NF0406: INTEGRATED CROP PROTECTION IN SRC WILLOW PRODUCTION

Dr Ming Pei
Project Leader in SRC Crop Protection
IACR – Long Ashton Research Station
Long Ashton
Bristol
BS41 9AF

INTEGRATED CROP PROTECTION IN SRC WILLOW PRODUCTION

Final project report: NF 0406

Overall objective: To provide the scientific foundation for an integrated, non-chemical strategy of disease and pest management, and associated best practice protocols, in UK renewable energy willow production.

INTRODUCTION

There will be a large increase of short-rotation coppice (SRC) production in the next 10 years in the UK to meet the growing demand for energy from renewable sources. Grown in SRC plantations, willow is the favoured biomass crop for renewable energy in the UK and western Europe. Diseases and pests can seriously affect crop productivity. The most widespread and damaging disease is rust caused by fungal pathogens of the genus *Melampsora*. Plantings of susceptible willow clones are frequently defoliated prematurely and yields can be reduced by as much as 40%. The leaf-feeding chrysomelid beetles are amongst the most common pests of coppice willows in Britain. In some years, they cause extensive defoliation, resulting in stunted growth and, in severe cases, the death of young developing shoots.

Disease and pest problems are likely to intensify as more and more large-scale plantings are established. Economic, technical and environmental considerations rule out the possibility of using pesticides for the control of these damaging organisms. Therefore, development of crop protection measures based on more fundamental methods is essential to the success of a young biomass-energy industry. These methods include the breeding and use of resistant willow clones, designing plantations based on host genotype diversity, and the effective deployment of biological control agents.

This research programme addresses the genetic basis of variation in rust pathogen populations, factors influencing the severity of beetle attacks, mechanisms by which mixed clonal plantings suppress rust and beetles and the potential for deploying aggressive strains of the biological control agent of rust in clonal mixtures.

1. PATHOGEN VARIATION AND DISEASE EPIDEMIOLOGY

1.1 Biology of rust pathogen

Two major species of rust, *Melampsora epitea* and *M. capraearum*, occur in SRC willow plantations in the UK. Of these, *M. epitea* is the most widespread and damaging. Within *M. epitea*, many pathotypes (defined as a group of genotypes showing the same virulence/avirulence patterns on a selected set of willow host differentials), occur naturally. These pathotypes can be assigned to three form species, *larici-epitea typica* (LET, occurring mainly *on S. viminalis* and *S. cinerea* % *S viminalis* hybrids in SRC plantations), *larici-retusae* (LR, mainly on *S. burjatica / S. dasyclados*) and *larici-daphnoides* (LD, on *S. daphnoides*).

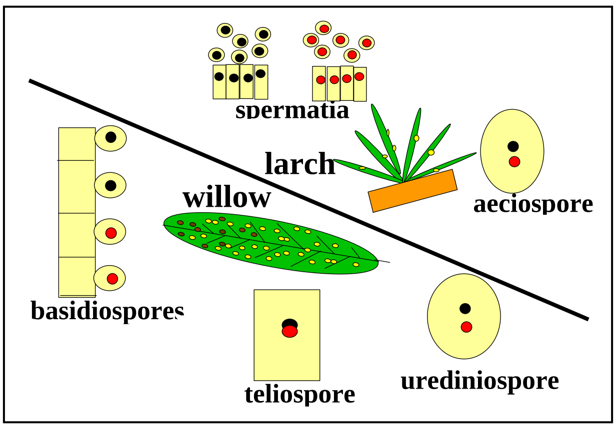

Fig. 1 Life cycle of *Melampsora epitea* and *M. capraearum*

Willow rusts have complex life-cycles. During their development they produce up to five spore stages (Fig. 1). Three spore stages occur exclusively on willows and the others on larch (called the alternate host). During late spring and summer, rust is seen on willows as yellow/orange pustules (uredinia) that produce urediniospores. The urediniospores are responsible for many cycles of disease in a growing season. In the autumn, teliospores form on previously infected willow leaves. Rust overwinters as teliospores on fallen leaves. In spring, they germinate to produce basidiospores that can only infect European larch. Infection by basidiospores results in formation of spermatia (sperm spores) and spermagonia (receptive structures). Fertilisation (gene exchange) occurs at this stage. The resulting aeciospores re-infect willows and produce urediniospores, hence completing the life-cycle.

1.2 Genetic interactions between rust pathotypes (Milestone 01/01)

The rise of new, virulent types of pathogens is the major cause of breakdown of disease resistance in crops. Since pathogenicity testing of field rust populations began in 1991, several previously unknown pathotypes have been detected. As most forms of rust undergo a sexual life-cycle, a major concern towards planting mixtures as a means of disease control is that the pathogen may be capable of accumulating various virulence genes through sexual recombination. This may increase the damaging potential of the pathogen and undermine the use of certain mixtures as a control strategy.

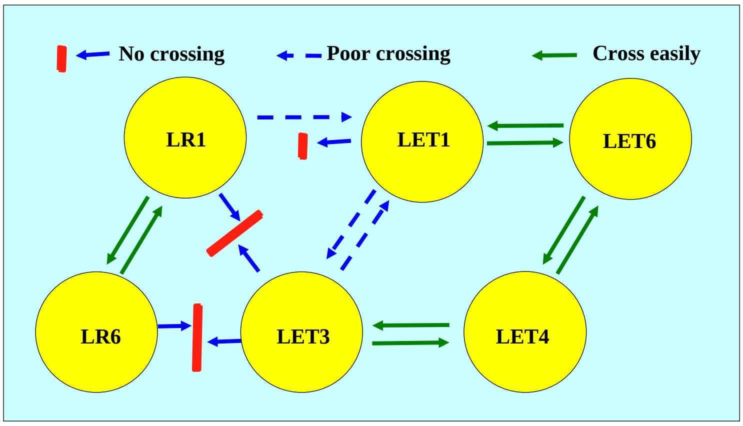

Fig. 2 Genetic interactions among LR and LET pathotyes

In this project, eight pathotypes (a pathotype is defined as a group of pathogen genotypes showing the same virulent/avirulent patterns on a set of host differentials), four found relatively recently (LET6, LET8, LR6 and LR8) and the other four most prevalent over the years (LET1, LET3, LET4 and LR1), were used to study genetic interactions between pathotypes. Using the alternate host European larch, crossing experiments were conducted to examine the likelihood of gene exchange between the recently found and the most prevalent pathotypes. Despite repeated attempts, the LET8 isolate and LR8 isolate failed to germinate and infect larch. The remaining LR6 isolate readily produced aecia (F1 hybrids) when paired with LR1. However, no crossing occurred when the LR6 isolate was paired with the LET3 and LET4 isolates. The LET6 isolate produced F1 when paired with LET1 and LET4 but no crosses were produced when paired with LR1 and LR6.

Previous studies suggested that, within LET, LET1 (predominant on *S. viminalis* clones) and LET3 (prevalent on *S. cinerea* % *S. viminalis* clones) do not cross easily, while LET1 and LET4 (on *S.* % *mollissima*) can exchange genes freely (MAFF, 1998). Results from our experiments showed that LET4 isolates are capable of hybridising readily with LET3. It indicates that gene flow between LET1 and LET3 may still be possible via other pathotypes such as LET4. The implications of our results are summarised in Fig. 2.

Our studies suggest that, in nature, many newly occurring pathotypes may be poor in fitness, as they may have been derived from hybridisation between genetically distant genotypes. Others, on the other hand, are reproductively fit and can exchange genes with existing pathotypes to produce various pathotypes, some of which may have accumulated virulences. Laboratory experiments and field population studies over the past 10 years suggest that virulence accumulation in willow rust is likely to be confined within a form species.

1.3 Genetic basis of pathogenicity in *M. epitea* (01/02)

One of the most important issues in breeding for resistance is how resistance genes are inherited. Plant disease is the consequences of the interactions between resistance genes in host and virulence/avirulence (pathogenicity) genes in pathogen. Therefore, behaviour of the genes for pathogenicity is as important as the behaviour of resistance genes in selection and breeding for resistance. Compared with the intensive studies on inheritance of rust resistance in crops, relatively little work has been done on the genetics of pathogenicity in rusts. With the rusts on woody plants, the first direct evidence of the genetic basis of pathogenicity came from our previous work (NF0402). Preliminary results from NF0402 suggested that one or two independently segregating genes may be responsible for virulence in LET1 and LR2.

In this project, carefully defined experiments were carried out to establish the genetic basis of pathogenicity in LET, using an LET6 isolate and five willow clones belonging to *S. viminalis* and its hybrids. Teliospores were produced from the isolate in a glasshouse and selfing was done using European larch. A total of 80 rust isolates were obtained from single aecia which developed as a result of selfing. By standardising inoculum dosage, each isolate was tested for pathogenicity on the five willow clones using the leaf disc method as described previously (Pei at al., 1996). The results show that the rust was homozygous highly virulent (all produced well-developed pustules) on *S.* % *stipularis* (*S. viminalis* % *S. cinerea*) and homozygous non-pathogenic or highly avirulent (none developed any pustules) on *S.* % *calodendron* (*S. viminalis* % *cinerea* % *caprea*). Pathogenicity patterns were segregated on *S. viminalis* 'Mullatin', *S. viminalis* 'Bowles Hybrid' and *S.* x *mollissima* 'Q83' (Fig. 3). The results show that two genes may be responsible for pathogenicity to 'Mullatin' and 'Q83', and one gene for 'Bowles Hybrid'. Unlike the preliminary results from our previous experiments using LET1 isolates (MAFF 1998), no obvious evidence was found with this population that pathogenicity genes for 'Mullatin' and 'Bowles Hybrid', both belong to *S. viminalis*, are linked. It appears that the genes are located at different chromosomes in the genome. The results also indicate that gene dosage may affect significantly the degree of virulence. For example, to 'Q83' or 'Mullatin', isolates having virulent genes in both loci are more virulent than those having virulent genes in one of the two loci.

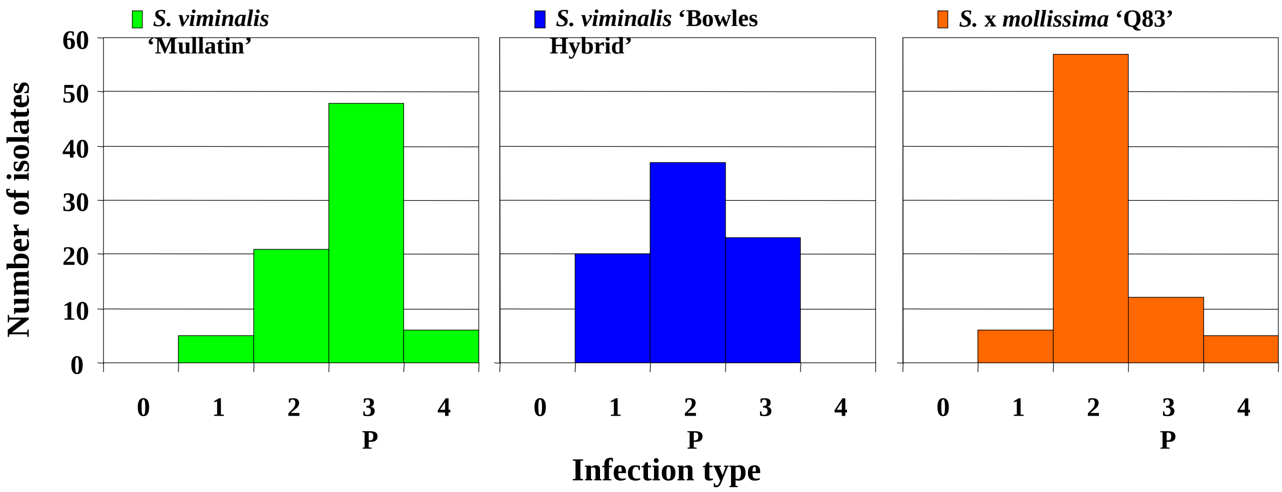

Fig. 3 Pathogenicity of the rust progeny from selfing of an LET6 isolate. Infection types 0 – 4: no pustules – well developed pustules. Infection types 2-4 are considered virulent. Positions of **P** indicate the infection type scores for the parental isolate.

An important implication of the results is that when the local pathogen population is less diverse and consists of only a few avirulent or less virulent genotypes (infection type 1 or type 2), willow plants would appear to be resistant in the field. Such resistance is likely to be short-lived because, through sexual reproduction, the pathogen may produce virulent genotypes (infection type 3 or 4), which, if become prevalent, would cause severe damage to the crops.

1.4 Heterogeneous origin of the new rust pathotype on 'Q83'

The rise and spread of new virulent types in plant pathogens, especially in highly specialised biotrophs such as rust fungi, can drastically breakdown host resistance and seriously affect crop productivity. For most rusts, rapid increase in population size is achieved through repeated cycles of asexual urediniospores. The urediniospores are thick-walled and known to travel long distances. To date, almost all the information on the spread of rusts is based on the combination of field observations, pathogenicity of rust, weather and spore-trapping data. Using these conventional methods, it is impossible to determine whether the new type has single or multiple origin. So far, hard evidence on the origin of new virulent types in rust pathogens has been lacking.

An outbreak of rust on *S.* % *mollissima* (*S. triandra* . % *S. viminalis*) 'Q83', an important biomass willow, was first observed at several locations in the UK in 1992. In the previous DTI-funded project (ETSU B/W6/00508), we developed AFLP protocol for willow rust and used it to examine, preliminarily, the rust collections obtained from 'Q83' in 1992 at Long Ashton, south west England and Markington, Northern England. In this project, a total of 54 isolates derived from the 1992 rust collections from Long Ashton, Markington and Loughgall, Northern Ireland, were examined using both the AFLP technique and the leaf disc inoculation method to determine whether the previously unknown pathotype was spread from a common source or from separate sources (Pei et al., 2000).

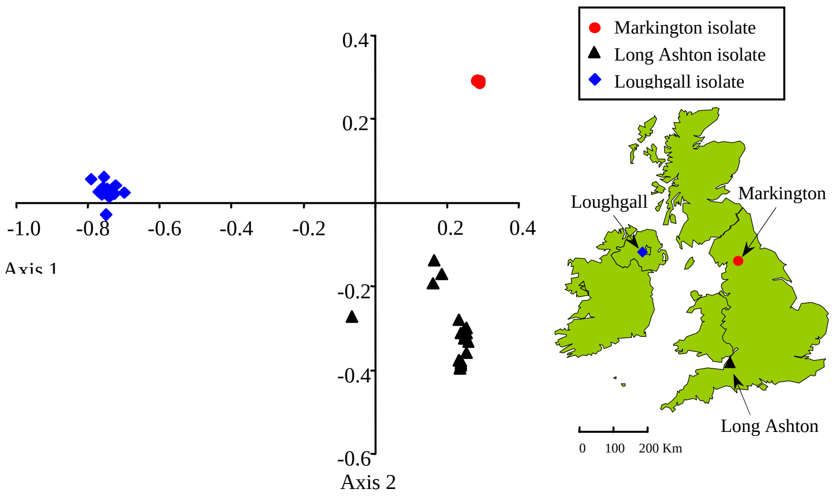

Figure 3. Principal coordinate analysis of *Melampsora epitea* isolates collected from *Salix* × *mollissima* 'Q83' at Markington ()), Long Ashton (э) and Loughgall (&) in 1992, based on 305 AFLP loci.

In the inoculation test, all isolates showed the same pathogenicity patterns on the eight willow differentials. However, when tested using AFLP, the three collections differed greatly in their genetic background. When the data were analysed, most diversity was partitioned between populations. Within site, the Markington population was least variable, 14 isolates being identified as the same clone. The results suggested that the rust on 'Q83' from Markington may have come from a relatively newly established source, while that from Long Ashton and Loughgall was from well-established sources. The results clearly indicate that, in 1992, this previously unknown pathotype was not spread from a common source but from separate sources. The AFLP analysis and early records on the host range of *M. epitea* indicate that the rust virulent to *S.* x *mollissima* may have existed in nature before 1992.

This work set an example of addressing important epidemiological issues using molecular tools in studies of rust diseases.

1.5 Gene flow of rust between geographical regions (01/03)

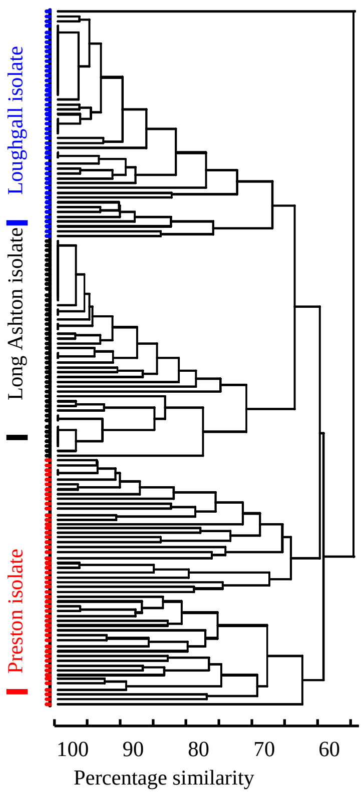

Fig. 5 Dendrogram of rust from 'Q83' at three sites in 1998

Willow clones free of disease in one location may become severely infected in another simply because pathogen populations differ between the two locations. The ability to spread in a plant pathogen crucially influences the durability of field resistance in a given geographical region.

This work was conducted to determine gene flow in the pathogen between different regions. Rust samples were collected from 'Q83' at Long Ashton, Loughgall and Preston (northern England) in 1998. The host clone and the three sites were selected because the work described in 1.3 gave background information on the rust infecting 'Q83' in these areas. Single pustule isolates were made from 50 samples from each site. Co-dominant microsatellites are still in the process of development and, therefore, AFLP was used to type the populations.

The results showed that the differences in AFLP patterns were greater between populations compared to that within a population (Fig. 5). Diversity within the Preston population was somewhat greater than that within Long Ashton or within Loughgall. Preliminary analysis on the gene flow between populations gave a prediction of Nm = 1.34. Theoretically, this is equivalent to an average of less than one spore (0.67 urediniospore, because urediniospores have two nuclei) migrating between the populations per generation. According to population genetics theory, if Nm <1, then local populations will differentiate; if Nm >1, then there will be little differentiation among populations. From our study, the estimate of the gene flow in the rust may be just enough to prevent local populations evolve separately.

1.6 Application of digital imaging technology in characterisation of resistance/pathogenicity

In this project, we made a breakthrough in the characterisation of rust resistance in willow/ pathogenicity in *Melampsora* by using digital imaging technology. Previously, we had used Infection Types (scales 0 – 4) to assess resistance/pathogenicity reactions in leaf disc inoculations (Pei et al., 1996). The infection types were scored based on pustule number counts and pustule size estimates. The main drawback of the previous method is that, because counting pustules and estimating pustule sizes are time-consuming, limited number of samples can be tested in an experiment. Moreover, estimates of pustule sizes given by different assessers may differ with the same samples.

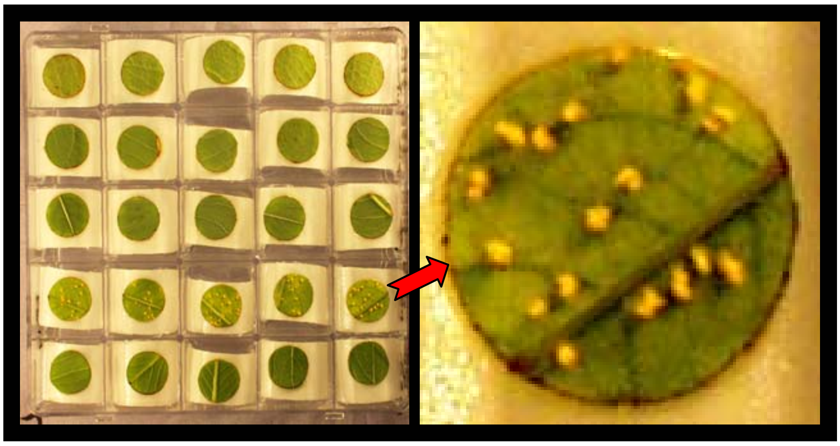

Fig. 4. Digital images of host/pathogen interactions in leaf disc inoculation experiments.

The new method uses a digital camera to record the disease reactions of leaf discs. Instead of counting pustules and estimating pustule sizes during experiments, the pustule number and sizes are measured later using the digital imagining software SigmaScan Pro 5. Using the new method, a large number of samples can be processed in a single inoculation experiment and the reactions on leaf discs can be quantified. Furthermore, a range of resistance reactions, which could not be properly recorded previously, such as discolouring and hypersensitivity, can be accurately profiled.

1.7 Willow dieback (01/04; 03/07)

Serious shoot die-back was noted on a number of willow types at two sites at LARS during the winter of 1998/1999, with the most obvious symptoms (shoot death) found on *S. burjatica* 'Germany' (Fig. 7). Associated with these shoots were numerous fruiting bodies of the fungus *Cryptodiaporthe salicella*, a bark-inhabiting fungus. *Phoma* sp., *Fusarium* spp., *Glomerella miyabeana* and some bacteria, including fluorescent *Pseudomonas* spp., were also found. Some 40 isolates of microorganisms were made from plants showing dieback symptoms, and willow cuttings were inoculated with florescent *Pseudomonas* and *Cryptodiaporthe* in three inoculation experiments. Limited development of lesions was observed but no typical dieback symptoms as seen in the field were produced. This suggests that the dieback may not simply be due to occurrence of exceptionally virulent strains of the two organisms which are most common on willow stems.

Fig. 8 Dieback on *S. burjatica* 'Germany'. Arrow indicates a stem without symptoms.

A further series of inoculations were done, during the autumn – winter, in the glasshouse on *S. burjatica* 'Germany' grown under stress (restricted water supply) or without such stress. As results, an isolate of *Phoma* sp., previously identified by CABI Bioscience as *Phoma samarorum* (IMI No. 385511), caused extensive dieback of shoots, with more severe symptoms on the plants grown under stress conditions during winter months.

Field assessment showed that *S. burjatica* ‘Germany’ was most severely affected. Other affected clones included (in the order of severe – slight) *S. dasyclados* ‘81090’, *S. burjatica* ‘Korso’, *S. dasyclados* ‘77056’, *S.* % *stipularis*, *S.* % *calodendron* ‘445 De Biardii’, *S.* % *sericans* ‘Coles’ *S.* % *mollissima* ‘Q83’, *S* % *dasyclados* Wimm., and *S. dasyclados* ‘79097’.

Through site visits and communication with growers, a survey was carried out in biomass willow plantings in the UK. The survey has shown that the dieback problem is not widespread in UK plantations. At present, the most likely underlying cause of the die-back appears to be the stress of the plants inflicted by, for example, the severe attack of rust on *S. burjatica* genotypes in the growing season. This, followed by a combination of environmental conditions at the time of leaf fall (resulting in exposed leaf scars), may have allowed a range of secondary invaders such as listed above to become established.

1.8 Field performance of willow clones against diseases and pests

Willows, as one-year-old shoots, in the National Willow Collection at Long Ashton were assessed annually for their resistance to diseases and pests. Disease and pest assessments were also carried out in several Forestry Commission sites (see 3.1) and in some commercial plantations. The assessments showed that all four major species of willow beetles occurred on the willows in the National willow collection and there were marked differences among the willow types in beetle damage. Results from the assessments and our observations suggest that current commercial clones (mostly *S. viminalis* clones plus its hybrids with *S. schwerinii*) have generally been resistant to leaf rust *M. epitea*. However, several of them are infected by the stem-infecting-form of rust in the UK. The new, highly promising biomass clone *S. viminalis* x *burjatica* ‘Stott 10’ (can yield twice as much as most biomass clones selected in 1980s) has been highly resistant to rust in most UK sites since it was bred in late 1980s. However, increased levels of rust on this clone have been found since 1997. Preliminary inoculation experiments suggested that the rust occurring recently on ‘Stott 10’ may be much more aggressive than those found previously.

2 ECOLOGY OF CHRYSOMELID BEETLES

There are four beetle species causing damage in SRC plantations, the major ones being the blue and brassy willow beetles (*Phratora* spp.). Beetle infestation is often severe locally, resulting in early defoliation due to foliar feeding. Feeding damage is caused firstly by overwintered adults early in the season, followed by larvae and then by newly developed adults later in the year.

2.1 Factors influencing beetle movement (02/01, 02/02, 02/03)

In this project, the following aspects were investigated to determine basic features of beetle migration and the mechanisms underlying beetle movement towards and within willow plantations.

Visual attraction: Willow plots surrounded by cereal crops and an experimental plot surrounded by a wide mesh screen were found to have significantly fewer beetles than those not hidden from view. This may indicate that beetle’s orient towards a plantation using vision. Sixty-four genotypes that differ in their susceptibility to the blue willow beetle had their light reflectance spectra compared. While genotypes clustered together for similar spectra, there were no correlations between susceptibility and spectra.

Pheromone/kairomone: Results from field experiments showed that beetles may be attracted to willows that already had a beetle presence, indicating the possibility of a sexual/aggregation pheromone. This was validated in the following year, during which extensive data were collected. Beetle densities were higher when adult beetles were present and willow leaves were damaged by beetle feeding (Fig. 8). The beetle numbers were similar on plants freshly damaged or damaged two days previously. These results suggest that two major factors, the presence of beetles and feeding damage, may affect willow beetle movements within plantations.

To investigate whether the presence of male or female beetles affects the density and the sex ratio of those attracted, male and female adults were confined in separate net cages suspended on potted plants and placed in willow plantings. Higher numbers of beetles landed on the plants with confined females. Of these beetles, there were significantly more females than males.

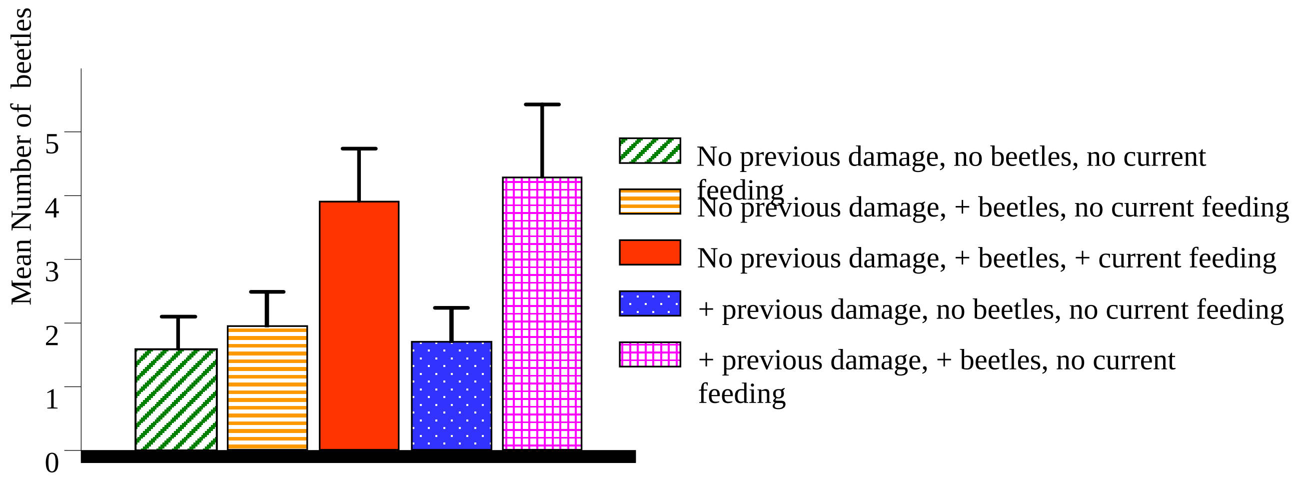

Fig. 8 Number of blue beetles on willows with or without leaf damage and with or without beetle presence

Volatile compounds: Volatile compounds emitted from plants are known to attract or repel insect species. To examine whether damage on leaves can affect emission of chemical volatiles by willow hosts, a chemical analysis was conducted using *S.* % *dasyclados* Wimm., which is favoured by beetles. It was found that two major green-leaf volatiles were increased upon mechanical damage. In further tests, 10 genotypes that differ in susceptibility to the blue willow beetle were examined. A significantly higher quantity of the green-leaf volatile, cis-3-hexenyl acetate, was found in the susceptible group of genotypes. As an exception, *S. viminalis* 'Bowles Hybrid', which is highly susceptible, emitted very low amounts of volatile compounds. The remaining nine genotypes collectively showed a significant positive correlation between the relative susceptibility of genotype to willow beetles and the yield of the volatiles trans-2- and cis-3-hexenyl-acetate from damaged plants.

2. 2 Factors influencing beetle mortality (02/01, 02/02, 02/03)

Three major factors affecting mortality were studied: plantation design, surrounding environment and willow genotype.

Plantation design (mono- and mixture plantings): For two consecutive years, targeted field experiments were done using potted plants with willow beetle eggs on leaves (before being put into the field, the plants were placed in a cage containing mated females). Results showed that egg mortality was higher on plants in a monoculture than in a mixture (strong or significant differences, depending on year). Preliminary work in the early part of the project showed that egg density per tree was higher in a monoculture. A possible explanation of higher mortality in monoculture would be that the higher number of eggs may attract more predators.

Surroundings: To assess effects of surrounding vegetation on beetle egg mortality, potted willow plants baited with beetle eggs were placed both inside and outside of willow strips surrounded by a cereal crop over two years. The egg mortality outside willow strips (in cereal plots) was significantly (marginally significant in the second year) higher than that inside the willow rows. Egg mortality was also higher when willow was surrounded by the cereals and was close to the willow strip. The EU-funded studies on combined food and energy farming system (1997-2000) showed that more natural predators can be found in cereal crops compared with willow strips. Our results suggest that the higher number of natural enemies in the cereal crop may be responsible for higher mortality of the eggs placed outside willow strips.

Willow genotypes: To examine whether and how willow genotype affects larval development of willow beetles, laboratory experiments were carried out using 11 current biomass clones (7 *S. viminalis* and 4 *S. viminalis* hybrids) and 35 clones from the National Willow Collection (NWC) that differ in susceptibility to the blue willow beetle. The results show that the genotypes on which larvae were reared significantly affected the rate of larval development, weight gain and mortality. With the NWC willows, genotype effects on fecundity were also tested. The results showed that the genotype on which adults were fed significantly affected the total number of eggs and eggs/clutch.

2.3 Sampling of willow beetles for population studies (02/04)

Population studies on the beetles using microsatellites (MAFF NF0414) are currently being conducted at Long

Ashton. In our NF0406, blue willow beetles were sampled from three different willow hosts from a range of sites covering N, S, W and E UK, and stored at –15 ^{o}C for future microsatellite analysis. Where possible, up to 60 beetles from each infested clone were collected from each site. Beetle samples were also collected from wild willows and natural surrounding habitats in several locations.

3 MIXTURES

Large-scale monoculture plantings are vulnerable to attacks by diseases and pests. A strategy to reduce such risks is to plant clonal mixtures to increase genetic diversity. The experience in the UK over the last 10 years suggests that, compared with monoclonal plantings, mixtures can reduce disease levels and pest damage. Because genotype mixtures are readily accepted for biomass production, SRC crops have a unique advantage over food or horticultural crops, in which flavour, colour, and shape may have prime importance.

3.1 Effects on rust severity (03/01, 03/02, 03/03)

In this project, the relative severity and spatio-temporal distribution (see 3.3) of stem cankers by *Melampsora spp.* were studied over two years (major experiments with one-year-old stems in 1997 and with 1-year shoots on 2-year-old stems in 1998). The work was done in the Long Ashton mixture trial which comprised random or regular mixtures of 3 or 5 willow clones and monocultures of each clone.

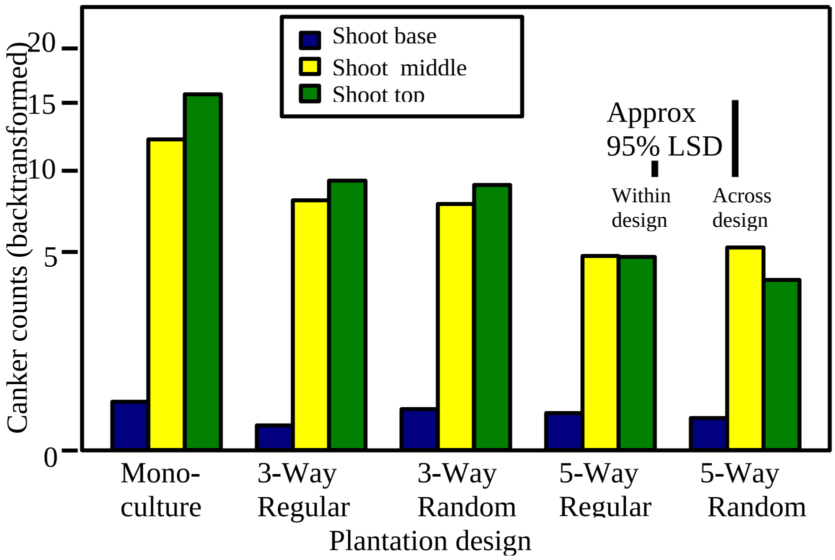

Fig. 9 Rust cankers on *S .viminalis* 'Bowles Hybrid' in monocultures and mixtures

Overall canker numbers in monocultures were significantly higher than those in mixtures ($P<0.01$ in 1997 and $P<0.001$ in 1998; see Fig. 9). The highest incidence was found in the monocultures with all mixture designs having lower values, significantly lower in the 5-way mixtures. Canker numbers were not significantly different between the designs of mixtures. However, there was a significant interaction between design and position of canker along stems. For example, the five-way designs had less number of cankers between the middle and top sections of stems, the monocultures had much more cankers between these two sections.

In this project, disease assessments in monoculture and mixtures were carried out in the Forestry Commission mixture trials, which were located across the country and planted with *S. burjatica* 'Germany', *S. % mollissima* 'Q83' *and S. viminalis* 'Jorrun'. The results obtained from various locations over three years (1998-2000) suggested that, compared with monocultures, mixtures had less or, in some cases, similar levels of disease. It was observed that mixtures had a delaying effect on disease onset and development early in the season. Such an effect was not so apparent late in the season because many leaves infected early either dropped or formed telia which are less conspicuous than uredinia.

3.2 Effects on beetle damage (03/01, 03/02, 03/03)

Previous observations suggested that there may be benefits of genotype mixtures against willow beetles, as individual component genotypes tended to support a reduced blue willow beetle population as the number of genotypes in a mixture increased and structural composition was random (Peacock & Herrick, 2000).

Beetle damage was assessed on 1- and 2-year old growth of *S. viminalis* 'Bowles Hybrid' over three growing seasons (1998-2000) and on 1-year growth of *S. % dasyclados* Wimm. for one year (1999) in the Long Ashton mixture trial. It was found that feeding damage was significantly more severe as the season progressed (by late June/July) in monocultures compared with mixtures. The *S.* % *dasyclados* Wimm., which is much more susceptible to beetles than 'Bowles Hybrid', did not always have a reduced number of beetles or damage in mixtures.

Salix viminalis and its hybrids are currently the dominant components in new commercial SRC plantations. We examined whether genetic variability occurs in beetle susceptibility among the commercial SRC clones. The results showed that there are significant differences in feeding preferences and larval development under the laboratory conditions among the 7 *S. viminalis* clones and 4 its hybrids.

The same 11 clones were also used to investigate the effects of leaf area damage on yield loss by artificially removing leaf areas. Preliminary results indicate that *S. viminalis* 'Orm' and its hybrids, *S. viminalis* x *S. schwerinii* 'Bjorn' and 'Tora', may be more tolerant to the leaf damage compared with the other clones. In practice, the clones tolerant to pests and diseases would be desirable in selection of SRC clones. Further work is needed to determine the tolerance of SRC clones to beetle feeding damage.

3.3 Spatio-temporal dynamics of beetles and rust (03/01, 03/02, 03/03)

Spatial processes of beetles: The data from three willow clones, *S.* % *dasyclados*, *S. burjatica* 'Korso' and *S. viminalis* 'Bowles Hybrid', at the Long Ashton mixture trial in 1997 were used to study spatial heterogeneity and spatial processes of blue willow beetles in mixtures and monocultures (Peacock et al., 1999). The negative binomial distribution (parameterised by the mean (m) and the variance ($m + m^2/k$)) was fitted to the data (the Poisson Index, 1/k, measures the degree of aggregation). The analysis showed that *P. vulgatissima* adults were spatially aggregated on favourable clones in the monocultures and the mixtures (Fig. 10). The degree of aggregation (measured by the k-parameter of the negative binomial) differed between willow clones. Beetles were highly aggregated at the start of the season, but less so later.

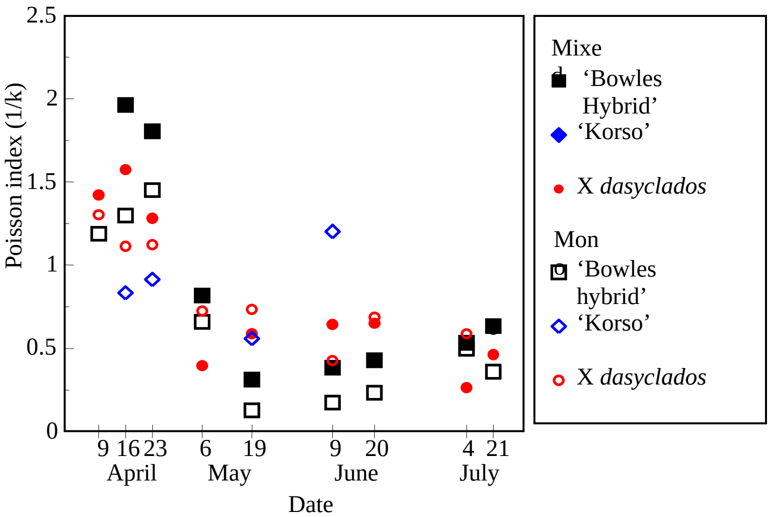

Fig. 1 Changes in the Poison index (1/k) over time for the three clones in mixtures and monocultures. All values are aggregated, the larger the value of 1/k, the higher of the level of aggregation.

Contour maps of log (beetle counts +1) were plotted for each date by plot combination using Mathcad (Version 5.0, Mathsoft Inc.; Fig. 11*)*. Contour maps give 'snapshots' of the beetle distribution over the season, and enable changes in distribution to be visually monitored. The dispersal studies of *P. vulgatissima* adults over time showed a pattern of beetle infestation along rows of the preferred clones in a regularly structured mixture. In the monocultures, movement was less directional.

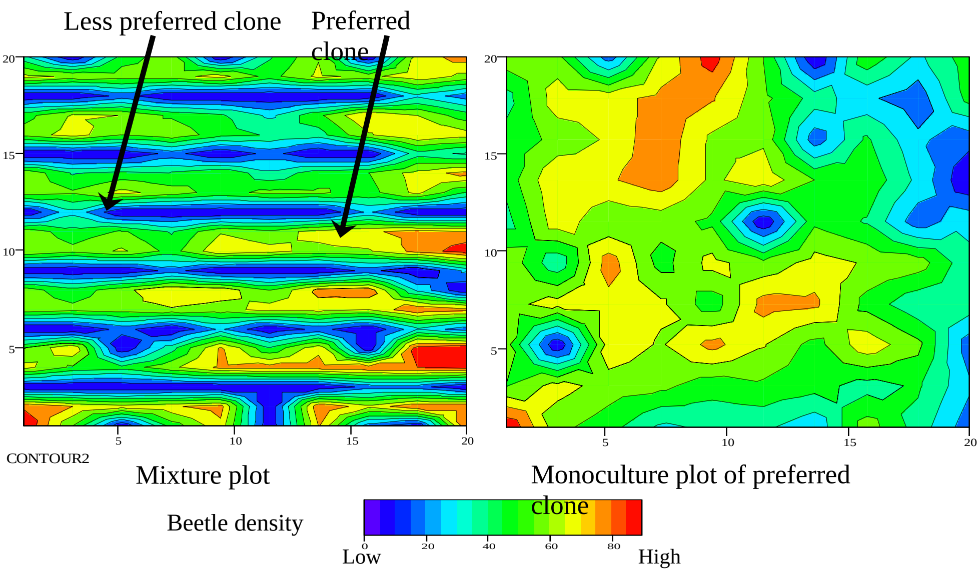

Fig.11 Density contour maps showing that beetles feed on preferred clones

A more detailed investigation of the spatial processes was done by evaluating semi-variograms (see, for example, Diggle, 1988) of log (beetle counts +1) in various directions across the plots. The results showed that, in mixtures, beetle numbers were higher along the rows of preferred clones than across the rows.

The stability of the spatial pattern of beetles over time was investigated in two ways: firstly between the presence/absence of beetles on a tree at each date and, secondly, a correlation between the counts themselves. A high positive correlation between dates suggests that a large number of beetles on one date is followed by a large number of beetles on the next date and vice versa, i.e. a degree of temporal stability. A negative correlation implies that a small number of beetles on one date result in a large number of beetles on the next date or vice versa, i.e. there are strong indications of movement. The results suggested that there was a tendency for the correlations to be positive early in the season and negative as the season progressed. In *S.% dasyclados* Wimm., beetle numbers tended to be stable both in the monoculture and the mixture early in the season. However, beetle movement appeared to have happened earlier (6 May) in monocultures than in mixtures (20 June).

Spatio-temporal dynamics of rust: Stem canker data from 1- and 2-year old stems of *S. viminalis* 'Bowles Hybrid' (see 3.1) were used for this study. The stem infection usually takes place on actively growing shoot tops and, therefore, the distribution of cankers along stems can reflect the extent of disease in time. The results showed that on 1-year-old growth (1997) the total number of cankers in all monoculture plots were significantly aggregated. In contrast, aggregation was found in only 1/3 of the regular, and 2/3 of the random, mixtures. Unlike the monocultures, no correlation was found in mixtures between aggregation on the basal section of stems and aggregation on the total stem. On the current growth of the 2-year-old stems (1998), cankers in all monoculture plots were randomly distributed, with the exception of only one plot having a significantly aggregated distribution for the basal section of stems. By contrast, the mixtures had similar patterns of distribution between years with significant aggregations in one third of the regular and five-sixths of the random mixtures.

Overall, the extent of stem infections in time was found different between monoculture and mixture designs. In monocultures, canker distribution differed greatly between years.

Spatial distribution of disease and pest damage: The degree and spatial distribution of damage by rust and beetle were studied concurrently in the mixed genotype trial at LARS for two consecutive seasons (on 1-year old shoots on 2-year-old stems in 1998 and on 1-year-old growth in 1999). The assessments were done on *S. viminalis* 'Bowles Hybrid' in both years and on *S. % dasyclados* Wimm. in 1999. A recently developed statistical programme, Spatial Analysis by Distribution Indices (SADIE), was used to model distribution patterns of pest and disease.

In 1998, rust was found to be aggregated in 75% of the mixture plots and only 33% of the monocultures. No significant differences were found in the spatial distribution of beetles between plantation design. There was a significant negative correlation between damage by rust and by beetle on individual stools in all but 3 of the 15 plots (two 3-way regular and one 5-way random). Spatially, however, distribution of rust disease was not negatively correlated with the distribution of beetle damage. In 1999, rust was aggregated in all monoculture plots, but only 8% of mixtures. The difference between years and design for rust is probably because of the late rust development in 1999 and the delaying effect of mixtures. Beetle damage distribution was similar between years 1998 and 1999. Beetles were found to be very mobile, their dispersal considered random rather than diffused. These results suggest that spatial design of willow plantations affects rust disease distribution to a greater extent than it does beetle distribution.

3.4 Induced resistance against rust (03/04, 03/05)

A more diverse range of pathotypes occur in mixtures compared with monocultures. A willow genotype in a mixed plantation is most likely to be exposed to both compatible and incompatible rust pathotypes, while that in a monoculture is likely to be exposed only to compatible ones. Studies in other crop systems have shown that the presence of incompatible pathotypes may cause less disease (referred as induced resistance). In this project, a major effort was made to determine whether and to what extent the presence of mixed inoculum affects the disease level. The following three inoculation experiments were carried out.

First experiment: Spores of a compatible pathotype and an incompatible pathotype were mixed and inoculated on to *S.* % *mollissima* ‘Q83’ using the leaf disc method. A total of seven pathotypes, two compatible and five incompatible, were used. As a result, all the mixed inoculations produced less disease, most of them significantly less in spore production compared with the controls which were inoculated with single compatible pathotypes.

Second experiment: Four biomass willows, *S. viminalis* ‘Mullatin’, *S.* % *calodendron, S. burjatica* ‘Germany’ and ‘Korso’ were inoculated with single and mixed inocula using the 7 pathotypes from the first experiment. Again, the results show that most mixed inoculations produced less disease, the majority showing a significant reduction in rust spore production compared with single, compatible pathotype inoculations.

Third experiment: The first and the second experiments used relatively high concentrations of spores. The third experiment was conducted to determine whether mixed inocula have the same effects when fewer spores are present. Four *M. epitea* isolates, representing pathotypes LET1, LET3, LET4 and LR1, were inoculated on to six biomass clones , *S. burjatica* ‘Korso’, *S.* % *calodendron*, *S.* % *mollissima* ‘Q83’ *S.* % *stipularis*, and *S. viminalis* ‘Mullatin’ and ‘78183’, using four levels of spore concentration. The inoculated leaf discs were photographed and pustule number and size were measured using SigmaScan as described in 1.6. Spores produced on each leaf disc were also counted. On all the tested willows, mixed inoculations resulted in less disease when the spore densities per leaf disc ($0.95cm^2$) exceeded 100 (examples are shown in Fig. 12). On *S.* % *calodendron*, the effect of ‘induced resistance’ was evident even when fewer spores were present.

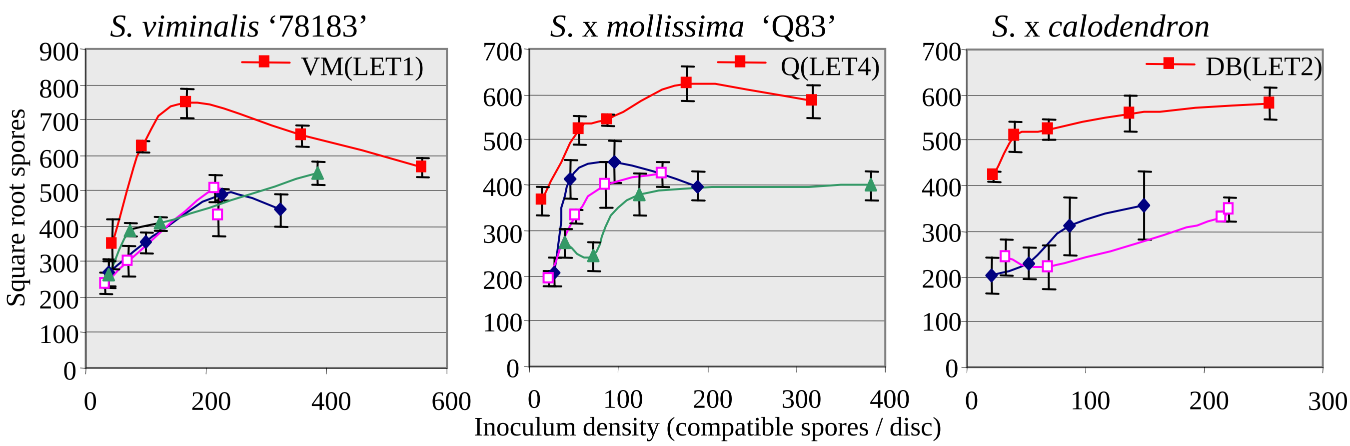

Fig. 12 Spore production on three willow clones after inoculations with single compatible pathotypes (■) and with a mixture of compatible and incompatible pathotypes (□, ●, ▲).

It can be concluded that, for a host genotype, the presence of both compatible and incompatible pathotypes, in general, results in less disease compared with the presence of single, compatible pathotypes in SRC willows. This ‘induced resistance’ becomes more effective when relatively large number of spores are present. The effectiveness of the induced resistance may vary according to host genotypes. Further work with high-yielding current/future biomass clones will provide useful information for the selection and configuration of mixture components.

3.5 Biomass yield and growth (03/06)

Fresh weight yield assessments were carried out at the Long Ashton mixture trial in 1999. All 400 stools within each plot were assessed for vigour (each stool was given a rating according to its overall growth). Ten healthy stools for each of the 5 clones were harvested from each plot and weighed to obtain a yield estimate for each plot. The results showed that the yields of all mixture plots (3-way, 5-way or random design) were greater than the average yield of the monoclonal plantings. For example, the mean fresh weight of the clones in monoculture was 16.14 t /ha whereas in the 3-way mixtures it was 20.36 t/ha and in the 5-way mixtures, which included lower yielding clones, was still 17.24 t/ha.

The data from the vigour assessments were analysed using a generalised linear mixed model (GLMM) with binomial error distribution and logit link function. This made it possible to compare the response of each clone in a mixture and in a monoculture. No significant differences were detected for healthy survival for *S. viminalis* ‘Bowles Hybrid’, ‘Mullatin’ or *S.*% *dasyclados*. However, both *S. burjatica* ‘Korso’ and *S.*% *stipularis* showed weaker growth in mixtures.

3.6 Effect of inter-plant competition on biomass yields (03/06)

The 1999 harvest data were analysed for the effect of stool competition on yields by using the residual maximum likelihood (REML) method. The five willow types used in the experiment were given scores for field vigour based approximately on their ranks for rust resistance, *S. viminalis* 'Bowles Hybrid' (1 = moderately resistant), *S.*% *dasyclados* Wimm. (2), *S. viminalis* 'Mullatin' (3), *S. burjatica* 'Korso' (4) and *S.* % *stipularis* (5 = highly susceptible). Planting design and willow types within design were declared as fixed effects; blocks, plots within blocks and stools within plots the random effects.

The analysis of the fresh weights showed that the higher-yielding willow types (*S. viminalis* 'Bowles Hybrid' and *S.* % *dasyclados*') perform better in mixtures. The performance of these two clones was consistent over a number of harvests (1994, 1996 and 1999). *Salix. viminalis* 'Mullatin' showed no difference in the 1999 harvest . The lower-yielding willows, *S. burjatica* 'Korso' *and S.*% *stipularis*, consistently showed reduced yields in mixture designs.

The overall score for the vigour of stool neighbours was included in the REML analysis as a covariate to allow testing of the hypothesis that neighbour competition affected stool weight. This covariate gave a positive coefficient that was statistically significant ($p<0.001$), showing that increased yield occurs in the presence of less vigorous neighbouring stools. Using the model, similar analyses were done with the data on willow heights, diameters (1996 only) and shoot numbers (1996 and 1999). The analyses of 1996 data showed that there is little effect on height of stools due to being in mixed or monoculture, nor is there evidence of effects on height due to different vigour of neighbours. However, stools surrounded by less vigorous neighbours produced significantly higher number of shoots compared with those surrounded by vigorous neighbours. This general pattern was repeated for the 1999 harvest, with the lowest yielding clones having significantly less shoots when grown in mixtures.

The large variation encountered in the stool vigour, even within monoculture plantings, suggests that, provided willow types are matched for approximately similar growth habit and yield potential, stool competition in mixtures may be no worse than in monocultures. It implies that well matched willows would perform better in mixed plantings.

4 BIOLOGICAL CONTROL OF RUST

Perennial crop systems, such as willows, are better suited for biocontrol compared with annual crops because of the potential carry-over effect on biological control agents, assuming a 3-5 year harvest cycles. *Sphaerellopsis filum* is a hyperparasite occurring naturally on willow rusts. Although *S. filum* occurs only on rusts in nature, it can be cultured on artificial media. This provides the opportunity of growing the fungus *in vitro* and then reintroducing it into the field. Previous NF0402 established that airborne sexual ascospores occur in *S. filum* (Yuan et al., 1999) and the fungus varies in pathogenicity to willow *Melampsora* (MAFF, 1998).

4.1 Pathogenicity to willow rust (04/01)

Following the findings in NF0402, two experiments were carried out to further examine pathogenic variation in *S. filum* to *M. epitea*.

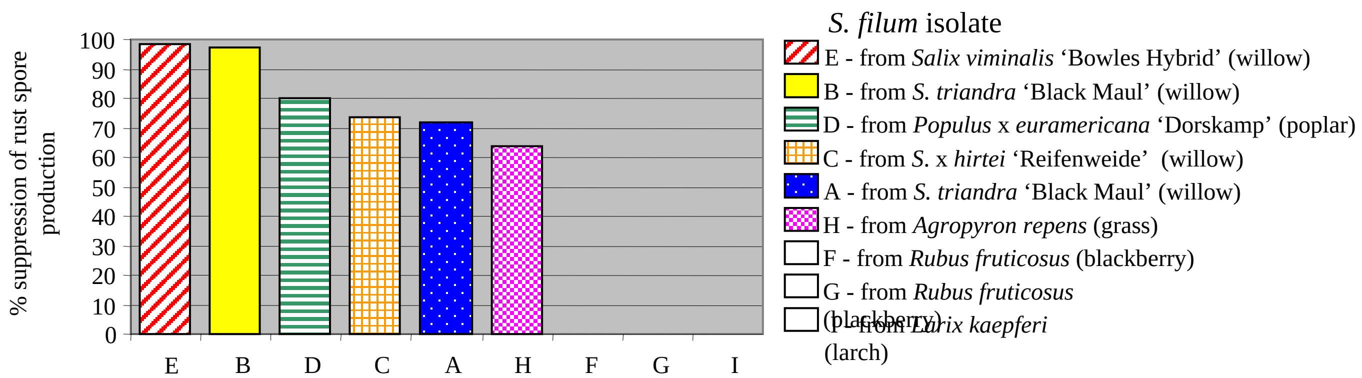

Fig. 13 Suppression of willow rust spore production by *Sphaerellopsis filum* from different sources in the first experiment.

First experiment: Nine isolates were tested using the method described in Yuan et al., 1999. Six isolates (five from *Melampsora* species on willows and poplar, one from rust on couch grass) infected *M. epitea*, while the remaining three (two from blackberry rust and one from larch rust) caused no infection (Fig. 13). The results conclusively demonstrated that *S. filum* is composed of pathogenically specialised populations, differing widely in their virulence indicating that the efficacy of biological control would be increased markedly by using highly virulent strains.

Second experiment: To examine the extent of variation in virulence, we conducted an inoculation experiment involving 13 *S. filum* isolates (12 from willow). While all proved to be effective against willow rust, the degree of virulence was variable. Some isolates reduced rust spore production by less than 90% while others by up to 99%.

4.2 Spread of *S. filum* in plantation (04/02)

How *S. filum* genotypes spread in a plantation is an important question to be answered in deployment of the hyperparasite for biocontrol in the field. Prior to this project, no such information was available. With asexually reproducing fungal pathogens, DNA fingerprinting provides the most efficient way of examining their spread. With willow rust, we have successfully developed an efficient protocol of the powerful fingerprinting method -AFLP. However, the AFLP method for willow rust cannot be directly applied to *S. filum*. Combined with the input from the EU project on willow rust (QOL 1585), we have developed a protocol to generate AFLP profiles from *S. filum* in this project

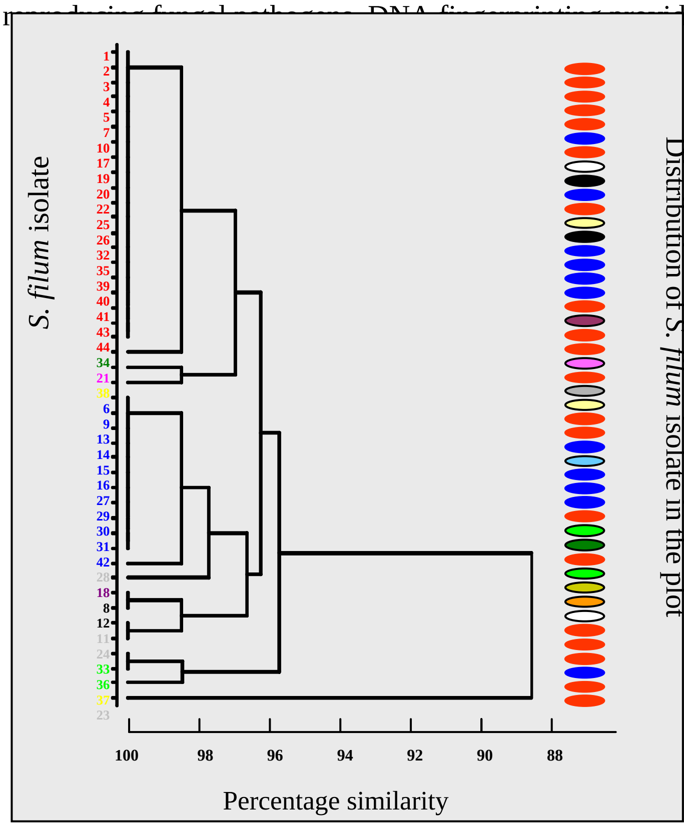

Fig. 14 Similarity and genotype distribution of *S. filum* isolates in a field plot, two genotypes (red and blue) were most common.

In this project, *S. filum* was sampled consecutively from 46 stools (0.5 m apart) of *S. burjatica* ‘Germany’ in a willow strip, which consisted of ‘Germany’, *S. viminalis* ‘Bowles Hybrid’ and *S. viminalis* x *burjatica* ‘Stott 10’, *S. viminalis* x *S. schwerinii* ‘Tora’, each in two rows, and was surrounded by cereal crops. Because natural infections were detected in the early stages of rust epidemics (July), no *S. filum* isolate was introduced into the planting. Single-spore isolates were made from each sample and fingerprinted using AFLP.

The preliminary results showed that two genotypes were most frequent (Fig. 14), one of which (red isolates in Fig. 14) on 43% of the stools. There was evidence that the same genotypes often occurred in adjacent stools, indicating that rain splash or leaf contact may be an important means of spread in *S. filum*. However, a number of genotypes and somewhat patchy genotype distribution appeared to suggest that other mechanisms, by insects or on rust spores for example, may play a significant role in the spread of *S. filum*. Further work involving larger number of samples is needed to establish the pattern and the extent of genotype spread in *S. filum*.

5 OUTPUT

NF0406 has accomplished a great deal in its three-year duration. The scientific achievements of NF0406, in many ways, have exceeded our original expectations. This is reflected by the quality and productivity of the project, as its contribution to the improvement of disease and pest management in SRC willow production.

Publications (05/01, 05/02)

During the project, 10 scientific papers have been published in refereed journals (most of which are top-rated in their fields, see List of Publications). Two papers have been submitted to refereed journals and two more are nearly completed. In addition, a total of 18 popular articles, symposium papers and lectures have been produced. For any research project, whether fundamental or applied, it is a remarkable achievement. These papers have described our findings and discussed the approaches to minimise the impacts of diseases and pests.

Information for willow breeding (05/01)

Throughout the project, we have been actively involved in breeding for resistance through regular meetings of the willow-breeding steering group. The field performances of willows in the National Willow Collection against disease and pest have been assessed annually and the results were made available to the European Willow Breeding Program. Various up-to-date information on diseases and pests, such as the preliminary results on some newly introduced breeding stocks and on the pathotypes present on elite clones, has regularly been communicated to the breeder throughout the project.

Information for general public (05/01)

An article titled *Integrated Rust Control in SRC plantations in the UK* was published in the journal Pesticide Outlook. The article was aimed at willow growers and the general public to raise the awareness of the integrated approaches to control the disease in SRC production.

An article titled *A millennium bug? The willow beetle* Phratora *(*Phyllodecta*) spp.* was published in the popular journal Antenna.

We also participated in the preparation of the booklet titled *Integrated Pest Management in SRC* by the Game Conservancy. This briefly described major diseases, pests and weeds in SRC crops and their control.

Recommendation of SRC clones (05/01)

The recommended list of SRC willows and poplars was prepared through collaboration with the Forestry Commission in the early stages of the project. Later (2000), we prepared a disease and pest information note, which describes the current situation of SRC diseases and pests in the UK and the up-to-date information on the performance of currently recommended biomass willow clones against diseases and pests. The note has been made available to the Forestry Commission for the preparation of new lists of recommended clones and to the European Willow Breeding Program for breeding purposes.

Position paper (05/02)

A position paper titled "Current status and future research on diseases and pests in SRC plantations in the UK", has been produced and submitted to MAFF. This document was presented to the IGEC (Inter-deparmental Group for Energy Crops) Committee in late 1999.

EU project on willow rust

This project has produced an EU project on non-fungicidal control of willow rust. The EU project runs for four years (2000-2004) and involves LARS, Department of Agriculture, N Ireland, Swedish Agricultural University, Sweden, and the Institute of Forest Genetics and Tree Breeding, Germany. The EU project adds an international dimension to the areas of research on pathogen populations, disease resistance, clonal mixtures and biocontrol of SRC willows.

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1. Peacock, L. & Herrick, S. (2000). Responses of the willow beetle *Phratora vulgatissima* to genetically and spatially diverse *Salix* spp. plantations. ***Journal of Applied Ecology*** **37:** 821-831.

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2. Peacock, L., Hunter, T., Turner, H. & Brain, P. Does host genotype diversity affect the distribution of insect and disease damage in willow cropping systems? (submitted)

Papers drafted for refereed journals

1. Peacock, L., Herrick, S. & Harris, J. Genetic variability of *Salix viminalis* in response to herbivory and in the feeding preference and development of willow beetle (*Phratora vulgatissima*)

2. Hunter, T., Peacock, L., Turner, H. & Brain, P. Stem-infecting rust in willow biomass coppice as affected by plantation design.

Popular articles published in journals for general public

1. Peacock, L. (1999) A millennium bug? The willow beetle *Phratora* (*Phyllodecta*) spp. ***Antenna* 23:** 143-149.

2. Pei, M. H. & Hunter T. (2000). Integrated rust control in renewable energy plantations in the UK. ***Pesticide Outlook* 11:** 145 – 148.

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1. Pei, M. H., Hunter, T. & Ruiz, C. Genetic variation in *Melampsora* rusts in renewable energy willow plantations in the UK. In the 6th International Symposium of Mycological Society of Japan. 26-27 November, 1998. Chiba University, Japan.

2. Pei, M. H., Hunter, T. & Ruiz, C. *Melampsora* rusts and the mycoparasite *Sphaerellopsis filum* in willow plantations in the UK. November 1998. Tsukuba University, Japan.

Position paper to MAFF

1. Pei, M. H. (1999). Current status and future research on diseases and pests in SRC plantations in the UK.

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1. Hunter T. (1998). Overview of pest problems in willow and poplar plantations. Task 17 IEA Biomass Programme. June 3-5, 1998, Dept. Short Rotation Forestry, Swedish University of Agricultural Sciences, Uppsala, Sweden.

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Executive summary

This project was carried out to provide the scientific basis for an integrated, non-chemical strategy of disease and pest management in UK SRC willow production. Implementation of such a strategy requires integrated utilisation of host resistance, genotype mixtures and biocontrol agents. Our research was focused on the genetics and behaviour of pathogens and pests, functions of willow mixtures and the biocontrol potential of a hyperparasite of rust. The project has been a great success and has firmly established the UK's leading status in SRC disease and pest research. Main results are summarised as the following.

Rust caused by *Melampsora* is the most damaging disease. The pathogen is variable and often gives rise to new pathotypes. While some new pathotypes are sterile, others are capable of exchanging genes with existing pathotypes to produce offspring having wide spectra of virulence. However, the gene exchange is likely to be confined within a form species. Within the form species LET of *M. epitea* (important on *S. viminalis* and its hybrids), some pathotypes do not cross easily. Yet, we found that the exchange of genes for pathogenicity between the reproductively distant groups may still be possible via a third group within LET. Further experiments on the genetic basis of pathogenicity confirmed that, in *M. epitea,* one or two genes may be responsible for pathogenicity to *S. viminalis* and its hybrid with *S. triandra.* These genes appear to be located at different chromosomes and, accordingly, segregate independently. This presents a strong challenge to the efforts to find universal markers for rust resistance. Virulence is dominant over avirulence, unlike the opposite pattern in most other plant pathogens. Gene dosage may significantly affect the degree of virulence. For example, the rust genotype having two virulence genes may be more virulent than that having one virulence gene. We examined gene flow between regions in the UK using the rust collected from SW England, N England and N Ireland. The theoretical estimate of the gene flow, calculated based on our preliminary results, appears to be just enough to prevent local populations evolving separately. The results suggest that gene flow in the rust pathogen between these regions may facilitate spread of virulence genes from one location to another.

The majority of current commercial SRC clones (mostly *S. viminalis*) are somewhat resistant to leaf rust *M. epitea*, but several of them are infected by the stem-infecting-form of rust. The newly released clone *S. viminalis* x *burjatica* 'Stott 10'can yield twice as much as most of the clones selected in the 1980s and has been highly resistant to rust since it was bred in the late 1980s. It appears that the rust currently occurring

on 'Stott 10' may be much more virulent than those found previously. Severe dieback of SRC willows was found in the winter of 1998/1999. The dieback caused severe damage on *S. burjatica* 'Germany' and several other clones. A survey showed that the dieback problem was not widespread in UK plantations. At present, the likely underlying cause of the dieback appears to be combination of plant stress and infection by secondary pathogens.

A breakthrough was made in characterisation of willow resistance / rust pathogenicity by the use of digital imaging technology. Using the new method, large number of samples can be processed in a single inoculation experiment and the reactions on leaf discs can be quantified. Furthermore, a range of resistance reactions, which could not be properly recorded previously, such as discolouring and hypersensitivity, can be accurately profiled. This technique will greatly improve the efficiency and accuracy in characterisation of host resistance / rust pathogenicity in future.

Chrysomelid beetles are the most important pest on SRC willow. Our results suggest that the sight of willow plantation, the presence of other beetles and damaged leaves may attract beetles. Egg mortality was higher on plants in a monoculture than in a mixture. The egg mortality was also higher when willows were surrounded by a cereal crop. It was possibly due to the higher number of natural predators in monocultures and in the cereal crop. In our experiments, the rate of larval development, weight gain and mortality were significantly affected by willow genotypes on which the larvae were reared. When adult beetles were fed on different willow clones, the total number of eggs and eggs/clutch laid by the beetles differed significantly. Results from our preliminary work suggest that some clones may be more tolerant to the leaf damage compared with the other clones.

Spatio-temporal analyses showed that blue willow beetle adults were spatially aggregated on favourable clones in both monocultures and mixtures. In mixtures, beetle numbers were higher on preferred clones. Beetle numbers tended to be stable both in the monoculture and the mixture early in the season. However, beetle movement occurred earlier (early May) in monocultures than in mixtures (late June). With rust, stem cankers on one-year-old growth was aggregated in all monocultures but less so in mixtures. On the two-year-old growth, the cankers were randomly distributed in monocultures but somewhat aggregated in mixtures. The results suggest that spatial design of willow plantations affects rust distribution, to a greater extent than it does beetle distribution.

Clear-cut evidence of mixture effects on rust was obtained by examining stem cankers caused by the stem-infecting form. Disease levels in mixtures were significantly lower than that found in monocultures, but not significantly different between the mixture designs. The yields of all mixture plots (5 clones, 3-way, 5-way or random design) were greater than the average yield of the monoclonal plantings. The higher yielding willow types performed better in mixtures while the lower yielding clones showed reduced yields in mixtures. Data analysis attributed this to the competition effects between stools.

We found that the presence of both compatible and incompatible pathotypes, in general, results in less disease compared with the presence of single, compatible pathotypes. This 'induced resistance' becomes more effective when more rust spores are present. Such effects may vary according to host genotypes. Exploitation of such resistance in mixture designing would improve the performance of mixtures against rust.

Sphaerellopsis filum is a hyperparasite occurring naturally on a wide range of rust fungi. It is clear now that *S. filum* is composed of pathogenically specialised populations, differing widely in their virulence. This indicates that the efficacy of biological control would be increased markedly by using highly virulent strains. Preliminary results suggested that some genotypes are widely spread in a plantation. Further work involving larger number of samples is needed to establish the pattern and the extent of genotype spread in *S. filum*.

Within the project, 10 scientific papers have been published in refereed journals (mostly highly-rated in their fields), 2 submitted and 2 drafted. In addition, a total of 16 popular articles, conference and symposium papers have been produced and 2 guest lectures given. The project has maintained active communication with the European Willow Breeding Programme, SRC growers and Government Bodies interested in renewable energy crops. NF0406 has also produced an EU project on willow rust to further our understanding of the major disease from a pan-European perspective. The EU project involves researchers in LARS, N