Phenotypic Differences Between Natural and Induced Variants of *Botrytis cinerea*

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Wild isolates of *Botrytis cinerea* were analysed to assess their suitability for genetic studies. When compared on synthetic and artificial media under laboratory conditions, the isolates differed in rate of growth, conidiation, sclerotia production and resistance to benomyl. Asexual progeny of individual cultures were sometimes phenotypically diverse, indicating that they could be heterokaryotic or heteroplasmic. Attempts to derive auxotrophic, nystatin-resistant and benomyl-resistant mutants from mutagen-treated conidia were not successful: only morphological and spore colour variants were recovered, and most of the morphological variants were phenotypically unstable. Cultures produced both macroconidiophores and microconidiophores. Macroconidia were about 10 μm long $\times$ 8 μm wide, contained 1 to 12 nuclei (mean 3.4) and usually germinated readily on agar media. Microconidia were about 3 μm diameter, uninucleate and rarely germinated on agar media. The perfect state, *Botryotinia fuckeliana*, was not recovered from mixed inocula of the wild strains. The significance of the results is discussed in relation to the use of *B. cinerea* for laboratory studies and the possible genetic basis of natural variations.

INTRODUCTION

Few fungal plant pathogens have been subjected to comprehensive genetic analysis (for example, see Day, 1974; Webster, 1974; Burnett, 1975). Fundamental genetic questions relating to most pathogenic species remain unanswered. For instance, what is the genetic basis for phenotypic variations among natural populations? What are the genetic factors controlling outcrossing, and how much genetic variation can be released by sexual or parasexual recombination? Are wild strains haploid or diploid, homokaryotic or heterokaryotic, homothallic or heterothallic? In the absence of genetic data, taxonomic divisions are often based solely on morphological criteria and host specificity, and simple genetic variants of interbreeding populations might be classified as distinct species.

The aim of this investigation was to assess the suitability of a common pathogenic filamentous fungus, *Botrytis cinerea* ('grey mould'), for genetic studies. *Botrytis cinerea* was chosen for four main reasons: it is a facultative pathogen that can be cultured on artificial media in the laboratory; wild isolates are abundant on a wide variety of host plants; a perfect state has been recorded (*Botryotinia fuckeliana*, an operculate discomycete; see Hennebert, 1973) which, even though it is rare, means that it is feasible to derive sexual progeny; it is economically important, causing losses to fruit and vegetable crops.

The relationship between *B. cinerea* and *Botryotinia fuckeliana* has been demonstrated by Groves & Drayton (1939), Groves & Loveland (1953) and Polach & Abawi (1975). The common conidial state, *B. cinerea*, consists of coenocytic mycelia that give rise to asexual spores and sclerotia (tough, black, irregular-shaped aggregations of hyphae). Sclerotia can withstand harsh environmental conditions, and the fungus probably overwinters in this

Table 1. *Strains of Botrytis cinerea*

Strain	Isolation no.	Source*
282	160.282	<i>Aesculus hippocastanum</i> , Kew, 1971
465	100.465	<i>Punica granatum</i> fruit, India, 1963
558	169.558	<i>Lycopersicon esculentum</i> , Perthshire, 1972
649	142.649	<i>Vicia faba</i> , Berkshire, 1958
1	WA3	Onion, Wellesbourne, Warwick, 1977
2	WA6	Onion, Wellesbourne, Warwick, 1977
3	WA8	Onion, Wellesbourne, Warwick, 1977
4	WB2	Onion, Wellesbourne, Warwick, 1977
5	WB4	Onion, Wellesbourne, Warwick, 1977
9	WC3	Onion, Wellesbourne, Warwick, 1977
10	GC	Greenhouse cucumber, Sheffield, 1977
11	GT1	Greenhouse tomato, Sheffield, 1977
12	HB2	Broad bean, Huddersfield, 1977
14	SB2	Broad bean, Sheffield, 1977

* Strains 282, 465, 558 and 649 were supplied by the Commonwealth Mycological Institute, Kew, Surrey. Strains WA, WB and WC were from three different fields of onion at the National Vegetable Research Station.

form to provide fresh colonies and spores in spring. The larger and more obvious spores (macroconidia) are easily disseminated to infect new host plants. The smaller spores (microconidia) probably function as spermatia in sexual reproduction (Drayton, 1932). The perfect state can be derived from sclerotia by applying conidia to their surface: cultures of *B. cinerea* are not self-fertile, indicating that it is a heterothallic organism, but the number of mating type loci and alleles is not known. Fertilized sclerotia occasionally give rise to stalked apothecia containing asci. The lengthy and involved procedure for producing apothecia under laboratory conditions (Groves & Loveland, 1953) could reflect an innate property of the organism or simply insufficient data on its requirements for sexual reproduction.

The inadequacy of our genetic knowledge of *B. cinerea* is exemplified by Webster's (1970) statement: 'It is probable that the name *Botrytis cinerea* is a collective name used to describe a number of closely similar, but genetically distinct, species, possibly with distinct apothecial states'. It is hoped that the information in this report will provide a foundation on which detailed genetic studies can be based and statements like the one above can be clarified.

METHODS

Strains. Cultures were obtained from the Commonwealth Mycological Institute, Kew, Surrey, and were isolated from diseased plant material. Their designations and sources are given in Table 1. The strains used for comparative studies were single conidial isolates of the original cultures, maintained on agar media at 5 °C or 20 to 25 °C.

Media. Stocks were transferred regularly to dishes or slants of synthetic media (MM and CM) and artificial medium (PDA). The minimal medium (MM; Vogel, 1964) contained 1.5 % (w/v) sucrose as the carbon source, and the complete medium (CM) consisted of MM supplemented with 0.5 % (w/v) yeast extract and casein hydrolysate. PDA contained 4 % (w/v) potato extract (Difco) and 1.5 % (w/v) glucose, and was adjusted to pH 6.0 with 10 M-NaOH.

Strains were compared for growth and morphology on MM, MM supplemented with benomyl (supplied as the garden fungicide Benlate by Pan Britannica Industries, Hertfordshire), PDA, *Neurospora* medium SC (Westergaard & Mitchell, 1947), *Aspergillus* media (Pontecorvo *et al.*, 1953), synthetic media X and Y (Last & Hamley, 1956), V8 agar (Peacock, 1966) and malt extract agar (Oxoid). Media were solidified with 2 % (w/v) agar.

Slow-growing, compact colonies were obtained on MS and CMS media, which comprised MM and CM, respectively, supplemented with 1 % (w/v) sorbose and 0.1 to 0.2 % (w/v) sucrose as carbon source; sorbose has a toxic effect on *Neurospora crassa* (Davis & de Serres, 1970). Compact colonies were obtained also on MM or CM supplemented with G3300 (alkyl aryl sulphonate; Honeywill Atlas, Surrey) at 0.25 g l⁻¹; G3300 causes reduced growth of *Aspergillus nidulans* (Majerfeld & Roper, 1978).

Mutagenesis. Conidia from young cultures on MM or FDA were suspended in 1 % (w/v) saline and exposed to ultraviolet (u.v.) radiation, ethyl methanesulphonate or the acridine mustard ICR-170 as described by Grindle (1974). Samples were removed at intervals, spread on dishes of CMS and incubated at 25 °C to determine survival curves. Additional samples, removed when about 50 % and 10 % of the conidia were viable, were used to select mutants.

To isolate morphological variants, conidia were incubated on dishes of CMS, and hyphae from the resultant colonies were transferred to slants of CM. Hyphae and conidia from the cultures in CM slants were inoculated on to dishes of MM and CM and incubated at 20 to 25 °C to obtain colonies that could be compared easily.

To select resistance mutants, conidia were suspended in cool (45 °C) CMS, poured into dishes and incubated for 24 to 36 h at 22 °C. The dishes of germinated conidia were overlaid with cool CMS supplemented with 3, 6 and 10 µg benomyl ml⁻¹ or cool CMS supplemented with 5 and 10 units nystatin ml⁻¹. Nystatin is a polyene antibiotic (supplied as mycostatin by E. R. Squibb & Sons, Speke, Liverpool). The overlaid dishes were incubated at 22 °C, and colonies that grew through the benomyl or nystatin layer were transferred to slants of CM to provide material for further analysis.

Attempts were made to select auxotrophic mutants using the method given above for isolating morphological variants, and also the technique of 'filtration enrichment'. Mutagen-treated conidia were incubated in flasks of liquid MM on a mechanical shaker at 26 °C; at 24 h intervals, the medium was filtered through glass wool to remove germinated conidia. After three or four filtrations, the MM was supplemented with sorbose, yeast extract and casein hydrolysate to permit limited growth of the remaining conidia. Colonies in the medium were removed with a Pasteur pipette and transferred to slants of CM to produce cultures for further growth tests.

Selection experiments. Mass-hyphal and mass-conidial inocula of wild strains were incubated on dishes of MM, CM and CM supplemented with *p*-fluorophenylalanine (PFA; Koch-Light) at 40 to 160 µg ml⁻¹. Colonies that developed after incubation at 20 to 25 °C were scrutinized for patches or sectors with unusual morphology. Tiny samples of conidia or hyphae were removed from the colonies with fine glass needles and incubated on dishes of MM, CM, X or Y media.

Suspensions of conidia from cultures of wild strains on MM and PDA were spread on dishes of CMS. Individual germinated conidia were transferred to dishes of MM and CM and incubated at 20 to 25 °C.

The colonies derived from conidial and hyphal inocula were compared with those of the parental wild strain to see whether they differed in growth rate or morphology.

Analysis of conidia. Spore suspensions consisting mainly of macroconidia were obtained from vigorous cultures on slants or dishes of MM and PDA. Suspensions with a high microconidial content were obtained from dishes of water agar and from flasks of dilute liquid MM (10 % of the usual concentration of salts and sucrose) inoculated with macroconidia. After 14 to 30 d incubation at 22 °C, conidia on the dishes of media were washed off with 1 % (w/v) saline, and conidia in the flasks were filtered through glass wool.

Microconidia were separated from the macroconidia and hyphal fragments in crude spore suspensions by filtration or centrifugation. They were trapped on Millipore filters of 0.45 µm pore size after passage through filters of 8 µm pore, or they were sampled from sucrose gradients in 5 ml centrifuge tubes. For centrifugation, the spore suspensions were layered on 10 to 40 % (w/v) sucrose gradients, accelerated to 1100 *g* and immediately run down without braking; fractions were removed with a hypodermic needle attached to a peristaltic pump (Watson-Marlow, Falmouth, Cornwall). The centrifugation system proved to be the more effective.

Conidia and conidiophores were examined with a Kyowa SMKE microscope fitted with an eyepiece micrometer and a Nikon EFM photomicrographic camera. Dry conidia were stained on cover slips with Azure A using the method of Huebschman (1952) or by the Feulgen reaction, with Carnoy's fixative, as outlined by Greenwood & Berlyn (1968). Macroconidia were germinated on slides of water agar incubated at 22 °C in Petri dishes containing deionized water and inorganic salts (Weast, 1967) to maintain relative humidities of 70 to 100 %; they were stained with cotton blue.

Crossing experiments. Individual strains and mixtures of strains were cultured at 15 to 25 °C on dishes of MM, CM and PDA and in flasks of sterile garden soil. Additionally, colonies were incubated at 22 °C on dishes of MM and PDA for 7, 14 and 28 d, and suspensions of microconidia and macroconidia from different wild isolates were applied to the sclerotia.

Table 2. *Rate of growth, density of conidia, number of sclerotia, and size of macroconidia of wild strains of Botrytis cinerea on agar media*

All colonies were incubated on Petri dishes of 85 mm diameter at 22 °C; growth measurements were commenced at day 2; conidia and sclerotia determinations were done at day 15.

Strain	Synthetic minimal medium (MM)				MM + benomyl Rate of growth* (mm d ⁻¹)
	Rate of growth* (mm d ⁻¹)	Density of conidia†	Number of sclerotia‡	Size of macroconidia§ (µm)	
558	23	****	*	9.4 (1.1) × 7.7 (0.6)	14
649	13	****	0	9.0 (1.0) × 7.3 (0.6)	14
1	25	**	**	9.0 (1.0) × 7.7 (0.7)	15
2	22	**	****	10.0 (1.4) × 7.9 (0.8)	10
3	21	*	****	9.6 (1.3) × 8.1 (0.8)	9
4	18	***	0	8.8 (1.0) × 7.0 (0.4)	17
5	23	***	***	10.6 (1.4) × 8.5 (0.7)	13
9	20	*	**	9.3 (1.2) × 7.8 (0.9)	22
10	24	***	**	9.9 (0.9) × 8.1 (0.7)	26
11	21	**	**	10.1 (1.6) × 7.9 (0.9)	22
12	23	*	**	10.2 (1.1) × 8.4 (0.7)	19
14	17	**	**	9.0 (1.1) × 7.5 (0.8)	12

Strain	Potato glucose agar (PDA)				Rate of growth* (mm d ⁻¹)
	Rate of growth* (mm d ⁻¹)	Density of conidia†	Number of sclerotia‡	Size of macroconidia§ (µm)	
558	27	****	*	9.4 (1.3) × 7.5 (0.6)	
649	16	****	0	9.4 (1.3) × 7.4 (0.7)	
1	33	**	**	9.6 (1.0) × 7.8 (0.7)	
2	33	*	****	10.2 (1.4) × 8.4 (0.8)	
3	34	**	****	10.8 (1.1) × 9.1 (1.0)	
4	31	****	***	8.1 (1.0) × 6.9 (0.5)	
5	30	****	**	10.7 (1.0) × 8.6 (0.8)	
9	31	*	**	9.9 (1.2) × 8.3 (0.6)	
10	29	****	*	10.4 (1.0) × 7.9 (0.7)	
11	29	***	**	10.8 (1.4) × 8.0 (1.0)	
12	23	**	**	9.9 (0.8) × 8.3 (0.7)	
14	30	****	**	9.7 (1.3) × 8.0 (0.8)	

* Increase in colony diameter; mean of three replicates.

† Amount of colony covered by conidiophores (sparse* to complete****); mean of three replicates.

‡ Number of sclerotia per colony (5–10* to 120–150****); mean of three replicates.

§ Length × width; means (and standard deviations) of 40 conidia. The overall means are 9.7 × 7.9 µm.

|| Benomyl [methyl(1-butylcarbamoyl)benzimidazol-2-ylcarbamate] at 0.1 µg ml⁻¹. Colonies on benomyl-supplemented MM were morphologically identical to those on MM.

RESULTS AND DISCUSSION

Growth conditions

Strains 282, 465, 558 and 649 were tested for growth on various media at 15 to 37 °C to establish the most convenient regime for manipulating strains in the laboratory. They grew poorly at the higher temperatures, on malt extract and V8 agar, and with glycerol or starch as carbon source. Vigorous cultures were obtained at 15 to 30 °C, on MM, CM, SC, X, Y, Aspergillus media and PDA, and with sucrose or glucose as carbon source. As a result of these tests, MM and PDA, and temperatures of 20 to 25 °C, were chosen for routine laboratory studies of *B. cinerea*.

The synthetic medium MM with sucrose as carbon source was chosen for five reasons: it is a defined medium prepared from simple materials that can be stored as a concentrated

Figs 1 to 6. Morphological differences between wild strains of *B. cinerea* on MM (top row) and PDA (bottom row). All colonies were derived from mass-hyphal inocula, incubated at 22 °C for 30 d. Petri dishes are 85 mm diameter. Strain 2 (Figs 1, 4); strain 11 (Figs 2, 5), the greater conidiation and size of sclerotia on PDA is typical of most wild strains; strain 14 (Figs 3, 6).

solution; it supports good growth, conidiation and sclerotia production; it can be supplemented with specific chemicals for investigating nutritional mutants; it enables the detection of minor phenotypic differences between strains; its properties as a growth substrate are well documented (Davis & de Serres, 1970). PDA was the best medium for rapid production of vigorous cultures with abundant conidia.

Media that promote rapid growth and conidiation are often not suitable for the isolation and analysis of genetic variants and sexual progeny. There is a need for media which limit growth substantially so that numerous isolates can be cultured on a Petri dish. Most of the wild strains formed neat, compact colonies on the sorbose media MS and CMS; the size of the colonies could be manipulated by varying the proportions of sorbose to sucrose. Strains that produced small diffuse colonies with irregular outlines on the sorbose media were easier to analyse on MM or CM supplemented with G3300.

Phenotypic differences between wild strains

Hyphal inocula, about 8 mm³, were transferred from the frontier of young cultures on PDA to the centre of dishes of agar media and incubated at 22 °C. The resultant colonies were analysed for differences in rate of growth, conidia and sclerotia development, and size of macroconidia (Table 2). There were substantial strain differences in morphology (Figs 1 to 6), growth rate, density of conidiophores and sclerotia, and resistance to benomyl, but only minor differences in macroconidial size (Table 2). Differences between strains were usually more pronounced on MM than on PDA, particularly with respect to their rates of growth and pigmentation (e.g. strain 649 produced a diagnostic red pigment on MM, but not on PDA). PDA was superior to MM for deriving vigorous cultures: growth was faster,

conidia were produced sooner and in greater numbers, and the sclerotia were larger (Figs 1 to 6). These phenotypic differences between strains were reproducible so long as large mass-hyphal inocula were used to derive colonies for the measurements; colonies derived from individual conidia or a few hyphae sometimes varied substantially (see later).

Three of the isolates were unaffected by the systemic fungicide, benomyl; strains 9, 10 and 11 continued to grow normally on MM containing 500 μg benomyl ml^{-1} . The concentrations of benomyl needed to prevent growth of the other isolates were 0.2 μg ml^{-1} (strains 558, 1, 2, 3, 5 and 14) and 0.3 μg ml^{-1} (strains 649, 4 and 12). Benomyl-resistant strains of various fungi have been obtained from field populations (see Dekker, 1977). The presence of three resistant strains in such a small sample of natural isolates of *B. cinerea* [and of one resistant strain among the three isolates of *Botrytis squamosa* that were recovered from the onion crop at Wellesbourne in 1977 (unpublished data)] suggests that extensive use of benomyl is applying heavy selection pressure in favour of spontaneous mutants. There is also the possibility that benomyl is inducing gene mutations (Dassenoy & Meyer, 1973) or chromosomal instability (Hastie, 1970).

Since the wild strains differ for most of the phenotypic characters, including quantitative traits under polygenic control, there must be considerable genetic diversity among wild populations of *B. cinerea*. Such diversity can be generated by mutation and by gene recombination between variants. But what are the relative contributions of these factors to genetic diversity in *B. cinerea* populations? Can fungicide resistance, for example, result from mutations in more than one gene, and can these genes be recombined via a sexual or parasexual cycle to produce strains with different levels of resistance? It was felt that the mutation versus recombination question would be answered most effectively by analysing laboratory mutants rather than natural variants. Accordingly, attempts were made to induce mutants carrying suitable genetic markers for monitoring gene flow and recombination between strains.

Induction of mutants

The conidia of *B. cinerea* wild strains were sensitive to all three mutagens, and unremarkable dose-effect curves were obtained as a prelude to mutant selection procedures. With u.v.-irradiation, for instance, the LD_{10} , LD_{50} and LD_{90} times were approximately 20, 120 and 360 s, respectively. Despite the lethal effects of the mutagens, very few useful mutants were selected from the surviving progeny.

In the search for morphological mutants among progeny on dishes of CMS, many variants of strains 558 and 649 were recovered that produced abnormal colonies on CM. The variants differed from the parental strains in colonial morphology, rate of growth, conidiation and pigmentation. On repeated subculture by mass-hyphal transfers, however, most of the variants proved to be phenotypically unstable and they soon resembled the original wild strain.

No resistance mutants of strains 649, 5 and 10 were recovered from a total of almost 3×10^7 viable, mutagen-treated conidia that were incubated in nystatin-supplemented media. Using the same mutagenic and selection methods with conidia of *Neurospora crassa*, nystatin-resistant mutants have been recovered with high frequencies (Grindle, 1973, 1974). Nystatin kills normal strains of *N. crassa* by binding to the ergosterol component of cell membranes so that the membrane is distorted and cell contents leak out. Resistant mutants carry recessive gene mutations causing blocks in ergosterol biosynthesis (Grindle & Farrow, 1978). Cell extracts of *B. cinerea* cultures, analysed as described by Grindle (1973), were found to contain ergosterol: it seems unlikely, therefore, that nystatin-resistant mutants have not been recovered because *B. cinerea* lacks appropriate mutable genes. It also seems unlikely that no mutants were obtained because multinucleate macroconidia were used for the mutagenic studies: the mutants of *N. crassa* were derived from macroconidia containing similar numbers of nuclei (the mean number of nuclei in stained macroconidia of *B. cinerea* was 3.4).

Morphological variants were isolated from nystatin media that had lost its antibiotic properties after 4 weeks incubation. Most of the variants were unstable and produced normal colonies after repeated mass-hyphal subculture on CM. Six phenotypically stable mutants with fawn conidia were derived from strain 649.

No benomyl-resistant mutants of strains 5 and 10 were recovered. This was surprising because they occur in natural populations (see above; Bollen & Scholten, 1971; Jarvis & Hargreaves, 1973; Miller & Fletcher, 1974) and as spontaneous mutants in the laboratory (Polach & Molin, 1975). Thus, at least some strains of *B. cinerea* must carry appropriate genetic material that is amenable to mutation.

All attempts to isolate auxotrophic mutants of strains 465, 558 and 649 were unsuccessful, and only morphological variants were recovered. Most of the variants were phenotypically unstable. A few of the unstable cultures appeared at first to be nutritional mutants in that they did not grow on unsupplemented media. On further analysis, however, it was found that hyphal subcultures varied considerably: some grew on MM and others were unable to grow on any supplemented media. This result suggests that the medium might be inadequate for selecting auxotrophic mutants. Accordingly, conidial suspensions were exposed to u.v.-irradiation and incubated on dishes of MS supplemented with 0.5% (w/v) Casamino acids, yeast extract and peptone, 0.2% (w/v) nucleic acids and 0.1% (w/v) caffeine. The additional nutrients were used to provide a very rich medium so that variants with complex nutritional requirements might have a better chance of survival. Caffeine was added because it is known to inhibit DNA repair and, consequently, it can enhance the effects of u.v.-irradiation (Hannan & Nasim, 1977).

No auxotrophic mutants of strain 558 were isolated from the enriched caffeine medium, and most of the morphological variants were unstable. Many of the variants were phenotypically identical, forming colonies with a pronounced red pigment in the mycelium. Similar variants were not obtained by incubating untreated conidia on the caffeine medium, or by incubating u.v.-irradiated conidia on medium without caffeine.

The inability to isolate nutritional and resistance mutants, and the phenotypic instability of most morphological variants, raises fundamental questions regarding the genetic constitution of wild isolates of *B. cinerea*. The results of the mutagenic studies can be accounted for by assuming that isolates are either heterokaryotic or diploid. If they are heterokaryotic, the phenotypic instability of morphological variants could be due to the reassortment of genetically different nuclei during hyphal growth, the phenotype depending on the kinds and proportions of nuclei present; in a balanced lethal system, only heterokaryons would be viable, and pure-breeding homokaryotic progeny would not be recovered. A balanced lethal system would make the isolation of recessive gene mutants very unlikely: a mutation in only one of the nuclear components of a heterokaryon would be masked by the normal allele in the other component (which must be present to retain viability). If wild strains are diploid, the phenotypic instability of induced morphological variants could be due to segregations of haploid, diploid and aneuploid nuclei in the hyphae; stable, recessive gene mutants would be difficult to obtain since the effects of a mutation on one chromosome would be masked by the normal allele on the homologous chromosome. There is evidence that both heterokaryosis and diploidy can contribute to natural variations among populations of fungi (Caten & Jinks, 1966; Caten & Day, 1977).

To assess whether the wild strains might be heterokaryotic, asexual progeny from individual cultures on MM and CM were compared for significant differences in morphology and viability. To assess whether they might be diploid, asexual progeny were taken from cultures on PFA-supplemented media; PFA can induce haploidization of diploid fungi (Lhoas, 1961).

Figs 7 to 12. Variations in growth rate, conidiation and sclerotia production among asexual progeny of *B. cinerea* strain 9. Each colony was derived from a single macroconidium, incubated on MM for 14 d at 25 °C. Only representative colonies are shown: macroconidial progeny exhibited a continuous distribution of phenotypes, from complete inviability to slight growth (Fig. 7) to rapid growth and dense conidiation (Fig. 12). Petri dishes are 85 mm diameter.

Selection experiments

Six of the wild strains were analysed for variations among macroconidial progeny. The progeny of strains 282, 465, 558, 649 and 5 differed only slightly in morphology and rate of growth (for example, progeny of strains 465, 5 and 558 had growth rates of 11.0 ± 0.05 , 21.6 ± 0.2 and 24.7 ± 0.2 mm d⁻¹ at 25 °C, respectively). The conidial progeny of strain 9, however, differed substantially with respect to general morphology, conidiation and rate of growth (Figs 7 to 12), and included isolates that were inviable (i.e. conidia produced only short germ tubes). Hyphal subcultures of strain 9 also differed substantially, although the phenotypic variations were less extreme than those illustrated in Figs 7 to 12; most of the hyphal tip isolates were inviable, and small groups of hyphae were usually required to establish colonies.

Strains 465, 649, 5 and 9 were grown on PFA media. They all formed colonies with abnormal morphology on CM containing more than 100 µg PFA ml⁻¹ (Fig. 14). With few exceptions, the hyphal progeny incubated on dishes of MM, CM, X and Y media were phenotypically wild type. The exceptional progeny produced sectors with unusual conidiation, pigmentation or mycelia, and repeated subculture of the sectorized progeny eventually resulted in colonies that differed from the parental strains. For example, the low-conidiation and high-conidiation derivatives of strain 9 (Figs 17, 18) were selected from a sectorized colony (Fig. 15) on CM; the selected cultures differed from the untreated parental strain (Fig. 13) that was maintained by mass-hyphal subculture.

Conidial variants of strain 9 were selected directly from colonies on CM containing 40 µg PFA ml⁻¹: colonies on this medium produced few conidiophores, which were distributed

Figs 13 to 18. Cultures of *B. cinerea* strain 9 before and after selection on medium supplemented with *p*-fluorophenylalanine (PFA). Petri dishes are 85 mm diameter.

Fig. 13. Typical mass-hyphal subculture on medium X, incubated for 21 d at 25 °C.

Fig. 14. Mass-hyphal subculture on CM containing 160 µg PFA ml⁻¹, incubated for 30 d at 25 °C. There are very few conidia and no sclerotia.

Figs 15, 16. Progeny derived from hyphae taken from a culture on PFA-supplemented medium. The hyphal inocula were incubated on medium X for 21 d at 25 °C, and produced colonies with low-conidiation and high-conidiation sectors. Sclerotia on the high-conidiation sectors are masked by dense conidiophores.

Figs 17, 18. Progeny obtained by selection of hyphae and conidia from the sectored colony in Fig. 15. The low-conidiation variant (Fig. 17) was derived from hyphae, and the high-conidiation variant (Fig. 18) from conidia. Inocula were incubated on medium X for 21 d at 25 °C.

unevenly as tiny clumps against an aconidial background. The hyphal and conidial inocula from these colonies gave rise immediately to low-conidiation and high-conidiation cultures (as in Figs 17, 18), respectively.

The results of the selection experiments do not point conclusively to heterokaryosis or diploidy as the genetic basis for strain variations and phenotypic instability. If strains are diploid, one might expect them to generate many kinds of variant due to haploidization on PFA media; in practice, however, most of the variants were recovered merely by sampling macroconidial and hyphal progeny from cultures on normal media. The phenotypic diversity of conidial isolates of *B. cinerea* was noted by Paul (1929) and Hansen & Smith (1932). Single conidial isolates studied by Hansen (1938) frequently fell into three phenotypic classes: mycelial (M), conidial (C) and 'intermediate' (MC). He referred to this as the 'dual phenomenon', and suggested that the three phenotypic classes represented the three genotypic classes of progeny of a heterokaryotic parent. Thus, if the parent mycelium contained two kinds of nuclei, A and B, the conidial progeny would comprise A homokaryons and B homokaryons (phenotypic classes M and C) and A+B heterokaryons (phenotypic class MC). A similar conclusion was reached by Webster *et al.* (1970) in their studies of

Figs 19 to 22. Photomicrographs of microconidia of *B. cinerea* strain 649. Microconidia attached to conidiophores are approximately $3\ \mu\text{m}$ diameter. Germinated microconidia are swollen, approximately $5\ \mu\text{m}$ diameter.

Fig. 19. Microconidiophore arising directly from a germinated macroconidium.

Figs 20, 21. Microconidiophores arising from hyphae, laterally (Fig. 20) and terminally (Fig. 21).

Fig. 22. Microconidia germinating on CM.

botran-resistant strains of *B. cinerea* selected in the laboratory; they implied that the different levels of resistance shown by conidial progeny were due to segregation of mutant (resistant) and normal (sensitive) nuclei during spore formation by a heterokaryotic parent.

Hansen's explanation can account for some of the variations among asexual progeny described here; in the case of strain 9, the 'inviable' isolates could represent the homokaryotic classes of progeny from a heterokaryon in which the A and B nuclei carry different, complementary lethal genes. However, there is an alternative explanation of the 'dual phenomenon': it could represent the selection of different cytoplasmic entities (such as virus-like

particles, or mitochondria) from a heteroplasmic parent. It is hoped that the heterokaryon versus heteroplasmon question will be resolved by applying the tests suggested by Caten & Jinks (1966), although progress in this respect will require further studies of the *B. cinerea* strains to clarify such issues as their sexual and heterokaryon compatibility.

The inconclusive data from the analysis of macroconidial progeny focussed attention on the microconidia as a possible source of uninucleate material. A supply of uninucleate spores is more suitable for determining whether homokaryotic progeny are genetically and phenotypically different.

Analysis of microconidia

The development of microconidiophores was observed on microscope slides coated with water agar, inoculated with macroconidia and incubated at 22 °C in chambers maintained at 70 to 100% relative humidity. High humidity (95 to 100%) was best for the production of microconidia. Microconidiophores developed aurally, on the surface of the agar, and within the medium: they arose from short germ tubes (Fig. 19), from short lateral hyphal branches (Fig. 20) and at the ends of long hyphae (Fig. 21). Mature microconidia were spherical, about 3 µm diameter and uninucleate. These studies of microconidia confirm the observations of Brierley (1918).

Microconidial suspensions were incubated at 22 °C on dishes of CM, CMS and water agar. Of the eight strains analysed, only 649 produced microconidia that germinated (Fig. 22), but when further samples of strain 649 microconidia were incubated to derive cultures for analysis, the conidia failed to germinate. Experiments are in progress to determine the precise conditions needed to provide a consistent supply of viable microconidia. The microconidia will be used to derive asexual progeny for comparative studies and as a potential source of induced mutants (it could be significant that the only spore colour mutants were obtained from the only strain, 649, whose microconidia have germinated on agar media).

Crossing experiments

Attempts to derive sexual progeny by applying conidia to the surface of sclerotia were not successful: no apothecia developed after 3 months. Also, no apothecia were formed in mixed cultures on MM, PDA or sterilized soil after 6 months incubation. This failure to obtain ascospore progeny could be due to the crude experimental method, so it might be necessary to utilize the elaborate system of Groves & Loveland (1953). It is possible, however, that the strains used here are not sexually compatible; that is, they carry the same mating type allele(s). Such genetic barriers to outcrossing are widespread in fungal populations. In extreme cases, such as *Phytophthora infestans*, compatible strains with different mating types occur in very few geographical areas (Gallegly & Niederhauser, 1959). In view of the rarity of the perfect state of *B. cinerea* in nature, it is conceivable that compatible strains of this organism co-exist in few populations. To assess whether this is so, more wild strains of *B. cinerea* will be screened for sexual compatibility. The frequencies and locations of compatible strains should provide a measure of the importance of sexual reproduction as a mechanism for releasing new genetic variants into natural populations.

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