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Pathogenic variation and sexual reproduction in Swedish populations of *Bremia lactucae*

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Summary. The host-pathogen interaction between lettuce (*Lactuca sativa*) and downy mildew (*Bremia lactucae*) is mainly differential and the resistance so far utilized in the host is vertical. As in many other obligate parasites, the introduction of cultivars with new vertical resistance has exerted a strong selection pressure on the pathogen resulting in significant changes in virulence frequencies and in the establishment of races with new combinations of virulence. Genetic diversity in pathogen populations may arise through mutation and gene flow, and new virulence genotypes may then be established through parasexuality and sexual recombination. In Swedish populations of *Bremia lactucae,* the pattern of variation in the parasite agrees well with that which might be expected in a diploid, outcrossing organism with frequent sexual reproduction. This is supported by: two or more isolates, different in virulence and mating type, may occur together on the same lettuce leaf; zygotes (oospores) are formed in all populations investigated and the frequency varies from 22% to 98%; oospores germinate rather frequently under suitable conditions. To breed for resistance in dynamic host-pathogen systems such as this one is difficult and the program should preferably be based on race-non-specific resistance.

Key words: *Bremia lactucae* – Pathogenic variation – Sexual reproduction – Recombination

Introduction

Downy mildew, which is one of the most severe diseases on lettuce (*Lactuca sativa*), is caused by the biotrophic fungus *Bremia lactucae* Regel.

In Sweden, lettuce crops are severely attacked by downy mildew which may result in serious losses in yield and reduced quality. Investigations on host-parasite interactions as well as breeding for resistance have been carried out (see Crute 1981; Matthews 1981). All results indicate that the interaction is differential and that the resistance in the host is vertical (race specific). Available data have been interpreted in terms of a gene-for-gene relationship, where twelve resistance factors of the host are matched by twelve virulence factors in the pathogen (Crute and Johnson 1976; Johnson et al. 1978; Bannerot 1980).

Several studies of pathogenic variation have been performed and *B. lactucae* shows a large variation and flexibility in its composition of virulence (Dixon and Wright 1978; Lebeda and Zavadil 1979; Osara and Crute 1981). In Sweden, pathogenic variation has been investigated for thirteen successive years (1971–1983), and the long period of observations has made it possible to study long term changes in virulence frequencies and the accumulation and fitness of virulence genes (Wellving and Crute 1978; Gustafsson and Larsson 1984). These investigations have shown that the pathogen, when exposed to new genes for race specific resistance, has responded quickly with a shift in its virulence gene composition.

One of the most important factors influencing the rate and direction of differentiation in host-pathogen systems is probably the degree of sexual reproduction and recombination followed by selection. However, little attention has been paid to the role of recombination, mostly due to the fact that little was known about sexual reproduction in *B. lactucae* until the studies of Michelmore and Ingram (1980, 1981). *Bremia lactucae* is heterothallic with two sexual compatibility types, designated B_1 and B_2. In Sweden at least, *B. lactucae* seems to be strictly outcrossing as all the sixteen investigated single-spore cultures are selfsterile (Gustafsson et al. 1983). The formation of sexual organs is initiated only when hyphae of different compatibility types come into contact. Gametangial contact is followed by karyogamy and formation of a zygote, oospore. A vegetative mycelium is developed after oospore germination. Cytological and genetical investigations show that *B. lactucae,* like several other Oomycetes, is diploid in its vegetative phase (Sargent et al. 1977; Tommerup 1981; Michelmore and Ingram 1981; Norwood et al. 1983).

The extent of sexual reproduction in natural populations of *B. lactucae* and the role of sexual recombination for pathogenic variations have not hitherto been well investigated. Precise results and evidence are difficult to obtain, but investigations concerning the simultaneous occurrence of different mating types and formation of oospores under natural conditions may provide valuable information. Such an investigation was initiated in 1982 with one of the main aims being to correlate the pathogenic variation to the extent of sexual reproduction.

Materials and methods

Studies of pathogenic variation of *Bremia lactucae* in Sweden was initiated by Wellving in 1971. Between 1971 and 1974 the isolates were tested by inoculating leaf discs from plants of a differential series of 10 cultivars following the procedure described by Tjallingii and Rodenburg (1969). However, it has been difficult to analyse the presence of virulence factors *v3* and *v6* with this tester set (Wellving and Crute 1978). From 1979 and onwards tests were made on leaf discs and seedlings according to the methods described by Crute and Dickinson (1976). The virulence factors of the isolates were determined by using a tester set including all known resistance genes (see Crute and Johnson 1976), which permits a detailed description of the virulence pattern of each isolate. Records were made 10–14 days after inoculation, following the usual classification system. The virulence pattern of the following number of isolates has been checked: 1979: 22 (MG); 1980: 25 (IG); 1981: 111 (IG); 1982: 206 (IG).

In our study, the presence of different mating types in commercially grown lettuce crops was investigated as follows: isolates of *B. lactucae* were sampled at different collecting sites. Each sample was kept separately and when necessary, stored by freezing newly sporulating leaf-pieces in sealed plastic boxes at a temperature of −20 °C. Each sample was inoculated onto at least 20 single, fully expanded, cotyledons or 15 leaf-pieces of the cultivar 'Hilde'. Inoculation was made by placing drops of a suspension of spores on the surface of each leaf. The suspensions contained about 10^5 spores/ml. The inoculum was prepared and the inoculations made in a laminar flow airbench in order to prevent contamination. The petri-dishes, containing inoculated leaves, were sealed and placed in a growth chamber at 15 °C, first in darkness for 24 h, and then in 16 h daylight. The leaves were kept until they were transparent. The formation of oospores was examined directly with a low power dissecting microscope (×50) at intervals of five days. In addition, leaf-pieces were fixed and cleared in 95% ethyl alcohol, then examined in a light microscope at a magnification of × 160 or more.

Calculation of virulence frequencies

As downy mildew is diploid in its infectious phase, a virulent isolate capable of attacking a host with the resistance gene *R1* is homozygous for the virulence gene *v1* and the genotype is described as *v1v1*. All the virulence genes known so far are recessive, non-allelic and consequently the genotype of an avirulent race must be either *+v1* or *++*. In a diploid organism, like *B. lactucae*, where random mating most likely occurs, different types of virulence frequencies can be calculated from the Hardy-Weinberg law. Thus, in a panmicitic population of *Bremia lactucae*, the total frequency of a recessive gene, q, and its dominant allele, p, will be 100% (or 1); and the frequency of the corresponding genotypes, $p^2 + 2pq + q^2 = 1$ (see Table 1).

Results

Pathogenic variation

Distribution of virulence phenotypes in Swedish populations of *Bremia lactucae* has been investigated with respect to pathogenic variation within populations, differences in virulence frequencies between populations, variation with time, and accumulation of unnecessary virulence.

Variation within populations

Isolates of *B. lactucae* have been sampled from individual host plants and their virulence studied (Table 2). The pathogenic variation concerns most virulence factors, only two (*v7* and *v10*) are distributed in all isolates, while one (*v2*) is absent. The number of virulence factors varies considerably from one isolate to another. Isolates taken from six of the plants contained between nine and eleven virulence factors, while those from two other plants contained only five and two, respectively.

Table 1. Different types of virulence frequencies used in the present study, based on the Hardy-Weinberg law

Symbol	Explanation
p	Calculated frequency of the dominant avirulence gene +
q	Calculated frequency of the recessive virulence gene v
q^2	Observed frequency of virulence phenotypes (homozygotes vv)
I_q	Calculated frequency of isolates which are homozygous or heterozygous for *v* ($= q^2 + 2pq$)

Table 2. Distribution of virulence factors in isolates collected on eight plants from one commercial growing. *v* indicates virulence, + avirulence, and n number of virulence factors present in a homozygous state

Plant no.	Virulence phenotype	*n*
1	*v1*, +, *v3*, *v4*, *v5*, *v6*, *v7*, *v8*, *v9*, *v10*, *v11*, *v12*	11
2	*v1*, +, *v3*, *v4*, *v5*, *v6*, *v7*, *v8*, *v9*, *v10*, *v11*, *v12*	11
3	+, +, +, *v4*, *v5*, *v6*, *v7*, *v8*, *v9*, *v10*, *v11*, *v12*	9
4	*v1*, +, *v3*, *v4*, *v5*, *v6*, *v7*, *v8*, *v9*, *v10*, *v11*, +	10
5	*v1*, +, *v3*, *v4*, *v5*, *v6*, *v7*, *v8*, *v9*, *v10*, *v11*, +	10
6	*v1*, +, *v3*, *v4*, *v5*, *v6*, *v7*, *v8*, *v9*, *v10*, *v11*, +	10
7	+, +, +, *v4*, *v5*, +, *v7*, *v8*, +, *v10*, +, +	5
8	+, +, +, +, +, +, *v7*, +, +, *v10*, +, +	

Other investigations show that isolates possessing different virulence patterns and compatibility types may grow together on the same host plant, even on the same lettuce leaf.

Pathogenic variation within single, commercial lettuce crops has also been studied by sampling several isolates throughout the entire growing season (I. Gustafsson, unpublished). The extent of variation in one population is illustrated in Fig. 1. In this case isolates were collected at eight different occasions throughout the growing period. The virulence phenotype of 76 isolates was determined. Throughout the sampling period, different virulence phenotypes occurred simultaneously in the population, with a minimum of two (sample 3) and a maximum of seven (sample 5). The same pattern of variation has been observed in other investigated populations.

Differences between populations

In 1982, isolates of *B. lactucae* were collected in three commercial crops of lettuce. The frequencies of 12 virulence factors were examined and the results are summarized in Table 3. The degree of variation is quite different for different virulence factors. The frequencies of *v2, v3, v9* and *v11* vary considerably with the population, while the frequencies of *v4, v5, v7* and *v10* seem to be more stable.

Pathogenic variation with time

Changes in virulence factor frequencies with time are shown in Fig. 2. The frequencies of virulence phenotypes, i.e. isolated homozygous (q^2) for virulence factors *v1* to *v11,* have been studied. Their frequencies have increased dramatically from 1976 to 1982, and as many as seven virulence phenotypes now have frequencies higher than 85%. Isolates possessing *v11v11* and *v2v2* continue to be maintained at low frequencies in the studied populations, but the frequency of *v11v11* has increased from 11 to 44%, while that of *v2v2* has risen from 15 to 48%. The factor *v7* seems to have reached a fixed level in the Swedish populations of *B. lactucae.*

However, in discussions about long term changes in the virulence pattern, information about the frequency of isolates carrying a particular *v*-gene, either in a homozygous or in a heterozygous state ($=I_q$ in Table 1), is more valuable. These calculations have been made and are summarized in Fig. 2 (upper curve). In 1976 the calculated frequency of isolates carrying *v11* was 55%, for *v2* 62%, and the corresponding figures for all other *v*-genes are 82% or higher. In 1982, 89% of the isolates are homozygous or heterozygous for *v11,* and 91% or more for all the other *v*-factors. Furthermore, in 1982 the calculated frequency of isolates which were homozygous or heterozygous for all eleven virulence genes was 69%.

Accumulation of virulence

In Sweden, pathogenic variation in *B. lactucae* is characterized by the evolution of greater complexity (Gustafsson and Larsson 1984). In 1976, the complex race S4 (with *v1* to *v10*) was identified, and later, races with all eleven virulence factors were isolated (I. Gustafsson, unpublished). Accumulation seems to comprise all virulence genes. During three successive years (1980–1982) 69 isolates were collected on the lettuce cultivar 'Great Lakes III', which possesses the resistance gene *R7.* The number of "unnecessary" virulence genes, i.e. all virulence genes which are not matched by any resistance factor in the host, was determined for each isolate. The results are summarized in Table 4. A high degree of accumulation is obvious, about 90% of the

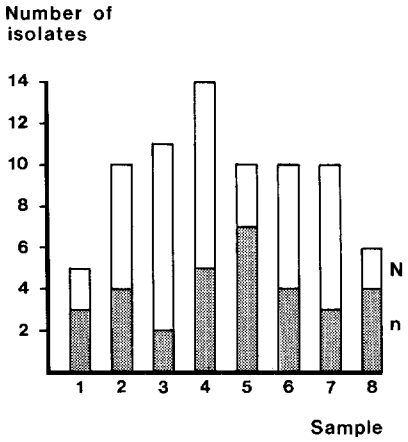

Fig. 1. Variation in virulence phenotypes within one commercial lettuce, sampled at eight different times in 1982. *N* indicates the total number of isolates checked, and *n* the number of different virulences phenotypes observed

Table 3. Frequencies of virulence phenotypes in *B. lactucae* isolates originating from three commercial lettuce crops

Virulence factor	Frequency in population		
	1	2	3
v1	0.79	0.95	0.78
v2	0.40	0.65	0.31
v3	0.64	0.84	0.52
v4	0.90	0.91	0.84
v5	0.94	1.00	0.93
v6	0.90	0.94	0.86
v7	1.00	1.00	1.00
v8	0.93	0.97	0.88
v9	0.64	0.81	0.43
v10	0.98	0.97	0.90
v11	0.57	0.49	0.19
v12	0.34	0.33	0.47
Isolates tested	58	79	58

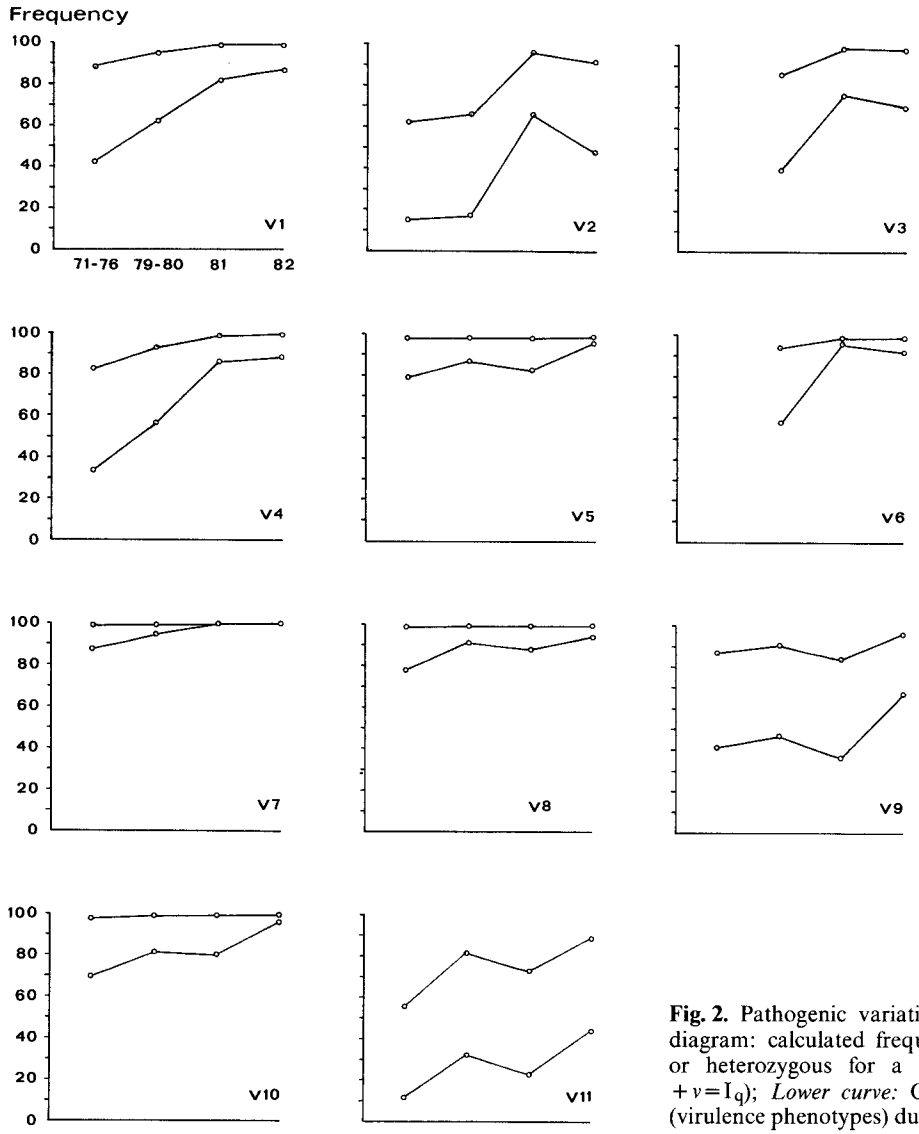

Fig. 2. Pathogenic variation with time. *Upper curve* in each diagram: calculated frequency of isolates being homozygous or heterozygous for a virulence gene (genotypes *vv* and $+v=I_q$); *Lower curve:* Observed frequency of homozygotes (virulence phenotypes) during the period 1971–1982

Table 4. Accumulation of "unnecessary" virulence genes in isolates of *Bremia lactucae*. All the isolates have been collected on the host variety 'Great Lakes III', which possesses *R7*. Therefore, the "necessary" virulence gene *v7* has been excluded from the figures. *n* indicates the number of isolates tested

Year	Total no. of virulence genes										*n*
	1	2	3	4	5	6	7	8	9	10	
1980	1	–	–	1	–	2	1	3	2	–	10
1981	–	–	–	–	–	–	1	3	1	10	15
1982	–	–	–	1	1	1	5	9	17	10	44
Total	1	–	–	2	1	3	7	15	20	20	69

isolates carry seven or more virulence factors in addition to *v7*. The slight differences between years may indicate that accumulation has occurred for a longer period of time.

Distribution of mating types

In 1982, an investigation was initiated with the purpose of studying whether different mating types occurred simultaneously within a lettuce crop. Thirty-two samples of *Bremia lactucae* were collected on single lettuce plants from five different crops, of which three were investigated in detail. Each sample was reinoculated on pieces of young lettuce leaves, following the procedure

described above. The formation of oospores was examined at five days intervals.

Of the thirty-two samples, oospore formation was observed in 14, indicating the presence of two mating types on the same host plant. At all three collecting sites intensively studied both compatibility types occurred simultaneously throughout most of the growing season. In 1983, the situation in twelve other populations was examined. In eleven of these two mating types were found.

Table 5. Spontaneous formation of oospores of *B. lactucae* in commercially grown lettuce crops

Population	Plants checked	Formation of oospores	
		Plants	%
83–40	47	19	40.4
83–41	26	7	26.9
83–42	49	11	22.4
83–43	46	38	82.6
83–44	50	43	86.0
83–45	59	58	98.3
83–46	62	19	30.6
83–47	75	35	46.7
83–49	48	38	79.2
83–50	55	35	63.6
83–51	47	35	74.5
Total	564	338	59.9

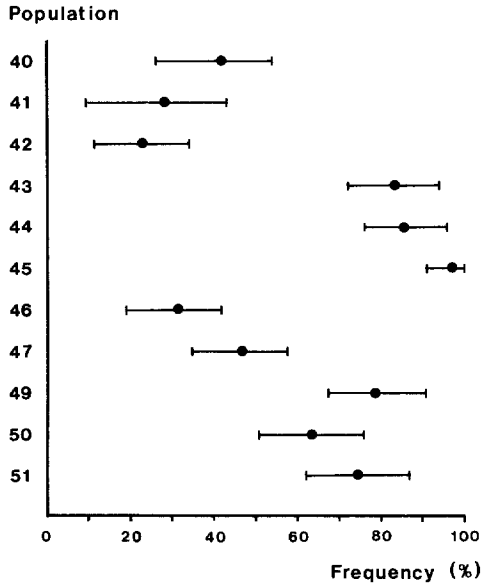

Fig. 3. Frequency (%) of oospore production in populations of *Bremia lactucae*. For each population the mean value is given and the confidence limits (95%) calculated

Spontaneous formation of oospores

Oospore production in commercially grown lettuce crops was investigated in 1983. Leaves attacked by *B. lactucae* were sampled from 11 sites in the southern parts of Sweden. Only one infected leaf was collected from each plant and samples were taken throughout the crop. The formation of oospores was examined at five day intervals. The results are summarized in Table 5 and Fig. 3.

In total, 564 plants were examined and oospores were formed in 338 (59.9%). Oospore formation was usually abundant when present. Oospores were formed in all populations, but the frequency varied from 22% to 98%. Statistical analysis indicates that the differences between populations are significant ($X^2 = 147.5$, $Df = 10$, $P = 0.0001$).

Calculations of confidence limits (95%) indicate that oospore production in, for instance, populations 43, 44, 45 and 49 deviates from that in populations 41, 42 and 46 (Fig. 3). Furthermore, no correlation exists between the sample size (number of plants checked) and the frequency of oospore formation ($r = 0.12$).

Discussion

The evolution of the Swedish *Bremia lactucae* population involves significant changes in virulence frequencies and the establishment of races with new combinations of virulence. This diversity is also present on single host plants, where several different virulence phenotypes may grow together on the same leaf. Very few isolates, which have been collected on the same host, show identical patterns of virulence. Most of the variation is due to the presence of one or two virulence genes, but in extreme cases, the virulence pattern may differ in as many as eight virulence factors. In those populations investigated here, all the twelve virulence factors were represented in the gene pool. The virulence frequencies may, however, vary with the population. Differentiation of the pathogen population can also show drastic changes in virulence frequencies with time. For instance, the number of virulence genes with frequencies higher than 85% increased from three in 1979 to seven in 1982.

Moreover, the virulence pattern of the isolates seems to be more and more complex, and a significant accumulation of virulence genes has occurred (Gustafsson and Larsson 1984). This has also been observed in other populations of *Bremia lactucae* (Dixon 1978; Dixon and Wright 1978; Lebeda and Zavadil 1979; Lebeda 1979). Rapid race differentiation, drastic changes in virulence frequencies and accumulation of virulence are common in many obligate pathogens, for instance in powdery mildew on barley (Wolfe and Schwarzbach 1978), *Phytophtora infestans* (Shattock et al. 1977) and wheat leaf rust (for references see MacKey 1981).

From an evolutionary and breeding point of view, the most interesting and important problems to be solved are how genetic diversity is maintained in a pathogen population and how new, well adapted complex races arise? Early workers assumed that races with new spectra of virulence simply arise by mutations (Jagger and Whitaker 1940; Channon et al. 1965). Of course, *Bremia lactucae*, like all other organisms, can undergo mutation resulting in new virulence genes in the gene pool. But, at least in Sweden, it is not a question of how this variability is obtained; it is already present in the population. Analyses of virulence frequencies showed that all 11 virulence genes were present in high frequencies in the Swedish *B. lactucae* populations at the beginning of the investigation. From 1971–1976, the frequency of isolates carrying *v11*, either in a homozygous or heterozygous state, was about 55%, *v2* – 62%, and all other virulence genes, higher than 82%. In 1982, these frequencies had increased up to 89% (*v11*), 92% (*v2*), and 97% (all others). It does not seem very probably that single mutations in virulence loci 1–11, from 1971 and onwards, have had any important effects upon the changes in gene frequencies observed in the Swedish population.

Successive introductions of new resistance factors have been performed in the Swedish lettuce population (Gustafsson and Larsson 1984), but very rapidly, new virulent combinations have been evolved by the pathogen. Previously, mutation has been considered as an important source of new virulence in *Bremia lactucae*. In order that the virulent phenotype is expressed, i.e. it is doubly recessive for the virulence factor, the same mutation must occur at the same locus on both homologous chromosomes. The chances of this occurring are infinitely small. Theoretically, single mutations can be expressed by fusion of hyphae followed by somatic recombination. Experiments have been performed in order to investigate the role of heterokaryosis, parasexuality and somatic recombination in *Bremia lactucae*, but so far, no positive results have been obtained (Crute 1981; Gustafsson, unpublished).

In 1980, Crute and Norwood suggested "that the sexual process is the most likely means, whereby new specific virulence combinations are produced and that heterozygosity and recombination could account for the race changes". *Bremia lactucae* is mainly heterothallic and may reproduce sexually when two compatibility types occur together (Michelmore and Ingram 1980, 1981). In Sweden outcrossing seems to be obligate as no self fertile isolates have been found (Gustafsson et al. 1983).

So far, very little evidence has been put forward to show that sexual reproduction and recombination really occur in natural populations of *Bremia lactucae*. However, the following results of the present investigation support the idea that sexual reproduction and recombination may be important: (1) Isolates occurring sympatrically are often different in virulence. (2) Different mating types are present in commercially grown lettuce crops. In all crops intensively studied, the two mating types occurred together throughout the entire growing season. In addition, both compatibility types have been found growing together on the same lettuce leaf. (3) Zygotes, oospores, are formed in all populations investigated. The frequency varies from 28% up to 98% with an average of 60%. The differences between populations are significant and no correlation exists between the number of plants checked in each population and the frequency of oospores formed. (4) Oospores may germinate rather frequently under suitable conditions and thus probably being an important primary source of inoculum. Similar results concerning oospore formation has been obtained by Michelmore and Ingram (1981) and Blok (1981).

These results are also in agreement with conclusions which can be drawn from the pattern of pathogenic variation. The great intrapopulational variation and flexibility, which is typical for many subpopulations of *Bremia lactucae*, fits well into the pattern of variation which might be expected in a diploid outcrossing organism with a high capacity of sexual recombination. Thus, the most important evolutionary processes influencing the rate and direction of differentiation in many obligate parasites like *Bremia lactucae* seem to be: (1) Mutation and gene flow, which are the ultimate sources of genetic variation. (2) Sexual recombination and genetic segregation, which make evolution of new virulence genotypes possible. (3) Selection, which sorts out the variability and maintains a high state of adaptation.

It is difficult to build up breeding programs for resistance to parasites with a high rate of sexual recombination. Strategies for utilizing vertical resistance are difficult to apply, a new combinations of virulence may arise very quickly. Alterations of resistance genes in space and time (Person 1966; Mac Key 1980) require an effective breeding program able to incorporate a large number of resistance genes. However, it should be noted that the higher the rate of recombination in the parasite, the less successful the breeding program will be. Probably, stable equilibria and long-lasting restrictions of the pathogen populations can only be reached through strategies utilizing race-non-specific resistance.

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