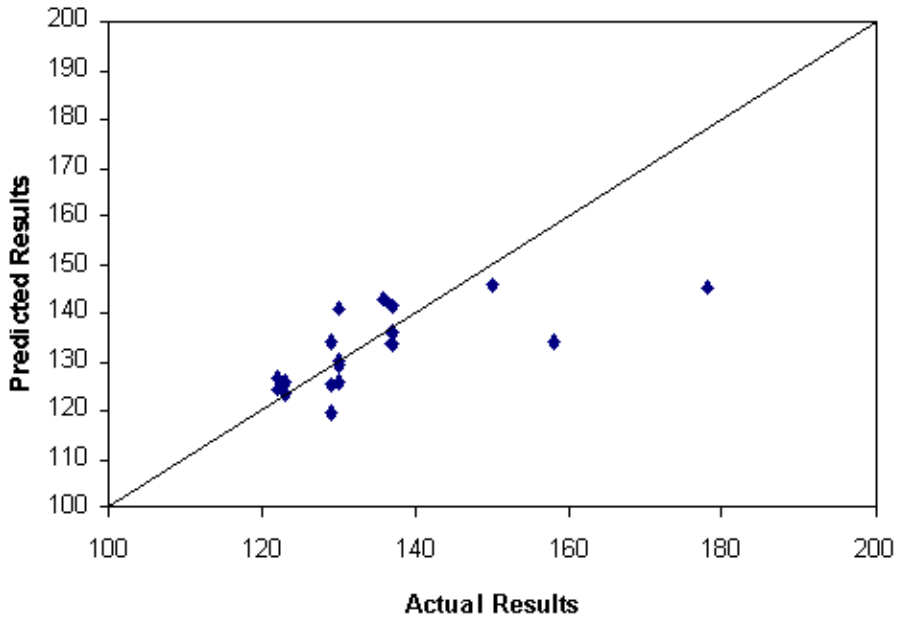



Figure 37. Correlations between actual and predicted results for Jan – Apr using multivariate regression

The best model Jan-May has 7/20 actual results lying outside the 95% confidence interval, but only three of the actual results were below the lower bound (Figure 38).

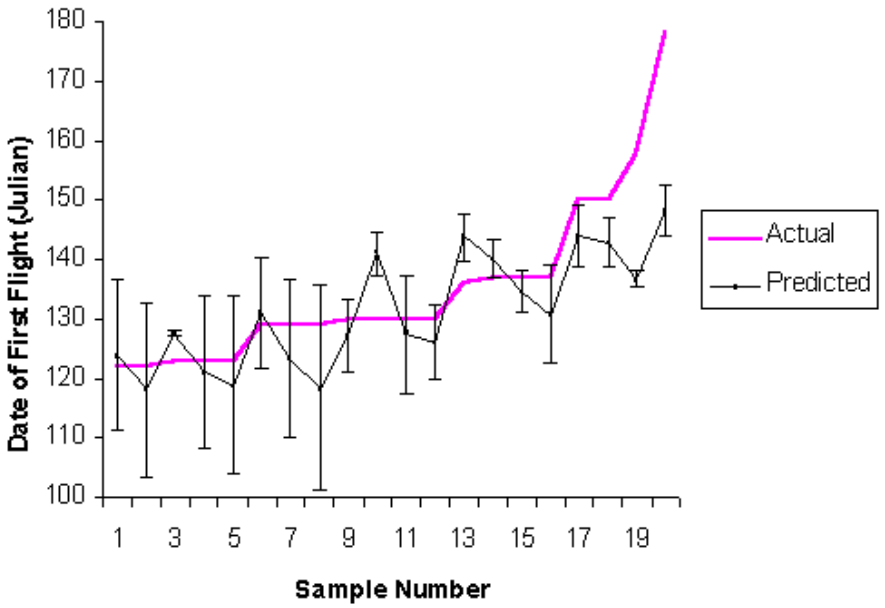



Figure 38. 95% Confidence interval showing actual results and predicted results for Jan-May using multivariate regression

*Neural Network Results.*

The stopping condition was met after an average of 1130 iterations, with a maximum of approximately 12,000 iterations for one particular trial. In Figure 39 it is

clearly shown that the total error decreases with the number of iterations until the stopping condition is met.


Figure 39. Errors plotted against iterations, found using Jan-May neural network

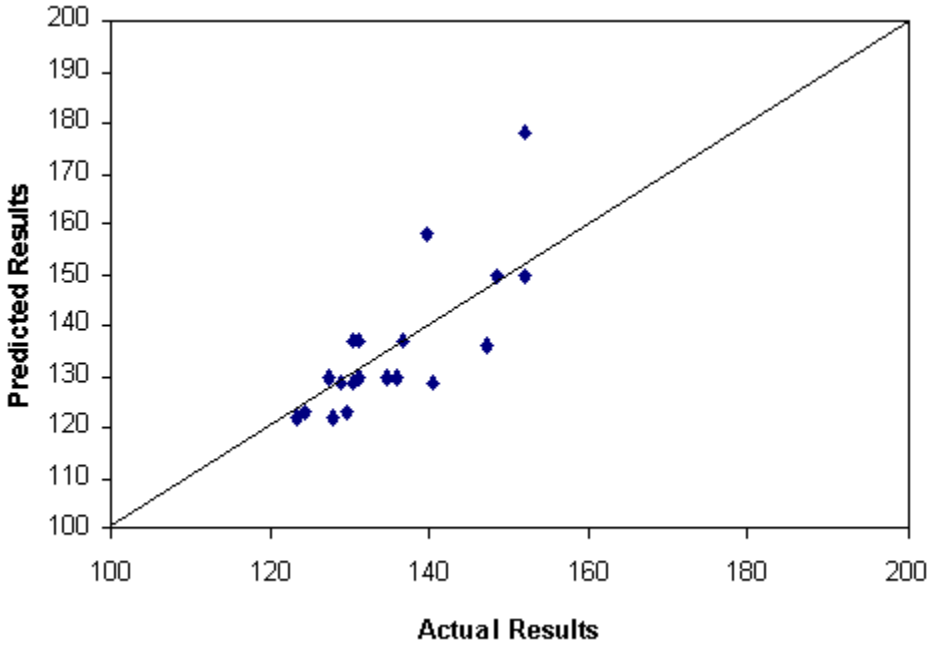



Figure 40. Correlations between actual and predicted results for Jan-Apr using artificial neural networks

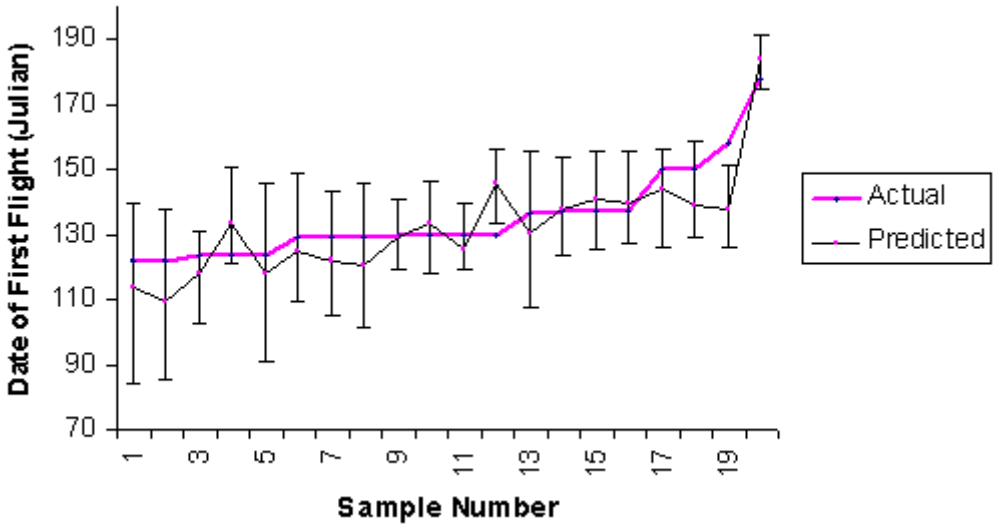



Figure 41. 95% Confidence interval showing actual results and predicted results for Jan-Apr using artificial neural networks

The coefficient of determination, $R^2$ and relative F values are shown in Table 38. As with the method of multivariate regression the best correlation between actual and predicted results are for the models using the inputs for Jan-Apr (Figure 40) and Jan-May. Using the neural networks method the best model appears to be Jan-Apr ($F = 14.930$; $P < 0.001$) indicating a highly significant result, with only two points lying outside the 95% confidence interval and one of the actual results occurring before the predicted results (Figure 41). However, this clearly suffers from the same problem as the multivariate analysis as the prediction cannot be made until after the end of April and so miss predicting early flights. However, in contrast to the multivariate analysis, all five models using the ANN method were significant and could be used in series (i.e, at the end of each month) to provide a prediction throughout the growing season.

It is interesting to note that all of the five models using neural networks not only have a higher coefficient of determination than their multivariate counterparts, but they also have a higher significance level.

*Discussion*

It is immediately apparent from the results that the method of neural networks provides more reliable results than the method of multivariate analysis.

There are two possible reasons for this. The first is possibly the loss of variability; in the principal components analysis only the principal components that had an eigenvalue greater than one were used in the stepwise regression. This is because the other principal components explained so little of the variance they wouldn't have made a significant improvement to the model. Also, the method of stepwise regression removes a lot of principal components from the model, so that in the end all five models only had two or three of the principal components as variables. This

leads to a loss of variability, which did not occur with the neural networks model, where all variables were used.

The second possible reason for the improved predictions of the neural networks is that the mapping between the weather data and the Julian date of first flight is almost certainly nonlinear (Chon *et al.,* 2000; Lankin *et al.,* 2001). The multivariate analysis is only capable of giving a linear mapping (Kendall, 1980), whereas the logistic equation (Equation 1) in the neural network implies that the neural network is nonlinear (Lek & Gu'egan, 1999; Russell & Norvig, 2003).

However, there are various problems that can occur when using neural networks. One of the main problems is where to set the stopping criteria. It has already been said that the error surface is nonlinear, and this results in local minima as well as the global minimum when minimizing the errors. If the final total error is too high, or the total number of iterations is too low, when setting the stopping condition, then there is the chance of the model finishing in a local minima, and never achieving the global minimum (Figure 36).

Overcompensating for this phenomenon can cause more problems known as overfitting. If the total error is too low, or the total number of iterations is too high when setting the stopping condition, then the training set will be continually shown to the network, until the network is able to find a way to "remember" the training set. This means that the network will be able to predict well for any test where the variables are identical to those in the training set, but will not be able to predict for values outside its range of experience.

Overfitting is also a problem that can occur in statistics when there is insufficient data with which to calibrate the regression model, and the model will only be useful in predicting values within its configuration data range. It is for this reason that a relatively large training set (over 150 samples) was used in this study and this should avoid the problem of overfitting the multivariate analysis. Also, with the neural networks approach, a number of trials were performed where the stopping conditions were changed and the average of these trials was used as the prediction. This avoided the problems of both overfitting and local minima.

It was found during the testing of neural networks that if two networks were trained on the same data, and all conditions were kept the same, then the network could produce different results because of the randomization of the weights upon initialization. A multiple number of trials were used to overcome this phenomenon (Melsen *et al.,* 1993). However, this was still a relatively small number of trials, and it may be possible that a greater number of trials could be required to find the most accurate prediction value.

There are three ways in which the methods described above could be extended. The first of these is to see how accurate predictions are based purely on maximum and minimum temperatures. Using only daily maximum and minimum temperatures, it is possible to calculate five of the ten weather variables used as inputs to the neural network (mean temperature, mean minimum temperature, minimum minimum temperature, degree days below zero and degree days above zero). If this method was able to produce accurate predictions then it would be possible to find the predicted Julian date of the aphid migration in individual fields.

Further work could also be done to see how well this method works not just for other aphid species but for other pests as well. Many different aphid species are

caught in the insect traps and timings for the first flights could be extracted from the database and used to test the neural network. It may also be interesting to see how the network fares against other pest species not only aphids, although this may require further work on the day-degree methodology

The final area of expansion would be to look at the population dynamics and insect phenology. However, this would be more challenging because of site-to-site variations in the environmental and ecosystem conditions. For instance, willow-carrot aphid over winters on willow trees, so a carrot field surrounded by willow trees is possibly more susceptible than one without. For this reason if the same network is applied to different sites, environmental factors may have to be taken into account. However, if the site remains constant there is no reason why this method cannot be extended to predict aphid population dynamics. Other issues would then come into play, such as the network learning false trends, and the issues concerning the quality of the data.

Overall, artificial neural networks have been shown to provide more accurate and useable results for the prediction of the Julian date of the first migration of willow-carrot aphid than the multivariate methods. These models could be used at the end of each month from January onwards to provide a series of predictions enabling carrot growers to incorporate this information into their decision-making processes.

## Conclusions

Overall, the project has made several significant scientific advances and has also facilitated the delivery of improved, robust diagnostics and much information on virus reservoirs and properties. Moreover, the greater understanding of the epidemiology of insect transmitted viruses gained through the project may deliver other approaches to study additional virus-vector interactions. Our understanding of virus epidemiology has been greatly enhanced; although it has been reported that PYFV is present in cow parsley, the project has shown that PYFV and AYV occurs widely in other weed hosts, notably hogweed. The results show that PYFV in hogweed forms a distinct clade (family) to that in cow parsley/carrot and hogweed cannot be source of PYFV for carrot crops. The relatively small number of isolates from celery and parsnip sequenced are very similar to the hogweed isolates and we can now say that in the UK there are two apparently entirely separate pools of virus *viz* those in carrot/cow parsley and those in hogweed/celery/parsnip. We have therefore established that, of the plants tested, only cow parsley carries the virus in a significant proportion of plants and carries PYFV of the right sequence family. We have also shown cow parsley carries the helper virus AYV. Secondly our understanding of vector migration and behaviour has been greatly improved; weekly use of insecticides against willow-carrot aphids have become routine but results from the networks of insect traps have indicated that the decisions to make sprays are not necessarily related to pest risk, for example in 2001 only a single aphid was caught in any of the traps but growers continued with their spray programme, and that more rational and effective management of PYFV and its vectors could be achieved based on the forecasting system developed in the project.

*Field-based vector phenology*:

Infield water trapping networks across all four years of the project have shown that the primary vector of PYFV, the willow-carrot aphid, arrives into the carrot fields between late April and late July and that these numbers peak between mid May and early June. There is a wide variation, particularly in the number of aphids, but also in the timing of first flight and peak, between fields both within and between regions. This field based variability agrees with previous studies of field specific trapping systems (e.g. Northing *et al*., 2004) and implies that the current method of relying upon the regional data from the Rothamsted Insect Survey (RIS) is sub-optimal and thus may lead to an inaccurate assessment of when to begin the spray programme. The main risk to the crop from this method occurs if aphids arrive into the field before they are first caught in the RIS suction traps. The use of in-field water traps could enable growers to ascertain when the willow-carrot aphid first arrives into the crop. These traps are easy to maintain, are cheap to run and there is already a commercial service in place for use within other cropping systems (Northing *et al*., 2004), mainly seed potatoes, but also in lettuce.

The results from this field trapping network also raised the possibility that a greater number of aphids were found in fields where there was a lower crop density. In the only year where investigation into this was possible (2003), the sites with a reduced seed rate had a significantly higher catch of the willow-carrot aphid. The lower seed rate would lead to a greater contrast between carrot seedlings and bare soil than in a conventionally grown crop. Evidence that suggests that the contrast between soil

and crop is important for aphid colonisation has been reported in other aphid-crop systems (Clements & Donaldson, 1997), where the numbers of cereal aphids were reduced in cereals grown through a permanent understorey of clover, when compared to a conventionally drilled crop. The clover crop provided a green background and camouflaged the emerging cereals. Care should be taken when interpreting our results, as comparison of seed rates was not possible within the same sites. However, the influence of crop density on aphid immigration into crops is worthy of further investigation. The implication of this evidence, if correct, is that crops drilled with a low seed rate are at a greater risk of aphid colonisation and should be monitored more closely for aphid arrival.

Over-wintering willow-carrot aphids were found on umbelliferous plants within the margins of the carrot crops at only one site of the twelve monitored (three aphids from 575 samples). This suggests that the umbelliferous plants within the field margins are not an important source of the aphids within the crop, however it remains possible that they could be a source of both aphid and virus at other times of the year.

*Virus epidemiology and molecular investigations*

Following extensive sample testing in 2000 and 2001 it became apparent that there was no clear association between the ELISA results and infection of herbaceous hosts in either year. This required that a separate technique was developed to ensure the accuracy of the diagnostic tests used within the project.

New TaqMan® assays for the detection of both AYV and PYFV in plant material were developed and proved invaluable for laboratory testing of plant material. Confirmation of infection in plant material with either virus was obtained conclusively within a short time prior to using source plants for vector transmission experiments, even when virus titre was very low. Although the assays were not designed for quantitative analyses, it was possible to extend analyses of results to determine the 'best' plants for use in these experiments where source plants with a high virus titre were desirable. In addition, the AYV and PYFV TaqMan assays are sensitive enough to detect virus in individual aphids but samples must be stored in a suitable preservative to ensure against RNA degradation.

Although SYBR® Green has clear uses as an alternative to PCR and TaqMan® detection assays, the complexity of PYFV with its high variability between isolates, and resource (time) constraints within the project, prevented further SYBR® Green method development for the detection of PYFV.

Although serology was not very effective, because of the molecular variability in PYFV, a range of PCR primers have been developed and it is now possible to detect virtually all sequenced isolates of PYFV in just one PCR. In addition to the detection element, automated extraction of RNA from plant samples and combining these extraction techniques with real time PCR will allow testing of 60-90 samples a day representing significant advances in the delivery of cost-effective virus detection to the horticulture industry. Furthermore, the initial primer sets developed during this project have been transferred to diagnostic laboratories and the assays are currently available to growers and the industry, providing a much more robust method for testing for PYFV than was available prior to the start of this project.

*Virus transmission*

In order to transmit PYFV to carrots, the willow-carrot aphid must have already acquired the helper virus, AYV. As carrot is resistant to AYV, the aphid must have acquired it from a non-crop host before arriving into the field. This means that there can be no spread of PYFV from carrot to carrot unless the aphid has already acquired AYV from a different host. Hence, it is the winged aphids arriving into the crop from AYV or AYV/PYFV infected hosts that will spread the virus. Experiments carried out to investigate the ability of the willow-carrot aphid to acquire and transmit the AYV helper virus have shown that it can be acquired and transmitted by the winged aphids after only two minute feeding periods at temperatures as low as 15ºC, but efficiency is low. Maximum virus acquisition efficiency was reached by 24 hours acquisition access period (AAP) at temperatures equal to or above 15ºC. Maximum virus transmission efficiency was reached by 24 hours (at 10-15ºC) or 30 minutes (20ºC). Aphid feeding periods for both virus acquisition and transmission appear to be more influential than temperature.

These data partly support and extend the work of Elnagar and Murant (1976a) who concluded that the frequency of transmission increased with increasing AAP and IAP. However, this study found that acquisition could occur after only two minutes compared to their minimum time of 15 minutes. Thus, contrary to the conclusion of Elnagar and Murant (1976a), that AYV is concentrated in deep-lying tissue and PYFV is distributed throughout the leaf, this study may indicate that AYV is not confined to the vascular regions but is also distributed throughout the leaf, like PYFV.

As the transmission of PYFV may only take place *after* the acquisition of AYV, and because carrot crops have immunity to AYV, reservoirs of the helper virus in the field are predominantly restricted to the non-crop hosts found in headlands and hedgerows. It appears that while a greater risk of virus spread will come from alatae feeding for periods of up to 24 hours at relatively high temperatures (e.g. 20ºC), aphids exposed to shorter feeding times and lower temperatures still have the ability to acquire and transmit AYV, thus maintaining at least a low level of virus pressure.

The evidence suggests that serial transmission of these semi-persistent viruses is also possible, with some willow carrot aphids able to transmit the virus up to 4 days after acquisition of the AYV/PYFV complex. The potential for serial transmission combined with the temperature effects on transmission time and the general principle that increasing temperature increases the probability of aphid movement between plants (Walters & Dixon, 1984) suggest that the risk of virus spread is increased in warmer weather due to greater within crop movement by viruliferous aphids.

*Maintenance of experimental cultures*

The maintenance of PYFV in laboratory cultures was consistently problematic, with stocks of PYFV alone or PYFV and AYV in complex periodically failing, a phenomenon experienced in other contemporary laboratories (J. Morris, pers.

com.). Repeatedly, stock plants infected with both viruses and subsequently used as source plants for vector transmissions in virus maintenance, yielded source plants with AYV only. This occurrence is not exclusive to the PYFV system. It has been observed elsewhere that when laboratory plants were infected with PYFV and Italian carnation mottle and after repeated passages, PYFV was eventually lost, although symptom expression remained unchanged. In each case, the most virulent virus flourished. Watson *et al*., (1964) observed attenuation of motley dwarf in field isolates that caused severe stunting in carrots but became less virulent after several months of glasshouse sub-culturing.

It appears that for naturally occurring PYFV to survive, infections of PYFV/AYV in host plants must maintain a balance, such that PYFV never totally disappears from a local area, a theory perhaps supported by Elnagar and Murant (1976a) who suggest that infection with either virus is unaffected by the presence of the other. While the experience of this study is that in chervil AYV out-competes PYFV in mixed infections in individual plants, the two viruses have evolved to co-exist naturally in cow parsley and other biennial or perennial non-crop hosts. Experience in maintaining laboratory cultures of AYV (singly) and AYV/PYFV in complex suggests that AYV replicates much more efficiently than PYFV. Following this assumption, and considering that PYFV relies exclusively upon AYV for successful transmission, the loss of PYFV in laboratory cultures may have been caused by the accumulative affect of AYV being vectored far more efficiently than PYFV. After several vector transmissions, with fewer PYFV particles on each occasion, a smaller reservoir of virus would be available, thus resulting in the eventual total loss of PYFV but high titre of AYV.

*Pesticide Efficacy against Willow-Carrot Aphid*

Preliminary trials showed that the three main insecticides commonly used in umbelliferous crops (Aphox (pirimicarb), Hallmark (λ-cyhalothrin) and Dovetail (pirimicarb and λ-cyhalothrin)) were all very effective against winged willow-carrot aphids with 100% mortality with doses as low as 25% (Aphox 48hrs; Dovetail 24hrs) and 10% (Hallmark 24hrs) of the standard field rate. Due to the possibility of serial transmission, it was also important to understand how effective the residual product was over time and hence its ability to continue to exert control of the virus some days after the initial treatment. These products were tested alongside two new products (neonicotinoids) and a toxic standard (Dursban WG – Chlorpyriphos). The results showed that, at full field rate, aphox, dovetail and the two neonicotinoid products, all had a 100% mortality after 15 minutes exposure. These products are therefore very effective at reducing virus spread by killing aphids with the potential for serial transmission. Further bioassays investigated the residual effect of the most effective products (a neonicotinoid (YRC+D OD) and Dovetail). This showed that the residual effects of YRC+D OD caused aphid mortality sooner than those of Dovetail. For example, a two hour exposure to three-day old residues led to 70-80% mortality with YRC+D OD and 50-60% with Dovetail. The 21 day old residues of both products caused 100% mortality after forty-eight hours exposure. The use of these products would appear to provide good long-term protection against aphids, with swift and considerable residual activity occurring up to a week after application and slower activity up to three weeks after application.

*Relationship between PYFV and other viruses in cultivated and non-crop hosts.*

PCR tests have been developed for carrot redleaf, carrot mottle viruses and carrot redleaf virus associated RNA. Moreover, project results have shown that for both carrot redleaf virus itself and CRLVaRNA English isolates are closer to the Californian than to either Belgian or Scottish isolates, whereas the English CmoV is more like the Belgian than Scottish sequence. The detection of the associated RNA is a first for carrots outside California and the first in any crop in the UK.

*Phenology Model*

A computer model has been developed to predict the first flight of willow-carrot aphids. Incorporating algorithms based on artificial neural networks (ANN), the system has been validated successfully against data observed in carrot crops at several sites in the UK. The development and validation of a model utilising ANN to forecast insect dynamics is a first worldwide and represents a highly significant strategic achievement. Five models were developed using environmental data from January to May and the models based on ANN's performed better than those developed using previously established multivariate techniques. A model that can provide a reasonable prediction of when the willow-carrot aphid is likely to start arriving into the crop can form an integral part of a new management strategy for PYFV.

*Rational Vector Management*

An investigation into the current management strategy of the industry was considered alongside the new information generated within this project to ascertain whether a new management strategy could be developed that maintained or improved the level of disease control whilst also providing a more rational approach to insecticide use. A more rational, evidence based approach will allow the industry, in low risk years, to reduce the amount of insecticide used without jeopardising their yield and in all years, provide a method of justification of the insecticide programme used for communication to the growers customers.

A management strategy has been formulated, based on targeted insecticide use, following predictions from the phenology model and data from in crop water traps.

**New Crop Management Strategy**

The advances made within this project have enabled the development of a new management strategy for the control of PYFV in carrots. The proposed new strategy is as follows:

1. Using the ANN derived phenology model, provide regional predictions (e.g. via the internet/press release etc…), at the beginning of February, March, April and May, of the first flight of the willow-carrot aphid based on environmental data from the major carrot growing regions.
2. Using the regional prediction date as a guide, growers should set up field specific water traps in their crops a week or two before the predicted date. The contents should be analysed weekly to ascertain the actual date of the beginning of the annual migration of the willow-carrot aphid into the growers crops.
3. Using the results from their field specific water traps, the grower should begin their spray programme once the first willow-carrot aphids have been trapped.
4. Growers should utilise products that have been shown to be effective both topically and residually e.g. the new neonicotinoid products or Dovetail (bearing in mind the requirements for carrot root fly control)
5. Where possible, growers should continue to use the water traps to monitor the incoming populations of willow-carrot aphids and potentially amend their spray programme once the end of the aphid flight has occurred (e.g. following two consecutive trap samples with no carrot-willow aphid)

The use of this new management strategy will provide a rational approach to the problem of PYFV, leading to better targeting of insecticides and, in low risk years, a reduction in insecticide use. This will benefit both the grower (reduced virus due to better targeting; reduced input costs due to lower insecticide use) and the environment (fewer chemicals in the environment and lesser effects on non-target organisms due to reduced insecticide use).

This new management strategy is relatively cost effective, but will require funding from the industry to put it in place. The development of the predictions will require the acquisition of the environmental data and some resource to cover the cost of reparamatising the ANN and producing the predictions. The use of field specific trapping requires a third party to sort and identify the willow-carrot aphids out from the rest of the trap catch. This cost could be borne either by individual growers or by the industry as a whole. Fortunately, there is a commercial service already in place for a similar scheme in Seed Potato crops, so negotiations with the service provider is recommended. Currently, the neonicotinoid products are still in development, so until they are marketed other insecticides will need to be used.

## Technology Transfer

Close informal interaction between consortium partners and with growers in general has occurred throughout the project. Samples of aphids from the networks of water traps and suspected virus infected plants were received from several sources and the results of studies utilising these materials have been presented to and discussed with the originators. In addition, several visits have been made to growers' farms and discussions regarding the project have taken place.

Prior to the onset of this project, no diagnostic methods of any kind were available for PYFV, AYV, CRLV, CMoV and CRLVaRNA. A small amount of polyclonal antiserum was available for the parsnip strain of PYFV (SCRI), which was used in IEM techniques, but this was found to be unsuitable for routine detection work. The molecular characterisation work carried out at HRI and CSL during the course of this project has led to the development of several sets of PCR primers for all of the viruses listed above. The PCR assays developed allow the specific detection of each of the viruses in single and mixed infections, something that was not possible prior to the start of this project. The assays developed are now in routine use in the diagnostic laboratory at CSL and have been used on a number of occasions to investigate suspect symptoms in carrot samples sent into the laboratory for diagnosis.

Since the ELISA tools developed for routine detection of PYFV were further modified using antibodies produced in HDC project FV228 "Carrots: diagnosis of PYFV" (Spence et al., 2000) the results from the original project have been transferred to HDC members via the appropriate commodity panel activities and form a basis of introducing the project to the wider industry audience.

An article was written for the LINK newsletter detailing the project, its objectives and potential benefits. Furthermore, preliminary results from the project were presented at an AAB conference on virus epidemiology and used to form the basis of a poster (Boonham *et al*., 2000).

An additional poster, depicting the development of TaqMan assays for the detection of PYFV and AYV, was presented at the Nordic-Baltic Congress of Entomology, resulting in an associated article being published in the Conference Proceedings (North *et al*., 2004).

This project also formed the basis of a Master of Philosophy degree (registered at Newcastle University), the thesis for which was submitted for examination in July, 2004 (North, 2004).

*Papers*

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*Presentations*

Boonham, N., Morton, A. Barbara, Spence, N., Barker, I., Morgan, D. 2000. Parsnip yellow fleck virus. Poster presented at the AAB Conference 'Plant Viruses', Dundee. 20-22 September 2000.

Spence, N. J., Barker, I., Mumford, R. & Wright, D.M. (2000). Carrots: diagnosis of parsnip yellow fleck virus. Final Report of project FV228, Horticultural Development Council, UK.

Julie North presented a poster 'Development of TaqMan® Assays for the Detection of Parsnip Yellow Fleck Virus and Anthriscus Yellows Virus' at The RES Postgraduate Forum, London, 2002 and AAB Postgradute Forum, Wellesbourne, 2002.

Derek Morgan presented a poster 'PYFV: Development of a Management Strategy' at The UK Onion and Carrot Conference & Exhibition, Spalding, 2001.

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