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Pathotype composition in *Melampsora epitea* populations occurring on willow (*Salix*) grown in mixed and monoculture plantations

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Abstract

Species/variety mixtures of *Salix* are used as a disease control strategy for rust (*Melampsora epitea* Thum. var. *epitea*) in short rotation coppice (SRC). Six LET (*larici-epitea typica*) pathotypes and two LR (*larici-retusae*) pathotypes of *M. epitea* var. *epitea* and also *M. caprearum* were detected in Northern Ireland plantations in 1994 and 1995. Three of these, LET7, LET8 and NI5 were detected in plantations for the first time. The predominant pathotype on most varieties was LET1. Logistic analysis showed that this pathotype was less prevalent on *Salix* × *dasyclados* Wimm. and more prevalent on *S. viminalis* L. 'Bowles Hybrid' than on *S. viminalis* '683'. The Shannon-Weaver Index of diversity indicated that there was greater pathotype diversity in mixtures than on the constituent varieties in monoculture. There was also a general trend of increasing diversity as the season progressed. The lowest pathotype diversity was recorded on *S. viminalis* 'Bowles Hybrid'. Pathotype predominance and diversity have an impact on the sustainability of the crop.

Introduction

Many *Salix* species and varieties currently grown in short rotation coppice (SRC) are highly susceptible to rust caused by *Melampsora epitea* Thum. var. *epitea* (McCracken and Dawson, 1992). The occurrence and severity of rust often varies between varieties and from site to site (Hunter et al., 1996). This variation may be due to different forms or 'pathotypes' of *M. epitea* var. *epitea*. Differing proportions of pathotypes within the population may cause further variation, which is also evident between sites and geographic region (Pei et al., 1992). Indeed, there have been concerns that the planting of large-scale stands of willow in the UK during the past 10 years may be imposing severe selection pressures on rust populations. These selection pressures may cause an increase in the proportion of virulent pathotypes occurring within the population. It is also possible that, similar to the situation occurring in *Populus* (Pinon, 1992), through time the pathogen may evolve to circumvent the resistance of many of the *Salix* varieties currently being grown.

Pei et al. (1996) reported the existence of seven pathotypes of *M. epitea* var. *epitea* within UK plantations in 1992, and by 1994 at least fourteen pathotypes had been identified (Pei et al., 1999a). These are designated as *larici-epitea typica* (LET), which occurs mainly on *S. viminalis* L. varieties, and *larici-retusae* (LR), which is associated with *S. burjatica* Nassorov varieties. This increase in numbers of pathotypes was particularly evident in N. Ireland plantations even from a limited survey consisting of single samples taken once during the season. Within a season there may be a succession of pathotypes and frequently several pathotypes are present simultaneously on the same variety or even the same leaf (C.Y. Allen unpublished data). Pathotype composition may also be correlated with variety and may be affected by the age of growth from a coppiced stool (Parker et al., 1995).

One of the most successful strategies currently being used to reduce the impact of rust disease on willow grown in SRC is the use of species and variety mixtures (McCracken et al., 1996). In mixtures comprising between four and six varieties, the onset of disease was delayed by as much as three weeks, disease development was slower and levels of rust at the end of the growing season were significantly reduced (McCracken and Dawson, 1998a). With the dynamic changes occurring in rust populations, concern has been expressed that in varietal mixtures, 'super-races' or 'super-pathotypes' may develop. These may be capable of infecting most or all of the varieties within a mixture, hence rendering the use of mixtures as a control strategy potentially more damaging than the original problem (Groth, 1976).

The primary objective of this investigation was to determine the diversity of rust pathotypes on individual varieties growing within mono-varietal plots and in mixtures during the growing season.

Materials and methods

Three adjacent large-scale (3 ha) willow sites were planted at Castlearchdale, Co. Fermanagh, N. Ireland in consecutive years beginning in 1986. At Site 1 the plant density was 16,000 ha^{-1} and at Sites 2 and 3 it was 20,000 ha^{-1}. Plot sizes at each of the three sites varied with the smallest mono-plot being 1500 m^2 in size and mixture plots being at least 5000 m^2.

At Site 1, four varieties – *Salix × dasyclados* Wimm., *S. viminalis* 'Bowles Hybrid', *S. burjatica* 'Germany' and *S. viminalis* '683' were included in mono-plots and mixtures. In 1994, growth was re-growth from freshly coppiced seven-year-old stools, which previously had been harvested twice. At Site 2, *S. mollissima-undulata* Hoffm. ex Elwert 'SQ83' was included with the four original varieties and in 1994 growth was in its third year from six-year-old stools which had been harvested once only. At Site 3, a sixth variety, *Salix × calodendron* Wimm., was added and in 1994 growth was in its second year from five-year-old stools which had been harvested once.

During 1994, leaves with actively sporulating rust were collected from Sites 1 and 2 in alternate weeks and from Site 3 every week starting in early June until late September. In 1995, sampling was carried out every two weeks from all three sites from June to September. On each occasion 10 stools were chosen at random from each of the varieties growing in mono-varietal plots and within the mixtures. All of the leaves from one shoot from each of the selected stools were sampled. The percentage rust cover on leaves was assessed using a key. A cumulative rust score was subsequently calculated in order to produce a disease progress curve of rust on each individual variety whether growing in mono-plots or mixture plots (McCracken and Dawson, 1992). Within 36 h of sampling, rust spores were 'hoovered' off the leaves using a vacuum pump into individual collection vials. The spores were air dried for 48 h prior to their storage at $-20\,^{\circ}$C. The spores obtained in this way remained viable for several months and could be used in subsequent pathogenicity tests. In preparation for each test the appropriate spore sample was removed from storage and, using a dry sable bristle paintbrush (size '0' artist brush), a 1 mg aliquot was used to inoculate three freshly detached glasshouse-grown leaves, corresponding to the host variety from which the sample had been obtained originally. Inoculated leaves were incubated in 13.5 cm diameter petri dishes on damp filter paper at 18–20 °C with 16 h of 300-lux light for 12 days. Following incubation the spores from a total of 20 different pustules per set of three leaves were collected and subsequently maintained as single-pustule isolates following inoculation of fresh leaves. This was necessary to obtain sufficient inoculum for subsequent tests.

Leaf discs (14 mm diameter) were cut from each of nine differential *Salix* host varieties (Pei et al., 1996) that had been grown in the glasshouse (Table 1). Spores of the single-pustule isolates were inoculated, as before, onto freshly cut leaf discs using a dry paintbrush. Replicate discs were floated on a solution containing 8 $mg\,l^{-1}$ gibberellic acid in 24-well microtitre plates. Plates were incubated at 18–20 °C with 16 h of 300-lux light for 12 days with subsequent assessment of pustule development. Pustule development was recorded as being either clearly present or absent. Results were expressed as a percentage of the 20 single-pustule isolates, e.g., if all 20 single-pustule isolates were identified as one pathotype, then that pathotype would be present at 100%. For a number of isolates, which did not fall clearly into defined pathotype groups, inoculations were repeated as necessary to confirm their reaction in the differentials.

The diversity of species (*M. epitea* and *M. capre-arum*) and pathotypes in each plot at each sampling

Table 1. Reaction of five pathotypes of *Melampsora epitea* var. *epitea* on a set of nine differential *Salix* spp. varieties (+ = susceptible reaction; − = resistant reaction)

Differential host	Pathotype										
	LET1	LET2	LET3	LET4	LET5	LET6	LET7	LET8	NI5	LR1	LR2
S. viminalis ‘Mullatin’	+	−	−	−	−	+	+	−	+	−	−
S. mollissima-undulata ‘SQ83’	−	−	−	+	−	+	−	−	+	−	−
S. viminalis ‘Bowles Hybrid’	+	−	−	−	−	+	−	+	+	−	−
Salix × *stipularis*	+	+	−	+	+	+	+	+	−	−	−
Salix × *calodendron*	−	+	+	−	−	−	−	−	−	+	+
S. burjatica ‘Korso’	−	−	−	−	−	−	−	−	−	+	+
S. disperma-himalayas	−	−	−	−	−	−	−	−	−	−	+
S. daphnoides ‘Meikle’	−	−	−	−	−	−	−	−	−	−	−
S. pupurea ‘Uralensis’	−	−	−	−	−	−	−	−	−	−	−

time was calculated using the Shannon-Weaver Index. This index is defined as:

$$H = -\Sigma p_i \ln p_i$$

The term p_i is the proportion of a particular species (pathotype) in a sample, which is multiplied by the natural logarithm of itself. H is derived by summing the product of all species (pathotypes) in the sample. The minus sign is to make the final value of H positive (Fowler and Cohen, 1993). An analysis of variance was then carried out on the diversity values using a split-plot analysis. The main plot treatments consisted of the three sampling times within each growing season and the two years were regarded as main plot blocks. The sub-plots were taken to be the combinations of the five varieties and two mixtures in each of the three sites.

There is a level of dependence between the main-plot observations resulting from samples being made on the same plots at different times. However, because of the large plot size requiring only 0.2% of stools to be sampled on any occasion, the dependence was not considered to be sufficiently strong to invalidate seasonal trends or their repeatability.

Following initial analysis it was obvious that the predominant pathotype on most varieties was LET1. The prevalence of LET1 was examined by means of a logistic analysis, whereby the log odds of LET1 samples were calculated over three sites on each of the three dates in each year. The log odds of LET1 was defined as:

$$\log(p/(1-p))$$

where p is the proportion of LET1 samples. The analysis was performed in order to determine differences in the LET1 log odds between willow varieties, between mono- and mixed plots and also any interactions between the two. The analysis was restricted to the three varieties on which LET1 was found, i.e., *S. viminalis* ‘683’, *Salix* × *dasyclados* and *S. viminalis* ‘Bowles Hybrid’.

Results

In both 1994 and 1995, disease progress followed similar patterns, with an initial lag phase followed by an exponential increase with a stationary phase and an apparent decline, due to the loss of infected leaves (data not presented). Defoliation was observed on some varieties, e.g., *S. viminalis* ‘Bowles Hybrid’, by mid August in both 1994 and 1995. In contrast, there was little leaf loss with *S. mollissima-undulata* ‘SQ83’ until normal leaf senescence in October.

Six LET pathotypes and two LR pathotypes of *M. epitea*, and *M. caprearum* were detected during the course of the study (Table 2). Three new pathotypes, designated NI5, LET7 and LET8, were detected in plantations for the first time. In 1994, LET4, LET5 and LET6 were all recorded in N. Ireland for the first time (Table 2). On *S. burjatica* ‘Germany’ only two pathotypes, LR1 and LR2 were detected.

There was no significant difference ($P = 0.05$) between the mean diversity indices for all varieties in 1994 (0.435) and 1995 (0.493). There was, however, a significant difference ($P = 0.05$) between mono-varietal plots (0.372) and mixtures (0.556), i.e., there was a greater diversity of pathotypes in mixtures. The diversity index of pathotypes on *S. viminalis* ‘Bowles Hybrid’ (0.255) was significantly ($P = 0.05$) less than on any of the other varieties (Figure 1). The diversity indices of the other four *Salix* varieties were

Table 2. Proportion (%) of *Melampsora epitea* var. *epitea* pathotypes: LET1, LET4, LET5, LET6, LET7, LET8 and NI5 on one-year-old growth of *S. viminalis* '683', *S. viminalis* 'Bowles Hybrid' and *S. mollissima-undulata* 'SQ83' recorded early, mid and late in the growing seasons, 1994 and 1995

Year	Time	Plot	Pathotype						
			LET1	LET4	LET5	LET6	LET7	LET8	NI5
Salix viminalis '683'									
1994	Early	Mono	100	—	—	—	—	—	—
		Mix	100	—	—	—	—	—	—
	Mid	Mono	70	20	—	—	—	—	10
		Mix	35	20	—	—	—	—	45
	Late	Mono	45	25	—	—	—	—	30
		Mix	25	40	—	—	—	—	35
1995	Early	Mono	100	—	—	—	—	—	—
		Mix	50	—	—	50	—	—	—
	Mid	Mono	85	—	—	15	—	—	—
		Mix	60	—	—	15	25	—	—
	Late	Mono	55	—	—	30	—	15	—
		Mix	80	—	—	—	20	—	—
Salix viminalis 'Bowles Hybrid'									
1994	Early	Mono	100	—	—	—	—	—	—
		Mix	100	—	—	—	—	—	—
	Mid	Mono	100	—	—	—	—	—	—
		Mix	100	—	—	—	—	—	—
	Late	Mono	100	—	—	—	—	—	—
		Mix	65	20	—	—	—	—	15
1995	Early	Mono	80	—	—	20	—	—	—
		Mix	—	—	—	—	100	—	—
	Mid	Mono	90	—	—	—	10	—	—
		Mix	—	—	—	—	100	—	—
	Late	Mono	90	—	—	—	10	—	—
		Mix	70	—	—	—	30	—	—
Salix mollissima-undulata 'SQ83'									
1994	Early	Mono	—	100	—	—	—	—	—
		Mix	10	40	—	35	—	—	15
	Mid	Mono	—	100	—	—	—	—	—
		Mix	15	35	—	30	—	—	20
	Late	Mono	—	100	—	—	—	—	—
		Mix	20	30	—	25	—	—	25
1995	Early	Mono	—	100	—	—	—	—	—
		Mix	15	65	20	—	—	—	—
	Mid	Mono	—	40	35	10	15	—	—
		Mix	—	60	—	—	—	30	10
	Late	Mono	—	100	—	—	—	—	—
		Mix	—	70	—	—	—	—	30

similar and were not significantly ($P = 0.05$) different (Figure 1). LR1 was the dominant pathotype on *S. burjatica* 'Germany' throughout the season and on all ages of growth in both years. The diversity indices for LR pathotypes, on individual varieties growing in mixtures were not significantly different ($P = 0.05$) from the same varieties when they were grown in monoculture. There was a general trend of increasing diversity as the season progressed (Figure 2). On *S. viminalis* '683', *S. viminalis* 'Bowles Hybrid' and *Salix* × *dasyclados* the diversity indices at the end of the growing season were significantly higher ($P = 0.05$) than early in the growing season.

On both *S. viminalis* '683' and 'Bowles Hybrid' on all ages of stem growth the dominant pathotype was LET1 in both years. Pathotypes LET4, LET6, LET7 and NI5 were also found on these two varieties. *M. caprearum* was common on *Salix* × *dasyclados*;

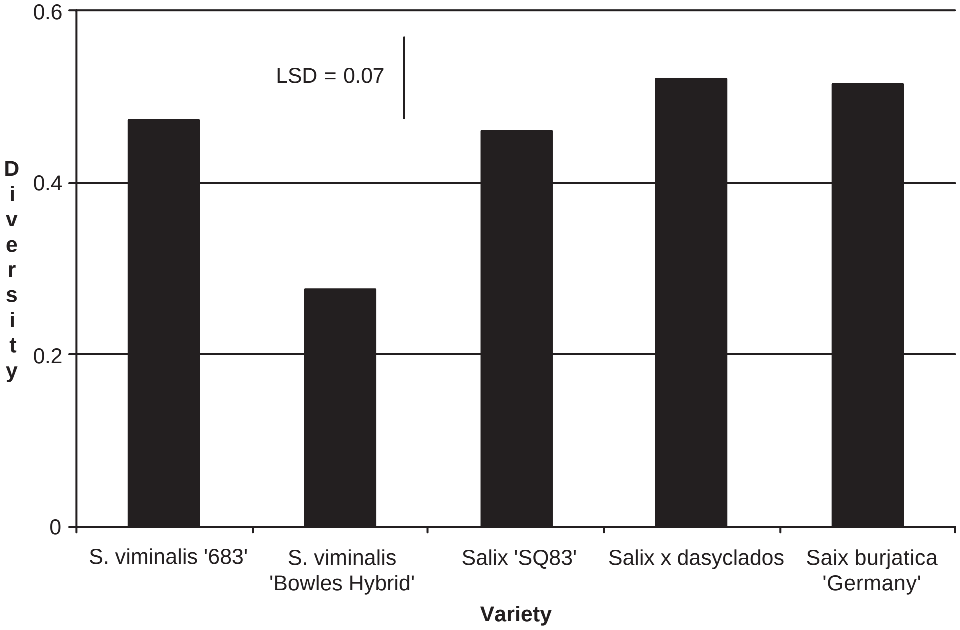

Figure 1. Diversity (Shannon-Weaver index) of *Melampsora* spp. and pathotypes on six *Salix* spp. varieties.

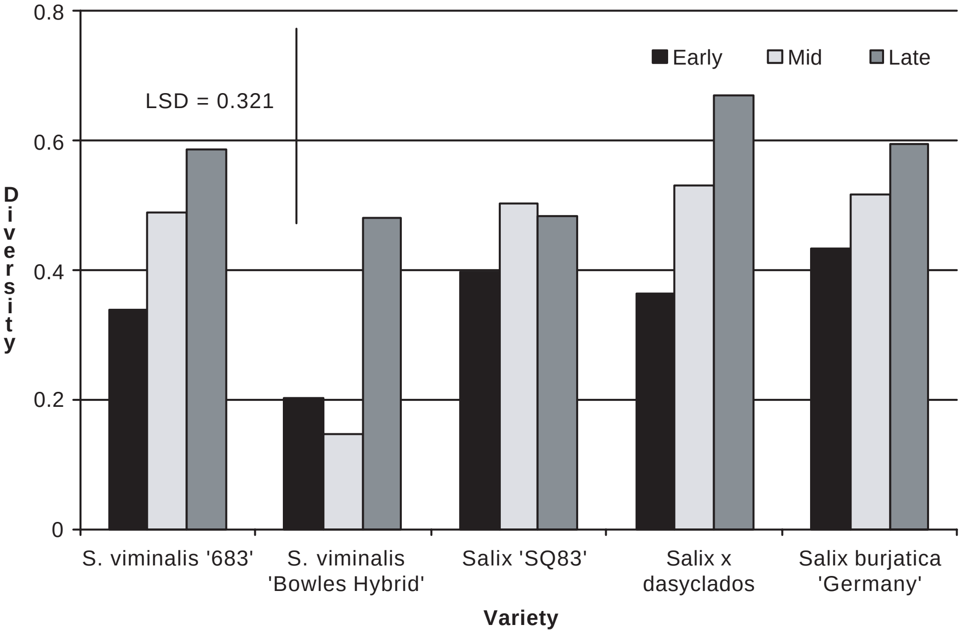

Figure 2. Diversity (Shannon-Weaver) index of *Melampsora* spp. and pathotypes on six *Salix* spp. varieties measured early, mid and late in the growing season.

Table 3. Parameters from the full model of LET1 log odds on each occasion and their significance on the normal distribution (* = significant at $P = 0.05$; ** = significant at $P = 0.01$; *** = significant at $P = 0.001$)

Parameter	Occasion					
	Early Year1	Early Year2	Mid Year1	Mid Year 2	Late Year 1	Late Year 2
Constant	0.547^{*}	1.19^{***}	0.847^{**}	1.494^{***}	0.769^{**}	0.475
Salix viminalis ‘Bowles Hybrid’	1.325^{**}	1.449^{*}	2.097^{**}	0.703	8.460	0.911^{*}
Salix × *dasyclados*	-0.815^{*}	-4.557^{***}	-2.341^{***}	-3.691^{***}	-2.113^{***}	-1.761^{***}
Mixture	1.325^{**}	−0.571	0	−0.647	−0.294	0.536
Salix viminalis ‘Bowles Hybrid’ × mixture	−1.00	-1.868^{**}	6.28	−1.145	−8.42	−0.911
Salix × *dasyclados* × Mixture	-2.156^{***}	2.066^{*}	0.647	1.458^{*}	1.091	−0.263

LET4 was the most frequently recorded pathotype on *S. mollissima-undulata* ‘SQ83’. Table 3 presents the parameters from the fitted logistic model of LET1 on each occasion, using the parameterisation whereby the first level of each factor and interaction are set at zero. LET1 was significantly less prevalent on *Salix* × *dasyclados* than it was on *S. viminalis* ‘683’ on every occasion. This pathotype was more prevalent on *S. viminalis* ‘Bowles Hybrid’ than it was on *S. viminalis* ‘683’, although this was only significantly so on two occasions. There was no consistent overall difference in the prevalence of LET1 between the mono- and mixture plots. Differences in pathotype occurrence between varieties in mono- and mixture plots were not consistent on the different occasions on which they were recorded.

Discussion

The interactions of *M. epitea* pathotypes with each other, with other *Melampsora* spp., with the willow variety on which they are growing, and with prevailing weather conditions are extremely complex. However, it is still very important to gain an appreciation of all these interactions and their influence on the composition of the rust populations, which in turn may affect the stability of SRC willow plantations.

Pathotypes LET4, LET5 and LET6, while present elsewhere in the UK and parts of Europe (Pei et al., 1999b), were recorded in plantations for the first time in N. Ireland in 1994. LET7 and LET8 were recorded in 1995 for the first time in plantations anywhere, although their existence had been predicted in the laboratory by genetic crosses using teliospores (Pei et al., 1999b). These field data have indicated that the number of pathotypes in N. Ireland willow rust populations appears to be constantly increasing. Similar increases have been reported between 1992 and 1994 in UK plantations (Pei et al., 1999b). The pathotype NI5, which also belongs to the *forma specialis* ‘*larici-epitea typica*’, has not previously been described elsewhere. It caused particularly severe damage to *S. mollissima-undulata* ‘SQ83’ resulting in leaf loss and reduced growth. These new pathotypes may have arisen by genetic exchange within the *f.sp.* ‘*larici-epitea typica*’ during the sexual cycle completed on its alternate host, *Larix decidua* (European larch) (Pei et al., 1999b). There is further supporting evidence (Pei et al., 1999b) for the importance of the sexual cycle in pathotype development. However, the role of *L. decidua* in rust disease epidemiolgy in N. Ireland is not clear. At sites where larch and SRC willow grow in close proximity the sexual stage of *M. epitea* has only been detected infrequently, despite intensive searching over a number of seasons (McCracken et al., 1996; A. Drysdale, Queen’s University Belfast, UK, personal communication). In addition to sexual recombination, other mechanisms for the introduction of genetic variation within a population, e.g., mutation, may be in action.

Throughout the growing season pathotype successions occurred with multiple pathotypes frequently present on the same variety or even the same leaf. One pathotype was clearly dominant on every willow variety investigated in this study, although different varieties had different dominant pathotypes. LET1 was dominant on *S. viminalis* ‘683’ at the start of the season when disease levels were relatively low. There is evidence to suggest that LET1 spores may over-winter in shoot buds and leaf litter (Parker et al., 1995) acting as an initial inoculum source. Furthermore, environmental conditions may have favoured LET1 growth at the start of the growing season. The frequency of LET1 decreased as other pathotypes, e.g., LET4 and NI5 appeared, although in the majority of cases LET1 remained the dominant pathotype.

Changes in the rust population affect the sustainability of certain varieties. There is evidence that varieties, which previously have been considered rust resistant, can become susceptible, to the point of being of no practical use (McCracken and Dawson, 1998b). Furthermore, with willow varietal mixtures being used as a disease control strategy (McCracken et al., 1996; McCracken and Dawson, 1998b) the potential for the rapid development of aggressive pathotypes is a major concern.

In general, the development of rust on leaves in the poly-varietal plots was slower than in mono-varietal plots, which was consistent with previous reports of the effect of mixtures in delaying disease onset and slowing its development (McCracken et al., 1996). Mixtures sustained a higher diversity of pathotypes than the same clones growing in mono-varietal plots. For example, on two-year-old shoots of *S. mollissima-undulata* ‘SQ83’ in 1994 a single pathotype, LET1, was recorded on mono-varietal plots, while on the same variety in mixture plots four pathotypes, LET1, LET4, LET6 and NI5, were present in significant proportions. This general pattern of pathotype diversity was similarly observed in other varieties grown in mixtures. This could have been due to more or different pathotypes being present in the mixtures. Hence the most aggressive pathotypes may have been diluted, allowing the less aggressive pathotypes to compete more successfully. There may have been a number of ways in which this was achieved, e.g.: the increase in the distance that spores had to travel between susceptible plants could have reduced the level of infection; a loss of spores due to unsuccessful infection attempts on non-susceptible varieties; or else numbers of susceptible plants successfully infected may have been low, resulting in a smaller number of spores for further infections.

Of the LET pathotypes that have been found, LET1 has been described as the most prevalent in the UK, causing severe infections on a wide range of *S. viminalis* varieties (Pei et al., 1996; 1999a). However, in the high disease pressure situations found in N. Ireland, pathotypes LET4, LET6 and NI5 were also common, especially on the *S. viminalis* varieties. Much of the new planting material currently being produced by willow breeding programmes is *S. viminalis* or *S. viminalis* hybrids. These varieties have differing levels of susceptibility to *M. epitea*, ranging from moderate susceptibility to complete resistance (McCracken and Dawson, 1997). Potentially however they are liable to attack by the same pathotypes (Dawson and McCracken, 1998) and hence it is still unclear as to whether *S. viminalis/ S. viminalis* hybrids constitute a genuine mixture in terms of disease reduction. However, differences in ‘pathogenicity factors’ such as latency period, size and number of pustules may be sufficiently significant to prevent major selection pressures being exerted on the components of the rust population (U. Gullberg, Swedish University of Agricultural Science, Sweden, personal communication).

It is possible that pathogens may be able to adapt to mixtures (Groth, 1976; Finckh and Mundt, 1992) resulting in the evolution of ‘super-races’ capable of infecting all or most of a mixture (Groth, 1976). The appearance of such complex pathotypes has been reported, often in association with biotrophs such as rust and powdery mildew of cereal crop mixtures (Finckh and Mundt, 1992). There is still some doubt as to whether the cultivar mixtures actually led to their selection and predominance (Groth, 1976; Huang et al., 1994). The present results would seem to indicate that the evolution of ‘super-races’ or ‘super-pathotypes’ is unlikely. Within varietal mixtures of willow there was a greater diversity of pathotypes with a possible increase in competition between them, which simultaneously may have decreased the evolutionary pressure on each pathotype. However, such ‘super-pathotypes’ may evolve on hosts having a narrow genetic base for resistance. It is uncertain whether resistance to rust in willow is controlled by a single or multiple genes. Within any rust population there is a wide diversity of individuals carrying virulence genes which are capable of overcoming their corresponding host resistance genes. Consequently, the continued success of mixtures is dependent on the component varieties containing as wide an array of multiple resistance genes as possible. Much of the most recent planting material has been selected on the basis of ‘field resistance’. Although never totally free of rust during the growing season, they may be able to tolerate the pathogen for long periods without significant loss in yield. This type of resistance is potentially more sustainable than total resistance (Pinon, 1992; Gullberg and Ryttman, 1993).

At present the most realistic strategy that can be recommended to reduce the impact of rust in willow plantations for energy production is the use of varietal mixtures (McCracken and Dawson, 1998b). Such mixtures are effective during at least the first three three-year-harvest cycles (McCracken and Dawson, 1997). However, it is envisaged that the life of a willow plantation will be at least eight cycles, i.e., up to 25 years. Varietal mixtures should increase the sustainability of such plantations over their full life span. To achieve more effective design and to use mixtures effectively an urgent need still remains to monitor pathotype changes occurring in the rust population over a number of growing seasons.

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