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Quantitative inoculations of poplars with *Melampsora larici-populina*

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Abstract

Four poplar clones were inoculated with four isolates of *Melampsora larici-populina* at seven spore concentrations (inoculum densities up to 680 spores cm^{-2}) using a leaf-disc method. Disease reactions were recorded using a digital camera. The number and size of uredinia were examined using image analysis software and the number of spores produced per leaf disc was counted. The infection efficiency was estimated in a range of 0.008–0.167 and the pustule diameter measured 0.75–0.94 mm. Rust resistance/susceptibility was expressed by the differences in both the number and the size of uredinia. Within a clone/isolate combination, pustule diameter and the number of spores produced per pustule did not differ significantly between different levels of inoculum density. There was a close correlation between the pustule area and spore yield. When Spearman rank correlation was tested between the disease variables, a close correlation was found between pustule number and pustule area per leaf disc (0.98) and between the number of spores produced and the pustule area/number per leaf disc (0.94 and 0.92, respectively). There was significant correlation between the number and the diameter of pustules (0.54, $P < 0.001$).

Introduction

Rust caused by *Melampsora larici-populina* is the most widespread and frequent disease of poplars (*Populus* spp.). In Europe, poplar hybrids between *P. deltoides* and *P. nigra* or *P. trichocarpa* are widely grown. Breakdown of resistance to *M. larici-populina* in some of these clones was detected in the early 1980s with appearance of races E1 and E2 (Steenackers, 1982; Pinon et al., 1987). Race E3 was detected in 1986 in Italy but was not considered to have a great impact on commercial clones (Pinon and Peulon, 1989). In the autumn of 1994, rust infections were encountered on *P. deltoides* × *P. trichocarpa* 'Boelare' and 'Beaupré', and on *P. deltoides* × *nigra* 'Primo', 'Ghoy' and 'Gibecq'. Subsequent studies revealed that the outbreaks of rust on these clones were caused by a new race, E4 (Steenackers et al., 1994). In the UK, poplar plantations sustained slight to moderate rust infections up to 1995 (Lonsdale and Tabbush, 1998). In 1996, rust outbreaks occurred in many poplar plantings and, in some cases, resulted in dieback and death of coppice stools. Since 1996, poplars in south west England have been severely infected by rust each year (M. Pei, T. Hunter and D. Lonsdale, unpublished observations).

Some of the most basic questions in plant disease epidemiology include what proportion of inoculum produces pustules/lesions (referred to as infection efficiency; see Zadoks and Schein, 1979) and how many spores are produced by a pustule/lesion. An understanding of infection efficiency is also important in characterisation of host resistance/pathogen virulence. Using a leaf disc method, Giorcelli et al. (1996) examined the influence of clonal susceptibility, leaf age and inoculum density on rust infections caused by E1 and E3 types of *M. larici-populina*. From their data, the average infection efficiencies on susceptible clones were 0.47 for E1 and 0.64 for E3, respectively. In their experiments, probably due to the large number of samples involved, only three leaf discs were used as replicates and only predicted figures were given for the number of spores deposited on the surface of leaf

discs. To date, critical examinations on the quantitative relationships between the inoculum densities and resulting disease in *M. larici-populina* on poplars are lacking.

Recently, digital profiling of disease in studies of *Melampsora* on *Salix* has greatly improved the efficiency and accuracy of disease assessments, and made it possible to test a large number of samples in a single experiment (Pei et al., 2002). In this study, the same procedure was used to determine, under defined experimental conditions, the relationships between the number of spores applied and the number of pustules produced, and between the number of pustules/pustule area and the number of spores produced.

Materials and methods

Poplar plants and rust isolates

Four poplar clones, 'Vereecken', 'Spijk', 'Trichobel' and '75028' (Table 1) were grown from 1-year-old cuttings in pots containing 'John Innes' Compost No. 3 in a glasshouse for 8 weeks. At the time of inoculation, plants from these cuttings were 60–90 cm tall and actively growing.

Four rust isolates were used (Table 1). Unpublished results from inoculation experiments involving 24 poplar clones showed that isolates BE and BO shared virulence/avirulence patterns similar to race E4 by causing infections on *P. deltoides* × *nigra* 'Ghoy' and 'Gibecq', and on *P. deltoides*× *trichocarpa* 'Beaupré' and 'Boelare' (Pinon, 1992; Steenackers et al., 1994). However, they appeared to differ in the degree of infections on several tested clones. Isolate SIM and VER shared certain virulence/avirulence patterns with E1–E3 by not infecting 'Ghoy', 'Gibecq', 'Beaupré' and 'Boelare'. Each rust isolate was cultured from a single uredinium and stored at −20 °C. Two weeks before inoculation, detached leaves of the host clone placed in Petri dishes containing water-soaked filter paper were inoculated with each isolate to produce fresh urediniospores.

Inoculation experiment

Incubation period

Leaf discs, 1.6 cm diameter (2.01 cm^2 area), were cut from 5–10th leaves on actively-growing poplar shoots. The discs were placed, abaxial surface uppermost, on blotting paper bridges soaked in tap water in 25 (5 × 5) compartments of 10 × 10 cm square Petri dishes. A 60 mm dia. Petri dish containing 1.2% water agar was also placed in the spray target area. Rust spores were suspended in tap water containing 0.004% Tween 20 (1 drop in 100 ml) and spore suspensions were adjusted to 40,000 spores ml^{-1}. The spore suspensions were sprayed on to the target area (1 ml per 10 × 10 cm area) using a Humbrol air brush (Humbrol Ltd.). Ten leaf discs, each from a different poplar leaf, were used as replicates for each isolate/clone combination.

After inoculation, the leaf discs were incubated in a growth chamber at 16 °C with 16 h/day illumination at an intensity of 80 $\mu E\, m^{-2}\, s^{-1}$. Inoculum density (viable spores applied per leaf disc) for each rust isolate was determined by examining the number of germinating spores on the water agar which had been placed in the target area and incubated at 16 °C for 24 h. For each isolate/density, ten randomly chosen microscopic fields (10 × 10 magnification, 2.4 mm^2 each field) or, in the cases that inoculum densities were very low, four 2.01 cm^2 agar discs cut from the agar plate were examined.

Inoculated leaf discs were examined daily from five to 13 days after inoculation and the latent period was recorded as days from inoculation until the appearance of ruptured uredinia on the leaf disc.

Table 1. Melampsora larici-populina isolates used

Rust isolate	Host species	Site and date of collection
BE	*P. deltoides* × *P. trichocarpa* 'Beaupré'	Markington, N Yorkshire, N England, September 1997
BO	*P. deltoides* × *P. trichocarpa* 'Boelare'	Long Ashton, N Somerset, SW England, September 1996
SIM	*P. simonii*	Alice Holt, Surrey, S England, September 2000
VER	*P. nigra* 'Vereecken'	Markington, N Yorkshire, N England, September 1996

Inoculations with different levels of inoculum

Rust spores were suspended in tap water containing 0.004% Tween 20 (1 drop in 100 ml) and spore suspensions were adjusted to 4, 8, 16, 32, 64, 128 and 256 × 1000 spores ml^{-1}. The spore suspensions were sprayed on to the target area (1 ml per 10 × 10 cm area) using a Humbrol air brush. Ten leaf discs, each from a different poplar leaf, were used as replicates for each isolate/clone/density combination. After inoculation, the leaf discs were incubated in a growth chamber. Inoculum density was determined, 24 h after inoculation, by examining the number of germinating spores on the water agar in ten 10 × 10 microscopic fields (2.4 mm^2each field) for the spore concentrations higher than 4 × 1000 spores ml^{-1}, and in four 2.01 cm^2 agar discs cut from the agar plate which had been placed in the target area for the concentrations 2 and 4 × 1000 spores ml^{-1}.

Thirteen days after inoculation, reactions on leaf discs were recorded using a digital camera (Olympus C-2500L). Experience suggested that most *M. larici-populina* isolates would take 7 or more days to produce pustules and a 13-day incubation period would exclude disease likely to have developed as a result of a second cycle of infection. After photographs had been taken, the leaf discs were gently transferred into 20 ml screw-top tubes (one disc per tube) using forceps. The tubes containing leaf discs were stored at −4 °C until spores were counted. Before spore counting, 1 ml distilled water containing Tween 20 was added to the tube containing the leaf disc and spores were gently brushed off from the leaf disc using a sable hair brush. The spore suspension was agitated using a vortex machine for 10 s and spores were counted using a haemocytometer. The number of spores on each leaf disc was estimated by averaging eight spore counts.

Image analysis software (SigmaScan Pro 5.0 (SPSS Inc.)) was used to examine the leaf disc images. For each leaf disc, pustule numbers were counted and the diameters of erumpent uredinia were measured manually with the 'Trace Measurement Mode' function. If there were more than 10 erumpent pustules on a leaf disc, only 10 randomly-selected pustules were measured to obtain an estimate of average pustule diameter on the leaf disc.

Data analyses

Uredinial pustule area on each leaf disc was estimated from the total number and average diameter of the pustules on the leaf disc. Infection efficiency was calculated as the number of pustules developed on a leaf disc divided by the number of viable spores deposited on a 2.01 cm^2 area. In a number of cases, the data were transformed in order to stabilise the variance of dependent variables and to spread out more evenly the independent variables. A logarithmic transformation ($\log_{10}$) was used for inoculum density, spore and pustule counts, and pustule area data.

A simple linear regression model was used to examine the relationships between pustule area and spore yield and between pustule number and spore yield. To determine whether there were differences between the relationships for the different poplar/rust isolate combinations, a series of models were fitted, i.e. a single overall line, parallel lines (i.e. a common slope but different intercepts for the different combinations), separate lines with different slopes but with a common intercept (intercept = 0) and finally separate lines with different slopes as well as intercepts. Spearman rank correlation was also tested for correlation between disease parameters. The analyses were carried out using GenStat 5 Release 4.2 (Genstat Committee, 2000).

Results

Incubation period

Inoculum densities were estimated as 500 spores per leaf disc for BE, 580 for BO, 220 for SIM and 600 for VER. Uredinial pustules became ruptured 9–10.6 days after inoculation (Table 2).

Inoculations with different levels of inoculum

More pustules developed as the inoculum densities increased in all poplar clone/rust isolate combinations (Figures 1 and 2). On 'Vereecken', the number of pustules produced by SIM was significantly higher than that of BO (Figure 1). On 'Spijk', BE produced significantly higher number of pustules compared with BO (Figure 2). On 'Trichobel', more pustules were produced by BE compared with BO at inoculum densities lower than 100 spores per leaf disc, but pustule numbers were similar at higher densities (Figure 2). Average incubation period, infection efficiency, pustule diameter and spores produced per pustule (irrespective of inoculum densities) were shown in Table 2. Average infection efficiency was 0.167 in BE/'Spijk' and 0.166 in BE/'Trichobel', significantly higher than in other combinations (Table 2).

Table 2. Average incubation period, infection efficiency, pustule diameter and spores per pustule of *Melampsora larici-populina* isolates

Rust isolate	Poplar clone	Incubation period	Standard error of means ($P < 0.05$)	Infection efficiency	Standard error of means ($P < 0.05$)	Pustule dia. mm	Standard error of means ($P < 0.05$)	Spores per pustule	Standard error of means ($P < 0.05$)
BE	*P. nigra* 'Vereecken'	9.4	0.44	0.075	0.038	0.756	0.046	1810	480
BE	*P. nigra* × *P. deltoides* 'Spijk'	9.0	0.00	0.167	0.042	0.943	0.03	1960	310
BE	*P. trichocarpa* 'Trichobel'	10.0	0.00	0.166	0.032	0.822	0.036	1280	230
BO	*P. nigra* 'Vereecken'	9.8	0.36	0.008	0.002	0.751	0.084	1850	670
BO	*P. nigra* × *P. deltoides* 'Spijk'	9.0	0.00	0.016	0.004	0.912	0.05	2020	440
BO	*P. trichocarpa* 'Trichobel'	10.6	0.72	0.067	0.016	0.828	0.06	1540	470
BO	*P. trichocarpa* × *P. deltoides* × *P. deltoides* '75028'	9.0	0.00	0.017	0.004	0.839	0.048	2260	550
SIM	*P. nigra* 'Vereecken'	9.0	0.00	0.07	0.018	0.928	0.058	2560	460
VER	*P. nigra* 'Vereecken'	9.0	0.00	0.068	0.028	0.887	0.08	2780	780

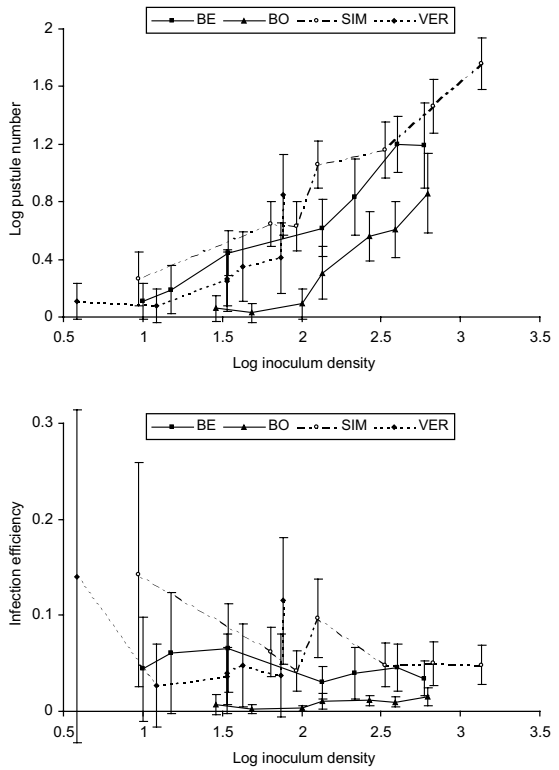

Figure 1. Number of uredinial pustules and infection efficiency of *M. larici-populina* isolates on poplar clone 'Vereecken' after inoculations with different levels of inoculum.

No significant differences in pustule diameter were found between different levels of inoculum densities within each poplar clone/rust isolate combination. However, between different clone/isolate combinations, pustule diameter differed considerably. For example, average pustule diameter of BE was 0.756 mm on 'Vereecken' and 0.943 mm on 'Spijk'. The number of spores produced 13 days after inoculation was estimated in a range between 1280–2780 spores per pustule (Table 2). The number of spores produced per pustule was lower in BE/'Trichobel' and BO/'Trichobel', on which pustules did not become ruptured until 10 and 10.6 days, respectively after inoculation. There were no significant differences in the number of spores produced per pustule at different levels of inoculum densities within a clone/isolate combination. Spore production per leaf disc in isolate BE was lower on 'Vereecken' than on 'Spijk' and 'Trichobel' at all the inoculum densities (Table 3). With BO, spore yield was higher on 'Trichobel' than on 'Spijk', '75028' and 'Vereecken'.

For the plot between log spore yield against log pustule number, the parallel lines model gave a significant improvement in fit compared to the single line ($P < 0.001$; %VAF increased from 71.7% for the single line to 74.0% for parallel lines). The model allowing individual slopes and intercepts did not give significant improvement ($P = 0.154$). Between the log pustule area and log spore yield, the model having individual slopes and intercepts and that having parallel lines (%VAF = 78.7 and 78.2, respectively) were slightly better in fit compared to the single line (%VAF = 77.1).

Spearman rank correlation coefficients between the disease variables are shown in Table 4. A close correlation was found between pustule number and pustule area per leaf disc (0.98, $P < 0.001$) and

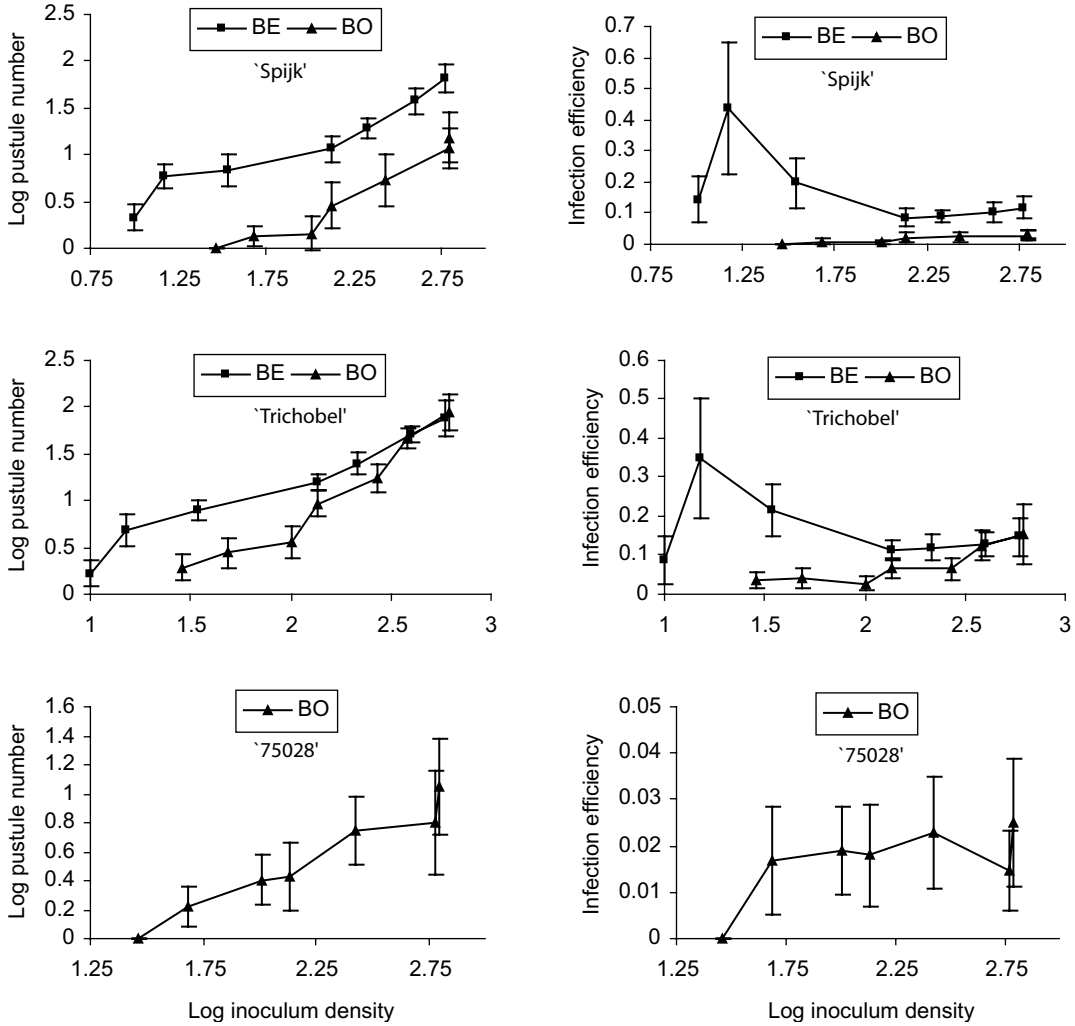

Figure 2. Number of uredinial pustules and infection efficiency of *M. larici-populina* isolates on poplar clones 'Spijk', 'Trichbel' and '75028' after inoculations with different levels of inoculum.

between the number of spores produced and the pustule area/number per leaf disc (0.94 and 0.92, respectively, $P < 0.001$). There was significant correlation between the number and the diameter of pustules (0.54, $P < 0.001$).

Discussion

All the nine clone/isolate combinations produced more pustules as higher doses of inoculum were applied, and there was a close linear correlation between inoculum density and the number of uredinia produced (%VAF = 73.9 when parallel lines were fitted). Average infection efficiency was highest for the combination BE/'Spijk' (0.167) and for BE/'Trichobel' (0.166) and lowest in BO/'Vereecken' (0.008). Hamelin et al. (1994), reported that infection efficiency in *M. medusae* was within a range of 0.011–0.075 in leaf disc inoculation experiments involving eight host genotypes and 11 rust isolates. When selected genotypes of F2 progeny of *P. trichocarpa* × *P. deltoides* were inoculated with *M. medusae* using a leaf disc method, infection efficiencies were 0.005 for resistant phenotypes and 0.06 for susceptible phenotypes (Newcombe, 1998). In willow rust *M. larici-epitea*, infection

Table 3. Log number of spores produced per leaf disc 13 days after inoculation with *M. larici-populina*

Rust isolate	Poplar clone		Levels of inoculum						
			A	B	C	D	E	F	G
BE		Inoculum density*	8.0	14	34	102	134	400	595
	'Vereecken'		0.77	1.31	1.36	1.36	3.88	4.41	3.84
	'Spijk'		2.87	3.63	3.81	3.81	4.55	4.76	4.97
	'Trichobel'		2.09	2.18	3.81	3.81	4.38	4.80	4.73
				Clone	Level	Clone × level			
		L.s.d.**		0.38	0.57	0.99			
BO		Inoculum density	28	48	100	134	268	385	620
	'Vereecken'		0.36	0.31	0.70	1.10	2.93	2.16	3.14
	'Spijk'		0.00	0.93	0.65	2.26	3.75	4.12	3.91
	'Trichobel'		2.96	2.23	2.40	3.80	4.36	4.56	4.80
	'75028'		0.00	1.77	2.50	1.89	3.53	3.40	3.84
				Clone	Level	Clone × level			
		L.s.d.		0.48	0.63	1.27			
SIM	'Vereecken'	Inoculum density	8.4	63	92	126	335	670	1360
			2.24	3.8	3.59	4.37	4.51	4.67	4.73
		L.s.d.		0.86					
VER	'Vereecken'	Inoculum density	2.8	11.2	34	34	67	42	75
			0.71	0.77	1.76	2.14	1.2	1.83	3.7
		L.s.d.		1.57					

*Estimated number of viable spores applied per leaf disc.
**Least significant difference ($P < 0.05$).

Table 4. Spearman rank correlation coefficients between disease variables

	Incubation period	Infection efficiency	Inoculum density	Pustule diameter	Pustule area per disc	Pustule number per disc	Number of spores per disc	Number of spores per pustule
Incubation period	—							
Infection efficiency	0.22**	—						
Inoculum density	0.15	0.06	—					
Pustule diameter	0.02*	0.61**	0.20**	—				
Pustule area per disc	0.16	0.73**	0.63**	0.67**	—			
Pustule number per disc	0.21*	0.71**	0.68**	0.54**	0.98**	—		
Number of spores per disc	0.12	0.66**	0.60**	0.61**	0.94**	0.92**	—	
Number of spores per pustule	0.00	0.52**	0.25**	0.71**	0.58**	0.52**	0.75**	—

*Significantly different from zero ($P < 0.05$).
**Significantly different from zero ($P < 0.001$).

efficiency was estimated as 0.11–0.68 in leaf disc inoculations (Pei et al., 2002). When wheat seedlings were inoculated with *Puccinia graminis* var. *tritici* at densities <2000 spores per cm^2, approximately 1% of the spores applied produced uredinia (Peterson, 1959). In bean rust *Uromyces appendiculatus*, infection efficiency was estimated as approximately 0.1 at inoculum densities 200–900 spores/cm^{-2} (Schein, 1964).

Under the present experimental conditions, pustule diameter was generally consistent for the different

inoculum densities within a clone/isolate combination. Pustule diameter is an important parameter in disease assessments. Conventionally, pustule sizes are measured using a stereomicroscope. As the disease has to be scored in a short period of time by the completion of inoculation experiments, it is not practicable to measure the pustules when a large number of samples are tested. Therefore, categorical scores are often used to give estimates of the size of pustules. For example, Prakash and Thielges (1987) described the size of pustules as minute, small, medium, or large and later, Hsiang and Castagner (1993) defined the pustules <0.4 mm in diameter as small and >0.4 mm as large. Disease scores obtained in this way can be subjective because the estimates given by different workers may vary. The present method of measurement of pustules using image analysis software provides a practical means of obtaining accurate estimates of pustule sizes in inoculation experiments involving large numbers of samples.

Disease resistance/susceptibility of a clone to different isolates was expressed by the differences in both the number and the size of uredinia. For example, infection efficiency in BO/'Vereecken' was low (0.008) and the size of pustules was small (0.75 mm) (Table 2). In BO/'Spijk', average infection efficiency was relatively low (0.016) whilst the average pustule diameter was relatively large (0.91 mm). When the data from all the leaf discs, irrespective of clone/isolate combinations, were examined, no significant correlation was found between the number and the size of pustules. In leaf disc inoculation experiments with *M* × *columbiana*, a possible hybrid between *M. medusae* and *M. occidentalis*, Newcombe et al. (2000, 2001) scored resistance/susceptibility according to infection types which were defined according to the size and the presence of chlorotic or necrotic flecking. The present results suggest that the size and the presence of flecking alone may not be sufficient for characterisation of rust resistance in poplars/virulence in *Melampsora* rusts.

The average number of spores produced per pustule 13 days after inoculation was within the range of 1280–2780. Dividing them by the number of days after the uredinia became ruptured (13 – incubation period), it can be estimated that some 430–700 spores may have been produced from each uredinium per day. Spore production is one of the most important driving variables in the development of disease epidemics. It is also one of the most accurate and least subjective ways of assessing the growth of pathogens and the susceptibility of hosts (Johnson and Taylor, 1976). In the present study, irrespective of the poplar clone/rust isolate combinations, the number of spores produced per leaf disc was closely correlated to the area and the number of pustules (Spearman rank correlation coefficients 0.94 and 0.92, respectively, $P < 0.001$). The area of pustules was also significantly correlated to the inoculum density (Spearman coefficients $=$ 0.63, $P < 0.001$, and %VAF $=$ 62.4–72.7 when regression models having individual lines for different clone/isolate combinations were fitted). Such relationships may be useful in improving the means of characterisation of disease resistance/pathogen virulence by taking inoculum densities into consideration.

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References

Genstat Committee (2000) GenStat 5 for Windows, Release 4.2, 5th edn. Numerical Algorithms Group, Oxford, UK

Giorcelli A, Vietto L, Anselmi N and Gennaro M (1996) Influence of clonal susceptibility, leaf age and inoculum density on infections by *Melampsora larici-populina* races E1 and E3. European Journal of Forest Pathology 26: 323–331

Hamelin RC, Ferriss RS and Shain L (1994) Prediction of poplar leaf rust epidemics from a leaf disc assay. Canadian Journal of Forest Research 24: 2085–2088

Hsiang T and Castagner GA (1993) Variation in *Melampsora occidentalis* rust on poplars in the Pacific Northwest. Canadian Journal of Plant Pathology 15: 175–181

Johnson R and Taylor AJ (1976) Spore yield of pathogens in investigation of the race-specificity of host resistance. Annual Review of Phytopathology 14: 97–119

Lonsdale D and Tabbush P (1998) Poplar rust and its recent impact in Great Britain. Information Note 7. Forestry Commission, Edinburgh, UK

Newcombe G (1998) Association of Mmd1, a major gene for resistance to *Melampsora medusae* f.sp. *deltoides* with quantitative traits in poplar rust. Phytopathology 88: 114–121

Newcombe G, Stirling B and Bradshaw HD (2001) Abundant pathogenic variation in the new hybrid rust *Melampsora* × *columbiana* on hybrid poplar. Phytopathology 91: 981–985

Newcombe G, Stirling B, McDonald S and Bradshaw HD (2000) *Melampsora* × *coulumbiana*, a natural hybrid of

M. medusae and *M. occidentalis*. Mycological Research 104: 261–274

Pei MH, Ruiz C, Hunter T, Arnold GM and Bayon C (2002) Quantitative relationships between inoculum of *Melampsora larici-epitea* and corresponding disease on *Salix.* Plant Pathology 51: 443–454

Peterson LJ (1959) Reactions between inoculum density and infection of wheat by uredospores of *Puccinia graminis* var. *tritici*. Phytopathology 49: 607–614

Pinon J (1992) Frequency and evolution of *Melampsora larici-epitea* Klebahn races in north-western France. Annales des Sciences Forestières 49: 1–15

Pinon J and Peulon V (1989) Mise en évidence d'une troisième race physiolosique de *Melampsora larici-populina* Kleb. en Europe. Cryptogamie Mycologie 10: 95–106

Pinon J, van Dam BC, Genetet I and De Kam M (1987) Two pathogenic races of *Melampsora larici-populina* in north-western Europe. European Journal of Forest Pathology 17: 47–53

Prakash CS and Thielges BA (1987) Pathogenic variation in *Melampsora medusea* leaf rust of poplars. Euphytica 36: 563–570

Schein RD (1964) Design, performance, and use of a quantitative inoculater. Phytopathology 54: 509–513

Steenackers V (1982) Nouvelle race physiologique de *Melampsora larici-populina* en Belgique (communication provisoire). FAO/CIP, 22e réunion de travail des maladies, Casal Monferrato, Italy, 6–10 Septembre 1982, 6pp

Steenackers M, Steenackers V and Delporte T (1994) A new race of *M. larici-populina* in Belgium. International Poplar Commission, Izmit, Turkey, 3–7 October, 8pp

Zadoks JC and Schein RD (1979) Epidemiology and Plant Disease Management. Oxford University Press, Oxford, UK