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HETEROTHALLISM IN *BREMIA LACTUCAE*

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It is shown that *Bremia lactucae* is capable of regular and predictable production of large numbers of oospores in lettuce tissues. Many isolates, while incapable of sexual reproduction when cultured alone, produced oospores in large numbers when cultured in combination with certain other isolates. This demonstrates the existence of heterothallism. In a survey of 39 isolates only two compatibility types were identified. These findings are discussed in relation to previous reports that sexual reproduction in *B. lactucae* is sporadic or does not occur at all.

There was an inverse relationship between the intensities of sexual and asexual sporulation.

Bremia lactucae Regel, the obligate biotroph responsible for the downy mildew disease of lettuce, is thought to survive in the absence of its host as thick-walled oospores formed as a result of sexual reproduction. The asexual cycle of the fungus and its interaction with lettuce have been studied in depth; until recently, however, little was known of its sexual cycle.

The existence of sexual reproduction in *B. lactucae* was a matter of controversy until clearly demonstrated by Humphreys-Jones (1971) and confirmed by Tommerup, Ingram & Sargent (1974). In these studies, and those of Ingram, Tommerup & Dixon (1975) and Fletcher (1976), oospores were produced in lettuce plants unpredictably and in low numbers. This could have been either because the experimental conditions used were not optimal for sexual reproduction or because of some genetically determined properties of the isolates studied. We have found that host genotype and nutritional and environmental factors have no marked effect on incidence of sexual reproduction by *B. lactucae* (unpubl.). Genetic aspects of oospore production are the subject of this paper.

MATERIALS AND METHODS

Isolates of Bremia lactucae

The origin of isolates of *B. lactucae* examined for their ability to produce oospores is given in Table 1. Those selected for detailed examination were five strains all apparently incapable of sexual reproduction when inoculated singly in lettuce cotyledons (IL4, NL6, IM43, IM44 and (N)W1), and by way of contrast, IM25, which produced oospores in single culture to a greater extent than any other isolate.

Maintenance of isolates of Bremia lactucae

Isolates of *B. lactucae* were cultured on detached, fully expanded cotyledons of *Lactuca sativa* L. (lettuce) cv. British Hilde. These were laid out in clear plastic boxes (125 × 80 × 20 mm) and were inoculated by spraying with a suspension of conidia in distilled water. They were incubated at 15 °C in the dark for the first 24 h then in a growth room lit for 12 h each day by fluorescent tubes giving a light level of 6·31 W m^{-2} at the surface of the boxes. Under these conditions asexual sporulation normally commenced about six days after inoculation. After a further two days of incubation the conidia were harvested by shaking the sporulating cotyledons in distilled water. The resulting suspension was washed once by pelleting in a bench centrifuge, to remove a water soluble inhibitor of germination, and resuspended in fresh distilled water ready for experimental use. This method of culture is based upon methods described by Tommerup *et al.* (1974).

When necessary, isolates of the fungus were stored for extended periods by freezing newly sporulating cotyledons, contained in sealed plastic culture boxes, at −20° Such treatment did not normally result in any major loss of viability, even after one year of storage. Frozen conidia were prepared for use in experiments by suspending in distilled water and washing by centrifugation as described above.

The viability of conidia was determined prior to inoculation of cotyledons by the fluorescein diacetate test of Ziegler, Ziegler & Witzenhausen (1975)

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Table 1. *Isolates of* Bremia lactucae *used in studies of heterothallism*

Code No.	Variety of origin	Geographical* origin	Supplier*	Compatibility† type	Virulence phenotype‡ 1	2	3	4	5	6	7	8	9	10	11
United Kingdom Isolates:															
IM25	Tina	NIAB Trial Ground	Authors	$B_2 \gg B_1$	1	.	.	4	5	6	7	8	9	10	.
IM43	British Hilde	NIAB Trial Ground	Authors	B_2	.	.	.	.	5	6	7	8	9	10	11
IM44	Winter Density	Garden, Totnes, Devon	Authors	B_2	1	2	.	4	5	?	(7)	8	9	10	11
74/T(Tv)	Larganda	Botany Field Station, Cambridge	N.V.R.S.	B_1	1	2	3	4	5	6	7	8	9	10	.
(N)W1	Miranda	Neasham, Co. Durham	N.V.R.S.	$B_2 \gg B_1$	1	2	3	4	(5)	(6)	.	(8)	.	(10)	.
(N)W3	Brioso	N.I.A.B. Trial Ground	N.V.R.S.	$B_2 \gg B_1$	1	2	.	4	5	.	7	8	(9)	10	.
WM1	Amanda plus	G.C.R.I.	G.C.R.I.	B_2	Not known										
WM7192	G.C.R.I. Breeding line No. 7192	G.C.R.I.	G.C.R.I.	B_2	Not known										
WM7718	G.C.R.I. Breeding line No. 7718	G.C.R.I.	G.C.R.I.	B_2	Not known										
WM7730	G.C.R.I. Breeding line No. 7730	G.C.R.I.	G.C.R.I.	B_2	Not known										
B/68/76	Avoncrisp	Not known	N.V.R.S.	B_2	1	.	.	4	5	6	7	8	9	10	.
B/17/78	Lobjoits	Banstead, Surrey	N.I.A.B.	B_2	?	2	3	4	.	6	7	(8)	.	.	.
B/29/78	136	Everton, Beds.	N.I.A.B.	B_2	?	2	3	4	(5)	6	7	.	.	.	.
B/30/78	Tina	Everton, Beds.	N.I.A.B.	B_2	?	2	3	4	.	?	7	.	.	.	.
B/31/78	Ithaca Great Lakes	Everton, Beds.	N.I.A.B.	B_2	?	2	3	4	(5)	6	7	(8)	.	.	.
B/32/78	Avondefiance	Everton, Beds.	N.I.A.B.	$B_2 \gg B_1$	?	.	3	4	5	6	7	8	9	.	.
B/33/78	Corelli	Everton, Beds.	N.I.A.B.	B_2	?	.	3	4	5	6	7	8	(9)	.	.
B/34/78	Amandine	Everton, Beds.	N.I.A.B.	$B_2 \gg B_1$	?	(2)	3	4	(5)	6	7	8	(9)	(10)	.
B/35/78	5140	Everton, Beds.	N.I.A.B.	B_2	?	.	3	4	5	6	7	8	9	(10)	.
B/36/78	Kares	Lyminge, Folkestone	N.I.A.B.	B_2	?	(2)	3	4	5	6	7	(8)	.	(10)	.
B/37/78	Reskia	Lyminge, Folkestone	N.I.A.B.	B_2	?	.	3	4	.	?	7	.	.	.	.
B/40/78	Avondefiance	E.H.S.	N.I.A.B.	B_2	?	.	3	4	5	6	7	8	(9)	(10)	.
B/41/78	Mildura	E.H.S.	N.I.A.B.	B_2	?	(2)	3	4	(5)	6	7	(8)	(9)	(10)	.
B/42/78	Sabine	E.H.S.	N.I.A.B.	B_2	?	2	3	4	(5)	6	7	(8)	(9)	(10)	.
B/43/78	136	E.H.S.	N.I.A.B.	B_2	?	2	3	4	(5)	6	7	(8)	(9)	(10)	.
B/44/78	7529 Jubilee	E.H.S.	N.I.A.B.	B_2	?	.	3	4	5	6	7	8	9	.	.
B/45/78	Cobham Green	E.H.S.	N.I.A.B.	B_2	?	2	3	4	.	?	7	.	.	.	.
B/46/78	Avoncrisp	E.H.S.	N.I.A.B.	B_2	?	2	3	4	5	6	7	8	(9)	.	.

Table 1 (*cont.*)

Code No.	Variety of origin	Geographical* origin	Supplier*	Compatibility† type	Virulence phenotype‡ 1	2	3	4	5	6	7	8	9	10	11
Non-United Kingdom Isolates:															
NL1	Not known	Holland	I.P.O.	$B_2 \gg B_1$	1	2	.	4	.	.	.	.	.	.	.
NL2	Not known	Holland	I.P.O.	B_2	1	2	3	4	(5)	6	.	8	.	.	.
NL3	Not known	Holland	I.P.O.	B_2	.	.	.	.	5	6	7	8	(9)	10	.
NL4	Not known	Holland	I.P.O.	B_2	1	2	.	4	5	.	7	8	.	(10)	.
NL5	Not known	Holland	I.P.O.	B_2	1	.	3	4	.	.	7	.	.	.	.
NL6	Not known	Holland	I.P.O. via N.V.R.S.	B_1	1	2	.	.	5	.	.	8	(9)	10	11
NL7	Not known	Holland	I.P.O.	B_2	?	2	3	4	5	6	7	8	?	?	.
CG1	Not known	Switzerland	Ciba-Geigy via N.V.R.S.	B_2	1	2	3	4	.	.	.	.	.	.	.
CG5	Not known	Switzerland	Ciba-Geigy via N.V.R.S.	$B_2 \gg B_1$	1	2	3	4	.	.	.	.	.	.	.
S1	Not known	Sweden	Swedish Seed Association via N.V.R.S.	B_2	1	.	3	4	5	6	7	8	9	10	.
IL4	Not known	Israel	Volcani Institute, Israel via N.V.R.S.	B_1	1	2	.	4	5	.	7	8	9	10	11

Notes

* N.I.A.B. = National Institute of Agricultural Botany, Cambridge, U.K. (I. Wright). N.V.R.S. = National Vegetable Research Station, Wellesbourne, Warwickshire, U.K. (I. Crute). G.C.R.I. = Glasshouse Crops Research Institute, Littlehampton, Sussex, U.K. (W. Morgan). E.H.S. = Experimental Horticultural Station, Luddington, Warwickshire, U.K. I.P.O. = Instituut voor Plantenzeiktenkundig Onderzoek, Wageningen, Netherlands (I. Blok).

† $B_2 \gg B_1$ indicates that oospores were formed more frequently and in greater numbers when the isolate was cultured with compatibility type B_1 than when cultured with B_2; the isolate therefore seems to be predominantly B_2.

‡ Virulence phenotypes were provided by the suppliers except for NL1–NL6 (provided by I. R. Crute) and NL7 (elucidated by authors from available virulence data). '?' in place of a number indicates that the presence of that virulence factor is not known. A number in parentheses indicates that lettuce seedlings containing the resistance factor corresponding to that virulence factor were infected only in very low numbers.

Isolates bracketed together were collected at one time.

and Söderstrom (1977) and confirmed by germination in distilled water on glass slides.

Determination of capacity for sexual reproduction

To determine the capacity of isolates or mixtures of isolates of *B. lactucae* to produce oospores, at least 25 single, fully expanded lettuce cotyledons were laid out, abaxial surface uppermost, in each of two plastic culture boxes and inoculated by spraying with a suspension containing a known number of washed conidia. A suspension containing 5×10^4 conidia ml^{-1} resulted in approximately 100 infections per cotyledon. Care was taken to achieve an even distribution of inoculum over the cotyledons. Incubation was in the culture room already described. Preliminary studies had shown that under such standard conditions, for a given concentration of conidia in the inoculum, the resultant oospore production was predictable and constant between replicate boxes.

The number of cotyledons containing oospores was ascertained by direct examination with a low power dissecting microscope (×50). Unfixed cotyledons were prepared for such examination either by infiltrating with water under vacuum or by expelling the air from intercellular spaces by rolling in a droplet of water on a microscope slide using a glass rod. Following these treatments cotyledons became translucent and the oospores within them clearly visible with the aid of the microscope. Alternatively, cotyledons were fixed and cleared in 95 % ethyl alcohol.

Precise counts of the total numbers of oospores per cotyledon were not possible. Estimates were made by mechanically comminuting batches of cotyledons in water using a 'Polytron' tissue comminuter (Kinematic Gmbh, Lucerne, Switzerland) and counting the oospores in the resulting suspension with a haemocytometer.

The intensity of asexual sporulation was determined by shaking the sporulating cotyledons in 10 ml of distilled water and making counts with a haemocytometer.

RESULTS

Effect of inoculum concentration on oospore production by isolate IM25

Pairs of lettuce cotyledons were inoculated with suspensions containing conidia of isolate IM25 of *B. lactucae* at the following concentrations: $2{\cdot}5 \times 10^3$, 9×10^3, $2{\cdot}5 \times 10^4$ and $2{\cdot}5 \times 10^5$ ml^{-1}. All cotyledons became infected, but the number containing oospores was dependent upon the con-

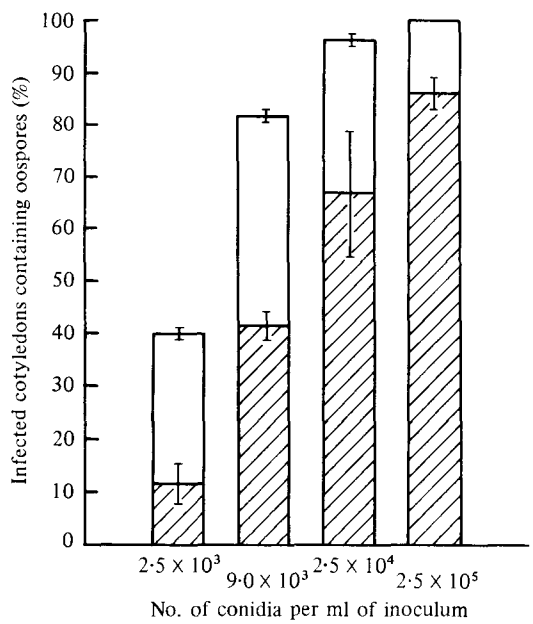

Fig. 1. Percentages of infected cotyledons of *Lactuca sativa* cv. British Hilde containing oospores following inoculation with different concentrations of washed conidia of isolate IM25 of *Bremia lactucae*. Each column represents the mean of counts from two replicate culture boxes, each containing 25 cotyledons. The shaded area of each column represents the percentage of infected cotyledons in which there were more than 5×10^8 oospores evenly distributed throughout the tissue. The bars represent the counts of the individual culture boxes and indicate the level of variation between replicates.

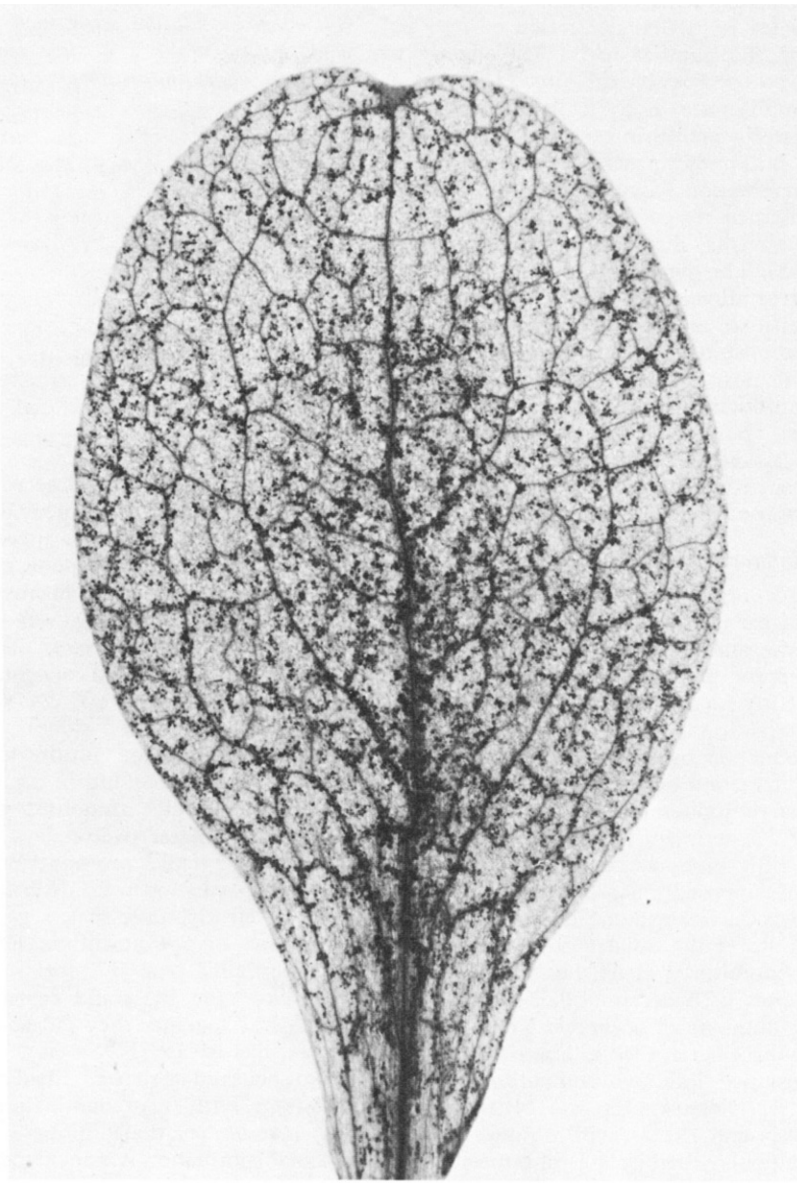

Fig. 2. A cleared cotyledon of *Lactuca sativa* cv. British Hilde fourteen days after inoculation with *Bremia lactucae* isolate IM25. The outlines of the veins and the dark patches of oospores, evenly distributed throughout the tissue, are clearly visible.

centration of conidia in the inoculum (Fig. 1). At the highest concentration 100 % of cotyledons contained oospores, at the concentration of $2{\cdot}5 \times 10^4$ conidia ml^{-1} the number had fallen slightly to 97 %, at $9{\cdot}0 \times 10^3$ conidia ml^{-1} to 82 % and at $2{\cdot}5 \times 10^3$ conidia ml^{-1} to 40 %. In all treatments the numbers of oospores in each cotyledon varied from a few hundreds to many thousands. At the highest concentration of inoculum 86 % of cotyledons contained large numbers of oospores ($> 5 \times 10^3$), evenly distributed throughout the tissue (Fig. 2). This figure fell to 67 % at the inoculum concentration of $2{\cdot}5 \times 10^4$ conidia ml^{-1} to 41·5 % at $9{\cdot}0 \times 10^3$ and to 11·5 % at $2{\cdot}5 \times 10^3$.

The relationship between concentration of conidia in the inoculum and frequency and density of oospore production was confirmed in subsequent experiments. Therefore, in experiments to determine the capacity of an isolate to reproduce sexually, suspensions containing high numbers of conidia were used as inoculum.

Interaction between isolates of Bremia lactucae

A survey of isolates of *B. lactucae* revealed that although some, such as isolate IM25, were capable of sexual reproduction whenever tested, others were apparently incapable of producing oospores even under conditions which supported abundant oospore production by isolate IM25. The possibility that interaction between such isolates might lead to sexual reproduction was therefore studied.

In the first experiment lettuce cotyledons were inoculated with high concentrations of conidia (10^5 ml^{-1}) of supposedly barren isolates from five different sources, either alone or in combination (Table 2). Four of the isolates did not reproduce sexually when cultured alone, but when cultured in combination with certain other isolates produced large numbers of oospores. This indicated that *B. lactucae* is heterothallic. The isolates tested could be grouped into two compatibility types, designated B_1 (isolates IL4 and NL6) and B_2 (isolates IM43 and IM44), with oospore production resulting only when isolates of opposite compatibility type became established on the same cotyledon.

In this experiment isolate (N)W1 produced oospores infrequently when cultured alone, but only because a high concentration of inoculum was used. Oospores were similarly formed in small numbers when this isolate was combined with isolates IM43 and IM44. In contrast, oospores were produced in all infected cotyledons when (N)W1 was combined with isolates IL4 or NL6. This indicated that (N)W1 had the reaction of both compatibility types, but gave a stronger B_2 reaction. Host senescence and necrosis was greater in cotyledons which contained oospores than in those which did not. There appeared to be a direct correlation between oospore numbers and the degree of senescence and necrosis.

Table 2. *Occurrence of sexual reproduction in cotyledons of* Lactuca sativa *cv. British Hilde following inoculation with high concentrations of conidia* ($> 10^5$ ml^{-1}) *of five isolates of* Bremia lactucae, *alone and in combination*

		Isolate no.				
		IM43	IM44	(N)W1	NL6	IL4
Isolate no.	IL4	+	+	+	−	−
	NL6	+	+	+	−	
	(N)W1	+/−	+/−	+/−		
	IM44	−	−			
	IM43	−				

\+ = Many oospores produced.
+/− = Oospores produced sporadically and in low numbers.
− = No oospores produced.

The conclusion that *B. lactucae* is heterothallic was critically examined by making a series of inoculations using standard concentrations of conidia. Thus, conidia of isolate IM25 (produced oospores readily when cultured alone) and isolates IM44 and IL4 (barren when cultured alone) were applied to cotyledons, alone and in combination, at three different final concentrations: $2{\cdot}5 \times 10^4$, 5×10^4 and 1×10^5 ml^{-1}. After twelve days of incubation the incidence of sexual reproduction and intensity of asexual sporulation were determined. The results (Fig. 3) clearly show that, regardless of the concentration of the inoculum, neither isolate IL4 (compatibility type B_1) nor isolate IM44 (compatibility type B_2) could reproduce sexually by itself, but together they did so profusely. Isolate IM25, like isolate (N)W1 in the previous experiment, behaved as though it had both compatibility reactions, with B_2 predominating. There was also an inverse relationship between intensity of asexual sporulation and incidence of sexual reproduction. At all inoculum concentrations tested, cotyledons in which sexual reproduction had occurred at high levels senesced more rapidly than cotyledons in which *B. lactucae* had produced only asexual spores.

The heterothallism of *B. lactucae* was further confirmed in a third experiment. A series of inoculations of cotyledons were made using standard final concentrations of inoculum (7×10^2, 7×10^3 and 7×10^4 conidia ml^{-1}), made up of compatibility types B_1 (isolate IL4) and B_2 (isolate IM44) alone and in the ratios detailed in Fig. 4.

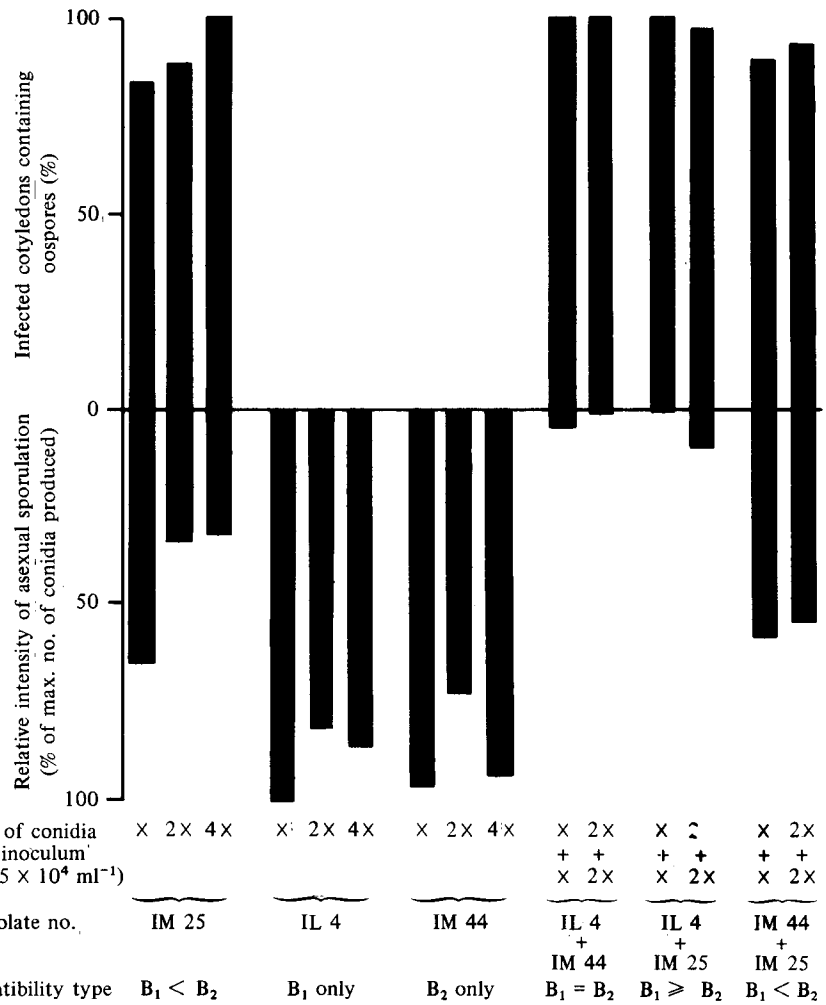

Fig. 3. Incidence of sexual sporulation and the intensity of asexual sporulation following inoculation of cotyledons of *Lactuca sativa* cv. British Hilde with suspensions of conidia of *Bremia lactucae* isolates IM25, IM44 and IL4, alone and in combination, at three different final concentrations ($x = 2{\cdot}5 \times 10^4$ conidia ml^{-1}; $2x = 5 \times 10^4$ conidia ml^{-1}; $4x = 1 \times 10^5$ conidia ml^{-1}). Figures for the incidence of sexual sporulation are percentages of cotyledons containing oospores based on counts of two replicate culture boxes, each containing 25 cotyledons. Figures for the relative intensity of asexual sporulation (% of maximum observed) are the means of 3 haemocytometer counts made on suspensions of conidia produced by washing all the infected cotyledons in each replicate culture box in 10 ml of distilled water.

Maximum oospore production for each of the inoculum levels occurred when conidia of the two compatibility types were present in inoculum in equal proportions. Equal concentrations of inoculum but with conidia of one compatibility type predominating resulted in markedly lower levels of oospore production. As in the previous experiment asexual sporulation was inversely proportional to sexual sporulation.

Finally, the interaction between compatibility types was examined in the following ways. Firstly, cotyledons were inoculated at opposite ends with droplets containing conidia either of compatibility type B_1 (isolate IL4) or B_2 (isolate IM44). These inoculum droplets were contained by 2 mm lengths of Vinyl tubing (internal diam 2 mm) secured to the surfaces of the cotyledons with petroleum jelly. This resulted in oospores being formed in bands across the centres of the cotyledons where the vegetative mycelia of the two compatibility types came into contact (Fig. 5). Asexual sporulation occurred at the ends of the

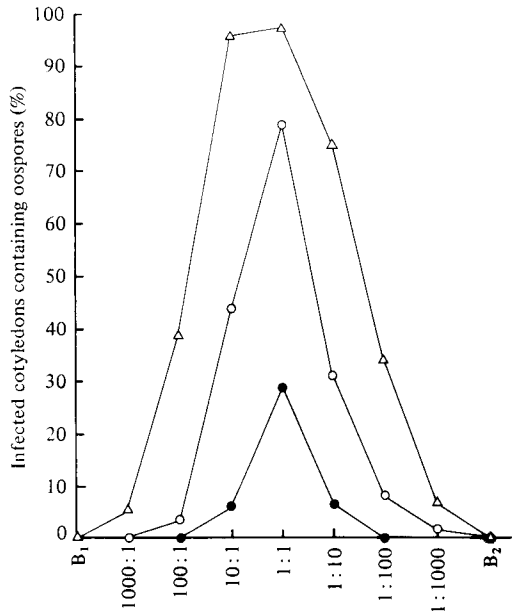

Fig. 4. Percentage of infected cotyledons of *Lactuca sativa* cv. British Hilde containing oospores following inoculation with suspensions containing different proportions of conidia of two compatibility types of *Bremia lactucae* (B_1 [isolate IL4] and B_2 [isolate IM44]). Suspensions were adjusted to contain final concentrations of 7×10^2 (●), 7×10^3 (○) and 7×10^4 (△) conidia ml^{-1}. Each point is the mean of the counts from three replicate culture boxes, each containing 25 cotyledons.

cotyledons but was absent in the zones of oospore formation. Secondly, a series of cotyledons was inoculated with conidia of compatibility type B_1 (isolate IL4) and B_2 (isolate IM44), the second inoculations being temporally separated from the first ones by a varying number of days. In this experiment oospores were still formed when inoculations with the two compatibility types were separated by up to four days. It was concluded from this and the previous experiment that close association between mycelia of opposite compatibility types is necessary for sexual reproduction, although such association need not necessarily occur during the early stages of the establishment of an infection.

In addition to the isolates of *B. lactucae* already described, a further thirty-four, from a wide range of sources, were analysed for compatibility type by inoculating lettuce cotyledons with conidia of each isolate alone, in combination with conidia of compatibility type B_1 (isolate IL4) and in combination with conidia of compatibility type B_2 (isolate IM44). Conidia of isolates IL4 and IM44 were applied to cotyledons 24 h after inoculation with conidia of the test isolates. Unfortunately, the isolates originally studied by Tommerup *et al.* (1974) were no longer available. Of the thirty-nine isolates analysed, 3 were of compatibility type B_1 and 29 of B_2, while 7 apparently reacted as both B_1 and B_2 (Table 1). All isolates were capable of sexual reproduction when mixed either with compatibility type B_1 or with B_2. Only isolates which were capable of reproducing sexually when cultured alone produced oospores when cultured both with B_1 or B_2. There was no evidence for any correlation between compatibility type of isolates and their virulence phenotype, geographical origin or host of origin.

DISCUSSION

This study has demonstrated that, in contrast to the findings of earlier studies (Tommerup *et al.*, 1974), *Bremia lactucae* is capable of regular and predictable production of oospores in large numbers. In those earlier studies where oospores were not found, the inoculum used may not have contained the appropriate mixture of compatibility types. In our survey of thirty-nine isolates, thirty-two were of one compatibility type only. Alternatively, the levels of infection in some earlier studies may have been too low to allow sexual reproduction to occur regularly, despite the presence of appropriate compatibility types in the population. Our first experiment demonstrated the importance of using large numbers of washed conidia as inoculum, to ensure high levels of infection.

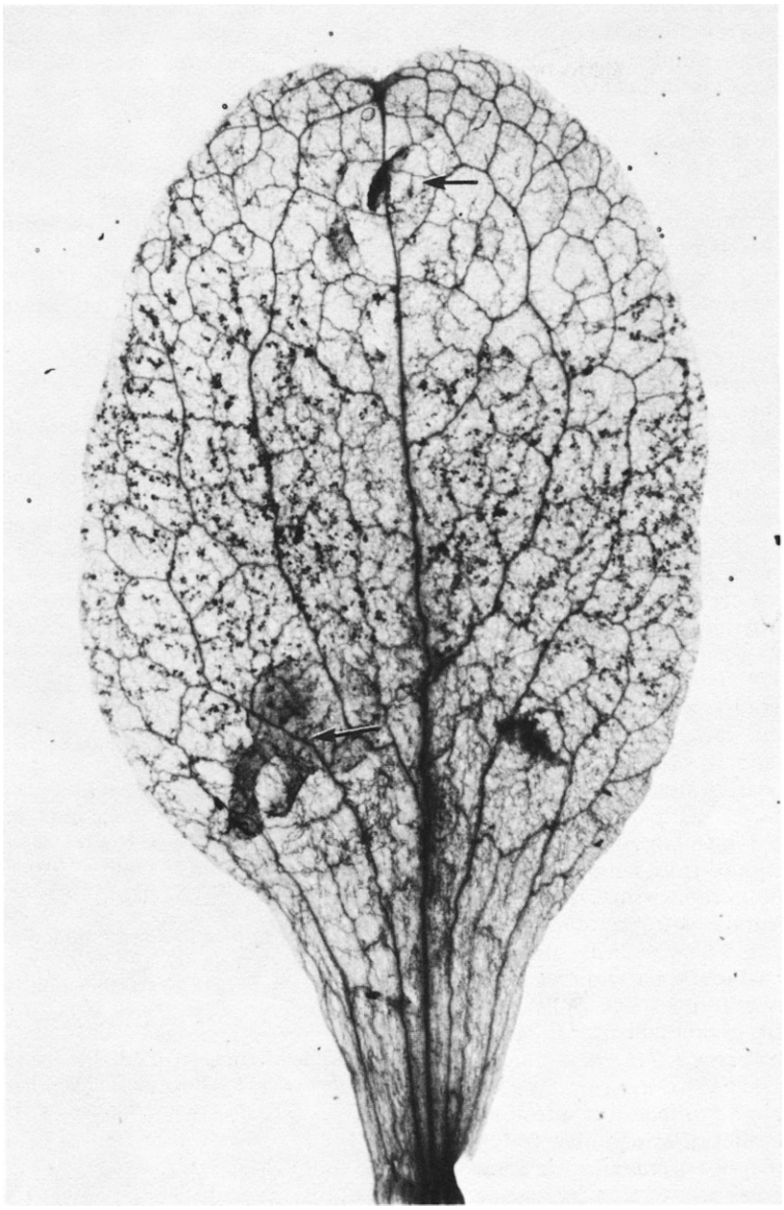

Fig. 5. A cleared cotyledon of *Lactuca sativa* cv. British Hilde ten days after inoculation at opposite ends with conidia of *Bremia lactucae* isolates IL4 (compatibility types B_1) and IM44 (B_2). The inoculation points are marked with arrows. A band of dark oospore-groups is clearly visible across the centre of the cotyledon.

It has been suggested (Fletcher, 1976) that oospore formation by *B. lactucae* is associated with necrosis of host tissue. Our observations suggest that production of large numbers of oospores leads to an increased stress on the host, resulting in enhanced senescence and necrosis, rather than oospores being formed in response to host senescence itself.

The demonstration of heterothallism in *B. lactucae* was surprising since in previous reports the antheridium has been interpreted as arising from the same hypha as the oogonium (Tommerup *et al.*, 1974). No other heterothallic Oomycetes have been reported as having their gametangia arising in this way. A re-investigation of the origin of the gametangia of *B. lactucae* using the scanning electron microscope has, however, now shown that the antheridia and oogonia arise from different hyphae (Michelmore & Ingram, unpubl.).

The existence of a mating system based on only two compatibility types in *B. lactucae* is the simplest explanation of the results reported here. The survey of thirty-nine isolates of the fungus from a wide range of sources did not reveal any further compatibility types. Since oospores of *B. lactucae* have been germinated on only one occasion (Morgan, 1978) it has so far been impossible to make any studies of the segregation of compatibility types; the designation of the compatibility types as B_1 and B_2 does not imply that compatibility is necessarily determined by two alleles at a single locus.

Several heterothallic *Phytophthora* species have now been shown to produce from single oospores secondarily homothallic isolates which react with both the A^1 and A^2 parental isolates (e.g. Mortimer, Shaw & Sansome, 1977). The possibility that the isolates of *B. lactucae* which apparently had the reactions of both compatibility types B_1 and B_2 had a similar kind of homothallism or, alternatively, were heterokaryotic, is now being studied.

Very little is known of the factors controlling sexual reproduction in other downy mildew fungi, despite the probable role of oospores in variation and survival of most members of this important group of plant pathogens. The observations reported here, however, do correlate well with reports that heterothallism may also occur in *Peronospora parasitica* (de Bruyn, 1935; McMekein, 1960). It is possible that heterothallism may be common among members of the downy mildew group.

The demonstration of heterothallism in *B. lactucae* has increased the potential of this organism as a model system for research on downy mildews. When oospores can be germinated readily it will be possible to elucidate the genetics of virulence in the fungus to complement the already extensive data (Crute & Johnson, 1976; Johnson, Crute & Gordon, 1977) on genetics of resistance in its lettuce host.

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