The Morphology, Ecology and Fungicidal Tolerance of *Botrytis cinerea* isolates

by

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1. ABSTRACT

Field isolates of *Botrytis cinerea* Pers. from tomato crops gave rise to two distinct morphological types after successive single spore isolations; type 1 (T1) characterised by regular, rapidly growing mycelium and type 2 (T2) characterised by sparse, often distorted, slow growing mycelium. The frequency with which isolates produced these morphological types varied considerably. The reaction to benomyl of both types obtained remained the same as that of the parent isolate through successive single spore isolations on fungicide free agar over 10 generations.

Benomyl tolerant field isolates could not be distinguished from sensitive isolates on the basis of colony morphology. Examination of 188 isolates of *B. cinerea* collected from 10 different tomato crops showed that sensitive isolates tended to be more pathogenic to detached tomato cotyledons and grew faster *in vitro* than tolerant isolates. *In vitro* and *in vivo* studies revealed no difference in the competitive ability of benomyl tolerant and sensitive isolates in mixed culture. In a study of 17 Lancashire tomato crops all were found to contain benomyl tolerant strains of *B. cinerea* although in some cases benzimidazole fungicides had not been used on the nursery for up to three years.

Examination of *B. cinerea* in tomato crops suggested that the majority of stem lesions arose from infected leaf scars and were non-aggressive. Side shoot stumps or scars were also liable to infection and the resulting lesions were more likely to become aggressive than lesions at leaf scars. Conidia were considered to be the major source of inoculum although lesions at leaf scars could take up to 61 days to develop after deleafing. This delay was attributed to latent infection. Artificial infection of the petiole with *B. cinerea* prior to deleafing greatly reduced the susceptibility of the resulting leaf scar to subsequent attack by *B. cinerea*. Extracts from infected stems were shown to delay the germination of *B. cinerea* conidia when compared to extracts from healthy stems. This inhibition of germination was attributed to a resistance factor (RF) produced by the fungus or host in response to infection.

2. INTRODUCTION

Botrytis cinerea Pers. is an ubiquitous pathogen capable of parasitising a wide range of plants (MacFarlane, 1968). This parasitism is severe in protected crops, where frequent cool, humid conditions ideally suit the growth of the fungus. The tomato, *Lycopersicon esculentum* Mill., one of the most important glasshouse crops, is particularly susceptible. *B. cinerea*, commonly referred to as grey mould, probably causes more loss to this crop than any other fungal pathogen (Fletcher *et al*, 1977; Fletcher and Harris, 1979). Tomatoes may be attacked at any growth stage with leaves, stems, flowers and fruit all susceptible to the pathogen. Stem lesions are one of the most common symptoms (Fletcher, 1965). In these lesions the characteristic grey brown mould can usually be seen sporulating profusely (Plate 1). Lesions may completely girdle the stem and kill the plant. Where foliar attack is severe, fruit can be rendered unmarketable because of ghost spotting symptoms caused by spores germinating on the fruit surface (Verhoeff, 1970) (Plate 2). Fruit may also be infected via the calyx, resulting in a soft rot. Infection of flowers reduces fruit set (Smith, 1970). In recent years much reliance has been placed on the chemical control of grey mould, particularly since the introduction of the systemic benzimidazole fungicides, of which benomyl is the most commonly used on the tomato crop (Smith and Spencer, 1971; Borecka and Millikan, 1973; McClellan *et al*, 1973). The rapid development of tolerance to benomyl shown by *B. cinerea* (Bollen and Scholten, 1971; Miller and Fletcher, 1974) has however,

resulted in a swift decline in the use of this fungicide (Fletcher et al, 1977).

This study was undertaken to examine the pathogenesis of tomato plants by B. cinerea and to investigate the significance of benomyl tolerance on the fungus. The existence of morphological and physiological variation between and within single strains of B. cinerea is well documented (Brierley, 1931; Menzinger, 1966; Lauber, 1971) and makes this fungus an extremely complex organism to study. An appreciation of the variation exhibited by the isolates of B. cinerea used in this project was therefore considered essential as a prelude to the study. A better understanding of the disease together with information about the consequences of fungicide tolerance could lead to the adoption of more efficient control measures.

Plate 1

A stem lesion of B. cinerea (grey mould) on tomato

Plate 2

Ghost spotting on tomato fruit infected by B. cinerea

3. LITERATURE REVIEW

3.1 Variability

Botrytis cinerea was almost certainly first described by Micheli in 1729, and the specific name *B. cinerea* given in 1771 (von Hailer, 1771); the genus *Botrytis* was validated by Persoon (1801). *B. cinerea* is considered to be the conidial form of a number of distinct *Botryotinia* species related to and including *Botryotinia fuckeliana* (de Bary) Whetz. This view is supported by the work of Groves and Loveland (1953) who obtained apothecia of *B. fuckeliana* by crossing isolates of *B. cinerea* from the Rhine valley, West Germany, with isolates from Canada. A comprehensive description of *B. cinerea* based solely on morphological characters has been made by Groves and Loveland, 1953 (Appendix I). Differences between isolates in culture have, however, led to many authors referring to *B. cinerea* as an aggregate or complex species or as "*Botrytis* of the *cinerea* type". Whetzel (1945) regarded *B. cinerea* as a collective species comprising numerous types which would, in time, be described as distinct species. Brierley (1931), however, claimed that *B. cinerea* is a single species containing a number of races or strains which are "morphologically congruent on the host plant but *in vitro* showing marked and constant cultural differences".

Differences between individual isolates of *B. cinerea* have frequently been reported. Paul (1929) described an isolate which produced large numbers of sclerotia, another which tended towards the development of aerial mycelium and a third characterized by the prolific production of conidia.

Saponaro (1953) divided isolates of B. cinerea from grape vine grown in different parts of Italy into seven morphological races according to conidial size. Nonaka and Morïta (1967) differentiated eight types on the basis of sclerotial characteristics and found that the optimum temperature for mycelial growth of 79% of all isolates examined was 20°C, for 15% 25°C and for 6% 15°C. Conversely Gorlenko and Manturovskaya (1971) reported that 19 strains of B. cinerea isolated from different hosts did not differ sharply in morphologial and cultural characteristics but did show considerable variation in pectolytic activity. Morgan (1971a) examined 33 isolates of B. cinerea and showed considerable inter-isolate variation but when this variability was subjected to numerical analysis it was considered insufficient to create new taxa. However, in a further analysis which included representative isolates of 12 Botrytis species, in addition to 39 isolates of B. cinerea, Morgan (1971b) was able to recognize two "forms" of B. cinerea. Type A was characterized by the production of grey colonies on a range of media and extensive sporulation with little or no sclerotial production. Type B produced cream/white colonies on arabinose, raffinose and sorbose media and showed enhanced sclerotial production at the expense of sporulation. When considering morphological features it should be noted, however, that cultural conditions may considerably modify the morphological characters frequently used in taxonomy. Vanev (1972) found that conidial size and form and colony characteristics in B. cinerea could be changed by altering incubation temperature and culture media. Menzinger (1965),

following an extensive study concluded, however, that provided there was an awareness of the effect of environmental conditions on morphological features they could still form a suitable basis for systematics in B. cinerea.

Changes in colony morphology can occur in successive generations of a single isolate grown under constant conditions. Brierley (1931) found that "pure-lines" (single spore isolates) of B. cinerea often produced sectors or "variants" when subcultured. Such variants either reverted to the original parent strain, remained stable, or gave rise to further variant strains. Work by Hansen and Smith (1932) established that apparently homogenous isolates of B. cinerea could be divided by single spore culture into two stable "homotypes"; a mycelial type (abundant mycleium and few conidia) and a conidial type (many conidia and reduced mycelial growth). A third unstable "heterotype" was also found, an intermediate between the two main types. Hansen (1938) termed this situation the "dual-phenomenon". He subsequently tested 900 isolates from 30 genera of imperfect fungi and found that more than 50% exhibited the dual-phenomenon. Menzinger (1966) confirmed the work of Hansen (1938), although he concluded that both the mycelial and conidial types could be further subdivided. A single isolate of B. cinerea obtained from Gloxinia hybrida produced 17 morpholigically identifiable strains following successive single spore isolation over four generations. Six of these strains were described as primary types because they remained stable and did not give rise to further variants in subsequent

single spore generations. Heterokaryosis is generally proposed as the explanation of within (or intra) - strain variation in B. cinerea (Hansen and Smith, 1932, 1934, 1935; Hansen, 1938; Menzinger, 1965, 1966; Lauber, 1971). Hansen and Smith (1932) reported that the conidia of B. cinerea are multinucleate. They found hyphal anastomosis to be common and suggested that this enabled nuclei of one strain to migrate into the hyphae of a second strain. Conidia and hyphae could therefore contain nuclei of more than one genotype. In 1934, Hansen and Smith demonstrated the combination of distinct homogenic strains into a single colony from which the component strains could be recovered by successive single spore culture. In a further experiment they grew isolates of B. ricini and B. allii in mixed culture. When 20 single spore isolates were made from the culture, six produced colonies identical with one parent, nine resembled the other and five were intermediate between the two. Mixing of hyphal contents by anastomosis resulting in heterokaryosis was proposed as an explanation. Menzinger (1965) confirmed that nuclei of B. cinerea could pass across hyphal anastomosis and moreover showed that nuclei were able to migrate freely within hyphae, passing through the septal pores. From these observations he suggested that in vivo heterokaryon formation was probably very frequent, although he was only able to demonstrate heterokaryosis in vitro on one occasion (Menzinger, 1966).

3.2 Benomyl Tolerance

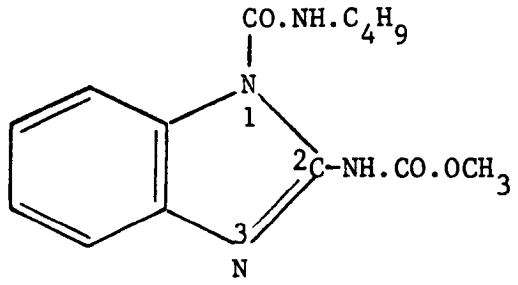
Benomyl [methyl (1-butylcarbamoyl) - benzimidazol - 2 -

ylcarbamate] was first released by the Dupont Company under the designation "Dupont fungicide 1991" in 1967. Preliminary studies showed it to have preventative, curative and systemic fungicidal action and to act as a mite ovicide (Delp and Klöpping, 1968). Benomyl has been shown to be toxic to a wide range of fungi but is, nevertheless, selective (Bollen and Fuchs, 1970; Edgington et al, 1971). Thus, Delp and Klopping (1968) reported that benomyl is effective against diseases caused by Cercospora Spp. Fusarium Spp. Penicillium Spp. Colletotrichum Spp. Botrytis Spp. and Verticillum Spp., but inactive against Pythium Spp. Phytophthora Spp. Alternaria Spp. and Helminthosporium Spp. Commercially available since 1971 as Benlate(R), a 50% wettable powder, benomyl can be applied as a spray, drench (Marsh, 1972), dip (Maggie and Wilfret, 1974), dust (Hide et al, 1969), or seed dressing (Schroeder and Provvidenti, 1968; Maude and Kyle, 1970).

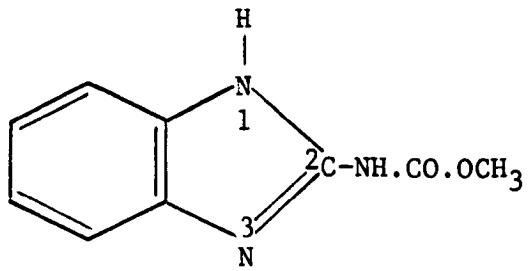
Benomyl is one of a number of closely related fungicides including carbendazim (methyl benzimidazol - 2 - ylcarbamate, MBC) and thiabendazole, which are based on the benzimidazole molecule. A further closely related fungicide is thiophanate methyl, a thiourea which depends on conversion to a benzimidazole ring for its activity (Figure 1). In aqueous solution benomyl is rapidly hydrolysed to MBC which is stable and fungitoxic (Clemons and Sisler, 1968; Sims et al, 1969; Peterson and Edgington, 1970). In addition butyl isocyanate is formed which has been shown to be toxic to Ustilago maydis and Saccharomyces cerevisiae (Hammerschlag and Sisler, 1973) (Figure 2). MBC is the principal fungicidal product of

Figure 1:

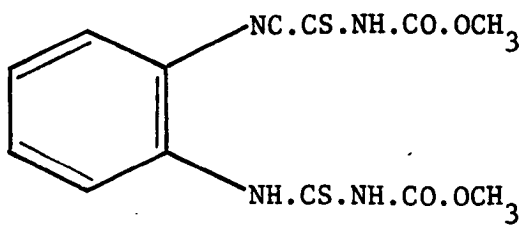
Benomyl
Methyl(1-butylcarbamoyl)-benzimidazol-2-ylcarbamate



MBC or Carbendazim
Methyl benzimidazol-2-ylcarbamate



Thiophanate-methyl
1.2-bis(3 methoxycarbonyl-2-thioureido)benzene



Thiabendazole
2-(Thiazol-4-yl)benzimidazole

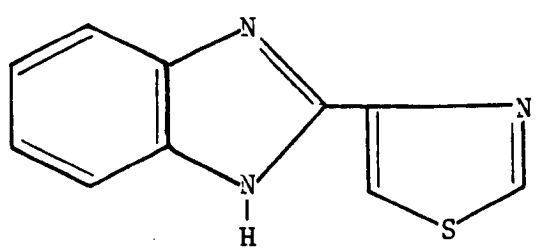
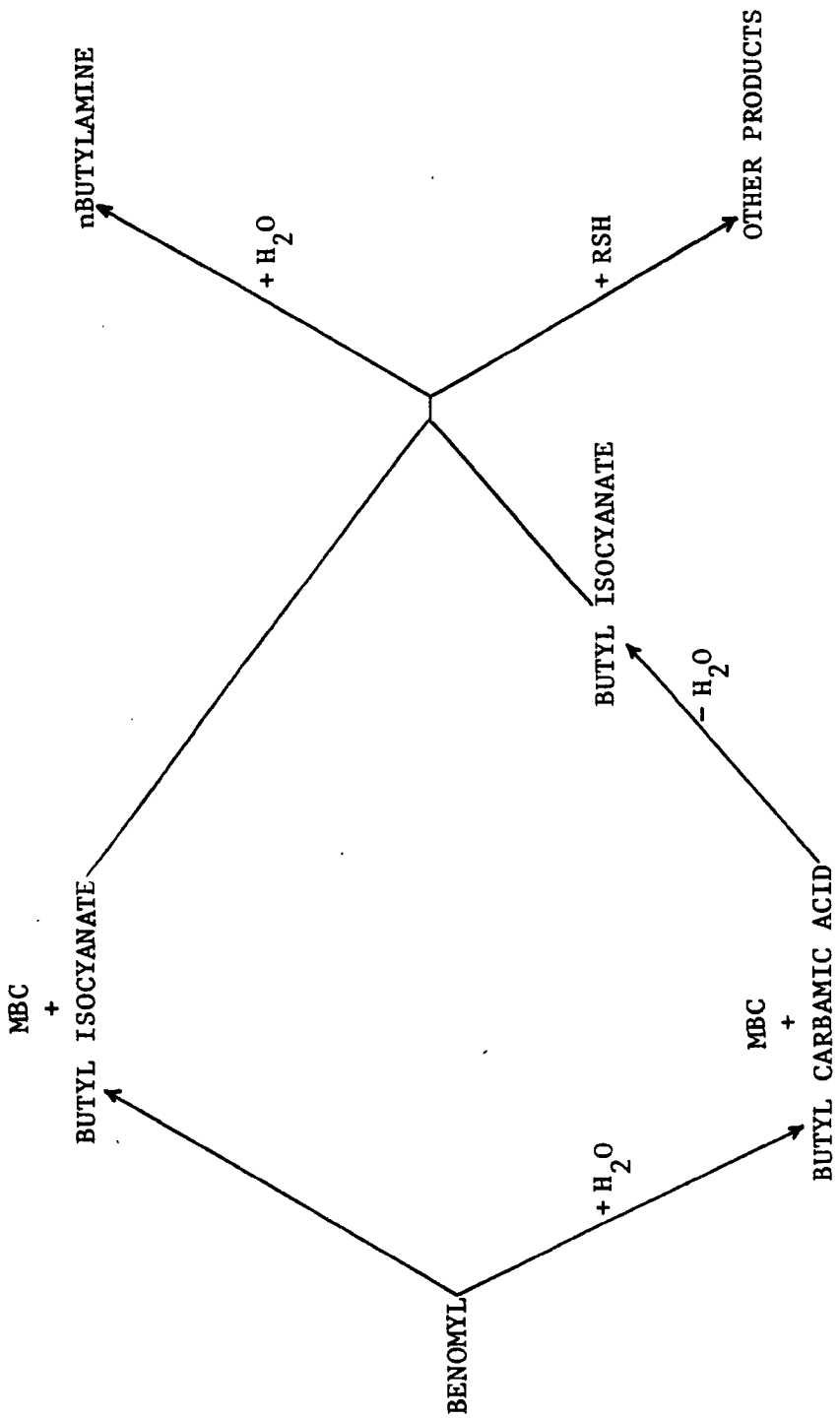


Figure 2: Proposed pathways of formation for various breakdown products of benomyl*



* after Hammerschlag & Sisler (1973)

benomyl which may be active at sites within the treated plant well removed from the point of application (Sims et al, 1969; Peterson and Edgington, 1970, 1971; Siegel and Zabbiá, 1972). Movement of MBC within plants is acropetal and accumulation occurs at the end points of the transpiration stream such as leaf margins (Gray and Sinclair, 1970; Ben-Aziz and Aharonson, 1974) and has been detected in seeds and fruit (Ellis and Sinclair, 1975). There is a rapid reduction in MBC concentration in new growth suggesting that plants may need repeated applications of the fungicide (Ben-Aziz and Aharonson, 1974).

Clemons and Sisler (1971) noted that only small quantities of MBC were required for fungitoxicity (< 1 p.p.m.) suggesting a specific mode of action. Using Neurospora crassa and Ustilago maydis they found that MBC was toxic only to actively growing mycelium; spore germination was not inhibited. In the presence of MBC, the rate of DNA synthesis declined in advance of any change in RNA or protein metabolism. Clemons and Sisler (1971) suggested that the site of action of MBC was either DNA synthesis or a related process such as nuclear or cell division. In 1973 Hammerschlag and Sisler reported that MBC interfered with mitosis. In synchronized U. maydis cultures, mitosis and cytokinesis failed in MBC treated sporidia even though the DNA required for replication had been synthesized prior to treatment, providing evidence of mitotic failure rather than a direct effect of MBC on DNA synthesis. Hammerschlag and Sisler (1973) later concluded that MBC interfered with the mitotic spindle. Davidse (1975)

suggested the formation of a complex between MBC and a subunit of the microtubuli, preventing normal assembly of microtubule subunits into spindle fibres, thus inhibiting spindle formation and preventing mitosis.

The effectiveness of benomyl as a broad spectrum fungicide coupled with its systemic activity, has ensured its use on a world wide scale. This is particularly true in the glasshouse industry where the crop values justify high cost treatments and because no interval is required between the application of benomyl and harvest (Marsh, 1972). The latter feature is particularly important in tomatoes and other sequential crops where fruit is harvested continuously over a long period. Prior to the introduction of systemic materials, tolerance (insensitivity) to fungicides was rare and created practical difficulties in only a few cases (Georgopoulos and Zaracovitis, 1967). Organic mercury compounds failed to control Drechslera avenae on oats in Scotland as a result of tolerance (Noble et al, 1966). Tolerance by Sclerotinia homoeocarpa on turf grass to cadmium containing compounds has been reported in the U.S.A. (Cole et al, 1968). Szkolnik and Gilpatrick (1969) showed that in New York State, U.S.A., dodine became less effective as a control for apple scab over a period of ten years. The introduction of systemic fungicides, characterized by highly specific modes of action, led to a rapid increase in the occurrence and importance of fungicide tolerance (Dekker, 1976). An early example was the development in Holland in 1969 of tolerance to dimethirimol by Sphaerotheca fulginea following the introduction of the

fungicide in 1968 (Bent *et al*, 1971). The first record of tolerance to benomyl was made in 1969 before the fungicide became commercially available; Schroeder and Provvidenti (1969) demonstrated tolerance to benomyl in *Sphaerotheca fulginea*. Tolerance to benomyl has now been reported in a wide range of fungi (Dekker, 1976). The majority of benomyl tolerant strains have been found in crops with a history of benomyl application. In many cases their presence has resulted in a breakdown of disease control with a resulting reduction in yield. Georgopoulos and Dovas (1973) reported that commercial applications of benomyl in Northern Greece gave excellent control of *Cercospora beticola* on sugar beet in 1970 and 1971. In 1972, however, control was poor with no significant difference in foliage destruction between treated and untreated plots. In Texas, U.S.A., Ruppel and Scott (1974) found benomyl tolerant isolates of *C. beticola* in crops treated with the fungicide whereas those from untreated fields were sensitive. They showed that five to six benomyl sprays failed to arrest the disease in commercial holdings where tolerance existed. Wicks (1974) reported that the failure of benomyl sprays to control *Venturia inaqualis* in a Jonathan apple orchard was due to the presence of tolerant strains. These strains had developed following three years of regular benomyl use. Excellent control of *Septoria leucanthemi* on chrysanthemum was given by benomyl from 1971 to 1973. During the latter part of 1973 and 1974, fields were found in which control was poor; all isolates of *S. leucanthemi* taken from these fields were found to be benomyl tolerant (Paulus *et al*, 1976).

Bollen and Scholten (1971) first reported the failure of benomyl to control disease caused by B. cinerea. This occurred in 1970 in glasshouse cyclamen where a strain of B. cinerea tolerant to 1000 mg/ml of benomyl was subsequently isolated. Since 1970 there have been frequent recordings of benomyl tolerance in B. cinerea. Jarvis and Hargreaves (1973) isolated tolerant strains of B. cinerea from mummified strawberry fruit treated the previous year with benomyl, they also reported that benomyl was failing to control strawberry and raspberry grey mould in some plantations. Dennis (1975) confirmed that the incidence of Botrytis rot of strawberries had increased with the development of tolerance. Miller and Fletcher (1974) and Fletcher and Scholefield (1976) monitored the extent of tolerance in B. cinerea isolates from tomatoes at one site following the introduction of high volume sprays of benomyl. They found that in 1972, 1973 and 1974, 0, 31 and 70% respectively of the isolates examined were tolerant to benomyl. In addition they noted that whereas benomyl treatment resulted in a significant decrease in disease incidence and an increase in yield when compared to untreated areas in 1972 and 1973, this was not apparent in 1974 (Fletcher and Scholefield, 1976). In 1977 Schüepp and Lauber conducted a detailed survey of 845 isolates of B. cinerea collected from vineyards in Northern and Eastern Switzerland. They concluded that the increase in the number of tolerant isolates collected could be correlated with an increased frequency of MBC application over the previous three years.

Three mechanisms have been proposed to account for the origin

of benomyl tolerance in the field:

(1) That strains of fungi potentially tolerant to the fungicide arise spontaneously without influence from the fungicide. In support of this theory Warren et al (1974) were able to demonstrate tolerance in a strain of Verticillium malthousei isolated in 1958, 10 years before the introduction of benomyl. Polach and Molin (1975) investigated benomyl tolerance in single ascospore isolates of Botryotinia fuckeliana obtained from areas not previously treated with benzimidazole fungicides. They found that benomyl tolerance occurred at a rate of 1 in 5×10^7 conidia.

(2) That benomyl acts as a mutagenic agent, artificially creating tolerant strains. The evidence of Hastie (1970) and Kappas et al (1974) shows the benomyl is mutagenic, demonstrating that the fungicide increased the instability of diploid cells of Aspergillus nidulans. Benomyl induced mutations have also been found in Fusarium oxysporum f.sp. melonis (Dassenoy and Meyer, 1973) and bacteria (Seiler, 1972). Bartels-Schooley and MacNeil (1971) have shown that a known mutagen, U.V. light can induce the formation of benomyl tolerant strains in F. oxysporum f.sp. melonis. Dekker (1976), however, notes that there are no unequivocal examples of mutation being induced by systemic fungicides at the concentrations used in commercial practice.

(3) That fungi gradually adapt to the fungicide. Abelentsev and Golyshin (1973) provided evidence for this theory when they increased the tolerance of B. cinerea isolates 25 times by successive subculture on increasing concentrations of benomyl over 290 days. They reported, however, that

tolerance was quickly lost after transfer to fungicide free agar. This is in contrast to the tolerance of field isolates which has remained stable after repeated subculture on fungicide free agar for 22 months (Jordan and Richmond, 1974) and three years (Geeson, 1976). Using Verticillium dahlie Talboys and Davies (1976) increased the tolerance of an isolate from less than 1 p.p.m. to 12 p.p.m. benomyl by successive subculture on to increasing concentrations of the fungicide and then found tolerance was retained in the absence of benomyl. In contrast, Geeson (1976) was unable to increase the tolerance of a benomyl sensitive isolate of B. cinerea using the successive subculturing technique. Strains exhibiting a small degree of tolerance (i.e. partially tolerant) can be found in the field, they are, however, comparatively rare (Miller and Fletcher, 1974; Geeson, 1976).

The existence of tolerant strains and the resulting poor disease control achieved with benzimidazole fungicides, has led to a decline in their use. This decline has been particularly noticeable in the glasshouse crops industry (Fletcher et al, 1977). Fletcher (1975) considered that "for tolerant strains to become important and cause crop loss they must be:

a. Genetically stable and become dominant in the pathogen population.
b. As aggressive and virulent as the sensitive strains.
c. Equally competitive as the sensitive strains in their saphrophytic phase.
d. Have no limitations in their epidemiology, e.g.

produce large numbers of spores and over-season effectively."

It is clear that tolerant strains can become dominant in the pathogen population in the presence of benomyl (Bollen and Scholten, 1971; Georgopoulos and Dovas, 1973; Ruppel and Scott, 1974; Dennis, 1975; Fletcher and Scholefield, 1976). Evidence of the ability of these strains to compete successfully with sensitive strains in the absence of benomyl is, however, conflicting. Jordan and Richmond (1974) reported no difference in pathogenicity between benomyl tolerant and sensitive isolates of B. cinerea from strawberry. In mixed inoculum experiments they found that benomyl tolerant strains could spread rapidly among plots in the absence of the fungicide. Following an examination of 28 single spore isolates of Cercospora beticola from sugar beet, D'ambera et al (1974) concluded that tolerance to benomyl did not effect pathogenicity of the fungus. Similar conclusions have been reached by Maggie and Wilfret (1974) working with isolates of Fusarium oxysporum f.sp. gladioli and by Bolten (1976) and Cho (1977) working with isolates of B. cinerea from Antirrhinum and Leucospermum cordifolium respectively. Griffee (1973), however, found that a tolerant isolate of Colletotrichum musae was less pathogenic to bananas than a sensitive isolate. Similarly, Wuest et al (1974) found that a benomyl tolerant isolate of Verticillium malthousei was neither as aggressive or virulent as sensitive isolates. Jones and Ehret (1976) observed that two benomyl tolerant isolates of Monilinia fructicola were similar to two

sensitive isolates in virulence on peach fruit, although a third tolerant isolate was less virulent. In contrast Bollen and Vanzaayen (1975) obtained a tolerant isolate of Verticillium fungicola which showed a higher virulence than the sensitive wild strain. Fletcher and Yarham (1976) reported that there was a tendency for benomyl tolerant isolates of V. fungicola to have a slower growth rate than sensitive isolates on unamended potato dextrose agar (PDA), suggesting a reduced saprophytic ability. A tolerant isolate of M. fructicola was also found to be slower growing than sensitive isolates, although two further tolerant isolates had similar growth rates to the sensitive isolates (Jones and Ehert, 1976). Dennis and Cohen (1976) reported considerable variation in the growth rates of both sensitive and tolerant isolates of B. cinerea grown on agar but found less variation in the ability of the isolates to cause fruit spoilage of strawberries and raspberries. Geeson (1976), in a study of 46 B. cinerea isolates from a wide host range and geographical location, concluded that differences in growth rate could not be correlated with benomyl tolerance or sensitivity. Dovas et al (1976) examined the persistence of benomyl tolerance in C. beticola in the absence of benomyl and found that three years after the cessation of benzimidazole use on sugar beet crops in Northern Greece, the frequency with which tolerant strains were isolated, had not changed. They concluded that sensitive and tolerant populations of the fungus were equal in fitness for survival in the absence of the fungicide. Jordan and Richmond (1974) showed that a tolerant strain of B. cinerea was able to overwinter on

strawberry leaf debris and produce sufficient inoculum for flower infection the following spring. Smith (1974), however, found that tolerant isolates of B. cinerea from tomato grown on unamended PDA produced less mycelium and fewer conidia than sensitive isolates. Similarly, Richmond et al (1974) reported that seven benomyl tolerant isolates of B. cinerea from strawberries were morphologically distinguishable from sensitive wild type isolates, although they found variation between the tolerant isolates in the degree of sporulation and in the number and types of sclerotia produced. Geeson (1976), however, concluded that benomyl tolerant and sensitive isolates could not be distinguished on morphological grounds.

3.3 Epidemiology

The optimum temperature range for growth and sporulation of B. cinerea is 22.5 to 25°C (Baker, 1946; Van den Berg and Lentz, 1968), while high humidity (> 95% R.H.) is required for spore germination (Snow, 1949). These conditions occur frequently in poorly ventilated glasshouses, particularly in unheated houses where a rapid drop in temperature at night is likely to cause condensation and high humidity within the crop. The link between B. cinerea infection and humidity has long been recognized. Thomas (1921) and Bewley (1923) recommended the manipulation of glasshouse ventilation systems to avoid this danger. Winspear et al (1970) showed that in heated glasshouses automatic humidity control, initiated when the relative humidity reached 75%, effectively decreased the incidence of Botrytis fruit spotting (ghost spot) in

tomatoes. Increasing the ventilation around the base of the plant by routine removal of the lower leaves (de-leafing) also helps discourage infection of the foliage (Smith, 1970; Kingham, 1973). The practice is also employed to improve light intensity around ripening fruit and to remove old leaves which are below their compensation point. Leaf scars resulting from deleafing can, however, be invaded by B. cinerea resulting in the formation of stem lesions (Fletcher, 1965).

B. cinerea is considered a weak parasite as it is unable to infect healthy plant tissue. De Bary (1886) and Brooks (1908) were among the first to observe that attacks of B. cinerea were often associated with prior colonization of dead or dying plant debris and that the fungus usually attacked senescent rather than healthy tissue. Thomas (1921), observing Botrytis infection of glasshouse tomatoes, considered it unlikely that the fungus had penetrated living tissue without first growing saprophytically on decaying organic matter. This conclusion is supported by the observations of Bewley (1923) and Wilson (1963) who reported enhanced infections of tomato stems when the fungus first established itself as a saprophyte from conidia germinating in badly pruned leaf scars. More recently Smith (1969) stressed the importance of a saprophytic base in the initiation of stem lesions of B. cinerea on tomatoes. This was clearly demonstrated in a deleafing experiment where the vast majority of B. cinerea stem lesions (94%) were associated with petioles infected prior to deleafing.

Young tomato fruits (< 30 mm diameter) are particularly susceptible to infection by conidia of B. cinerea. Spores on the fruit germinate and the germ tubes penetrate the epidermal cells under conditions of high humidity. In the majority of cases the fungus fails to develop further and the fruit continues normal development, although a small lesion, known as a ghost spot, marks the point of penetration of the fungus. Ghost spotting greatly reduces the economic value of the tomato fruit (Ainsworth et al, 1937; Verhoeff, 1970).

B. cinerea conidia are considered unable to establish infection on healthy stem and leaf tissue. Both Thomas (1921) and Smith (1970) have shown that tomato foliage sprayed with conidial suspensions of B. cinerea can remain healthy for two weeks under conditions of high relative humidity. Louis (1963) considered that the plant cuticle is the major barrier against infection by conidia. He found that Fuchsia leaves, which have a much thinner cuticle than the tomato, were susceptible to infection when sprayed with conidia, whereas tomato leaves were not. Similarly a decreased susceptibility of tomato fruit to conidial infection has been correlated with increased cuticle thickness (Ainsworth et al, 1938). There is also considerable evidence that the surface microflora of healthy leaves may be antagonistic to B. cinerea. Sztejnberg and Blakeman (1973) showed that bacteria on leaves may develop in close association with conidia of B. cinerea. This could result in a water gradient away from the conidia, causing leaching of nutrients and a decreased ability

to germinate. Kashyap and Levkina (1977) found that most microorganisms isolated from tomato leaves reduced mycelial growth of B. cinerea in culture. In addition, leaf extracts taken from the plants prior to flowering have been shown to inhibit conidial germination (Kovacs and Szeoke, 1956). B. cinerea conidia can, however, remain viable on tomato leaves for up to three months (Ilieva, 1970) and may therefore initiate infections as leaves become senescent or damaged. Baker (1946) and Smith (1970) found that flowers, unlike leaves and stems, could be directly infected by conidia. Smith concluded that infected flowers were a major source of inoculum in tomato crops. They are frequently observed on leaves and she suggested that they could act as a saprophytic base for invasion of healthy plant tissue. A similar pattern of host penetration has been demonstrated for B. cinerea in other crops. Jarvis (1963) showed that if spores of B. cinerea were placed between an adhering petal and the intact surface of a ripe strawberry fruit the petal became fully colonized before invasion of the fruit occurred. In peas infection has been shown to develop from B. cinerea colonizing petals which in turn can fall into the axils of nodes (Ford and Haglund, 1963). Harthill and Cambell (1974) found that removal of flower heads markedly decreased the incidence of B. cinerea attack of tobacco. This was directly correlated with the density of corollas falling on the leaves. The susceptibility of flowers may be due to their reduced cuticle thickness or to the presence of a higher density of pollen. Chou and Preece (1968) and Borecka and Millikan (1973) found that

pollen grains from tomato flowers greatly stimulated the germination of B. cinerea conidia. Smith (1970), however, concluded that naturally occurring pollen loads on tomato plant foliage were insufficient to overcome the resistance of leaves and stem to conidial inocula.

There is considerable controversy regarding the origin of B. cinerea stem lesions on tomato plants. The majority of reports (Thomas, 1921; Bewley, 1923; Smith, 1970) suggest that natural disease arises from the prior colonization of moribund tissue or flowers. Wilson (1962) and Verhoeff (1967), however, have shown that B. cinerea conidia placed on leaf scars, either dry or in water suspension can initiate stem lesions. Wilson (1962) claimed that when infection by conidia was successful a well established mycelium could be observed after 72 hours, penetrating to a depth of at least one centimetre. No mention was made of the severity of resulting lesions. He reported that an increased vascular pressure, caused by an increase in humidity, led to exudation from the cut ends of xylem vessels at leaf scars. Spores present on the scars became suspended in the exudate and were drawn into the xylem as humidity and vascular pressure decreased. In sectioned material he observed spores germinating at depths of over two millimeters from the cut surface of the scar. Where lesions failed to develop the spores remained viable for considerable periods. Wilson referred to this as "latent" infection and suggested that lesions could subsequently develop at leaf scars up to 11 weeks after inoculation. Verhoeff (1967) confirmed that conidia could

give rise to lesions at freshly exposed leaf scars, although he was unable to demonstrate that conidia could be drawn into the vascular system. In contrast Smith (1970) failed to induce lesions by artificially inoculating leaf scars with conidia.

The physiological condition of tomato plants greatly influences their susceptibility to B. cinerea. Wilson (1964) showed that soil moisture was important in influencing host response. Following inoculation more lesions developed at leaf scars when crops were grown in permanently wet soils than in those kept relatively dry. Smith (1970) similarly reported that more Botrytis lesions developed and that a higher proportion were aggressive on inoculated plants grown in soil maintained at field capacity than those grown in drier soils. Wilson (1962) noted that leaf scars at the base of the plant were less susceptible to B. cinerea than those at nodes higher up the stem. This has since been supported by Verhoeff (1965) who considered that the degree of xylem development in older stems leads to an increased dry matter content and therefore reduced succulence. Smith (1970) reported that restricting the root growth of plants before transplating into border soil resulted in an initial increase in the percentage of dry matter by a factor of x 1.7 and a reduction in susceptibility to B. cinerea infection. Horsfall and Dimond (1957) classified Botrytis spp. as "high-sugar" pathogens. Grainger (1962) suggested that B. cinerea needs large amounts of carbohydrate, particularly reducing sugars, when parasitising its host. Kamoen (1976) found that Begonia leaves

with a high sugar content were particularly susceptible to B. cinerea. Disease incidence was reduced in plants shaded to reduce photosynthetic efficiency and therefore sugar production. Van Die (1962) reported that the sucrose, glucose and fructose were less concentrated in older stems and this has been proposed as a possible reason for their greater resistance to Botrytis attack (Verhoeff, 1965). Verhoeff (1965) found that higher fertiliser rates decreased the rate of lesion development on tomatoes infected with B. cinerea. Nightingale et al (1928) showed that the carbohydrate content of tomatoes is reduced when the soil nitrogen content is increased. Verhoeff (1965) therefore suggested that the effects of nitrogen supply on B. cinerea lesion development may be attributed to differences in carbohydrate. Smith (1970) failed to support this claim, although she found that the incidence of Botrytis stem lesions was significantly increased by treating plants with high levels of phosphorous and low levels of potassium. Potassium deficiency also increases plant carbohydrate (Wall, 1940) and succulence (Richards and Shih Sheng-Han, 1940; Johnson, 1947). Similarly, Stall et al (1965) reported that Botrytis was more severe in tomato crops grown with high levels of phosphorous combined with low levels of calcium.

4. MATERIALS AND METHODS

4.1 Terminology

To avoid possible confusion the terms population, strain and isolate are defined as follows for the purposes of this investigation:

A population of B. cinerea is a collection of strains existing within defined limits (e.g. a glasshouse). An isolate is a colony of B. cinerea obtained from the field by brushing spores from a lesion on an infected plant onto agar. It is, therefore, possible for an isolate to contain a mixture of strains. An isolate can only be called a strain in vitro if it is derived from a single spore or hyphal tip.

4.2 Culture of isolates

Isolates were grown in the dark on potato dextrose agar (PDA, Difco Ltd) at 20°C ± 1°C, unless stated otherwise. 9 cm Petri dishes were used for culture, each containing 10 ml of media dispensed with a sterile syringe to ensure uniformity. Isolates were stored on PDA in Petri dishes sealed with cellotape which were kept in the dark at room temperature. Stored cultures were sub-cultured every three months. An 8 mm disc cut from the edge of the colony was transferred to a fresh PDA plate which was sealed with cellotape when growth covered the plate.

4.3 Determination of benomyl tolerance

Isolates were classified as tolerant if they grew on PDA amended with 100 p.p.m. benomyl and sensitive if they failed

to grow on PDA amended with 1 p.p.m. benomyl. Amended agar was prepared by aseptically adding a sufficient volume of an aqueous suspension of Benlate(R) (50% a.i. benomyl) (Dupont (UK) Ltd) to molten, sterile PDA at 45°C to give the required fungicide concentration.

4.4 Collection of isolates

Isolates of B. cinerea were obtained from a large number of glasshouse tomato crops growing in the Yorkshire/Lancashire region of the Agricultural Development and Advisory Service (A.D.A.S.). Pieces of sporulating stem lesion were cut from plants using sterile instruments, placed in clean paper envelopes, returned to the laboratory and stored at -18°C. Isolates were prepared by lightly brushing the infected tissue against the surface of a PDA plate and incubating at 20°C. Pure cultures were obtained by transfer of mycelium from the resulting colony to a fresh culture plate.

4.5 Detection of tolerant strains in B. cinerea stem lesions

The procedure outlined in 4.4 was followed using PDA amended with 100 p.p.m. benomyl; mycelial growth indicated the presence of tolerant strains.

4.6 Single spore isolation

A crude spore suspension was prepared by placing the tip of a sterile glass rod against the surface of a sporulating B. cinerea culture or stem lesion and transferring conidia to a drop of sterile distilled water (SDW) on a glass slide. The droplet was subsequently spread over the surface of a

PDA plate using a sterile glass spreader. After overnight incubation at 20°C individual germinated spores could easily be removed from the plate with the aid of a mounted needle and dissecting microscope.

4.7 Hyphal tip isolation

An 8 mm disc of agar bearing mycelium was cut from the leading edge of an actively growing culture and placed onto 1.5% tap water agar. After incubation at 20°C for 48 to 72 hours single hyphae could be distinguished and hyphal tips removed using a mounted needle under a dissecting microscope.

4.8 Estimating the proportion of tolerant to sensitive spores in stem lesions

100 single spore isolates were prepared from each lesion as described in 4.6 and placed onto PDA amended with 100 p.p.m. benomyl (25 spores/plate). Spores from tolerant strains produced distinct colonies within two days (Plate 3), those from sensitive strains germinated to produce highly branched and distorted germ tubes which ceased growth after 24 hours (Plate 4).

4.9 Propagation of plants

Tomato seed used throughout the study was the variety Moneymaker (Harrisons re-selected, Asmer Seeds Ltd).

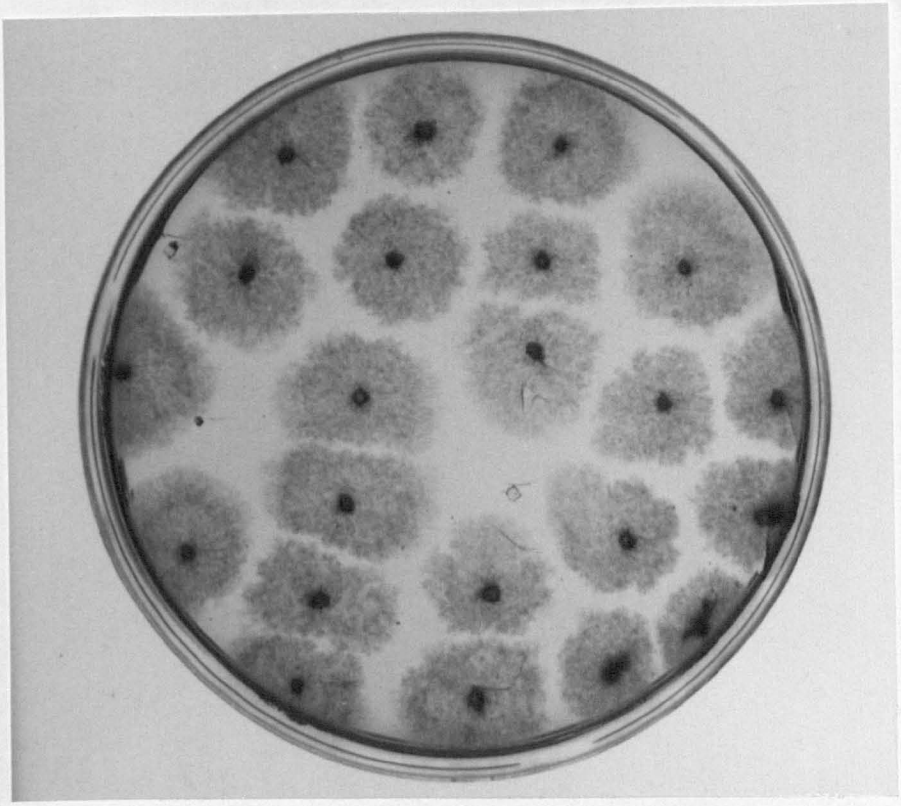
(a) Cotyledons: Tomato seed was sown in John Innes seed compost in 36 x 22 x 5 cm trays and placed under fluorescent lights (5000 x 1.1 lux) (16 hour day) at room temperature. On emergence seedlings were thinned to approximately 50 per tray and after two weeks the

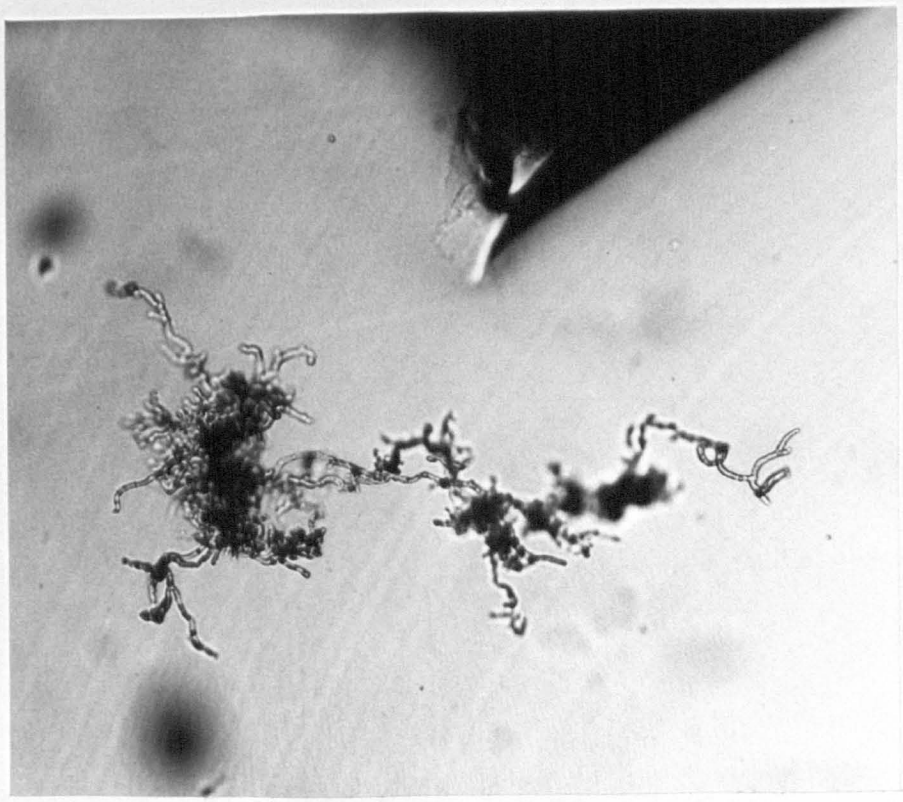
Plate 3

Spores from a benomyl tolerant strain of B. cinerea producing distinct colonies on PDA amended with 100 p.p.m. benomyl.

Plate 4

A spore from a benomyl sensitive strain of B. cinerea germinating on PDA amended with 100 p.p.m. benomyl.





growing point of each seedling was removed to encourage enlargement of the cotyledons. After a further week the cotyledons were suitable for use in pathogenicity tests (4.15).

(b) Pot Plants: Young seedlings were pricked out into 14 cm plastic pots containing John Innes No. 2 compost and placed onto a glasshouse bench. A minimum temperature of 15°C was maintained and when necessary mercury vapour lamps provided supplementary lighting to ensure a 16 hour day.

4.10 Preparation of tomato stem extract

Lengths of tomato stem were frozen in liquid nitrogen and ground using a pestle and mortar. Phosphate buffer Ph 6 was added at a rate of 10 ml/100 g fresh stem weight. The homogenate was filtered through four layers of muslin and twice through filter paper (Whatman No. 2) under suction using a Büchner funnel. The resulting extract was either used immediately or stored in 20 ml universal bottles at -18°C until required.

4.11 Preparation of tomato stem broth

300 g fresh weight of 12 week old tomato stems were blended with one litre of tap water. The homogenate was autoclaved for 15 minutes at 121°C and then stored at -18°C until required.

4.12 Preparation of fungal inoculum

(a) Mycelial inoculum: 6 mm discs of agar plus mycelium were removed from the margin of an actively growing three day

old culture on PDA.

(b) Spore inoculum:

(i) In aqueous suspension: 10 ml of a sterile 0.1% solution of Tween 80 were added to a nine day old sporulating culture. Spores were dislodged with a glass spreader and the resulting suspension passed through four layers of muslin to remove mycelial fragments. The spores were washed in three changes of sterile distilled water and the final concentration determined using a haemocytometer.

(ii) Dry: Spores were transferred directly from naturally sporulating stem lesions with a camel hair brush.

4.13 Spore germination studies

A known concentration of spores was incubated at the required temperature in 0.2 ml of the appropriate substrate on a cavity slide; each test was replicated twice. To prevent desiccation the slides were enclosed in 9 cm Petri dishes containing glycerine agar (1% (w/v) agar and 5% (w/v) glycerine in distilled water). Glassware for germination studies was washed in a non-ionic wetter (0.01% Dicon 90) and rinsed in at least three changes of glass distilled water.

4.14 Inoculation of mature plants

(a) Petiole inoculation: The petiole was wounded by removing a leaflet with a sterile scalpel prior to securing with sellotape a disc of inoculum, mycelial face to the leaflet scar.

(b) Stem inoculation

(i) With mycelium: A petiole was removed (either by hand or with a knife) and a disc of inoculum placed against the freshly exposed leaf scar with the mycelial face adjacent to the stem. This was secured with sellotape for 48 hours, after which time the sellotape was removed leaving the disc in place.

(ii) With spores: Dry spores were brushed onto the freshly exposed leaf scar with a camel hair brush.

4.15 Pathogenicity tests

(a) Mature tomato plants: Petioles in the central region of 10 week old tomato plants (approximately 2m tall) were inoculated with fungal mycelium at a distance of 10 to 12 cm from the stem. Ten replicates were prepared for each isolate tested. The extent of subsequent lesions measured from the point of inoculation towards the stem was recorded at regular intervals (Plate 5).

(b) Excised tomato cotyledons: A rapid pathogenicity test was developed using excised cotyledons (see Appendix II for an evaluation of the test). A 3 mm disc was cut from the margin of a colony growing on quarter strength PDA. Each disc was dissected into two equal halves, each of which was placed mycelial face down on the tip of a detached cotyledon. Preliminary experiments had shown that use of a low nutrient base enabled differences in pathogenicity between isolates to be observed more easily. Inoculated cotyledons were placed on glass slides supported by U-tubes in a moist chamber consisting of

Plate 5

Pathogenicity test - using petioles on mature plants

9 cm Petri dishes containing 10 ml of tap water; 10 replicate cotyledons were used per isolate. Inoculated cotyledons were maintained in an illuminated incubator (800 - 1000 x 1.1 lux) with a 16 hour day length. Lesion extent was measured after 4 days (Plate 6).

4.16 Measurement of fungal growth rate on agar

PDA plates were each inoculated with a 6 mm mycelium/agar disc and the extent of mycelial spread recorded after three days; five replicates were prepared for each isolate. Two diameters, at right angles, were measured on each plate and the mean value determined.

4.17 Recording colony morphology

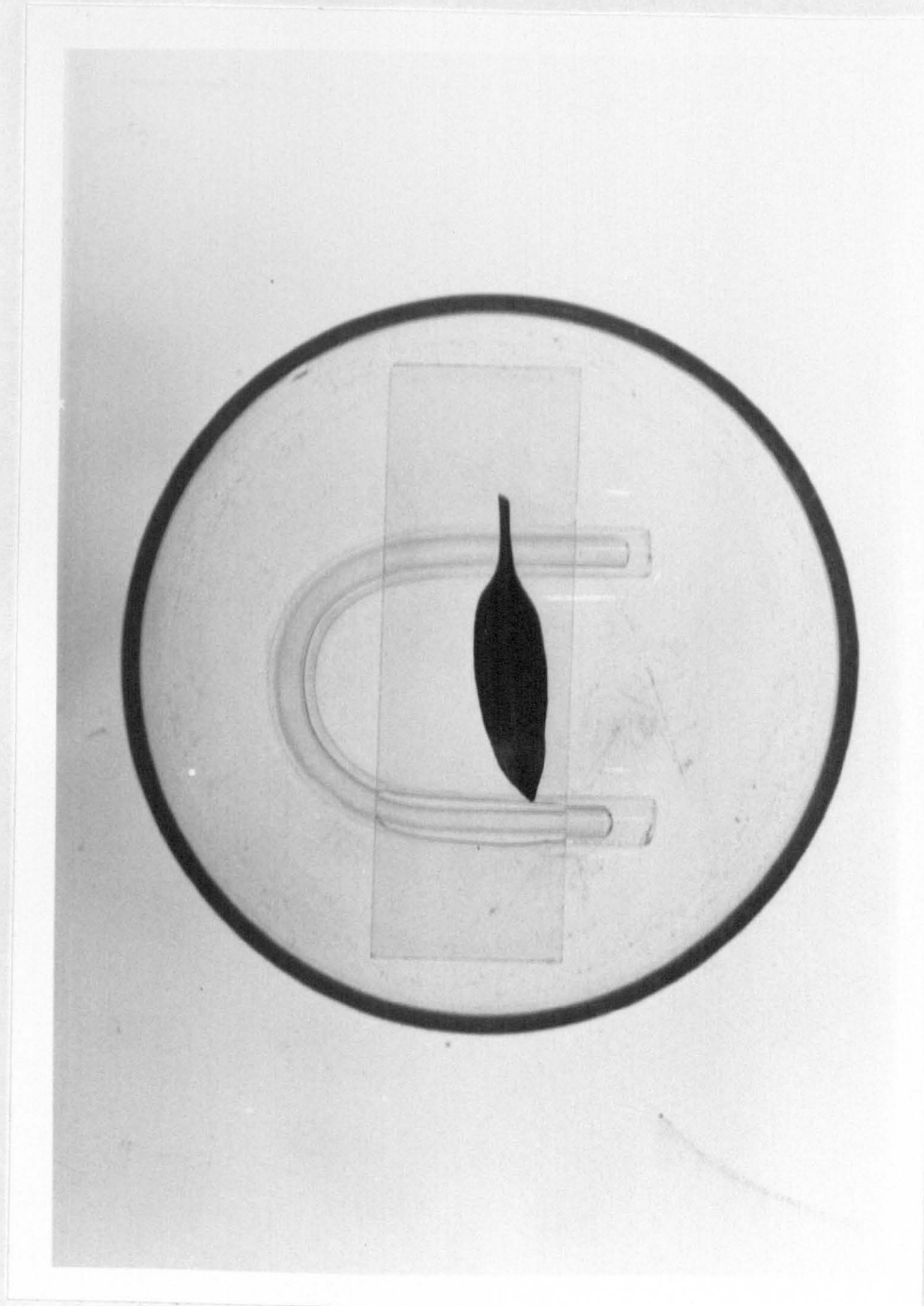
Five replicates of each isolate were subcultured onto PDA and incubated at 20°C. Plates were examined at regular intervals and gross morphological features recorded. After 14 days, sporulation and sclerotial production was scored on a 0 (nil) to +++++ scale.

4.18 Field observations

Detailed observations were made of natural *B. cinerea* infections in crops grown under commercial conditions. These were done in commercial glasshouses throughout the Yorkshire/Lancashire region of A.D.A.S. Fungicide applications to these crops were applied by the grower according to his own disease control programme.

Plate 6

Pathogenicity test - using excised tomato cotyledons



4.19 Field experiments

Inoculation experiments were undertaken in tomato crops grown under commercial conditions. Normally plants in single or double rows positioned at the edge of the glasshouse were used. Cultural treatments applied to these plants, including fungicide treatments, were usually in keeping with commercial practice but were varied if required. All experimental treatments were replicated at least ten times and fully randomized.

4.20 Statistical analysis

Experimental data were analyzed using appropriate statistical techniques as outlined by Duncan (1955) and Bishop (1971).

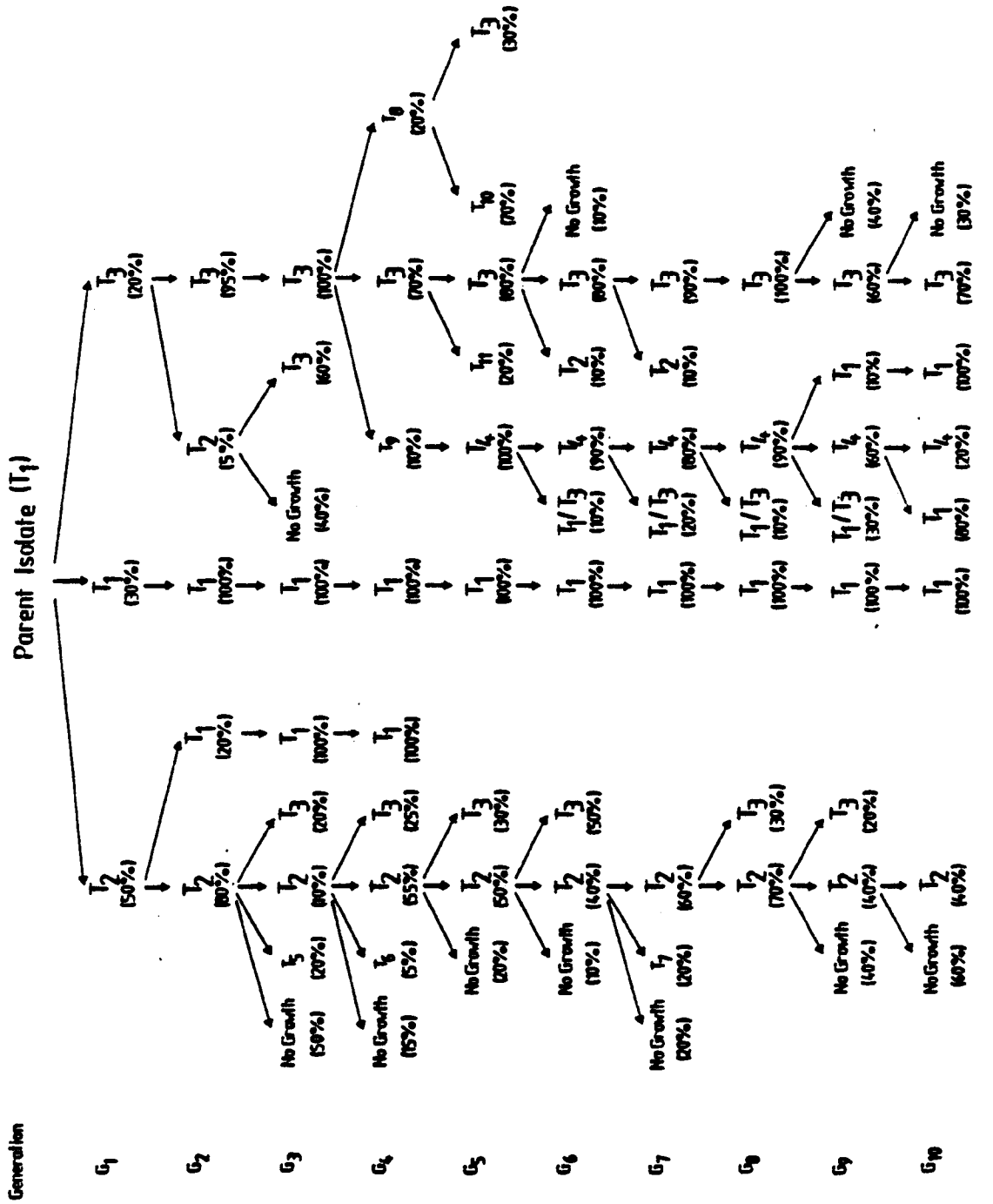
5. VARIABILITY EXHIBITED BY ISOLATES OF *B. CINEREA* COLLECTED FROM TOMATO CROPS

An aim of this project was to compare benomyl tolerant and sensitive isolates of *B. cinerea*. In order that *in vitro* observations were not to be misleading, a study of the variation exhibited by randomly selected isolates was undertaken. This study also allowed a comparison of the relative stability of benomyl tolerant and sensitive isolates.

5.1 An examination of the morphological variability exhibited by four isolates of *B. cinerea*

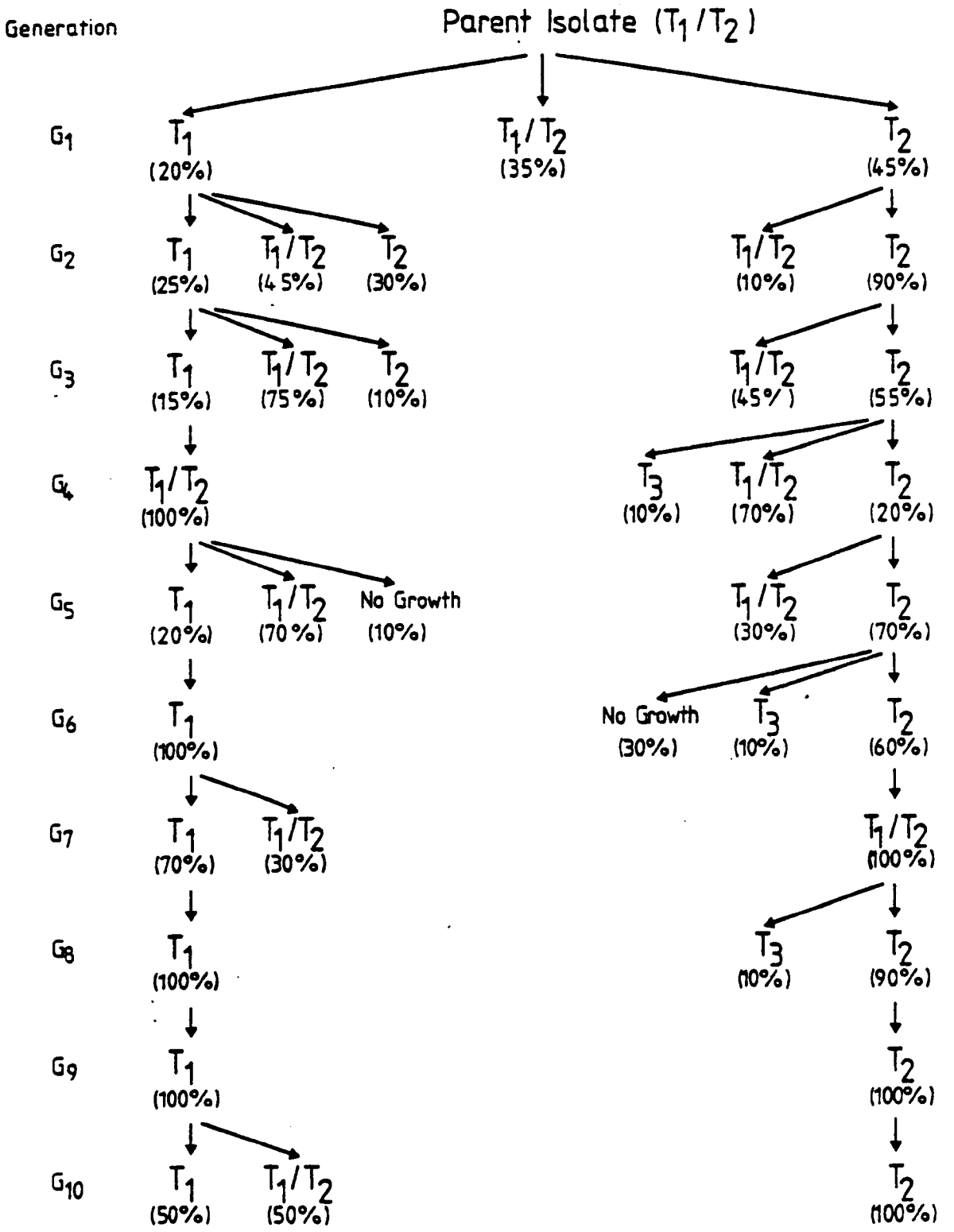
Twenty single spore isolates prepared from each of the following isolates: 24 AiiS and 45 AvS (benomyl sensitive) and 23 AiR and 32 AiiR (benomyl tolerant) were incubated on PDA at 25°C. The resulting cultures (termed generation one [G1]) were examined regularly until sclerotial development was complete (10 to 20 days) and their morphological features recorded. 20 sub-cultures were then prepared from any morphologically distinct G1 colonies. Where possible sub-cultures were produced by single spore isolation; non sporulating cultures were propagated by hyphal tip isolation. By this procedure 10 generations of each parent isolate were examined; however, after the fourth generation the number of sub-cultures taken from each morphological type was reduced to 10. Morphologically distinct types were termed T_1, T_2 ... etc., any intermediate types were designated T_1/T_2, T_2/T_3 ... etc. In addition to recording morphological variation, sensitivity to benomyl was also examined in the cultures of each successive generation. The results are expressed as dendograms in Figures 3 to 6. Morphological descriptions are given in Table 1.

Figure 3: Isolate 24 AiiS: Morphological types obtained over 10 generations



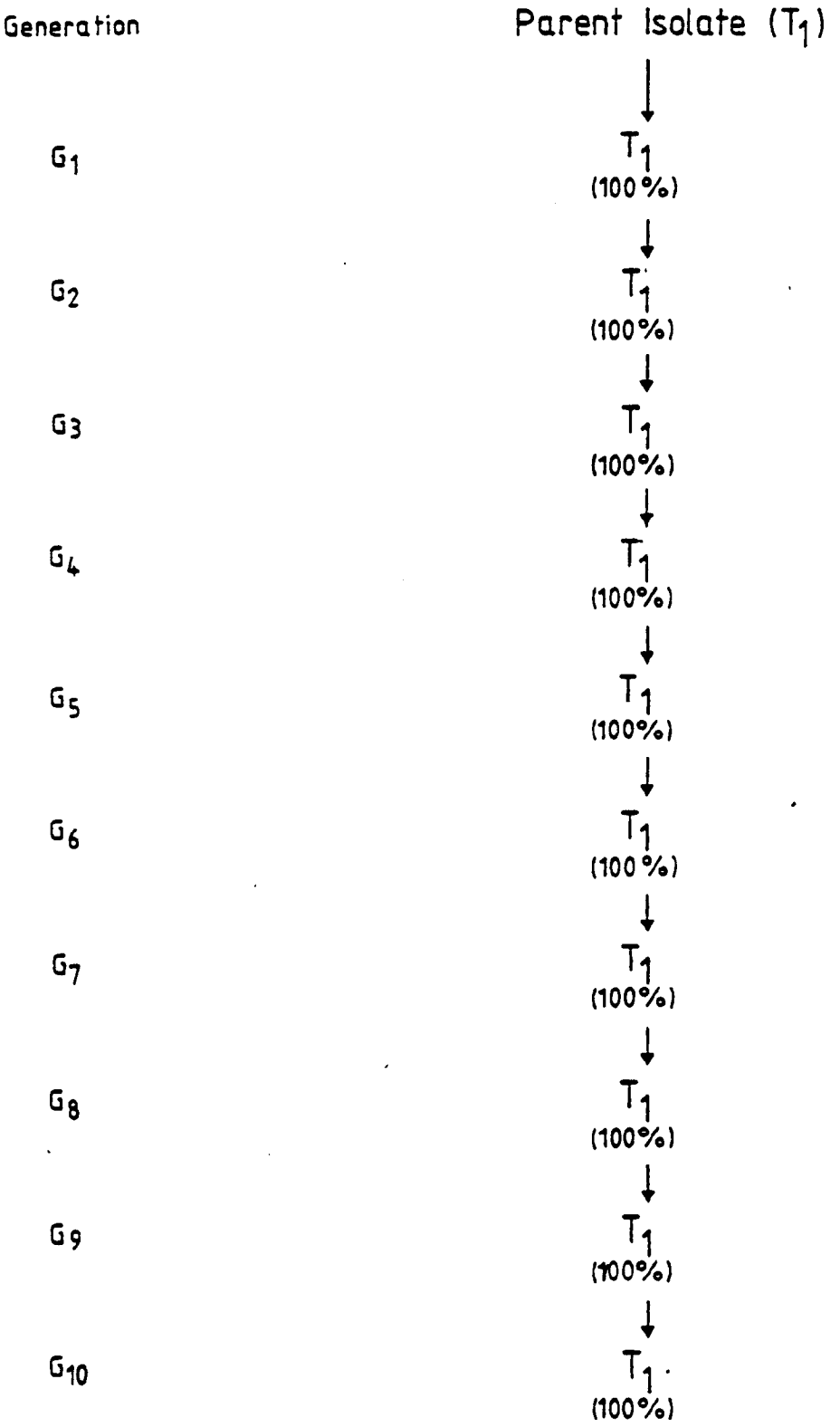
Percentage in brackets indicates the number of each type in that generation: G_1 - G_3 out of 20 single spore cultures; G_4 - G_{10} out of 10 single spore cultures.

Figure 4: Isolate 45 AvS: Morphological types obtained over 10 generations



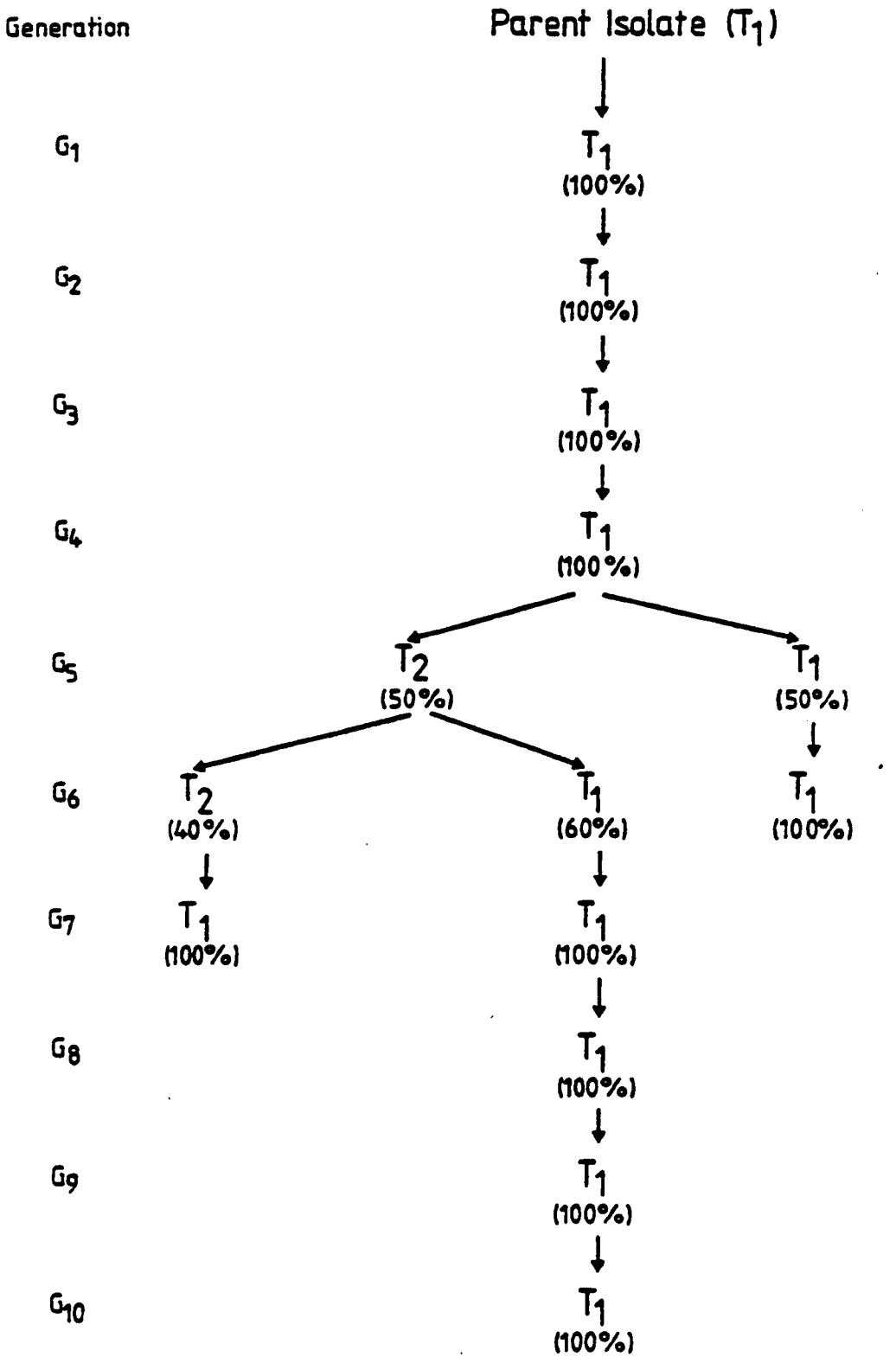
Percentage in brackets indicates the number of each type in that generation: G_1 - G_3 out of 20 single spore cultures; G_4 - G_{10} out of 10 single spore cultures.

Figure 5: Isolate 23 AiR: Morphological types obtained over 10 generations



Percentage in brackets indicates the number of each type in that generation: G_1 - G_3 out of 20 single spore cultures; G_4 - G_{10} out of 10 single spore cultures.

Figure 6: Isolate 32 AiiR: Morphological types obtained over 10 generations



Percentage in brackets indicates the number of each type in that generation: G_1 - G_3 out of 20 single spore cultures; G_4 - G_{10} out of 10 single spore cultures.

Table 1: Morphological types obtained from isolates 24 AiiS, 45 AvS, 32 AiiR and 23 AiR over 10 generations

ISOLATE	TYPE	MYCELIUM		SPORES			SCLEROTIA			SECTORS[1]
		Growth/form	Colour	Quantity	Position	Colour	Quantity	Position	Diameter	
24 AiiS	T_1	Rapid, regular, flat	Yellowish white	Dense	Edge	Umber	O/+	Edge	≤ 1 mm	✓[2] Ochreous
	T_2	Slow, sparse, distorted, flat	Sepia with irregular black & white pigmentation	Dense	Centre	Umber	++	Through-out	≤ 1 mm	-
	T_3*	Slow, sparse, edge distortion, flat	Yellowish white	Dense	Throughout showing density rings	Olivaceous	O/+	Through-out	≤ 1 mm	-
	T_4	Slow, sparse, distorted, fluffy centre	Yellowish white	Dense	Centre	Dark green	O	-	-	-
	T_5	Slow, sparse, thread like, irregular, flat	Umber	Moderate	Patchy	Umber	O	-	-	✓[3] White
	T_6	Slow, very limited, flat	White	Moderate	Centre	Dark green	O	-	-	-
	T_7	Slow, feather like, flat	Fuscous black	Dense	Centre	Hyaline	O	-	-	-
	T_8	Slow, sparse, delicate, flat	White	Moderate	Centre	Dull green	O	-	-	-
	T_9	Slow, sparse, distorted, flat	White	Dense	Centre	Olivaceous	O	-	-	-

Table 1: - continued

ISOLATE	TYPE	MYCELIUM		SPORES			SCLEROTIA			1 SECTORS
		Growth/form	Colour	Quantity	Position	Colour	Quantity	Position	Diameter	
24 AiiS	T_{10}	Slow, sparse, distorted, flat	Ochreous	Slight	Centre	Hyaline	0	-	-	✓ 4 Fuscous black
	T_{11}	Slow, very limited, flat	White	Absent	-	-	0	-	-	-
45 AvS	T_1	Rapid, regular, fluffy at edge	Greyish white	Dense	Edge	Umber	0	-	-	-
	T_2	Slow, sparse, distorted, flat	Yellowish white	Sparse	Through-out	Umber	++++	Through-out	< 2 mm	-
	T_3	Slow, sparse, distorted, flat	Greyish white patches of umber	Sparse	Patchy	Umber	0	-	-	✓ 5 Yellow-ish white
23 AiR	T_1	Rapid, regular, fluffy at edge	Greyish white	Moderate	Edge	Umber	++/++++	Edge	1 - 5 mm	✓ 6 Ochreous
32 AiiR	T_1	Rapid, regular, fluffy	Greyish white	Moderate	Through-out	Ochreous	0	-	-	-
	T_2**	Slow, fluffy, becoming flat	Yellowish white	Slight	Through-out	Ochreous	++++	Through-out	< 2 mm	-

* The growth vigour of this type was greatly reduced over successive generations.

** Initial colonies appeared as T_1 types. Loss of aerial mycelium and yellowish colouration tended to occur after four days. Subcultures of mycelium onto fresh PDA produced typical T_1 colonies.

1. ✓ sectors present; - sectors absent.

2. Sectors occurred as indentations of the border, felt like, ochreous, lacking in sporulation. Subcultures of mycelium onto fresh PDA produced identical colonies but with a small zone of hyaline spores in the centre. Single spore isolations from this zone all produced T_1 colonies.

3. Numerous sectors of fine white mycelium.

4. Single fuscous black sector similar to T_2 type.

5. Sectors resembled T_2 type.

6. Sectors identical to 2.

All colours have been described according to Rayner (1970).

Two major morphological types were recognized in this investigation. The first, designated T_1, was characterized by regular, rapidly growing mycelium. This took one of three forms: flat, flat becoming fluffy at the edge of the plate or uniformly fluffy; colour was either yellowish white or greyish white. Spores varied in density and were either uniformly distributed or concentrated at the edge of the plate, ochreous or umber in colour. Sclerotia varied in size, number and distribution. The second major morphological type, designated T_2 was characterized by sparse, often distorted, slow growing mycelium. The mycelium was generally flat, although occasionally fluffy at the centre of the plate. The colour was very variable (Table 1), often showing irregular patches of pigmentation. Spores varied in colour, density and distribution. Sclerotia, although variable in number and distribution, were always small (< 2 mm in diameter). The variation in mycelial form, colour, sporulation and sclerotial formation enabled T_1 and T_2 types originating from one parent isolate to be morphologically distinguishable from any other isolate. The reaction to benomyl of all cultures in each generation remained the same as that of the parent isolate, regardless of morphological type. The variation seen in each isolate is summarized below.

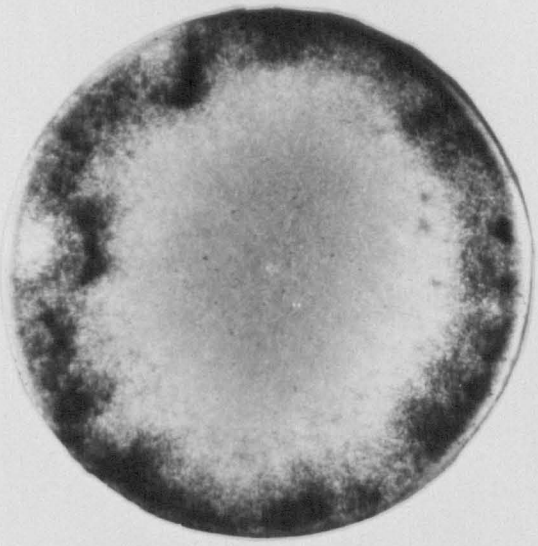
24 AiiS (Figure 3, Table 1, Plate 7)

The most variable isolate examined. In addition to the T_1 and T_2 types two further types, T_3 and T_4, were prominent and others, T_5 to T_{11}, were identified. T_3 to T_{11} inclusive, however, all had a sparse, distorted mycelium, so whilst they were each morphologically distinguishable, they could be broadly

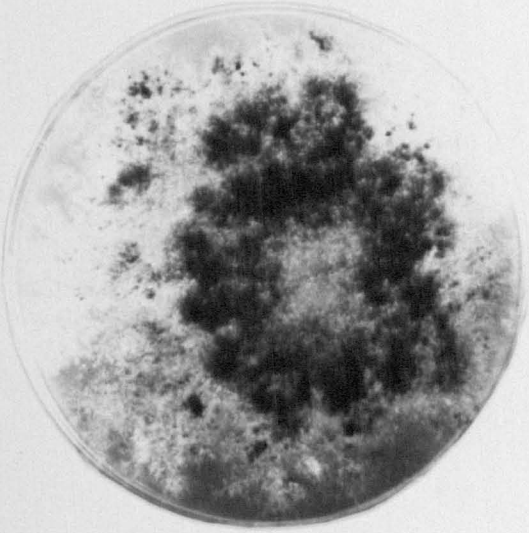
Plate 7

Principal morphological types obtained from isolate 24 AiiS

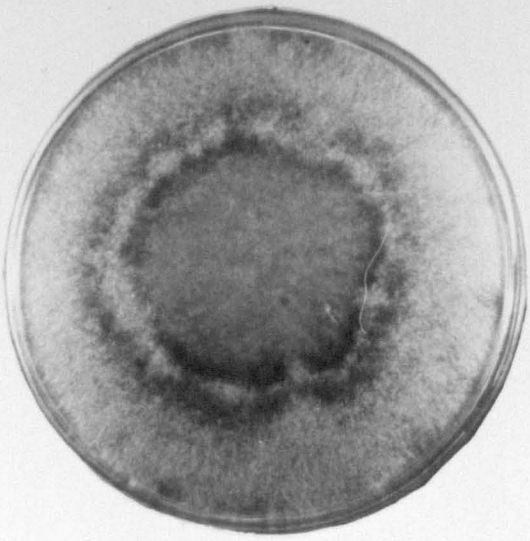
ISOLATE 24AiiS



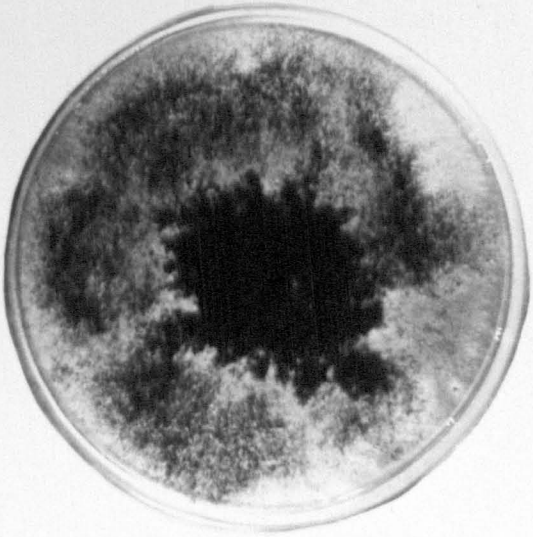
T_1



T_2

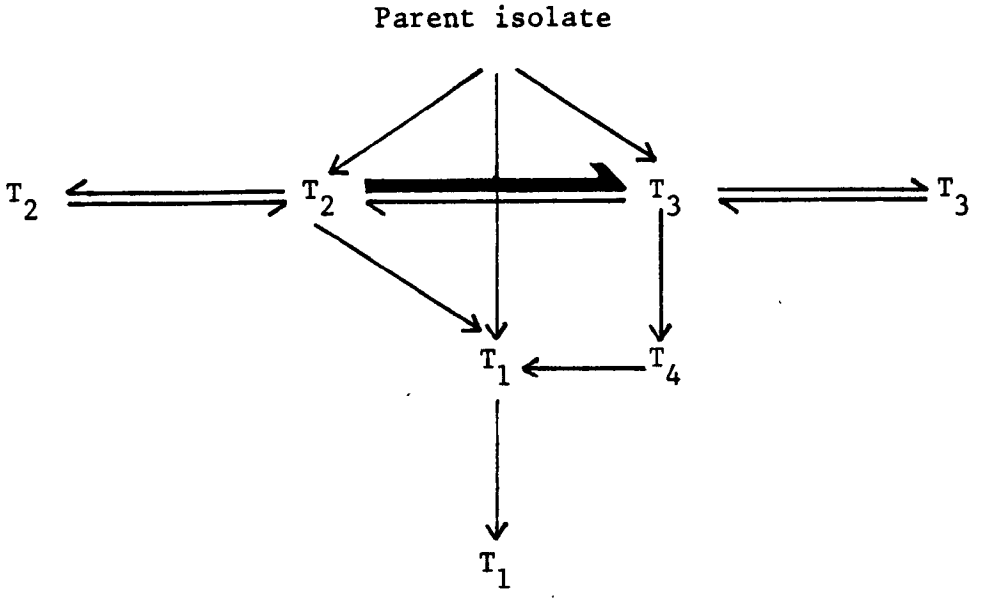


T_3



T_4

classified as T_2 types. The T_1 type was characterized by its stability over 10 generations which would suggest homogenicity. The T_2 types demonstrated varying degrees of instability which can be summarized by the following diagram:



45 AvS (Figure 4, Table 1, Plate 8)

The two characteristic types, T_1 and T_2, were produced. A third type, T_3, was recognized, its distorted growth suggesting that it could be broadly classified as a T_2 type. In addition a T_1/T_2 intermediate was obtained; the frequency of this intermediate tended to decline after G5.

23 AiR (Figure 5, Table 1, Plate 9)

This isolate proved stable throughout 10 generations, producing only the T_1 type.

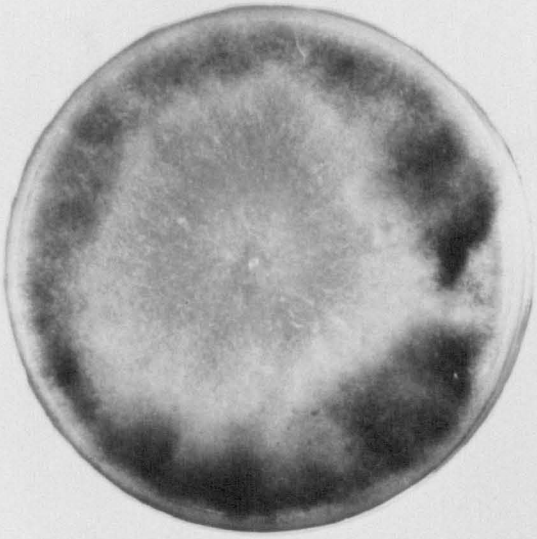
32 AiiR (Figure 6, Table 1, Plate 10)

T_1 and T_2 types were observed, although the T_2 type cultures obtained from this isolate were atypical. When sub-cultured by mycelial disc transfer, T_2 types from isolates 24 AiiS and

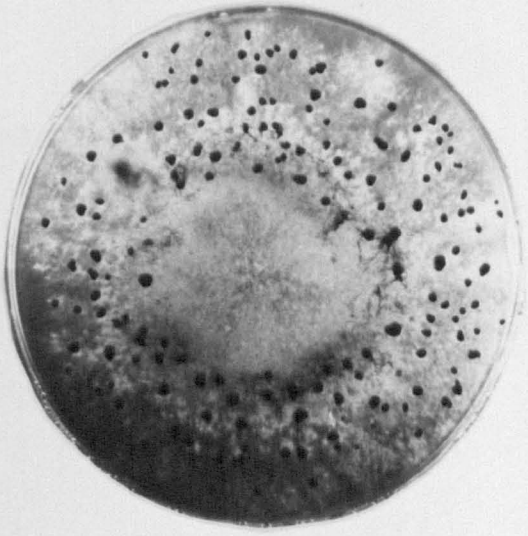
Plate 8

Principal morphological types obtained from isolate 45 AvS

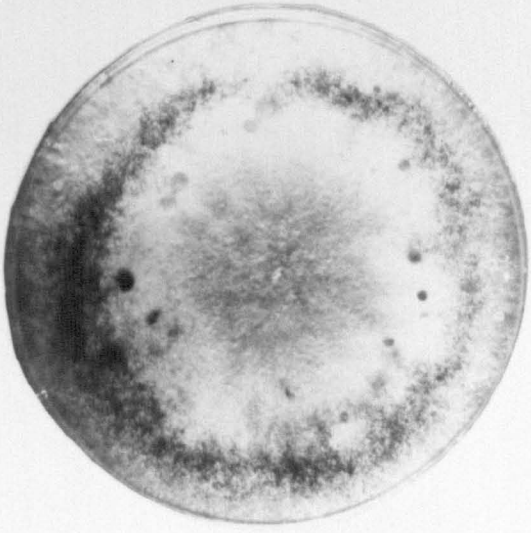
ISOLATE 45AvS



T_1



T_2



T_1 / T_2

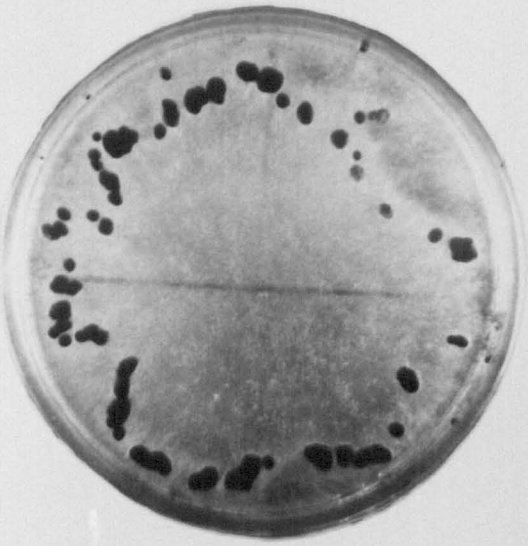
Plate 9

Morphological types obtained from isolate 23 AiR.

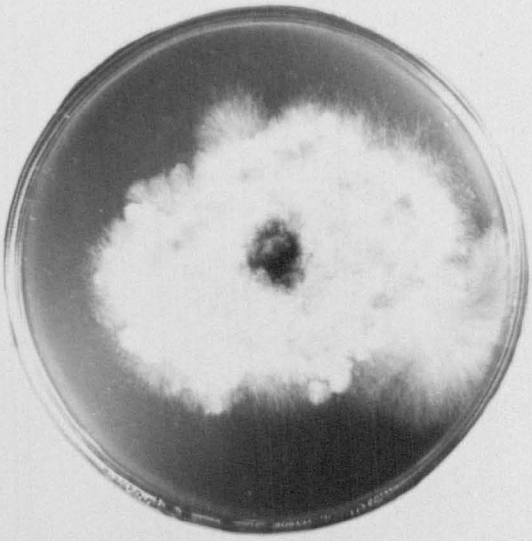
Plate 10

Morphological types obtained from isolate 32 AiiR.

ISOLATE 23AiR

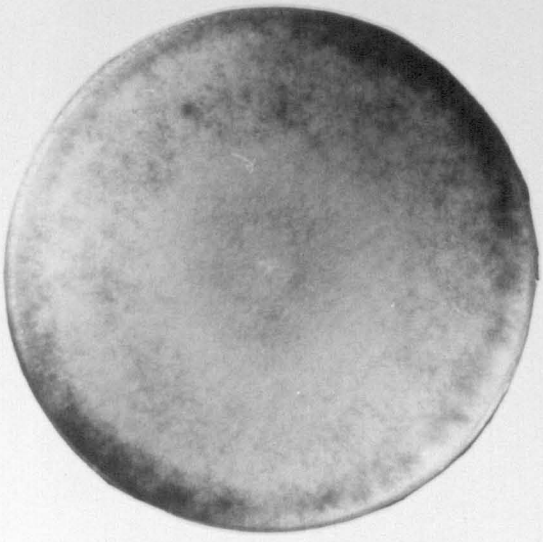


T_1

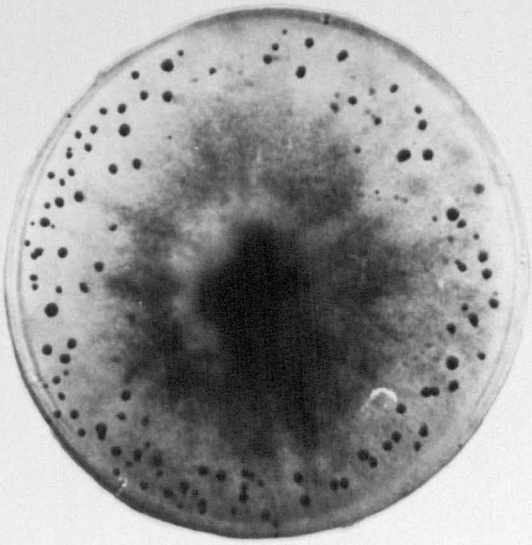


T_2

ISOLATE 32AiiR



T_1



T_2

45 AvS showed reduced vigour. Transfer of an 8 mm disc from the margin of a T_2 colony of this isolate resulted, however, in a vigorous growth identical to the T_1 type. It is possible, therefore, that in this case staling factors may have been responsible for the occurrence of the T_2 type growth.

5.2 The effect of increasing the number of single spore isolations on the appearance of morphological types in successive generations

In experiment 5.1 23 AiR had proved stable throughout 10 generations of subculturing. In order to test this stability more stringently the number of single spore isolations made from each generation was increased to 100. An eighth generation (G8) culture was used and this was subcultured for a further two generations.

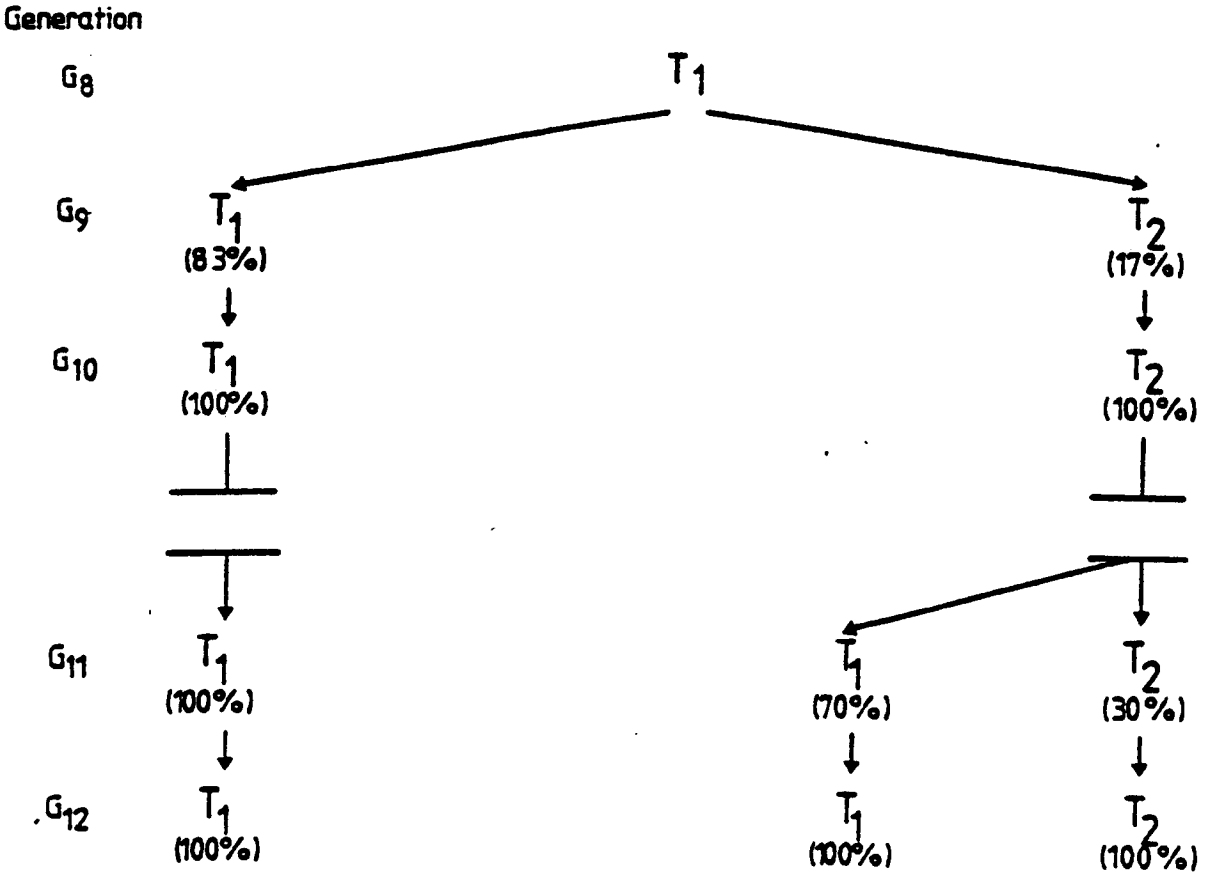
A T_2 type was found (Figure 7); this was greyish white with a limited, feather like growth (Plate 9) and remained stable when subcultured.

It is possible, therefore, that other morphological types may have been overlooked in the previous experiment, 5.1, because an insufficient number of single spores were examined. This consideration must apply to all isolates tested.

5.3 The effect of storage on the stability of morphological types obtained in experiment 5.2

The T_1 And T_2 types obtained from 23 AiR in the tenth generation (G10) were stored on PDA slopes at $4^{o}C$. After storage for nine months, 100 single spore isolations were made

Figure 7: Morphological types obtained from isolate 23 AiR (G_8)



Percentage in brackets indicates the number of each type in that generation out of 100 single spore cultures.

: isolates stored for nine months at 4°C

from each type and two further generations, G11 and G12, produced.

In G11 all single spore cultures from the T_1 type were identical to the parent colony (Figure 7). 70% of the T_2 type cultures, however, produced colonies of the T_1 type. This points to the hererogeneity of the sample and suggests that segregation of T_1 and T_2 components can be influenced by a period of cold storage.

5.4 An examination of the morphological variability exhibited by 20 isolates of B. cinerea

Experiments 5.1, 5.2 and 5.3 indicated that single spores taken from the four isolates examined tended to produce colonies of two distinct types, T_1 and T_2. This experiment was undertaken to ascertain comparatively rapidly whether the production of T_1 and T_2 types was characteristic of a sample of 20 isolates used in this project. 10 single spore isolates were prepared from each of the 20 parent isolates (10 benomyl sensitive, S and 10 benomyl tolerant, R). Resulting colonies were examined and recorded as in experiment 5.1. 10 single spore isolations (hyphal tip isolations in the case of non-sporulating cultures) were prepared from any morphologically distinct first generation cultures. All the progeny in the second generation were cultured on 100 p.p.m. benomyl and classified as benomyl tolerant or sensitive.

70% of isolates produced the two principal morphological types, T_1 and T_2, observed and described in experiment 5.1

(Figure 8). The remaining 30% produced only T_1 type cultures over two generations. The T_2 type cultures could be further subdivided, having either

(i) an ochreous central region surrounded by a zone of sporulation from which distorted hyphal growth occurred; or

(ii) a sporulating central region from which irregular hyphal growth occurred.

Many of the T_1 type cultures showed varying degrees of sectoring, recognized by indentations of the colony border. Sectors took the form of sparse mycelium with an ochreous tinge and were typically non sporulating. The experiment indicates (Figure 9) that T_1 morphological types are dominant over T_2 types and would appear to have a much higher degree of stability. It was noted, however, that mycelial distortions observed in T_2 type colonies was greatly reduced when cultured at the lower temperature of 15°C or 20°C. Examples of T_1 and T_2 types are illustrated in Plate 11. Morphological descriptions of types exhibited by the 20 isolates are given in Appendix III.

All second generation cultures retained the same sensitivity to benomyl as their parent isolate. Benomyl tolerant isolates appeared to have a similar degree of variability when subcultured as their sensitive counterparts.

Figure 8: Morphological types exhibited by 20 isolates of B. cinerea over two generations

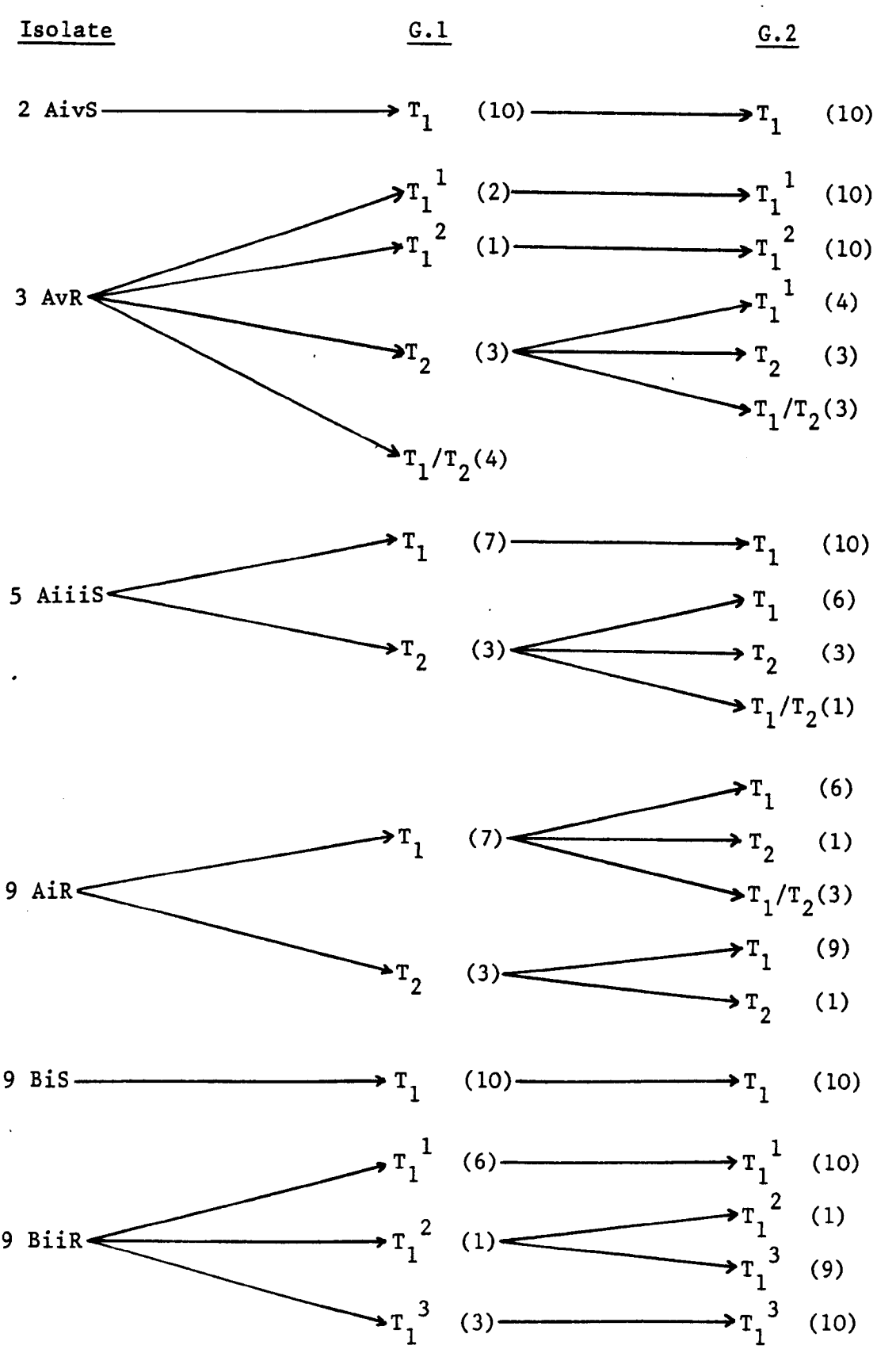


Figure 8: - continued

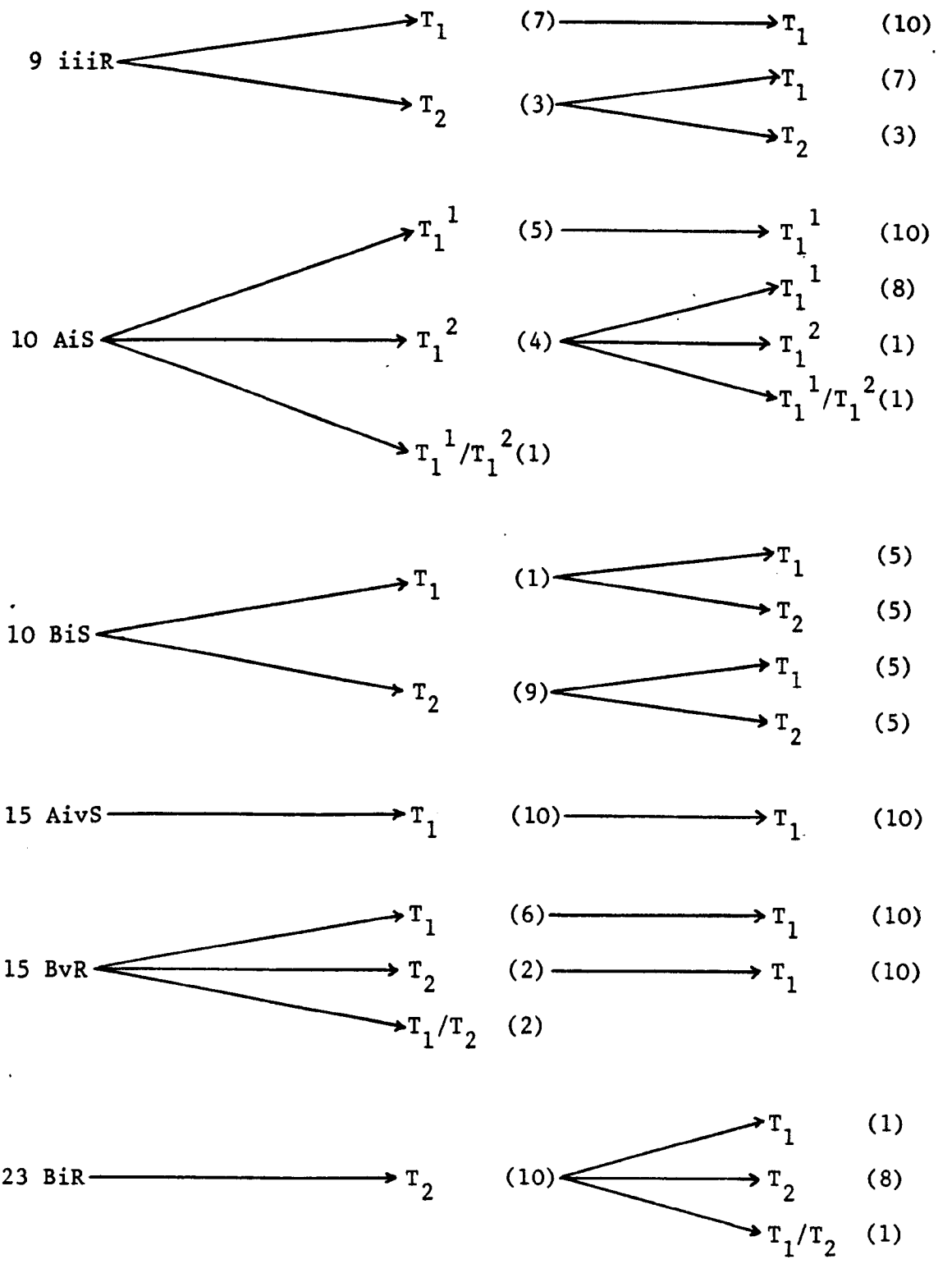


Figure 8: - continued

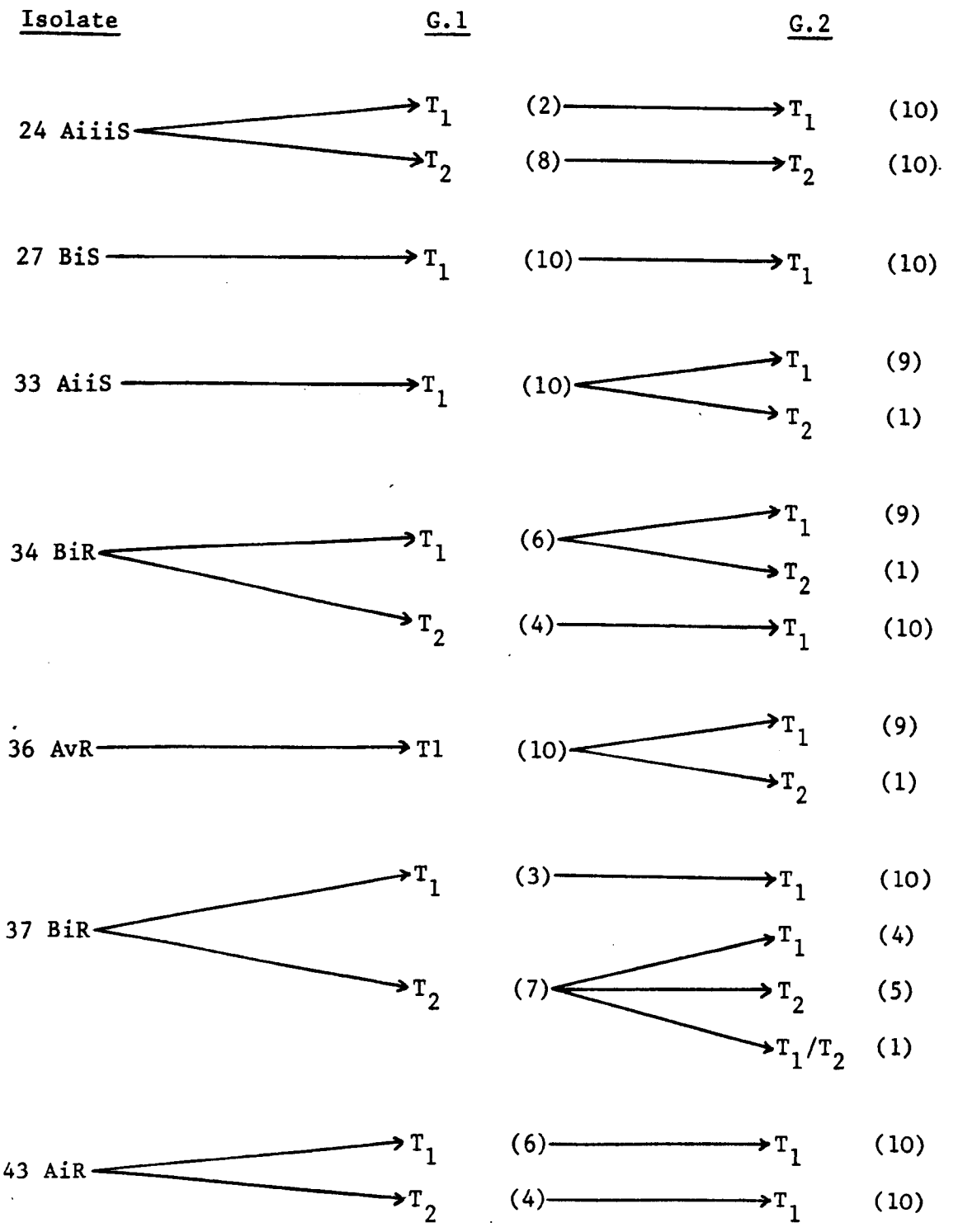


Figure 8: - continued

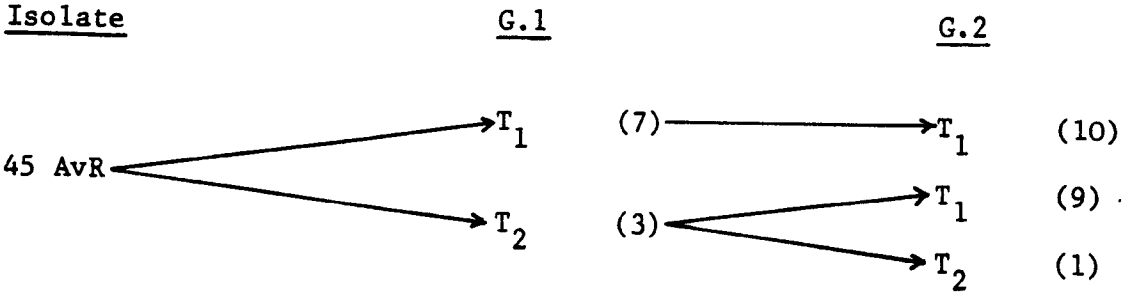
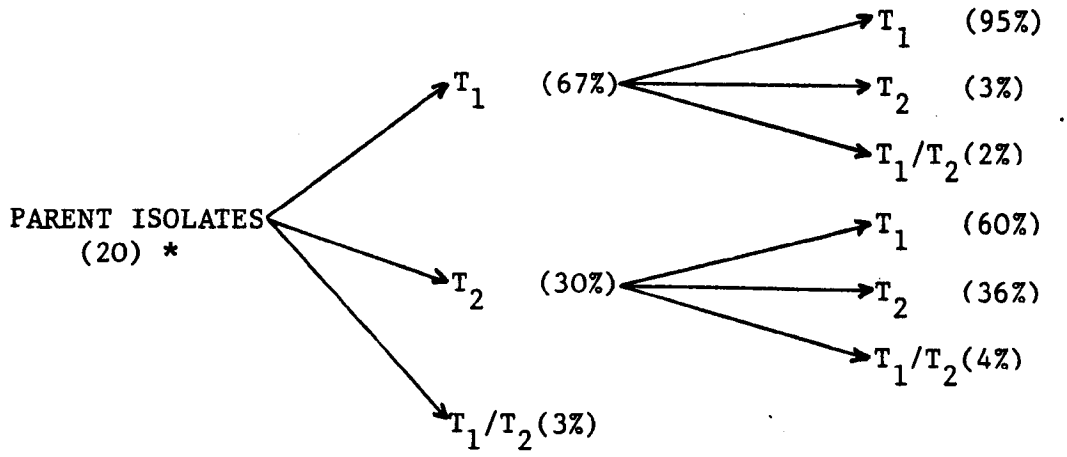


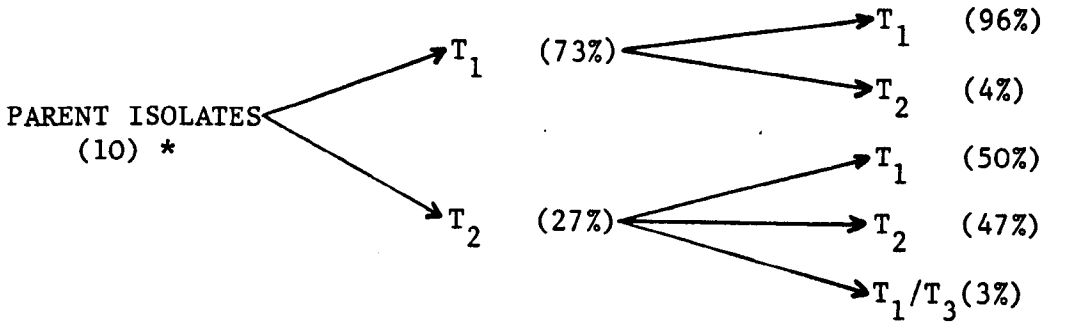
Figure in brackets after type designation is the number of isolations out of 10 giving rise to that type.

Figure 9: A summary of culture types obtained in experiment 5.4

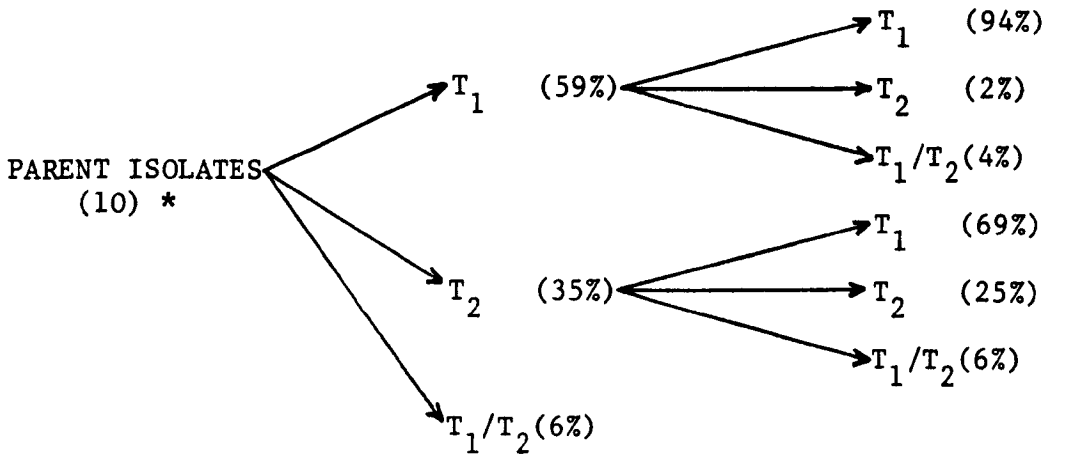
1. All isolates



2. Benomyl sensitive isolates



3. Benomyl tolerant isolates

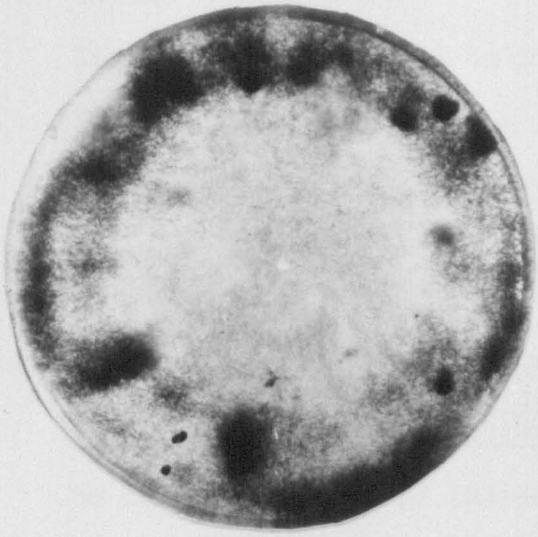


* Number of parent isolates

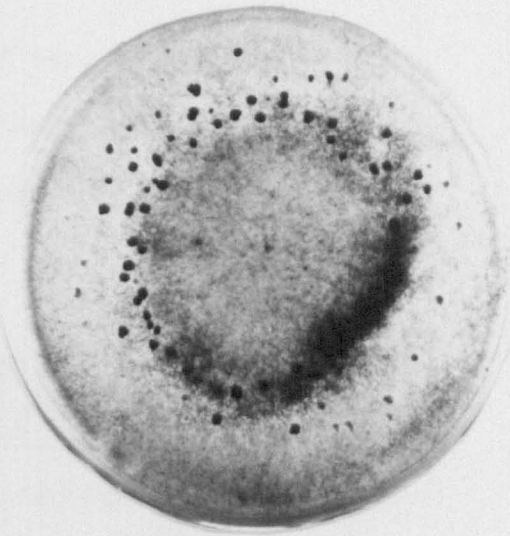
Figure in brackets after type designation is the percentage of isolations giving rise to that type.

Plate 11

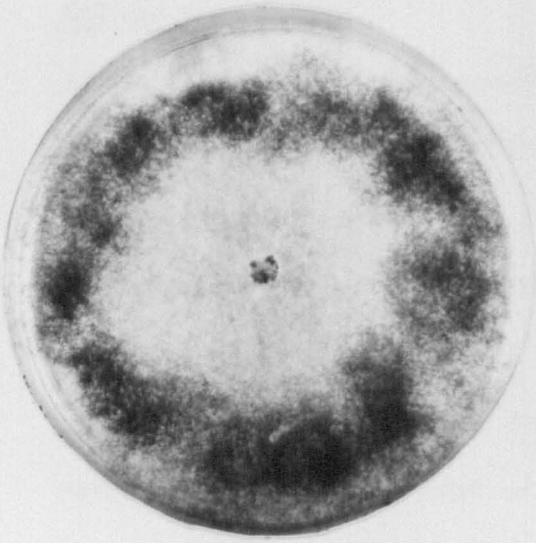
Examples of T_1 and T_2 morphological types exhibited by isolates of *B. cinerea*



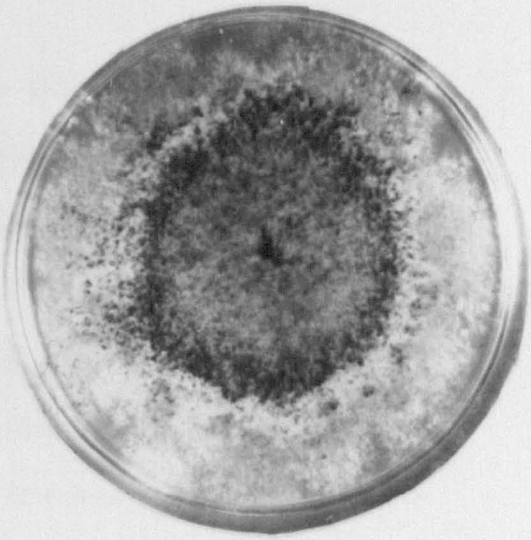
9Aiii T_1



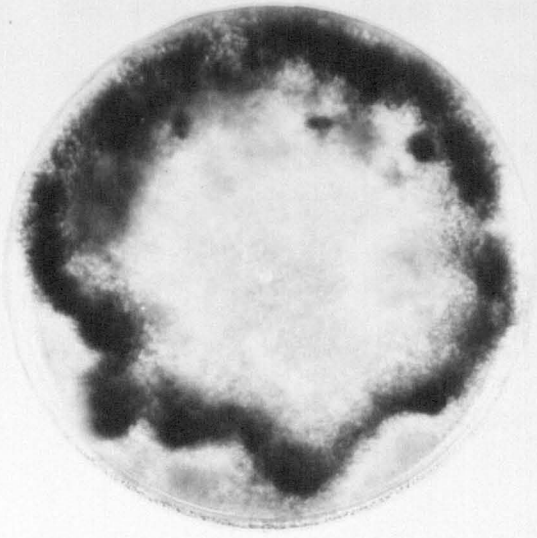
9Aiii T_2



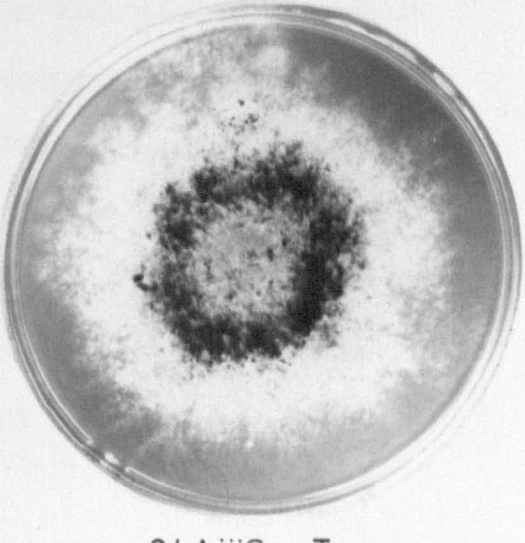
15Bv T_1



15Bv T_2



24AiiiS T_1



24AiiiS T_2

6. BENOMYL TOLERANCE IN ISOLATES OF B. CINEREA COLLECTED FROM TOMATO CROPS

The biology of benomyl tolerant and sensitive isolates of B. cinerea from glasshouse tomato crops was studied to ascertain if any fundamental differences existed between the two groups.

6.1 A comparison of benomyl tolerant and sensitive isolates of B. cinerea collected from different glasshouses on a number of tomato nurseries in Yorkshire and Lancashire

In this experiment 28 isolates from widely spaced sites were compared, all having a common host, the tomato.

Colony morphology

Isolates collected, as described in 4.3, were subcultured onto PDA, incubated at 20°C and a record made of their gross morphology (Table 3). The two morphological types, T_1 and T_2, were recognized as described in 5.1. Regular T_1 mycelium was produced by the majority of isolates (21) and was usually flat and greyish white in colour. Occasionally colonies became fluffy at the edge of the plate and in isolated cases the whole colony was fluffy. The intensity of sporulation varied considerably but was usually densest at the edge of the plate. Sclerotial production was also variable, both in the number formed and in their arrangement on the plate (Plate 12). The formation of appressoria like structures (small clusters of swollen hyphae) on the base of the plate was characteristic of a few of the isolates. The sparse distorted mycelium, T_2 type, was produced by seven of the isolates; four benomyl sensitive and three benomyl tolerant.

Table 3: The gross morphological characteristics of benomyl tolerant and sensitive isolates of B. cinerea collected from different glasshouses in the Yorkshire/Lancashire region

Key no. of isolate	Isolate	Appearance of mycelium *	Pattern and degree of sporulation		Pattern and degree of sclerotial production		Presence or absence of appressoria like structures	Plate no.
	Benomyl sensitive							
1	2 AivS	Flat	Edge	++++	Edge	+	-	12
2	5 AiiiS	Slightly fluffy with floculations	Edge	+++	Edge	+	✓	12
3	7 AiiiS	Sparse, distorted, flat	Uniform	++	Tending edge	++	✓	12
4	9 BiS	Flat	Uniform	+	Throughout	+++	-	12
5	9 AiiiS	Sparse, distorted, flat	Uniform	++	Throughout	+++++	-	12
6	15 AivS	Flat	Edge	+++	0		-	12
7	19 BiS	Sparse, distorted, flat	Uniform	++	Edge	++	-	12
8	24 AiiS	Flat	Edge	+++	Tending edge	+++	✓	12
9	24 AiiiS	Flat	Edge	++++	Tending edge	+	-	12
10	24 BivS	Flat, slightly fluffy at edge	Edge	++	Throughout	++	-	12
11	27 AiS	Slightly fluffy	Edge	++++	Throughout	+	-	12
12	33 AiiS	Flat	Edge	++++	Throughout	+	-	12
13	45 AvS	Flat	Edge	++	Tending edge	+++	-	12
14	46 AiiiS	Sparse, distorted, flat	Uniform	++	Throughout	++++	-	12

Table 3: - continued

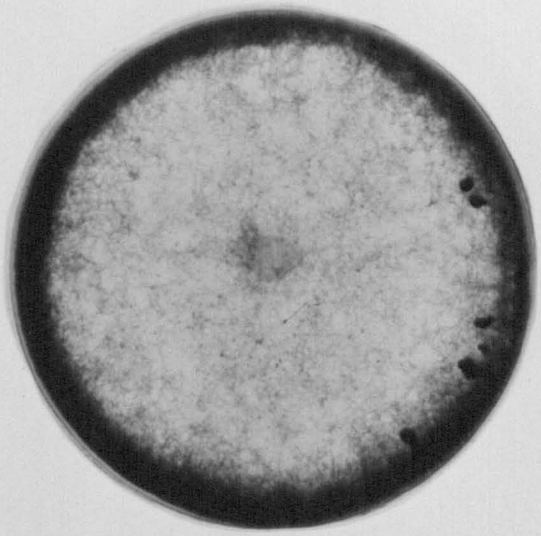
Key no. of isolate	Isolate	Appearance of mycelium *	Pattern and degree of sporulation	Pattern and degree of sclerotial production	Presence or absence of appressoria like structures	Plate no.
	Benomyl tolerant					
15	4 BiiR	Slightly fluffy	Edge +++	Edge +	✓	12
16	12 AvR	Flat	Uniform +++++	Throughout +	✓	12
17	15 BvR	Slightly fluffy	Edge +++	Edge +	-	12
18	18 AiiR	Flat	Edge +++	Tending edge ++	-	12
19	21 AvR	Flat	Edge ++	Tending edge ++	✓	12
20	23 AiR	Flat	Edge ++++	Throughout ++	-	12
21	23 BiR	Sparse, distorted, flat	Uniform ++	Throughout +++	-	12
22	25 AiR	Slightly fluffy	Tending edge +++	Edge +	-	12
23	32 AiiR	Very fluffy	Edge ++	Edge ++	-	12
24	34 BiR	Slightly fluffy	Uniform +++	0	-	12
25	36 AvR	Flat	Tending edge ++++	Edge ++	-	12
26	37 BiR	Slightly fluffy	Uniform +++	Edge ++	-	12
27	43 AiR	Sparse, distorted, flat	Uniform +	Throughout +++	-	12
28	44 BiiR	Sparse, distorted, flat	Uniform ++	Throughout ++++	-	12

* All observations based on an examination of five replicate plates per isolate.
✓ present; - absent

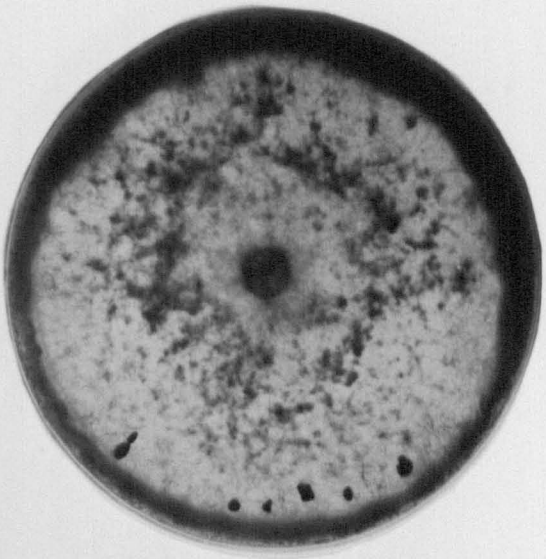
Plate 12

B. cinerea isolates collected from different glasshouses in Yorkshire and Lancashire

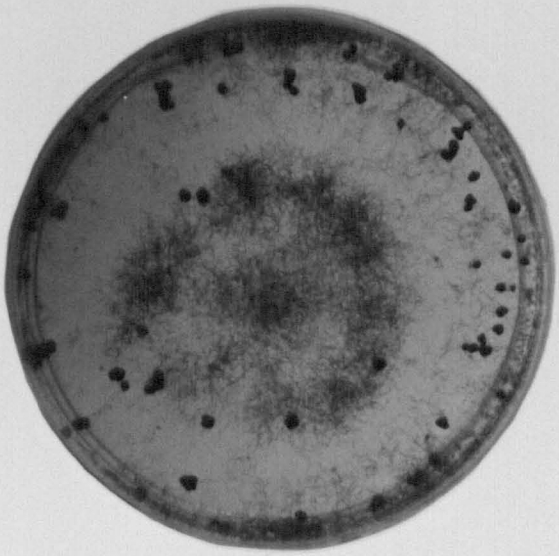
Benomyl sensitive isolates:	2 AivS,	5 AiiiS,	7 AiiiS,
	9 AiiiS,	9 BiS,	15 AivS,
	19 BiS,	24 AiiS,	24 AiiiS,
	24 BivS,	27 AiS,	33 AiiS,
	45 AvS,	46 AiiiS.	
Benomyl tolerant isolates:	4 BiiR,	12 AvR,	15 BvR,
	18 AiiR,	21 AvR,	23 AiR,
	23 BiR,	25 AiR,	32 AiiR,
	34 BiR,	36 AvR,	37 BiR,
	43 AiR,	44 BiiR.	



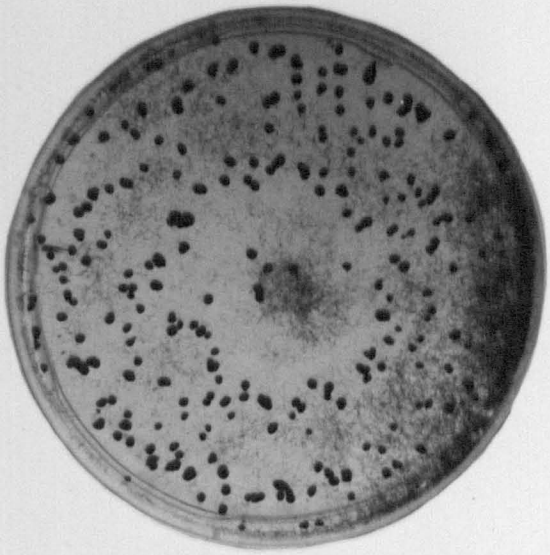
2AivS



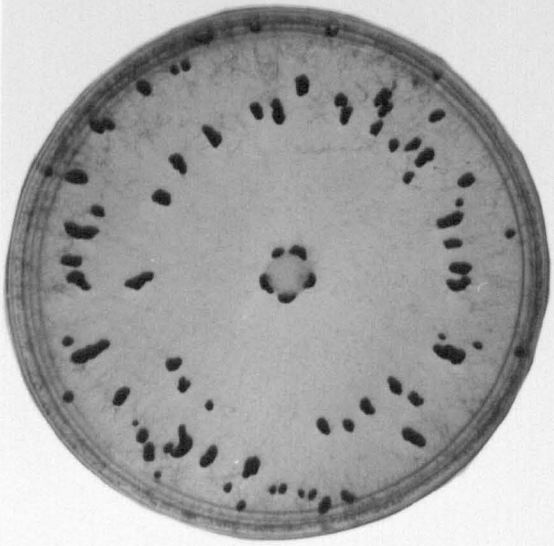
5AiiiS



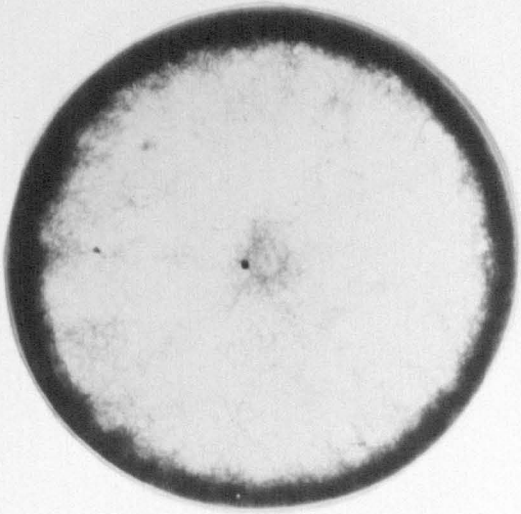
7AiiiS



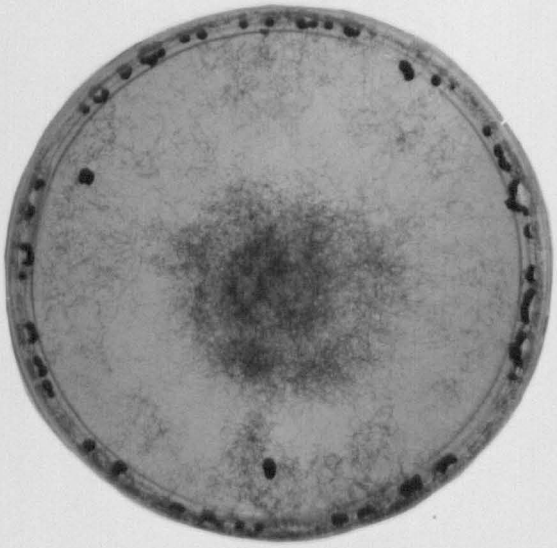
9AiiiS



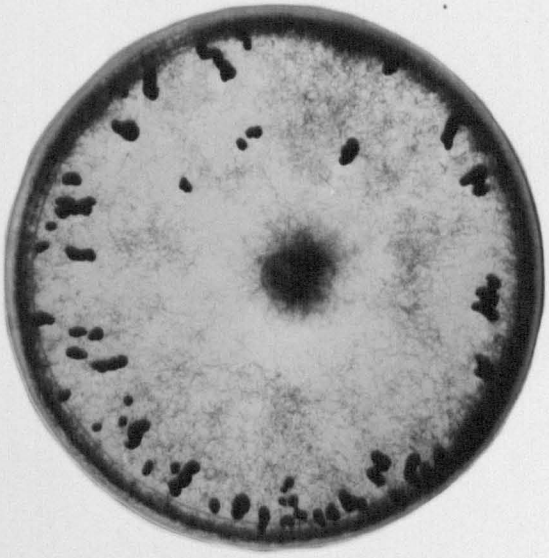
9BiS



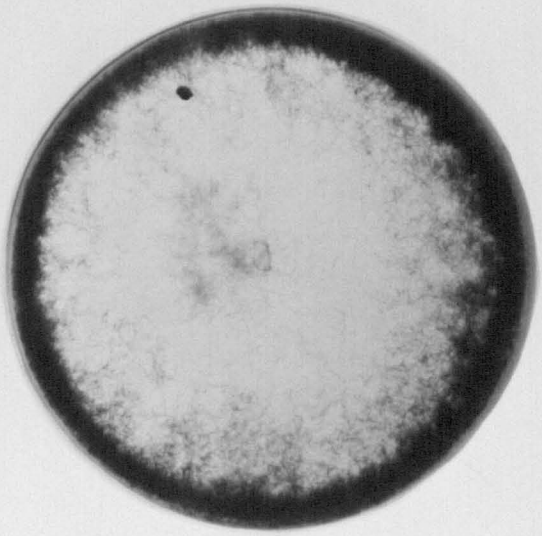
15AivS



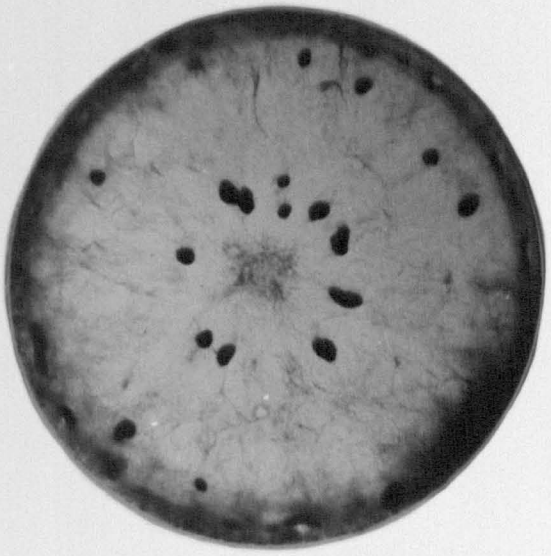
19BiS



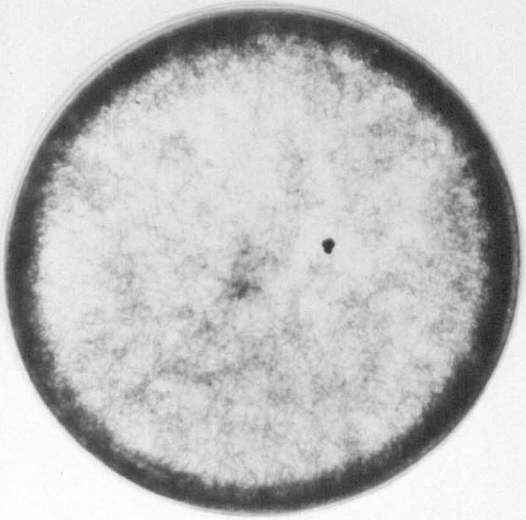
24AiiS



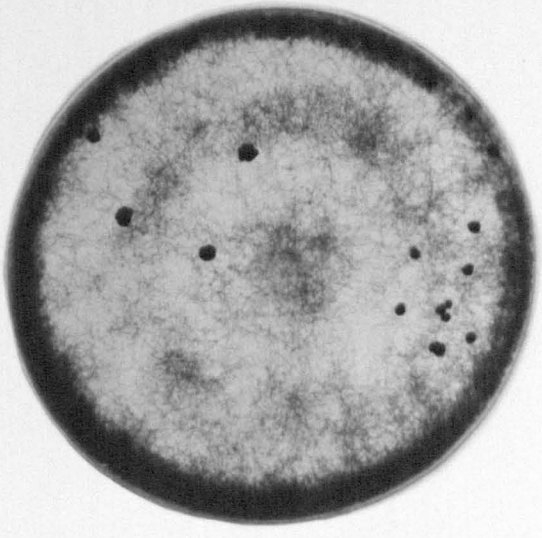
24AiiiS



24BivS

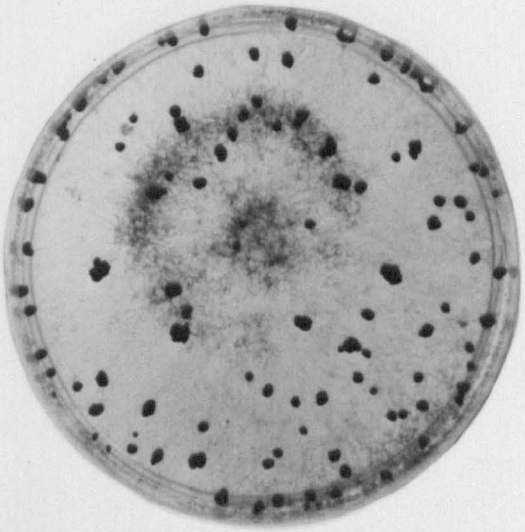


27AiS

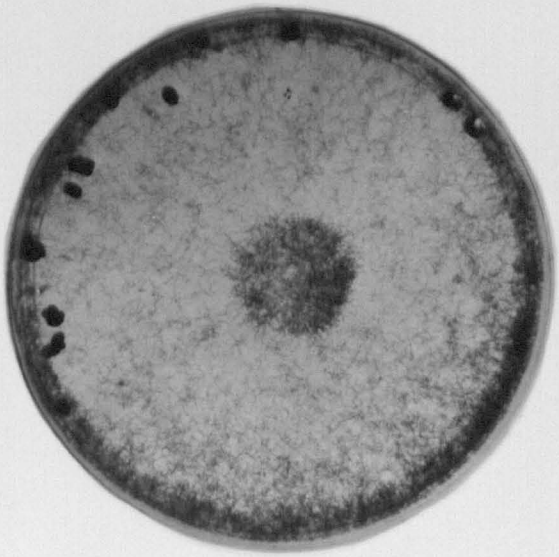


33AiiS

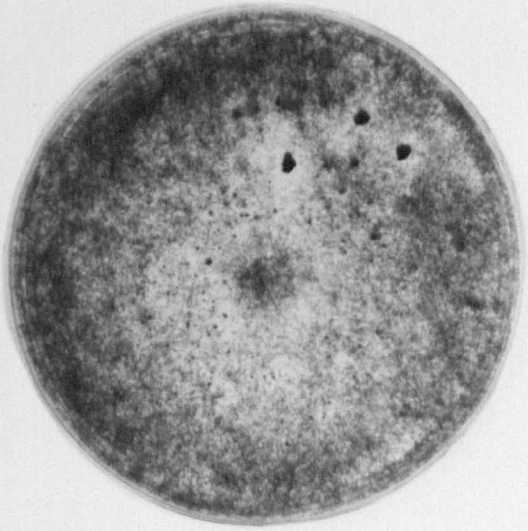
45AvS



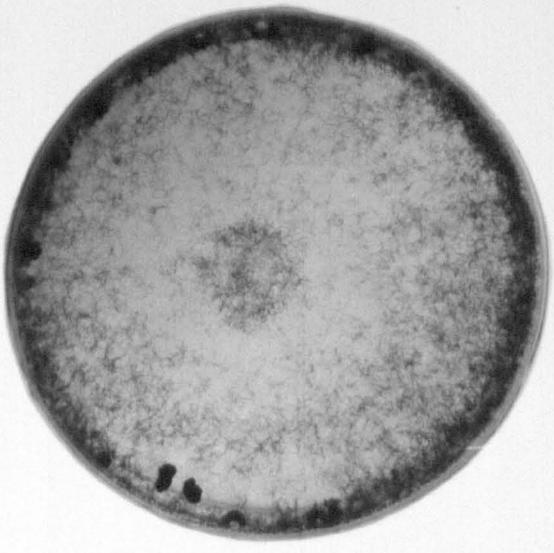
46AiiiS



4BiiR

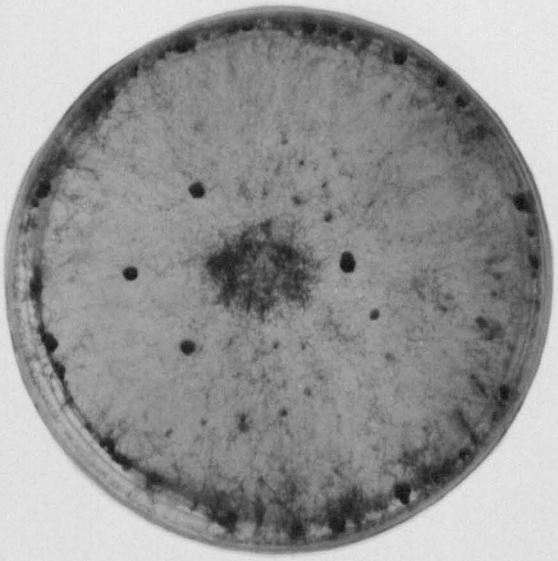


12AvR

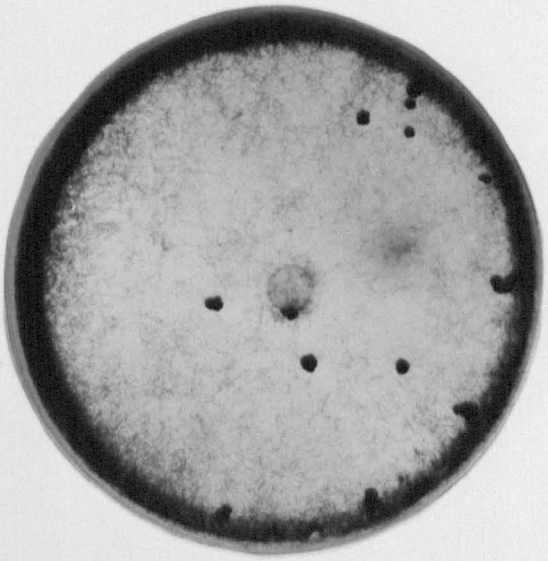


15BvR

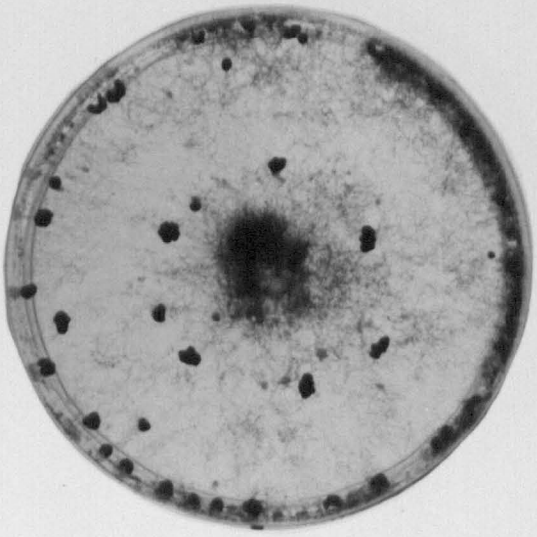
18AiiR



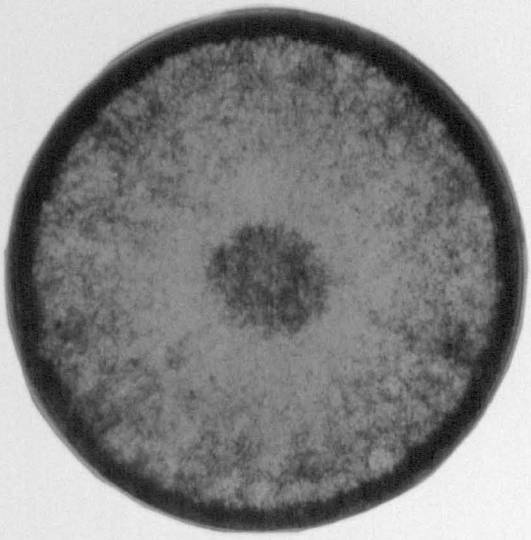
21AvR



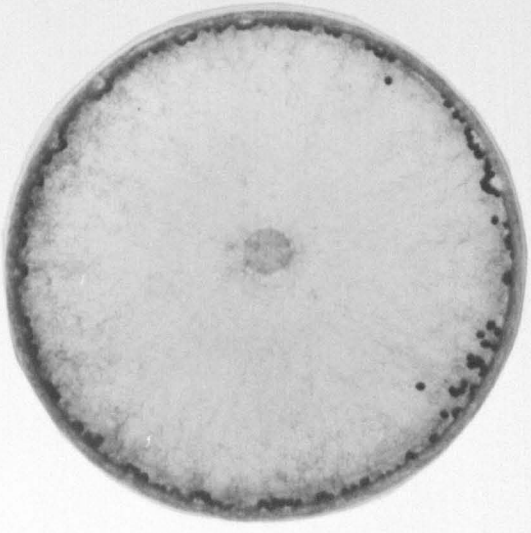
23AiR



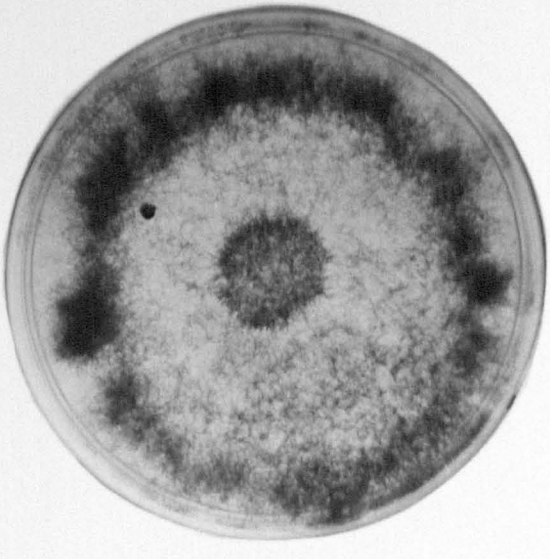
23BiR



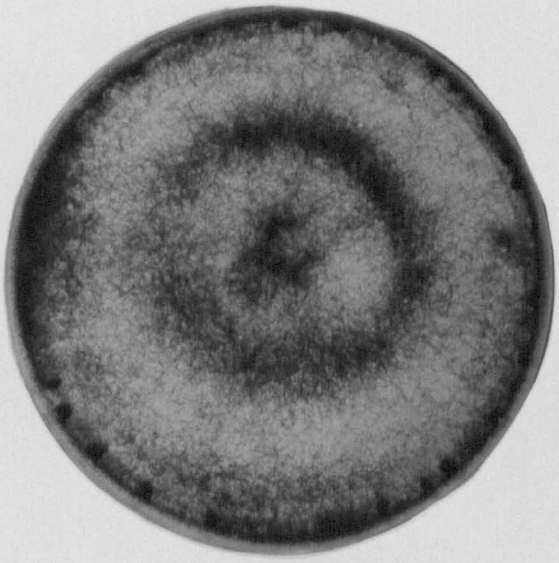
25AiR



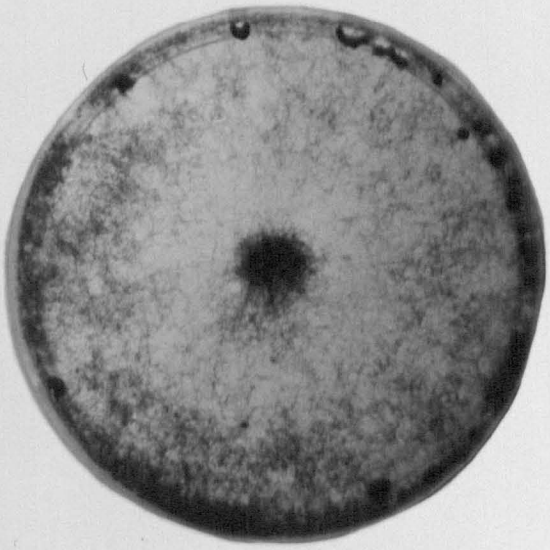
32AiiR



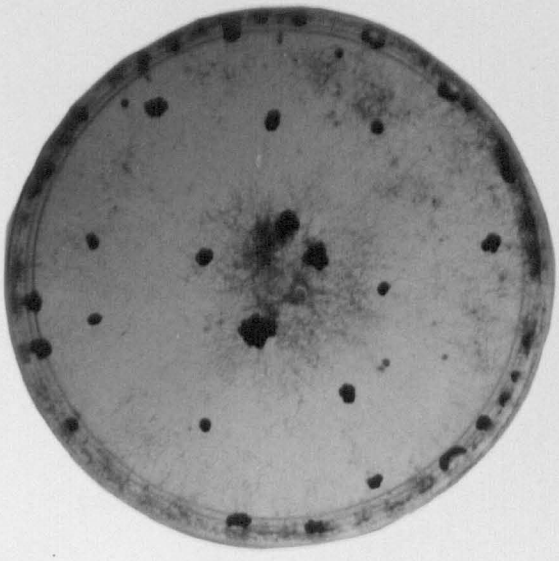
34BiR



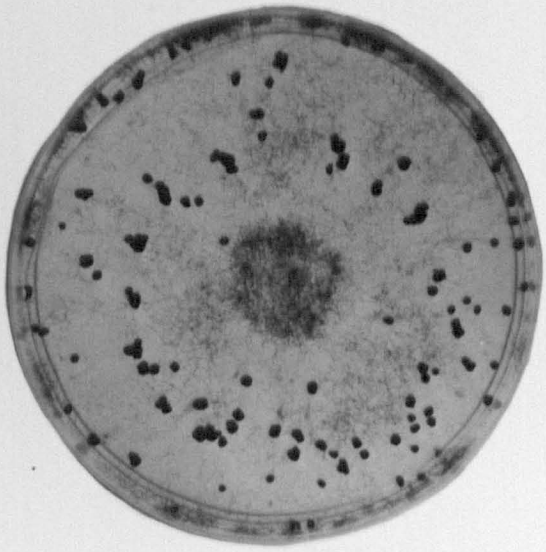
36AvR



37BiR



43AiR



44BiiR

It was concluded that although there was considerable variation between isolates, benomyl tolerant and sensitive isolates could not be separated on the basis of the gross morphological features examined.

Growth rate on PDA

The degree of mycelial spread was recorded after three days at 20°C (Figure 10). Significant differences were found in the growth rates of isolates; the majority of benomyl tolerant isolates were found to be slower growing than their sensitive counterparts.

Pathogenicity

The pathogenicity of the isolates was measured using the excised cotyledon test (Figure 11). Significant differences were found in the rate of lesion extension. These differences, however, could not be correlated with isolates sensitivity or tolerance to benomyl. Similarly growth rate on PDA was not significantly correlated with pathogenicity.

6.2 A comparison of benomyl tolerant and sensitive isolates of B. cinerea collected from a single tomato glasshouse

In the previous experiment (6.1) the isolates examined were collected from a number of glasshouse tomato crops spread over a wide area. In this experiment isolates from single glasshouses were compared. Two nurseries, two miles apart were selected for study: Chartwell growers of Hesketh Bank, Lancashire (B1) and North West Growers of Banks, Lancashire (L1). An A.D.A.S. investigation in 1974 showed the presence of benomyl tolerant

Figure 10: The growth rate of B. cinerea isolates collected from different glasshouses in the Yorkshire/Lancashire region on PDA

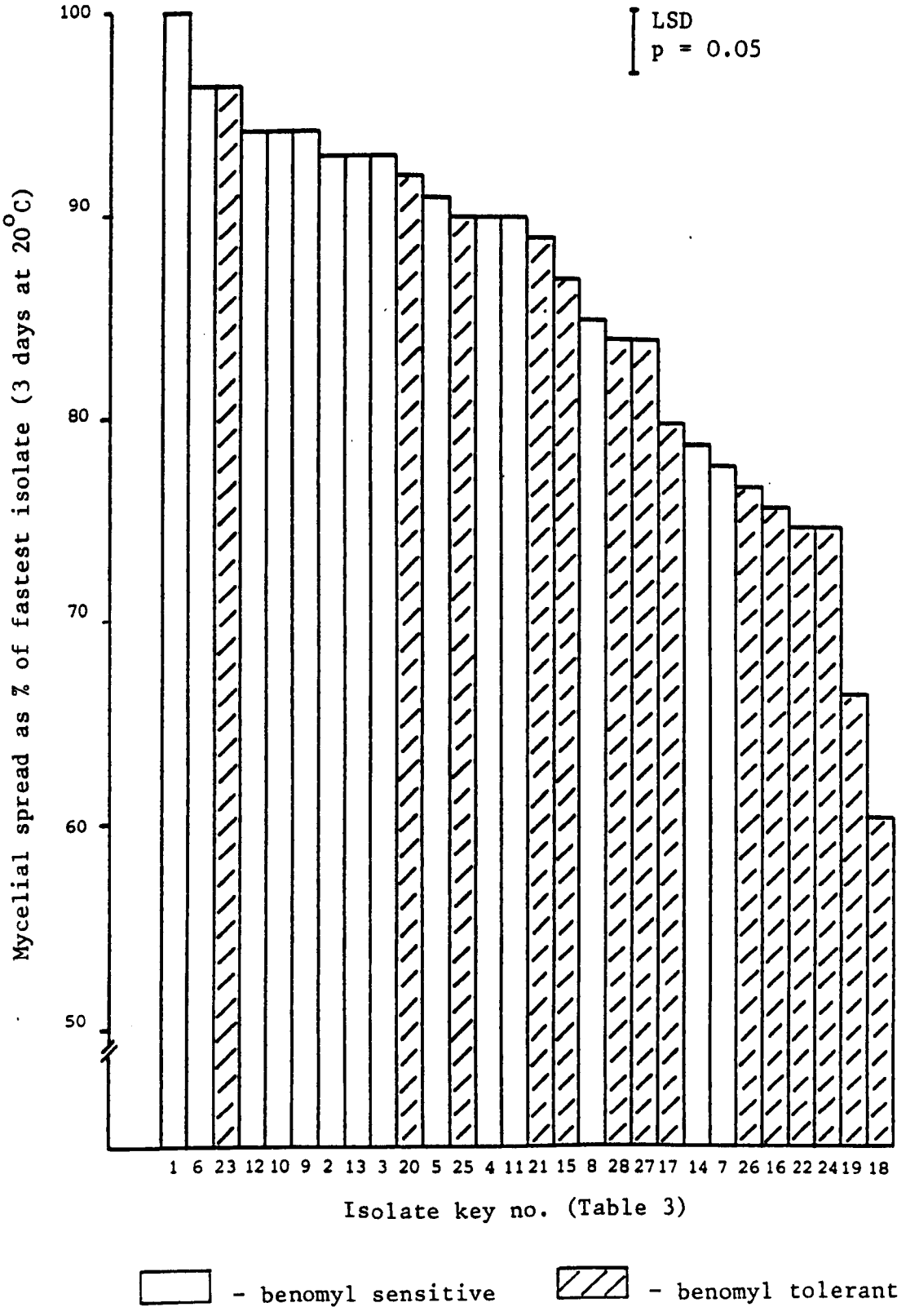
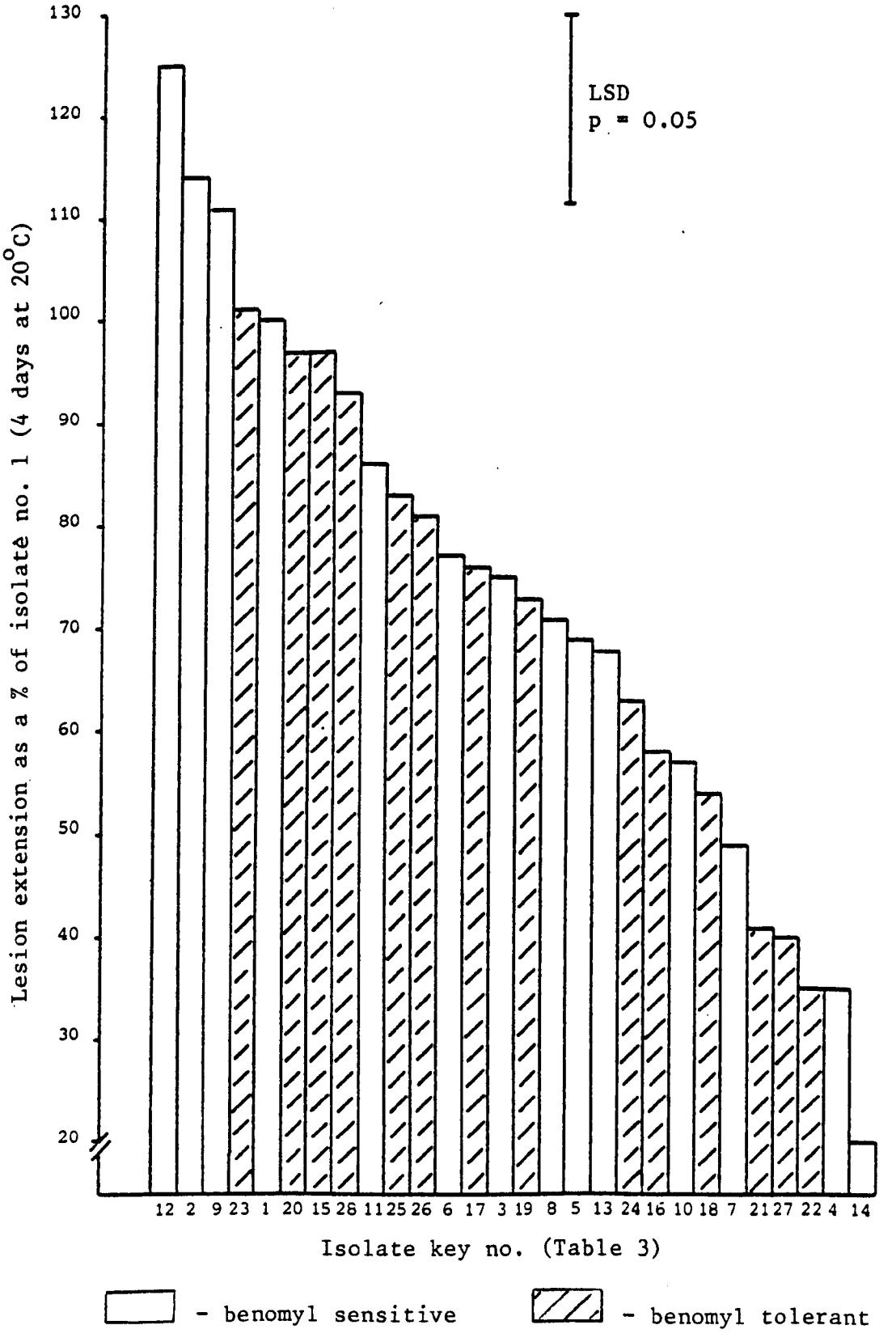


Figure 11: The growth rate of benomyl tolerant and sensitive isolates of B. cinerea collected from different glasshouses in the Yorkshire/Lancashire region on excised tomato cotyledons



strains at both nurseries. Benzimidazole fungicides, however, had not been used at either nursery since 1974, two years prior to this investigation. 40 lesions were sampled at random from one glasshouse at each nursery.

Glasshouse B1

All lesions produced tolerant isolates. One hundred spores from each lesion all produced colonies on benomyl agar, indicating that 100% of the spores in each lesion were benomyl tolerant. It was therefore impossible to make a comparison between tolerant and sensitive isolates at this site. The tolerant isolates were examined, however, to determine the degree of variation existing within the group. Isolates were subcultured on both unamended PDA and PDA containing 100 p.p.m. benomyl.

Colony morphology

Unamended PDA: Isolates were very similar, all having a rapidly growing greyish white mycelium (T_1 type). The majority of spores were formed at the edge of the plate, although there was considerable variation in density. Similar variation occurred in the number and size of sclerotia (Table 4).

PDA containing 100 p.p.m. benomyl: The addition of benomyl caused the mycelium to become slightly fluffy. Sporulation tended to occur uniformly over the colony. The production of sclerotia was delayed and fewer formed than when isolates were grown on unamended PDA (Table 4).

Table 4: Conidial and sclerotial production by benomyl tolerant isolates of *B. cinerea* collected from a single glasshouse (B1), in the presence and absence of benomyl

Isolate	Conidial production		Sclerotial production	
	Unamended PDA	PDA + 100 p.p.m. benomyl	Unamended PDA	PDA + 100 p.p.m. benomyl
1	++	++++	++	+
2	+++	++++	+	0
3	+++	++	+	++
4	++	+++	+	+
5	++	+++	+	0
6	++	++++	+	0
7	++	+++	++	+
8	+	+++	+++	++
9	++	++++	+	+
10	++	++++	+++	+
11	++	+++	+	0
12	++	++++	+++	0
13	+	+++	++	++
14	+++	+++	0	0
15	+++	++++	++	0
16	++	+++	+	0
17	+++	++++	+++	0
18	+++	+++	+	++
19	++	++++	+	0
20	++	+++	+	0
21	+	+++	++	0
22	++	+++	+	0
23	++	+++	++	+
24	+	++	+++	++
25	++	++++	+++	++
26	+	+++	+++	+
27	+++	++++	+	0
28	++	+++	++	+
29	+++	++	+	+
30	+++	++++	++	0
31	+	+++	+++	+
32	++	++	+++	+
33	++	+++	+	+
34	+++	++++	+	0
35	+++	+++	+	0
36	+	+++	+	0
37	++	++	+++	++
38	++	++++	+++	0
39	++	++++	+	+
40	++	++++	+	0
Mean score	2.1 +	3.5 +	1.7 +	0.7 +

Growth rate on PDA

The rate of mycelial spread of isolates differed considerably at 20°C (Figure 12). The addition of 100 p.p.m. benomyl to the agar reduced spread by about 50%, although the degree of inhibition varied considerably between isolates (Figure 13).

Glasshouse L1

12 of the 40 lesions sampled produced benomyl tolerant isolates. One hundred single spores taken from each of the 12 lesions giving tolerant isolates revealed that in nine lesions less than 1% of the spores were tolerant, whereas in the remaining three lesions 100% of the spores were tolerant. This was the first evidence obtained that lesions could contain mixtures of tolerant and sensitive spores. 10 benomyl tolerant and 10 sensitive isolates were selected at random for further examination.

Colony morphology

Benomyl sensitive isolates all produced a very similar flattened mycelium which became slightly raised at the edge of the plate (T_1 type). Sporulation was concentrated in this region although density varied considerably. Sclerotia also varied in size, number and position on the plate. Isolate 22S produced appresoria like structures on the base of the plate (Table 5). Benomyl tolerant isolates were more variable in morphology, with three mycelial forms being recognized:

(i) very flat;

(ii) flat becoming fluffy at the edge of the plate;

Figure 12: The growth rate of isolates of B. cinerea collected from glasshouse B1 on PDA

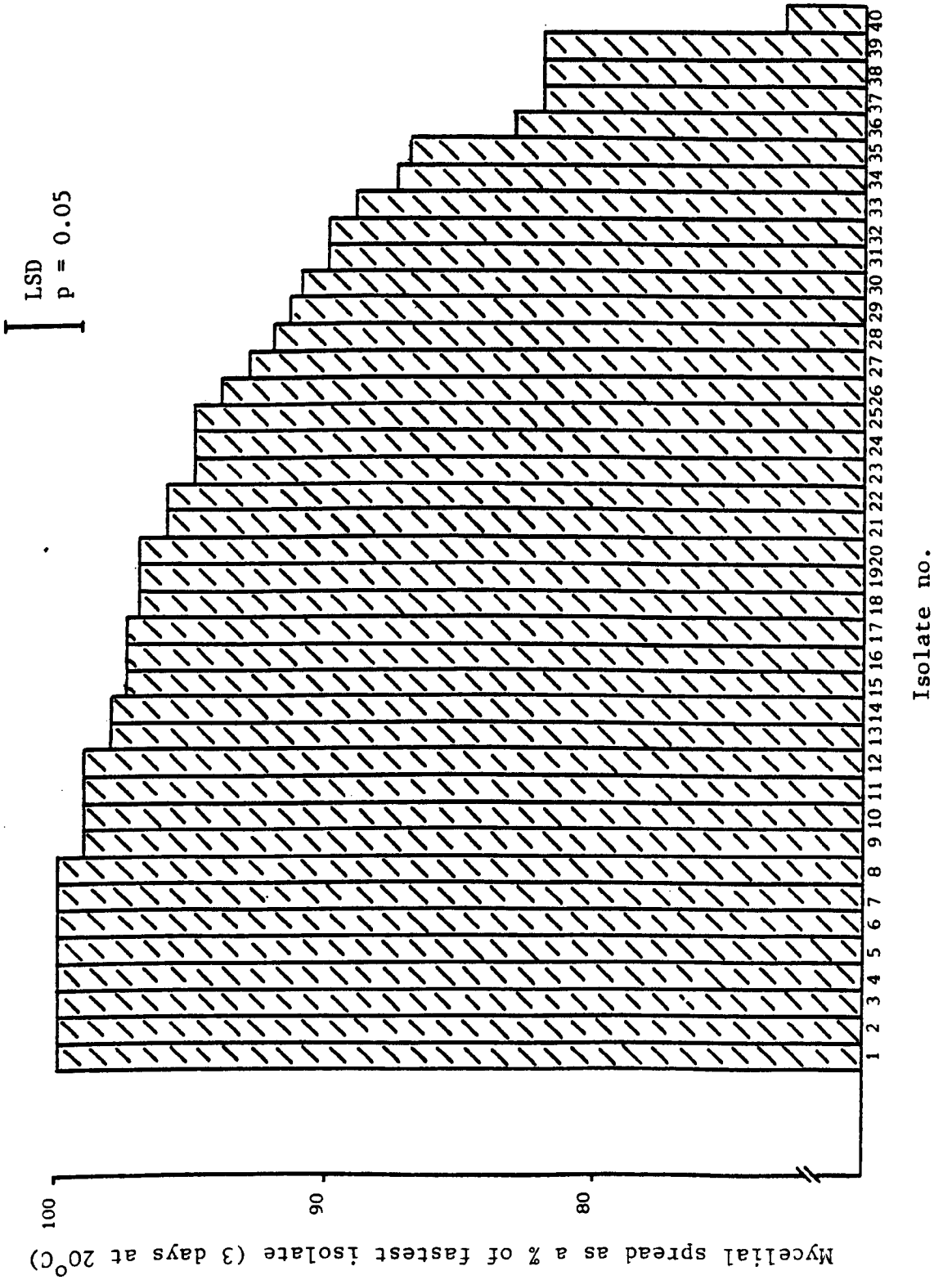
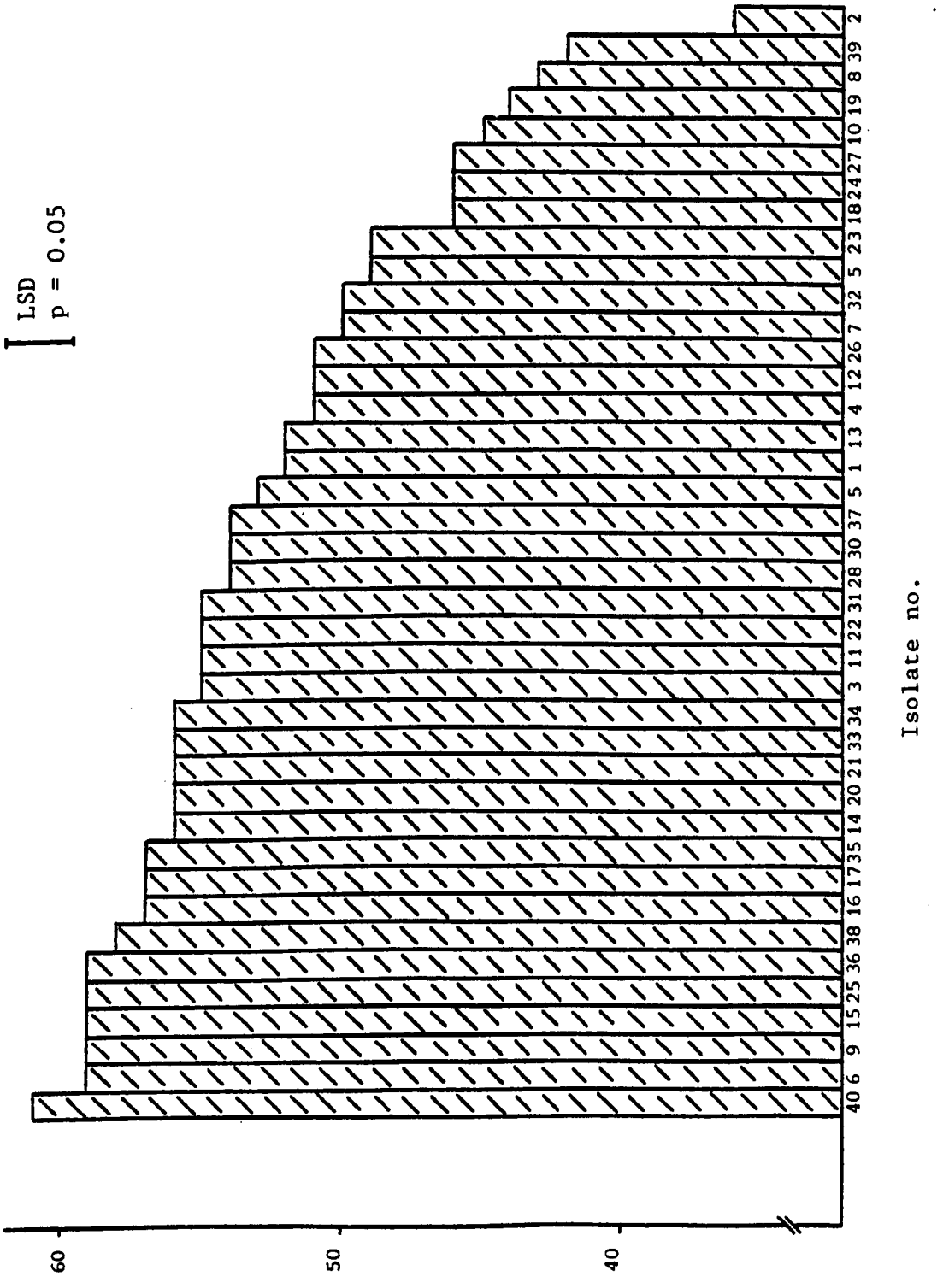


Figure 13: Percentage inhibition of growth of benomyl tolerant isolates of B. cinerea collected from glasshouse B1 on PDA containing 100 p.p.m. benomyl when compared to growth on unamended PDA



(iii) slightly fluffy.

All were of the T_1 type. The pattern and density of sporulation and sclerotial production was very variable and 17R, 30R, 32R and 34R also produced appresoria like structures (Table 5). Morphological differences were sufficient to allow visual separation of seven of the ten isolates examined. 30R, 32R and 34R, however, were morphologically indistinguishable.

Growth rate on PDA

Significant differences in the rate of mycelial spread were observed between isolates at 20°C. In addition the majority of tolerant isolates were slower growing than their sensitive counterparts (Figure 14).

Pathogenicity

The pathogenicity of the isolates was compared using the excised cotyledon test (Figure 14). Significant differences were found in the rate of lesion extension and there were indications that the majority of tolerant isolates were less pathogenic than their sensitive counterparts.

6.3 An examination of the persistence and fitness of benomyl tolerant strains of B. cinerea isolated from 17 glasshouse tomato crops in Yorkshire and Lancashire

Observations indicated that differences existed between isolates with respect to their growth rate on PDA and their pathogenicity. Experiments (6.1 and 6.2) suggested that tolerant isolates may be slower growing than sensitive ones on PDA and, within a single glasshouse, may also be less pathogenic. The value of comparing isolates from over a

Figure 14: The growth rates of benomyl tolerant and sensitive isolates of *B. cinerea* collected from glasshouse L1 on excised tomato cotyledons and PDA

Table 5: The gross morphology of benomyl tolerant and sensitive isolates of B. cinerea collected from a single glasshouse (L1)

Isolate key no.	Isolate	Appearance of mycelium *	Pattern and degree of sporulation		Pattern and degree of sclerotial production		Presence or absence of appressoria like structures
	Benomyl sensitive						
1	1 S	Flat, slightly raised at edge	Edge	++	Edge	++	-
2	5 S	Flat, slightly raised at edge	Edge	++	Edge	+++	-
3	9 S	Flat, slightly raised at edge	Edge	+++	Edge	++	-
4	11 S	Flat, slightly raised at edge	Edge	+++	0		-
5	13 S	Flat, slightly raised at edge	Edge	+++	Throughout	+	-
6	22 S	Flat, slightly raised at edge	Edge	++	Edge	++++	✓
7	24 S	Flat, slightly raised at edge	Edge	++	Edge	+++	-
8	27 S	Flat, slightly raised at edge	Edge	+++	Throughout	++	-
9	33 S	Flat, slightly raised at edge	Edge	+++	Throughout	++	-
10	37 S	Flat, slightly raised at edge	Edge	++++	Tending edge	+	-
	Benomyl tolerant						
11	3 R	Flat	Uniform	++	Throughout	++++	-
12	4 R	Flat	Uniform	+++	Throughout	+++	-
13	14 R	Flat	Edge	+	Throughout	++	-
14	17 R	Flat, very fluffy at edge	Edge	++	Throughout	+	✓
15	19 R	Flat	Uniform	++	Tending edge	++	-
16	20 R	Flat	Uniform	++++	0		-
17	29 R	Flat	Edge	+++	Edge	++	-
18	30 R	Slightly fluffy	Tending edge	+++	Throughout	+	✓
19	32 R	Slightly fluffy	Tending edge	+++	Throughout	+	✓
20	34 R	Slightly fluffy	Tending edge	+++	Throughout	+	✓

* All observations were based on an examination of five replicate plates per isolate
✓ present; - absent

wide area can be questioned since distinct populations of the fungus may exist within the confines of a single glasshouse. Owing to inherent variability, strains of B. cinerea making up one population are likely to differ from strains in another population if the environmental conditions in both glasshouses are not the same. Consequently, it was decided to examine sets of isolates, each set collected from a single glasshouse.

In 1977, 40 B. cinerea isolates were collected from each of 17 glasshouse tomato crops on 15 nurseries in the Yorkshire/ Lancashire region. Details of fungicide use at all sites were recorded (Table 6). Benomyl tolerant isolates were obtained from all the glasshouses sampled (Table 6). Populations were usually mixed, both tolerant and sensitive strains being found within the same glasshouse. Stem lesions from eight glasshouses also contained both tolerant and sensitive spores (Table 7). The history of benomyl application varied but there was no apparent relationship between the date of the last reported fungicide use and the ratio of tolerant to sensitive isolates.

In 1976, 1977 and 1978 isolates were obtained from two nurseries, the same glasshouse being sampled each year. The percentage of tolerant isolates collected from the two glasshouses sampled in these years showed only small changes (Table 8). Benomyl had not been used in these houses since 1974.

10 glasshouses, each containing mixed populations of tolerant

Table 6: Number of benomyl tolerant isolates of B. cinerea collected from tomatoes in different glasshouses.

Nursery and glasshouse	Date sampled (1977)	Cultivar and area (ha)	Fungicides applied in 1977	Year benomyl last applied	Tolerant isolates (out of 40)
B1	21 Aug.	Sonato (0.1)	Dichlofluanid	1974	40
B2	21 Aug.	Sonato (0.2)	Thiram	1974	38
L1	18 Aug.	Curabel (0.1)	Chlorothalonil	1974	18
L2	18 Aug.	Sonato (0.3)	Dichlofluanid	1974	14
A1	23 Aug.	Erasta (0.1)	Dichlofluanid	1975	1
J1	15 Aug.	Sonato (0.1)	Dichlofluanid Iprodione	1975	24
K1	23 Aug.	Extase (0.1)	None	1975	8
M1	15 Aug.	Sonato (0.2)	Dichlofluanid Iprodione	1975	16
G1	5 Sep.	Curabel (0.1)	Thiram	1976	33
I1	18 Aug.	Curabel Verosa (0.1)	Iprodione	1976	9
N1 *	22 Aug.	Curabel (0.1)	Dichlofluanid Iprodione	1976	17
C1	18 Aug.	Curabel Extase Stacos (0.1)	Benomyl	1977	40
D1	24 Aug.	Curabel (0.1)	Benomyl Dichlofluanid Iprodione	1977	40
E1	20 Jul.	Sonato (0.3)	Benomyl Dichlofluanid	1977	40
E2	20 Jul.	Verosa (0.1)	Iprodione Thiram	1977	39
F1	15 Aug.	Extase Kingsley Cross (0.1)	Benomyl Dichlofulanid	1977	34
H1	6 Feb.	Sonato (0.1)	Benomyl	1977	26

* New glasshouse constructed in 1977 on previously uncultivated land

Table 7: Percentage benomyl tolerant spores in samples of 10 lesions containing tolerant strains of *B. cinerea*

Glasshouse	Benomyl tolerant spores in each lesion (per cent) 1	2	3	4	5	6	7	8	9	10
B2	100	100	100	100	100	100	100	100	100	100
C1	100	100	100	100	100	100	100	100	100	100
D1	100	100	100	100	100	100	100	100	100	100
E1	100	100	100	100	100	100	100	100	100	100
F1	100	100	100	100	100	100	100	100	36	100
G1	100	100	90	100	100	100	100	< 1	100	100
H1	100	100	100	< 1	100	100	100	100	100	100
I1	100	< 1	100	< 1	100	< 1	< 1	38	100	-
J1	100	100	100	100	100	100	1	100	100	100
K1	100	100	100	43	100	< 1	< 1	-	-	-
L1	100	100	100	100	100	100	100	100	100	100
M1	100	100	70	100	< 1	100	100	100	100	100
N1	2	100	< 1	100	4	100	100	100	6	94

Table 8: Percentage benomyl tolerant isolates from two glasshouses sampled in consecutive years in which benomyl had not been used since 1974

Glasshouse	Benomyl tolerant isolates 1976	1977	1978
B2	100	95	85
L2	25	35	34

and sensitive strains, were selected from the 17 examined for further study. For each house, the growth rate on PDA and pathogenicity of isolates (maximum of 20) were compared (Figures 14 to 23). Each isolate was ranked in order of vigour (1 = most vigorous, 20 = least vigorous) for both growth rate on PDA and pathogenicity. The mean rank number for tolerant and sensitive isolates was then calculated. For example:

Glasshouse I_1	Mean rank number	
	Growth rate on PDA	Pathogenecity
Sensitive isolates	7.9	10.3
Tolerant isolates	11.1	7.8

This particular example indicates that sensitive isolates tended to grow faster on PDA but that tolerant isolates were more pathogenic than sensitive isolates.

In all the glasshouses examined significant differences in growth rate on PDA and pathogenicity existed between isolates, both tolerant and sensitive (Figures 14 - 23). When the mean rank number was calculated it was found that:

(i) Sensitive isolates tended to grow faster on PDA than tolerant isolates in eight out of ten glasshouses.

(ii) Sensitive isolates tended to be more pathogenic than tolerant isolates in six out of ten glasshouses, equal in pathogenicity in one glasshouse and less pathogenic in

Figure 15: The growth rates of benomyl tolerant and sensitive isolates of B. cinerea collected from glasshouse F1 on excised tomato cotyledons and PDA

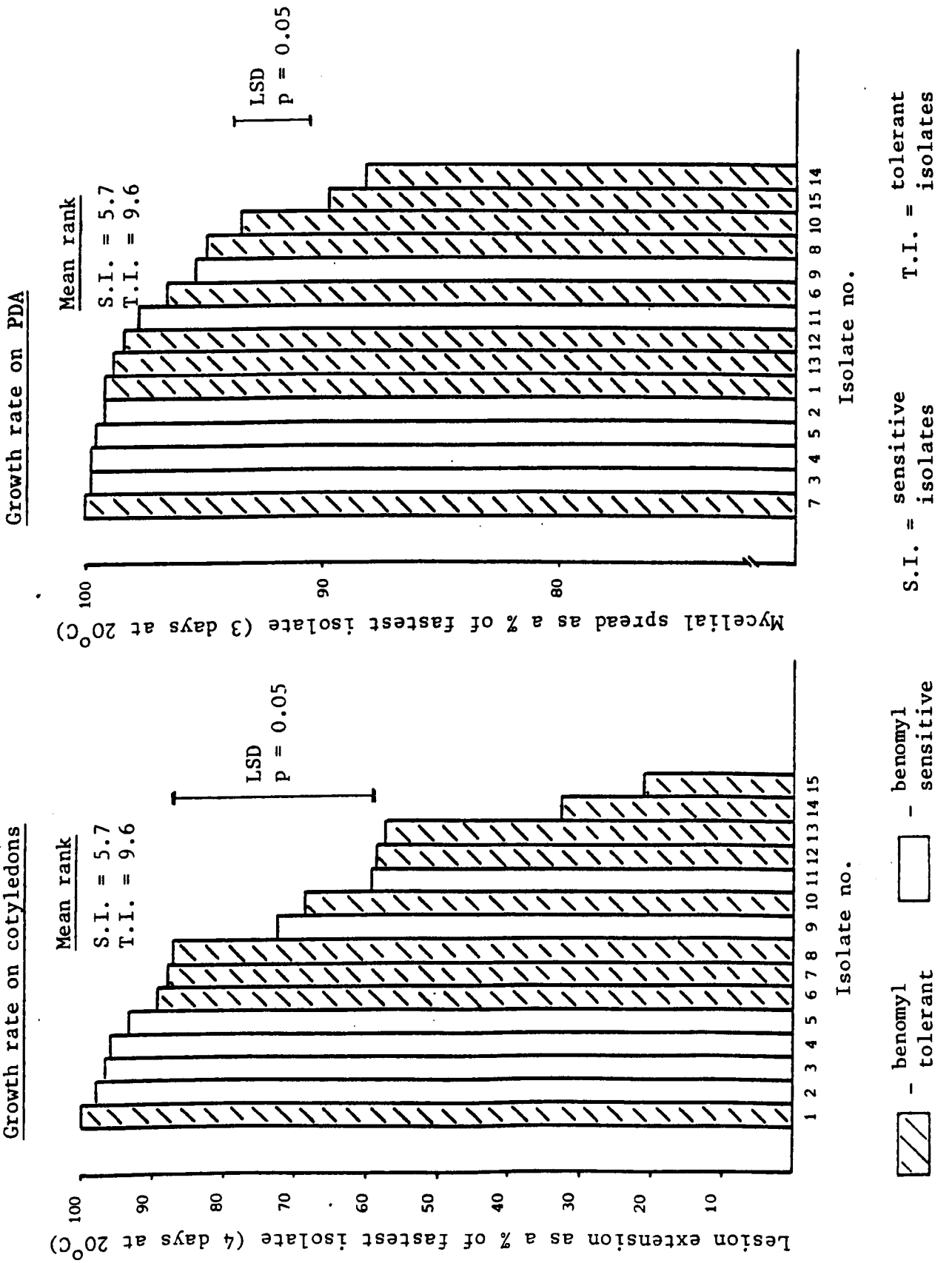


Figure 16: The growth rates of benomyl tolerant and sensitive isolates of B. cinerea collected from glasshouse G1 on excised tomato cotyledons and PDA

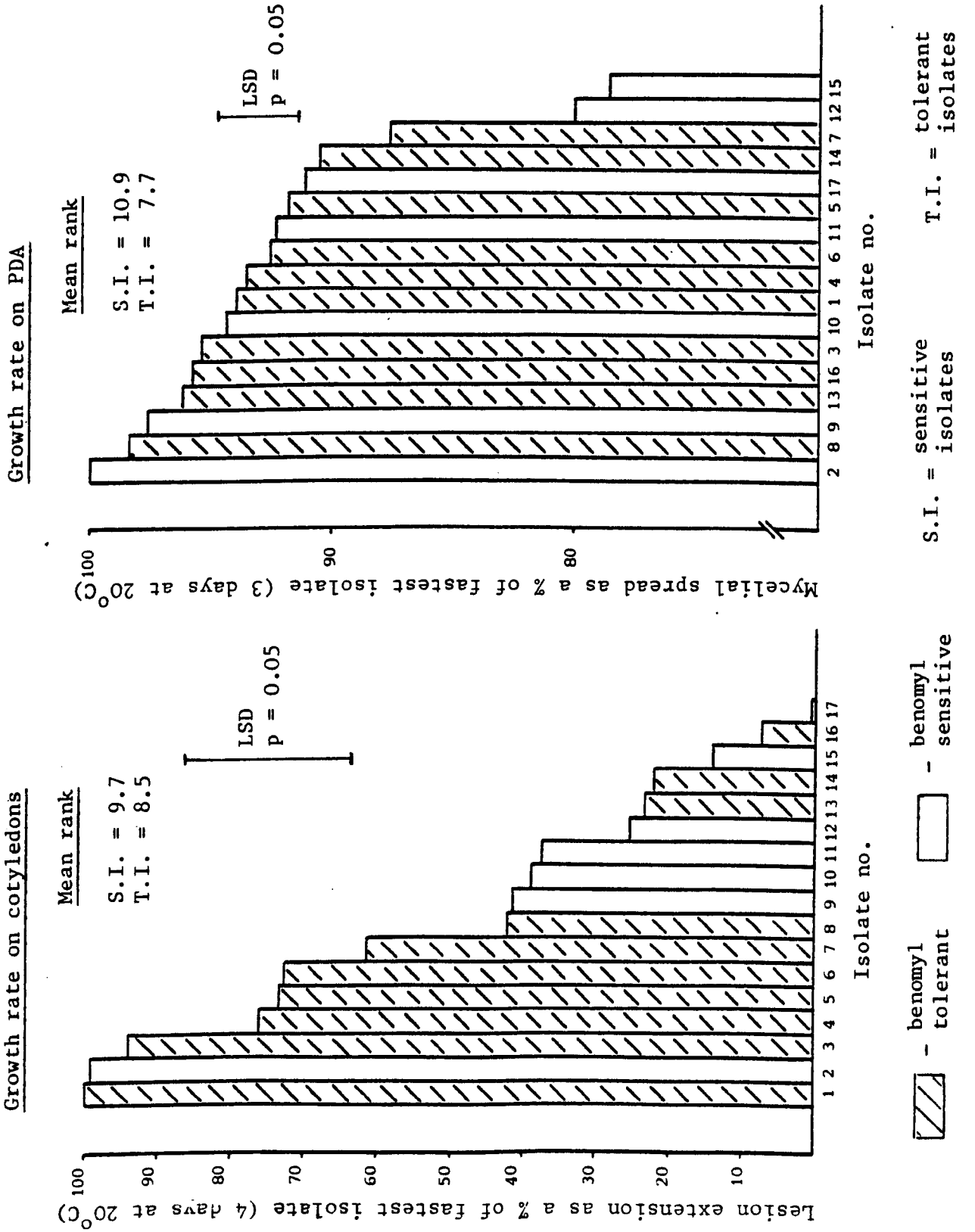


Figure 17: The growth rates of benomyl tolerant and sensitive isolates of B. cinerea collected from glasshouse H1 on excised tomato cotyledons and PDA

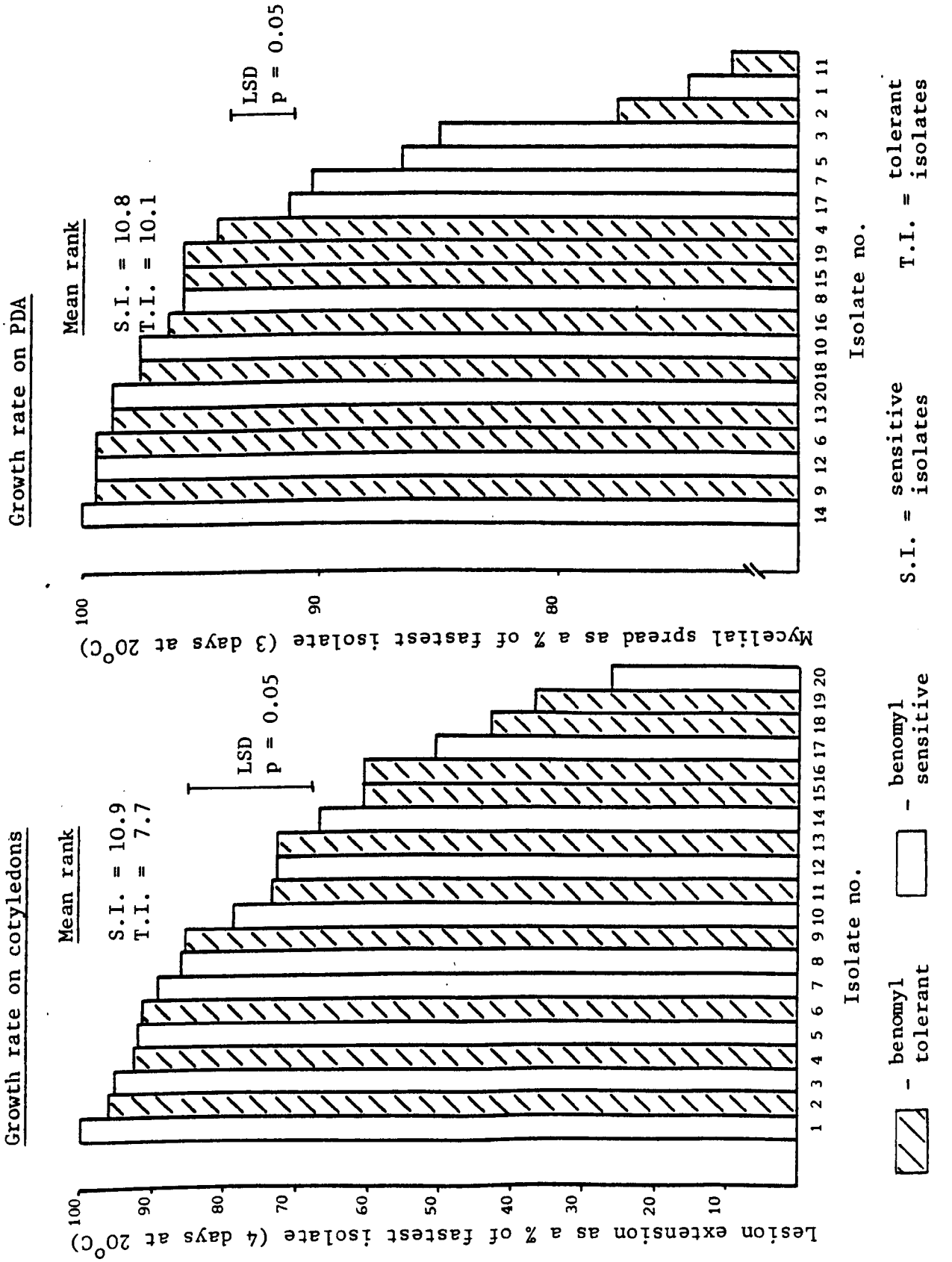


Figure 18: The growth rates of benomyl tolerant and sensitive isolates of B. cinerea collected from glasshouse I1 on excised tomato cotyledons and PDA

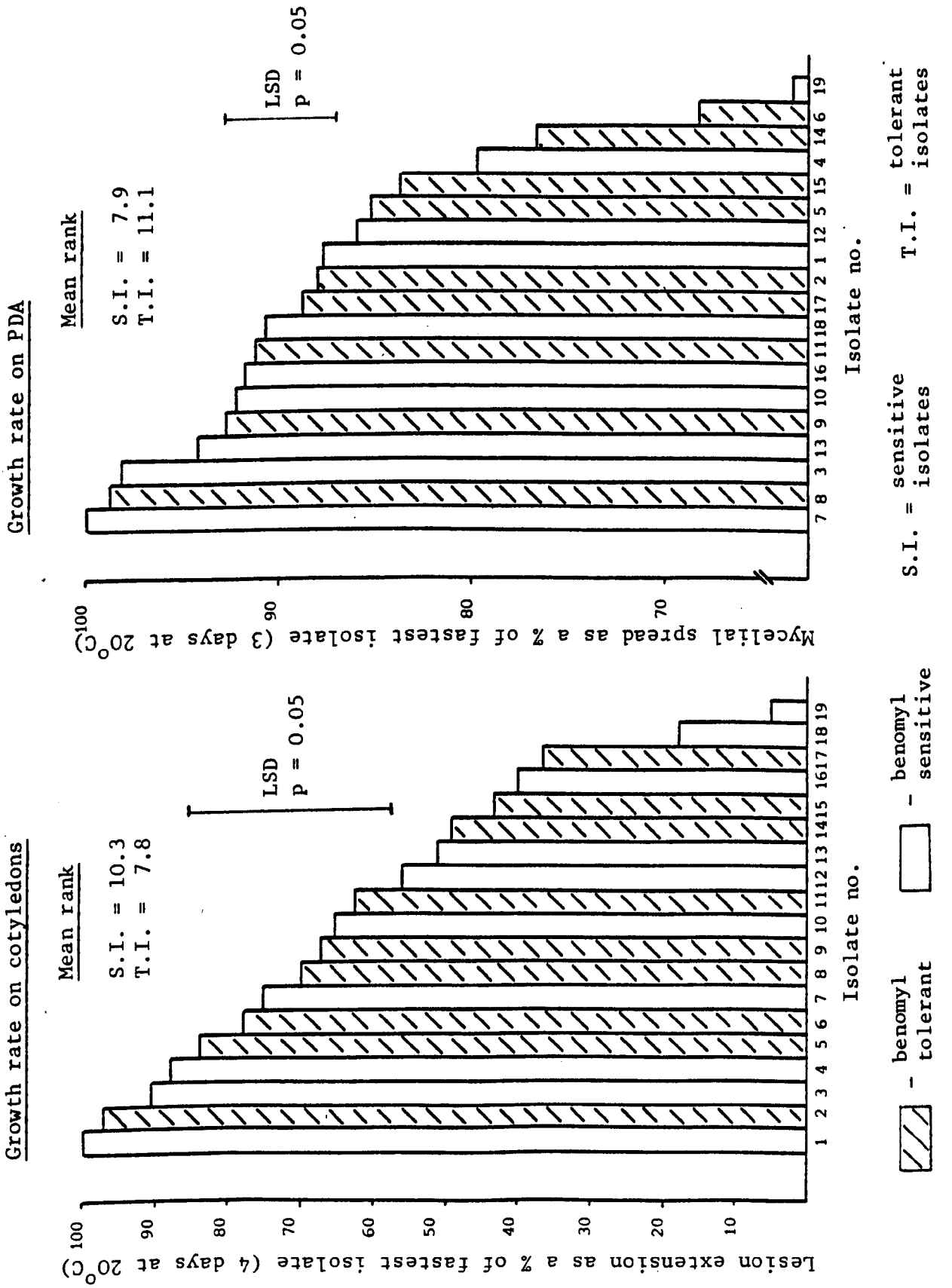


Figure 19: The growth rates of benomyl tolerant and sensitive isolates of B. cinerea collected from glasshouse J1 on excised tomato cotyledons and PDA

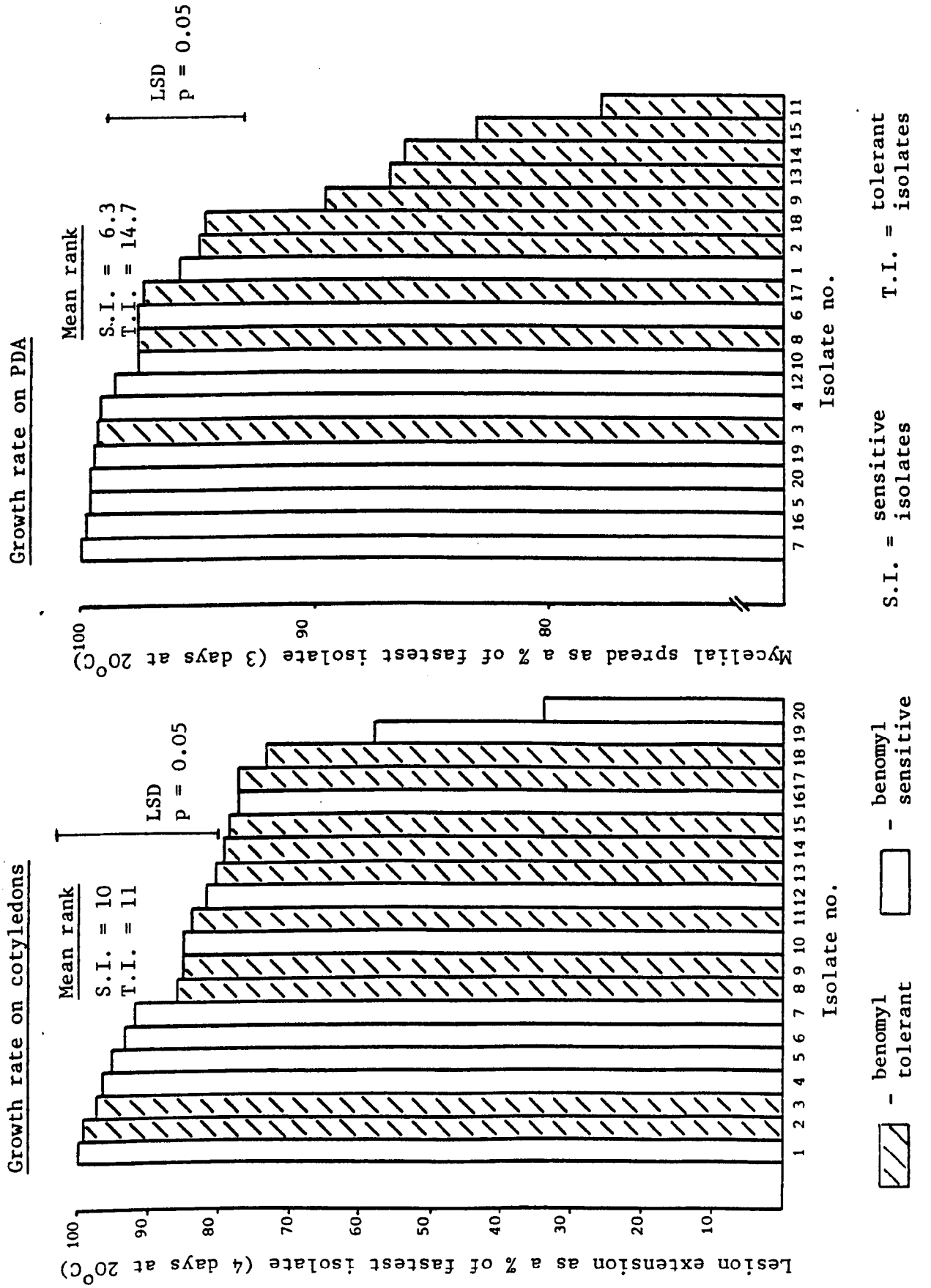


Figure 20: The growth rates of benomyl tolerant and sensitive isolates of B. cinerea collected from glasshouse K1 on excised tomato cotyledons and PDA

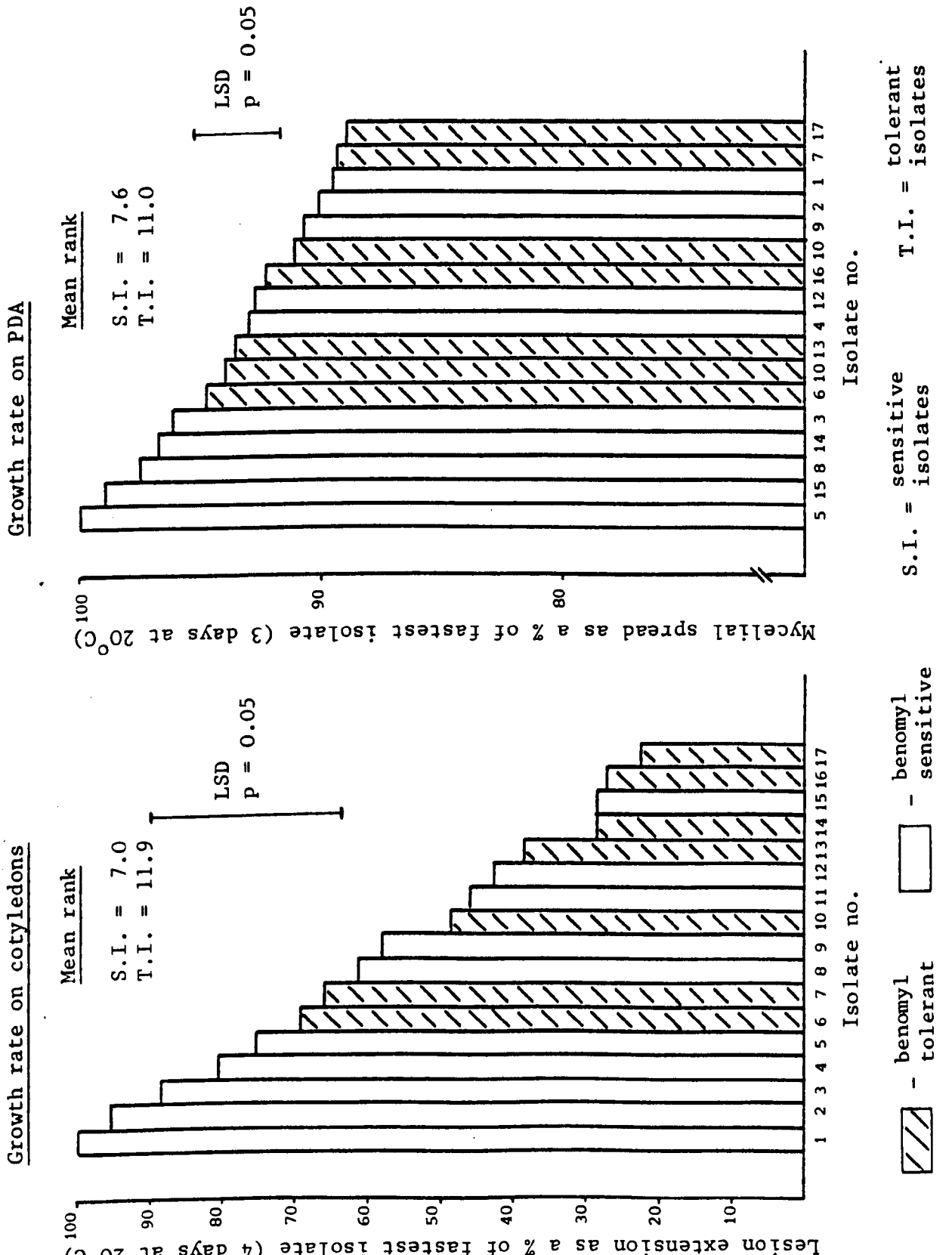


Figure 21: The growth rates of benomyl tolerant and sensitive isolates of B. cinerea collected from glasshouse L2 on excised tomato cotyledons and PDA

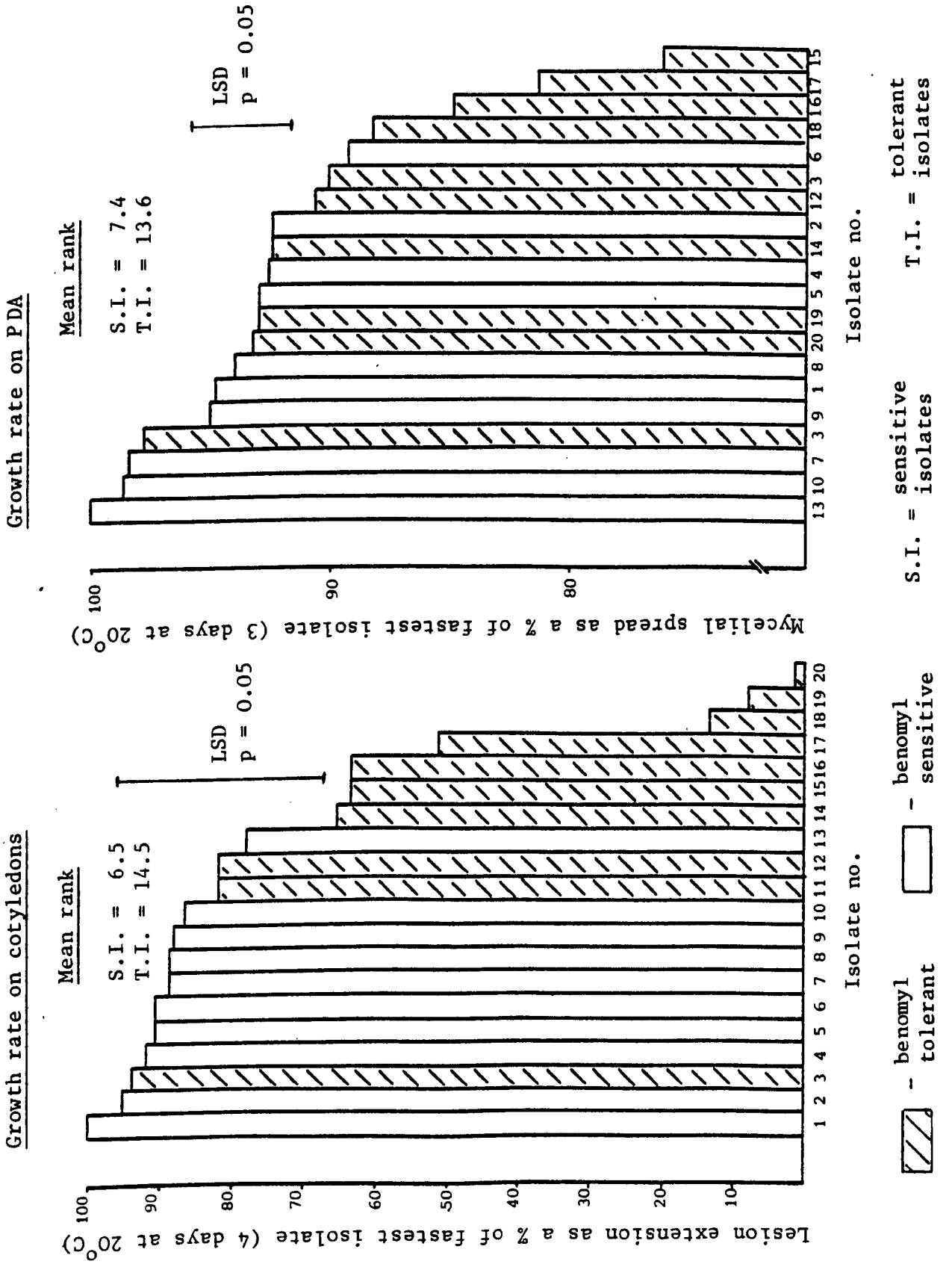


Figure 22: The growth rates of benomyl tolerant and sensitive isolates of B. cinerea collected from glasshouse M1 on excised tomato cotyledons and PDA

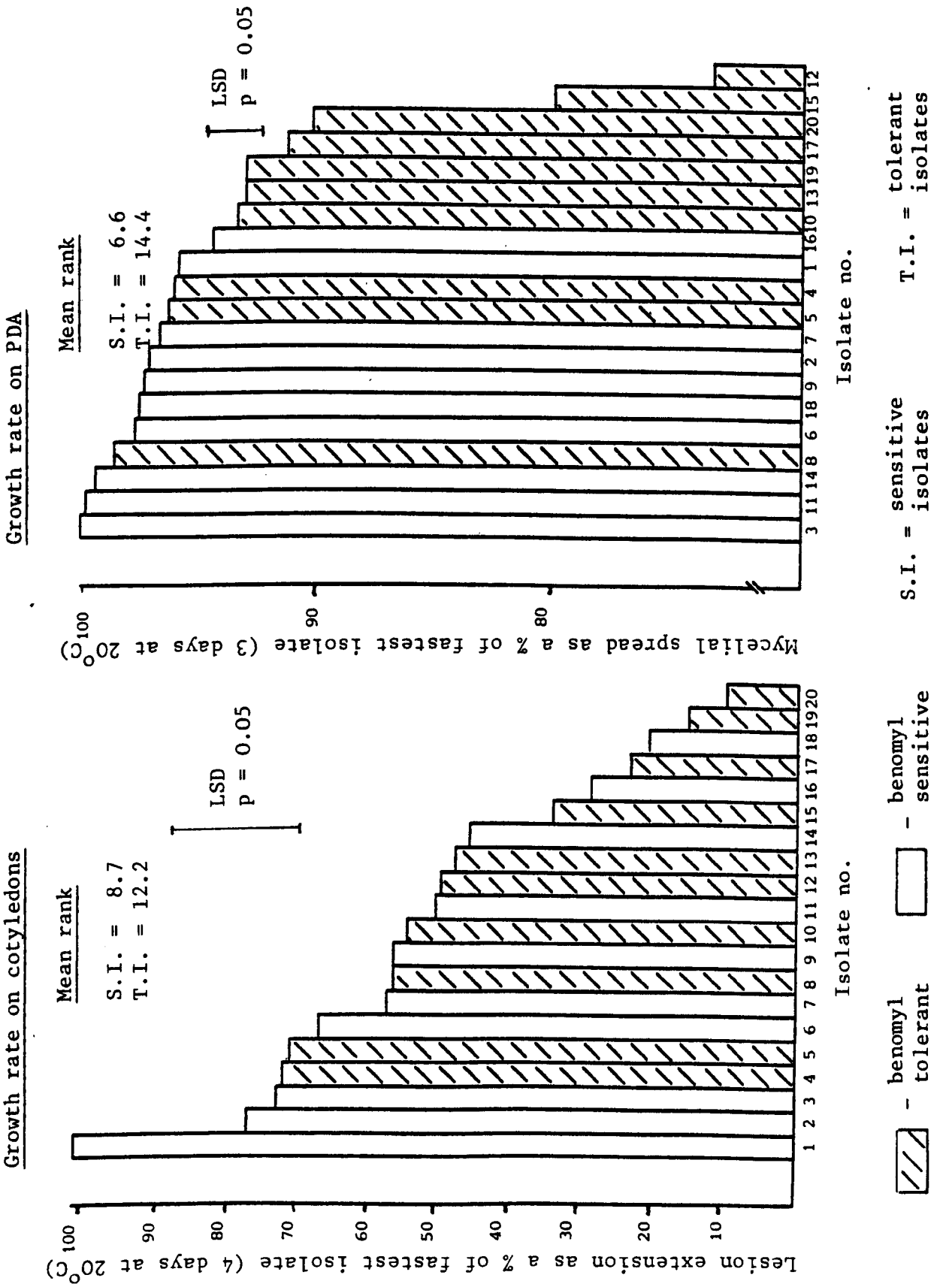
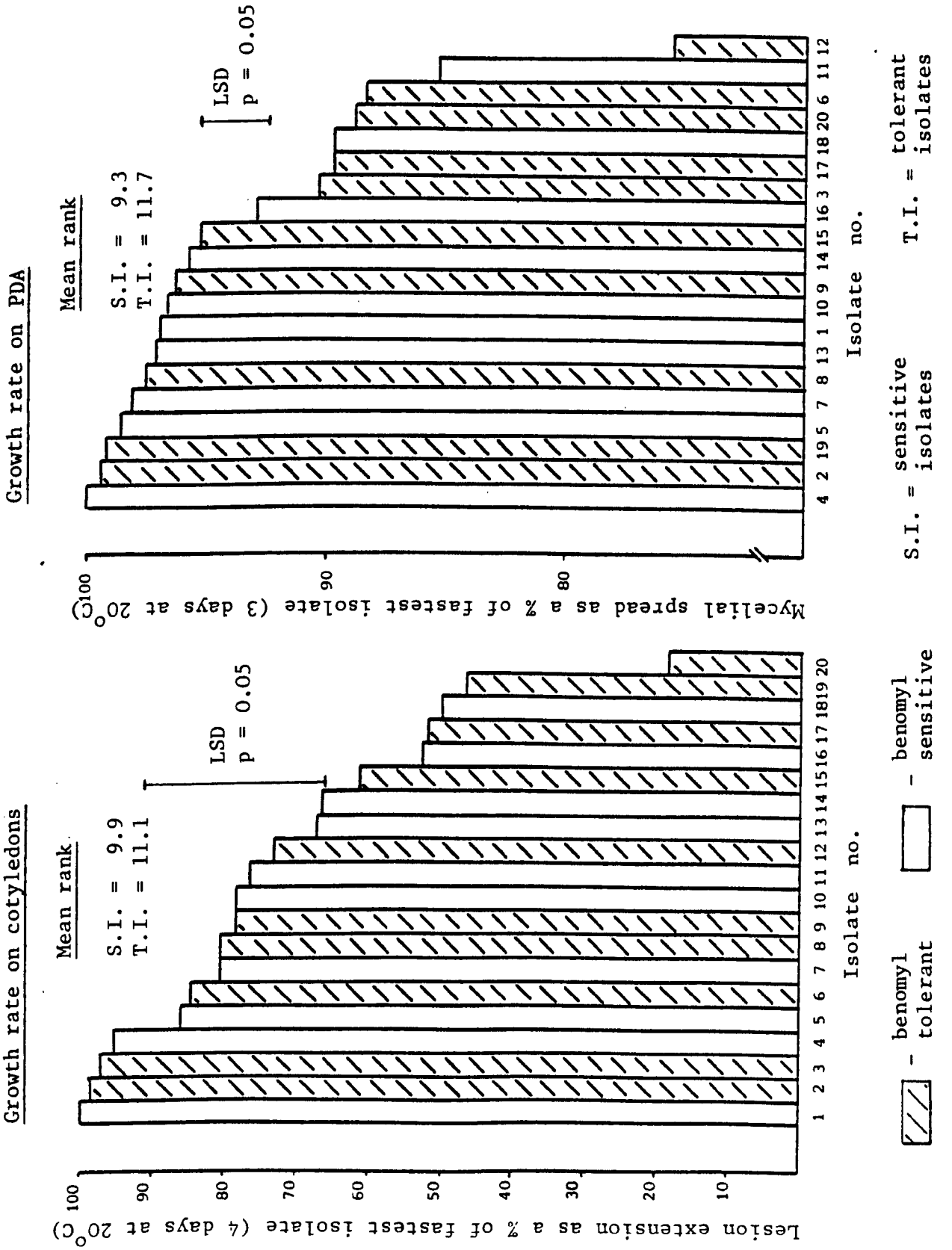


Figure 23: The growth rates of benomyl tolerant and sensitive isolates of B. cinerea collected from glasshouse N1 on excised tomato cotyledons and PDA



three glasshouses. Combining all the data there are indications that the majority of sensitive isolates:

(a) have a faster growth rate on PDA than tolerant isolates; and

(b) are slightly more pathogenic than tolerant ones.

6.4 An investigation into the ability of tolerant and sensitive isolates of B. cinerea to compete in mixed cultures

A series of experiments were undertaken to determine the ability of tolerant strains of B. cinerea to compete with sensitive strains under the same conditions. In the field this competition would presumably occur in two distinct phases:

(i) as saprophytes, when both are competing on moribund plant materials; and

(ii) as parasites, when both are attempting to invade the host plant.

In order to exist in the field in the absence of the selection pressure of benomyl, tolerant strains must be able to compete successfully with sensitive strains in both these phases.

6.4.1 Competition between tolerant and sensitive isolates of B. cinerea as saprophytes

Isolates of B. cinerea collected from a single glasshouse (L1) in 1976 were examined using PDA as the saprophytic medium. A series of 9 cm petri dishes were prepared containing 10 ml unamended PDA or PDA amended with either 0.1, or 1 p.p.m. benomyl respectively. Spore suspensions of each isolate (10^4 spores/ml) were added to the plates to give mixtures of spores on the plates in the following

ratios:

Sensitive isolate + Tolerant isolate	Unamended PDA	PDA amended with 0.1 p.p.m. benomyl	PDA amended with 1 p.p.m. benomyl
1S + 20R	90:10, 50:50, 10:90	90:10, 50:50, 10:90	90:10, 50:50, 10:90
5S + 14R	" " "	" " "	" " "
33S + 17R	" " "	" " "	" " "
13S + 19R	" " "	" " "	" " "
27S + 20R	" " "	" " "	" " "
37S + 14R	" " "	" " "	" " "

A combined total of 0.5 ml of spore suspension (i.e. 50,000 spores) was evenly distributed over the agar using a sterile glass spreader. Plates were incubated at 20°C under 16 hour day illumination (8 to 10,000 x 1.1 lux). After eight days the resulting colonies were flooded with 10 ml of sterile distilled water, the spores dislodged with a sterile glass spreader and the suspension obtained passed through four layers of muslin to remove mycelial fragments. 0.5 ml of the filtrate was transferred to fresh plates of PDA amended with the appropriate amount of benomyl. This procedure was repeated at eight day intervals for a maximum of 56 days. After 8, 16, 24 and 56 days respectively a sample of the filtrate was analyzed and the ratio of tolerant to sensitive spores determined. The experiment was not replicated but was repeated to check the validity of the results using unamended PDA only.

In general one of the two isolates in the mixture became

dominant, although the time taken varied (Figures 24-29). This was usually regardless of the initial ratio of tolerant to sensitive spores. There were two exceptions, however. 19R achieved dominance over 13S when the ratio of tolerant to sensitive spores was equal to or greater than 50:50 (Figure 27). When the ratio was as low as 10:90, 13S achieved dominance (Figure 27). Similarly 20R became dominant over 27S only when the ratio was as high as 90:10 (Figure 28). 0.1 p.p.m. benomyl had no effect on competition, whereas 1 p.p.m. completely inhibited the sensitive isolates.

When the experiment was repeated similar results were obtained with the exception of two of the mixtures:

(i) 27S+ 20R: Whereas 20R had previously dominated over 27S at a spore ratio of 90:10, on this occasion 27S rapidly achieved dominance (Figure 30); and

(ii) 5S + 14R: Whereas 14R had previously maintained dominance over 5S at all spore ratios, on this occasion neither isolate achieved complete dominance over the other (Figure 30).

6.4.2 The relationship between the capacity of an isolate of B. cinerea to sporulate in pure culture and its ability to compete in mixed culture

The number of spores produced by an isolate in a given time was considered to be a very important factor in determining its ability to compete in mixed culture. For this reason spore production by each of the isolates used in the previous experiment was examined. 9cm Petri dishes containing 10 ml of PDA were each inoculated as in experiment

Figure 24: The percentage of tolerant spores recovered from mixed cultures of benomyl tolerant and sensitive isolates of B. cinerea after repeated subculture on PDA.

Isolates 1S + 20R

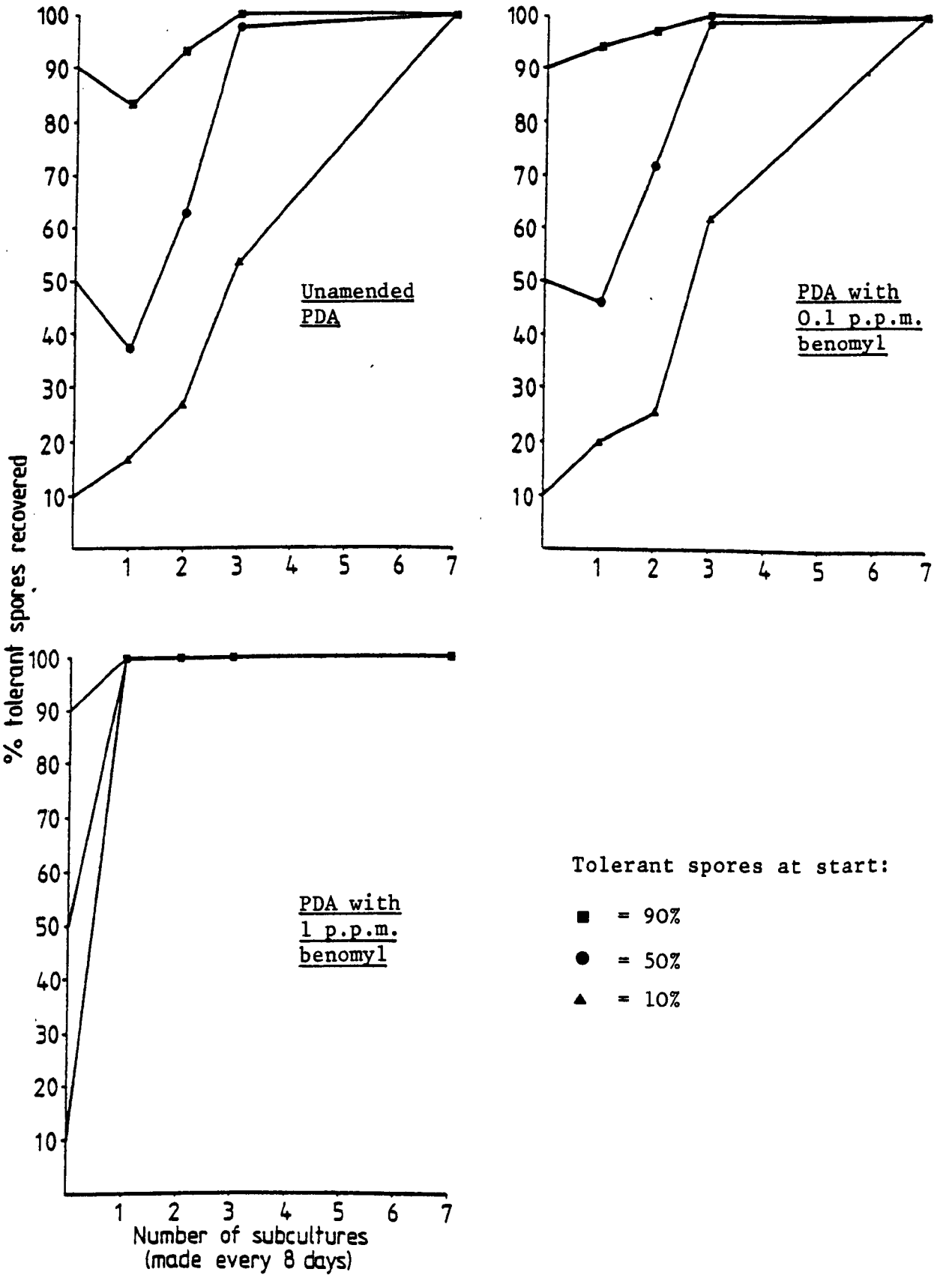


Figure 25: The percentage of tolerant spores recovered from mixed cultures of benomyl tolerant and sensitive isolates of B. cinerea after repeated subculture on PDA

Isolates 5S + 14R

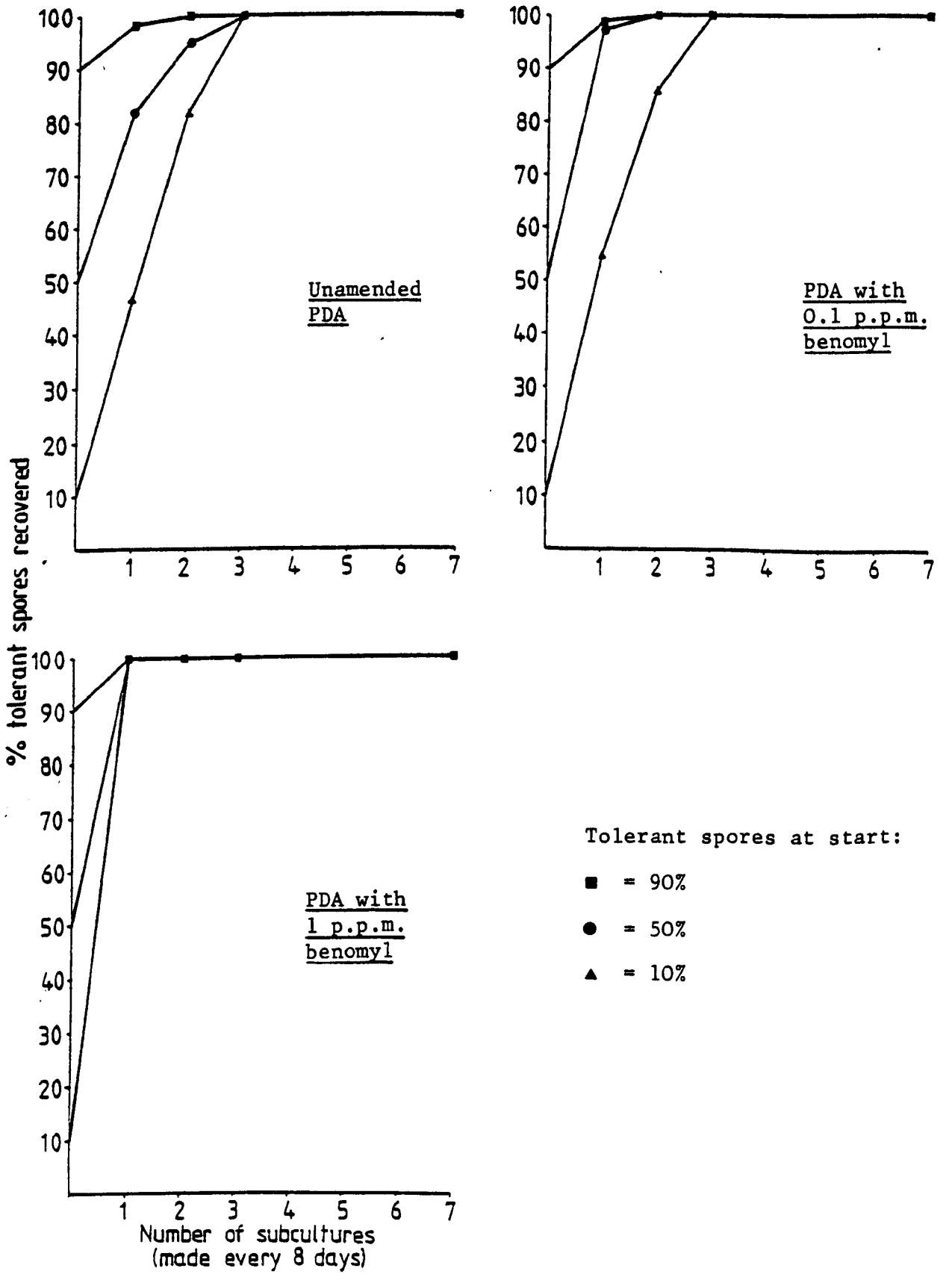


Figure 26: The percentage of tolerant spores recovered from mixed cultures of benomyl tolerant and sensitive isolates of B. cinerea after repeated subculture on PDA

Isolates 33S + 17R

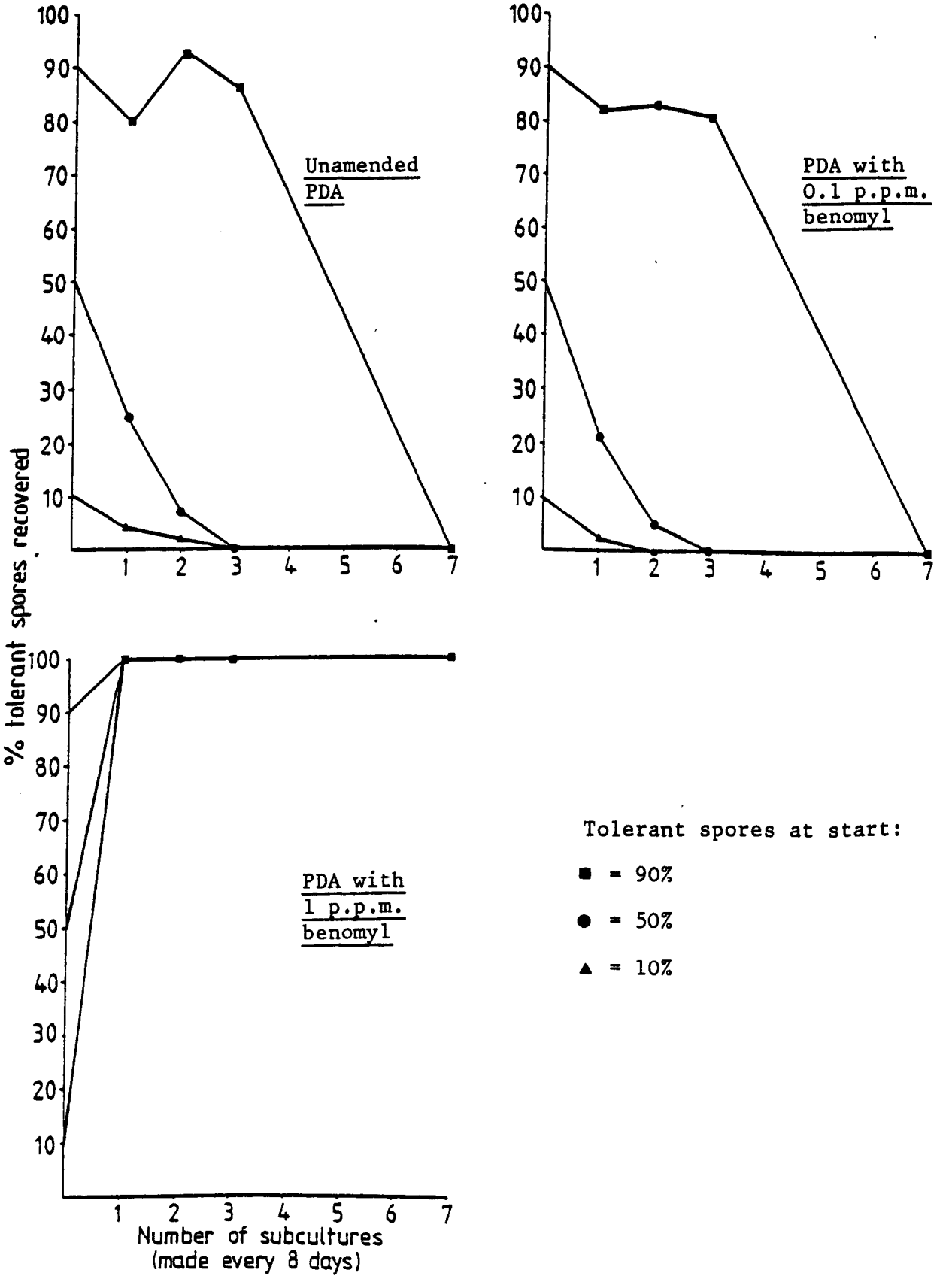


Figure 27: The percentage of tolerant spores recovered from mixed cultures of benomyl tolerant and sensitive isolates of B. cinerea after repeated subculture on PDA

Isolates 13S + 19R

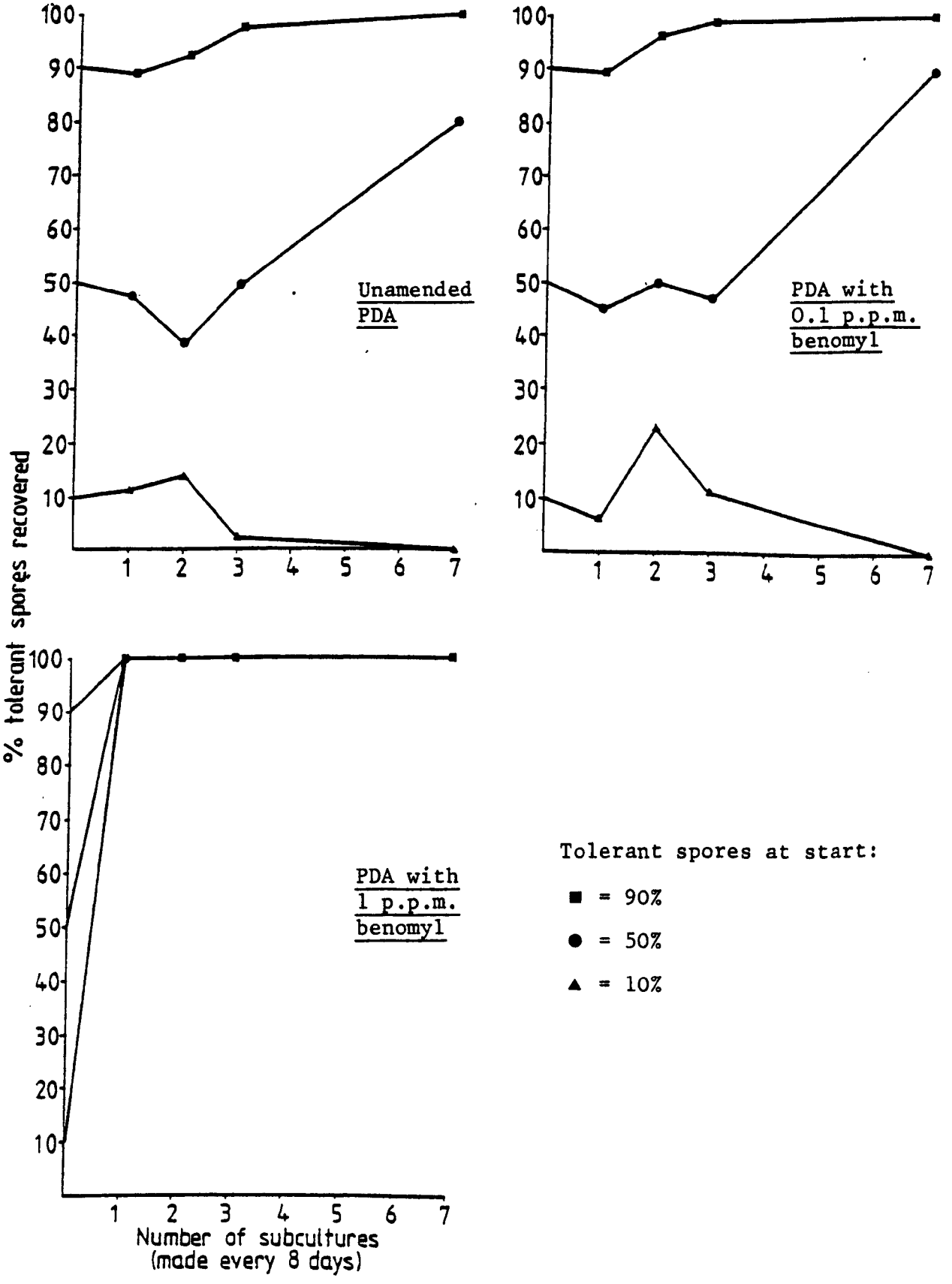


Figure 28: The percentage of tolerant spores recovered from mixed cultures of benomyl tolerant and sensitive isolates of B. cinerea after repeated subculture on PDA

Isolates 27S + 20R

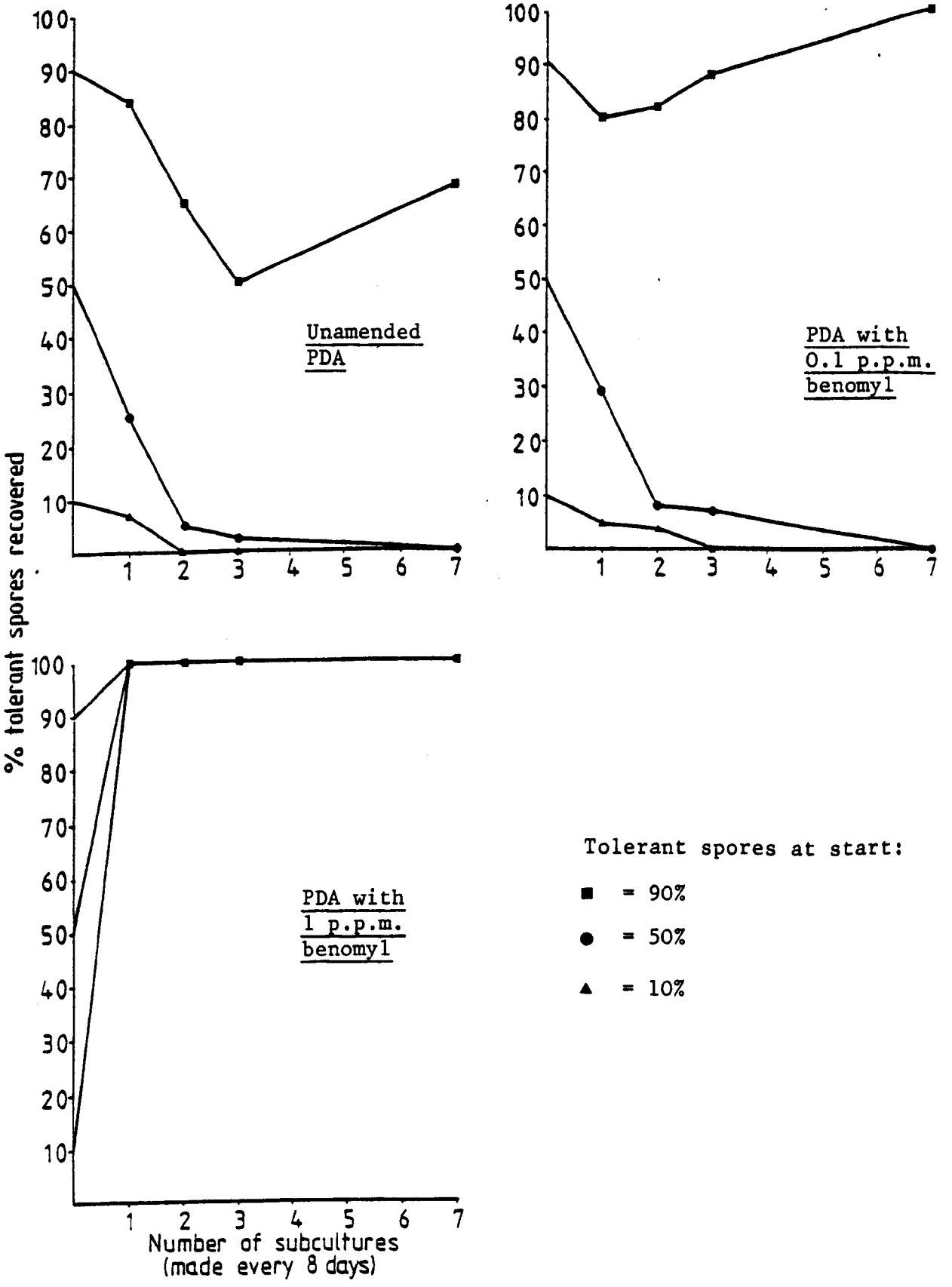


Figure 29: The percentage of tolerant spores recovered from mixed cultures of benomyl tolerant and sensitive isolates of B. cinerea after repeated subculture on PDA

Isolates 37S + 14R

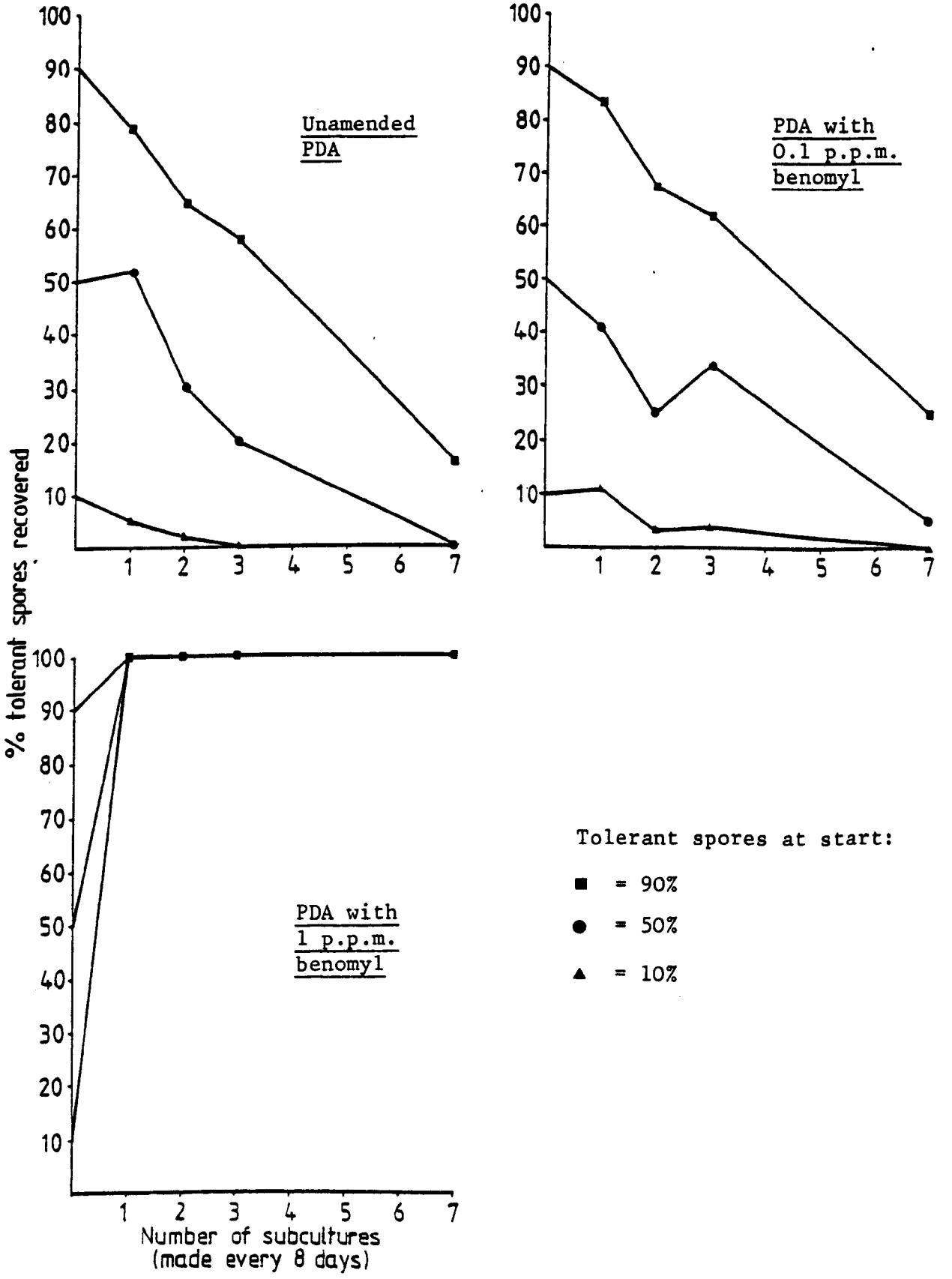


Figure 30: The percentage of tolerant spores recovered from mixed cultures of benomyl tolerant and sensitive isolates of B. cinerea after repeated subculture on PDA - a repeat experiment

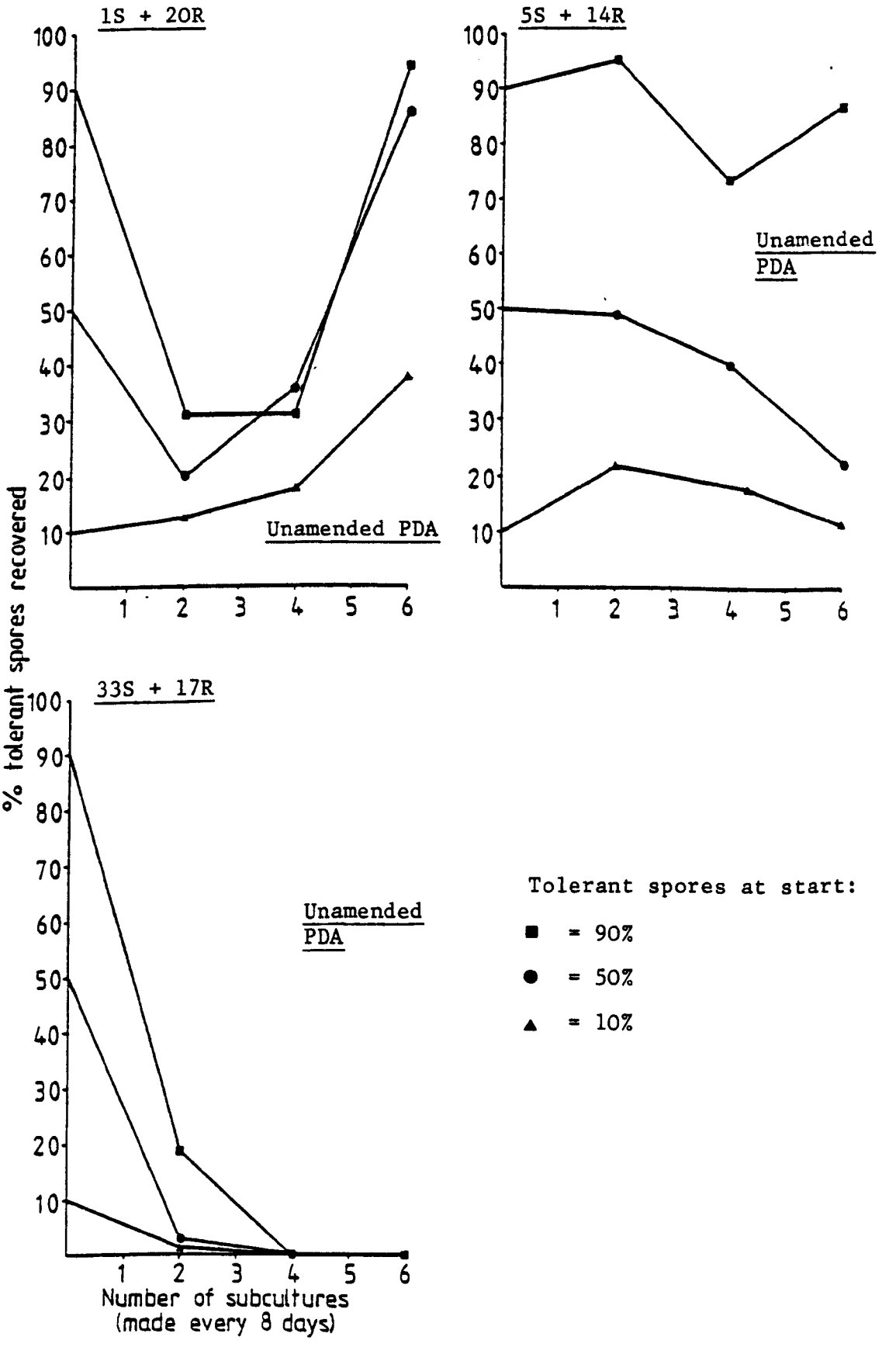
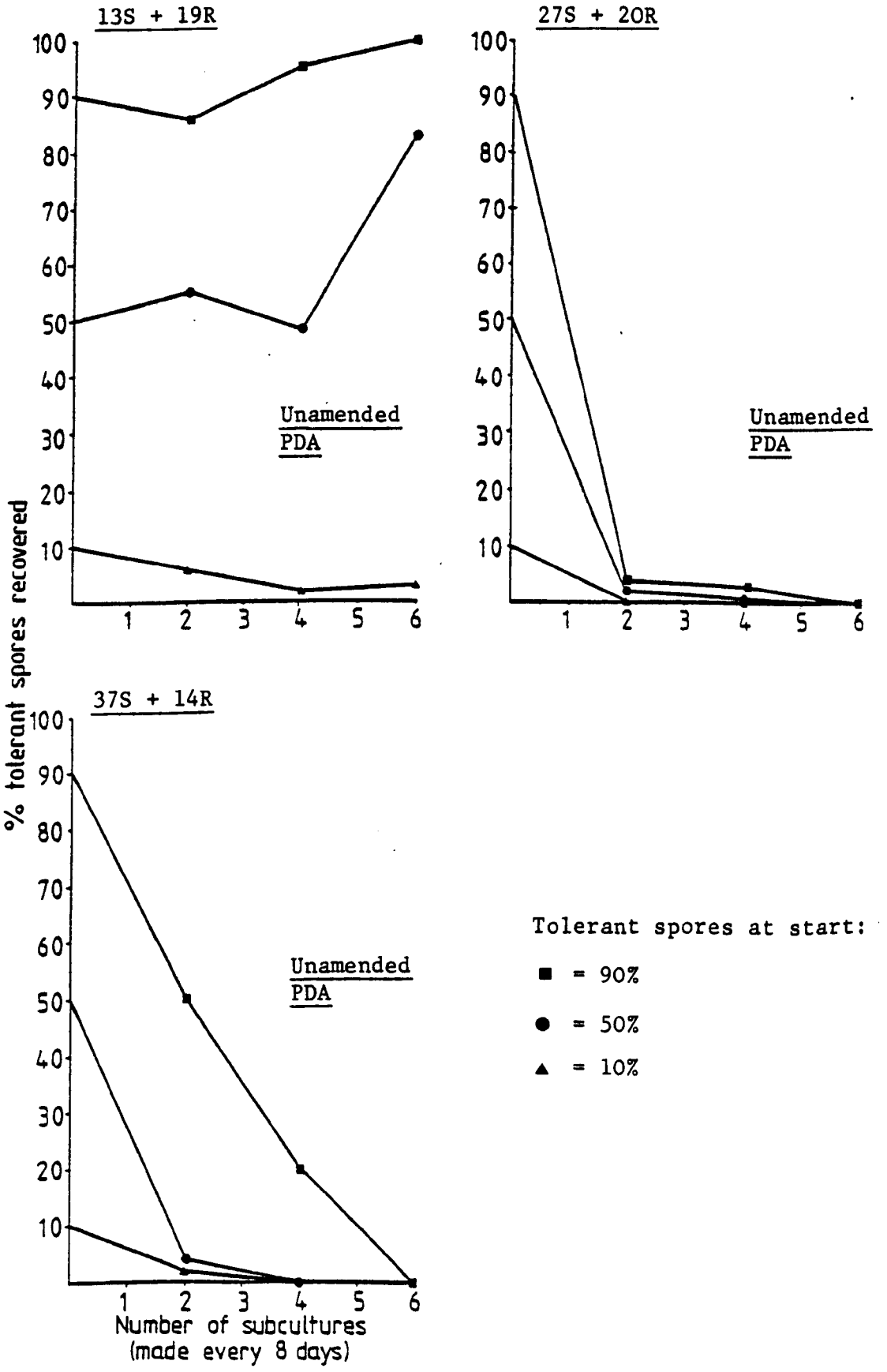


Figure 30: - continued



6.4.1 with 50,000 spores (0.5 ml of suspension) of one isolate. Six replicate plates were prepared for each isolate and incubated at 20°C under 16 hour illumination. At intervals of 4, 8 and 12 days two plates of each isolate were flooded with 10 ml of sterile distilled water, the spores dislodged with a glass spreader and the suspension filtered through four layers of muslin. The concentration of spores in the filtrate was then determined. The experiment was repeated to determine whether the isolates exhibited any variation in their ability to sporulate.

In four out of six cases isolates which had become dominant when mixed in a ratio of 50:50 also produced the largest numbers of spores when cultured alone (Table 9). The two exceptions were the mixtures:

(i) 33S + 17R: The dominance of 33S was particularly apparent in this mixture despite 17R producing the larger number of spores in pure culture; and

(ii) 13S + 19R: Similarly 13S produced the largest numbers of spores in pure culture whereas 19R was the dominant isolate in the mixture.

When the experiment was repeated a number of isolates produced quite different numbers of spores indicating that isolates were inconsistent in their ability to sporulate, even under identical cultural conditions.

6.4.3 Factors affecting the survival of B. cinerea strains as saprophytes

The previous experiments (6.4.1 and 6.4.2) using mixed

Table 9: Number of spores produced by different isolates of *B. cinerea* on PDA

Isolate \ Days after seeding	MEAN SPORE PRODUCTION PER PLATE (x 10^{-3})				
	4	8	12	Repeat 8 weeks later	12
1S [1,2,3, *]	102	130	165	1S *	965
20R	21	56	104	20R [3]	1,240
5S [2]	85	1,650	1,385	5S [3 *]	1,370
14R [1,3 *]	160	625	2,310	14R	560
33S *	48	52	66	33S [3 *]	1,430
17R [1,2,3]	156	1,055	1,510	17R	670
13S [1,2,3]	610	1,440	2,060	13S	650
19R *	226	1,040	1,835	19R [3 *]	1,385
27S [1,2,3 *]	65	256	1,215	27S [3 *]	1,500
20R	21	56	104	20R	1,240
37S [1,2 *]	240	1,610	2,155	37S *	560
14R [3]	160	620	2,310	14R	560

* Dominant isolate in 50 : 50 mixed inoculation.

1 Isolate producing most spores after 4 days.

2 Isolate producing most spores after 8 days.

3 Isolate producing most spores after 12 days.

populations of spores had been undertaken under well defined and constant environmental and cultural conditions. Observations made in these experiments suggested, however, that slight changes in cultural conditions could greatly influence spore production and therefore possibly the behaviour of isolates in mixtures. This experiment was designed to examine the effect of certain changes on the sporulation of two isolates, 33S and 17R. All tests were replicated three times.

(a) Changes in media: Three media were compared: PDA, malt extract agar (MEA, Difco Ltd) and Czapek Dox agar (CDA, Difco Ltd). Plates were seeded as in experiment 6.4.2 with 50,000 spores of the appropriate isolate and incubated at 20°C under 16 hour illumination. Spore production was assessed after nine days.

The degree of sporulation differed according to the media used, although both isolates responded in the same way (Table 10):

Degree of sporulation CDA > MEA > PDA

(b) Changes in spore concentration: Four spore suspensions of each isolate were prepared: 1×10^3, 1×10^4, 1×10^5 and 1×10^6 spores/ml. PDA plates each seeded with 0.5 ml of a spore suspension were incubated at 20°C under 16 hour day illumination. Spore production was assessed after nine days.

The initial concentration of the spore inoculum influenced the ability of the isolates to produce spores (Table 10). The isolates differed in their response, however. 33S produced the largest number of spores when the concentration of the spore inoculum was low and 17R when the concentration was high.

(c) Changes in temperature: PDA plates each seeded with 50,000 spores of one isolate were incubated at either 15°C, 20°C or 25°C under 16 hour day illumination. In a repeat experiment the plates were incubated in darkness, each plate being wrapped in aluminium foil. Spore production was assessed after nine days.

Incubation temperature influenced the ability of an isolate to sporulate (Table 10). The two isolates examined did not, however, respond in the same way:

Degree of sporulation - 33S : 15°C < 20°C < 25°C

" " " - 17R : 15°C < 20°C > 25°C

Incubation in darkness also influenced spore production; 33S gave an opposite response to 17R (Table 10).

Degree of sporulation - 33S : 15°C > 20°C > 25°C

" " " - 17R : 15°C < 20°C < 25°C

6.4.4 Competition between tolerant and sensitive isolates of B. cinerea as parasites

The isolates previously compared as saprophytes (experiment 6.4.1) were examined as parasites. The pathogenicity had

Table 10: Factors affecting the sporulation of *B. cinerea* in vitro

ISOLATE		TEST 1				TEST 2			TEST 3			TEST 4		
		SPORE CONCENTRATION (16 hour day)				MEDIA (16 hour day)			TEMPERATURE (16 hour day)			TEMPERATURE (dark)		
		$1x10^3$	$1x10^4$	$1x10^5$	$1x10^6$	CDA	MEA	PDA	15°C	20°C	25°C	15°C	20°C	25°C
33S	Mean spore production per plate ($x\ 10^{-3}$)	A	A	B	B	C	B	A	A	B	C	D	CD	A
		18	25	47	70	1,500	245	35	13	35	75	275	160	0
17R	Mean spore production per plate ($x\ 10^{-3}$)	B	B	B	A	C	B	B	A	D	BC	B	B	C
		60	65	48	25	1,470	215	175	1	175	40	45	35	150

For each test means with the same letter not significantly different ($p = 0.05$)

previously been determined using the excised cotyledon test (Figure 14) and the following mixtures were examined:

	Sensitive		Tolerant	Pathogenicity of isolates
1	1S	+	20R	Equal pathogenecity
2	5S	+	14R	" "
3	33S	+	17R	33S stronger pathogen
4	13S	+	19R	13S " "
5	27S	+	20R	20R " "
6	37S	+	14R	14R " "

Mixtures were prepared by macerating together 6 mm agar/mycelium discs of the respective isolates. Petioles were then inoculated as described in 4.14. The experiment was undertaken in a commercial glasshouse using tomato plants c.v. Sonato, approximately 2 m high. Three fungicide treatments were incorporated in the experiment:

0 p.p.m. benomyl; 0.1 p.p.m. benomyl and 10 p.p.m. benomyl. A single spray was applied to run off using a hand sprayer three days after inoculation. Inoculations and fungicidal treatments were replicated ten times and fully randomized. The pathogenicity of individual isolates on mature plants was also assessed; no fungicides were applied to these plants. Three weeks after inoculation the infected petioles were removed aseptically and sealed in clean paper envelopes. Spore suspensions were prepared from the leading edge of the sporulating lesion and the ratio of tolerant to sensitive spores determined. The results are expressed in the form

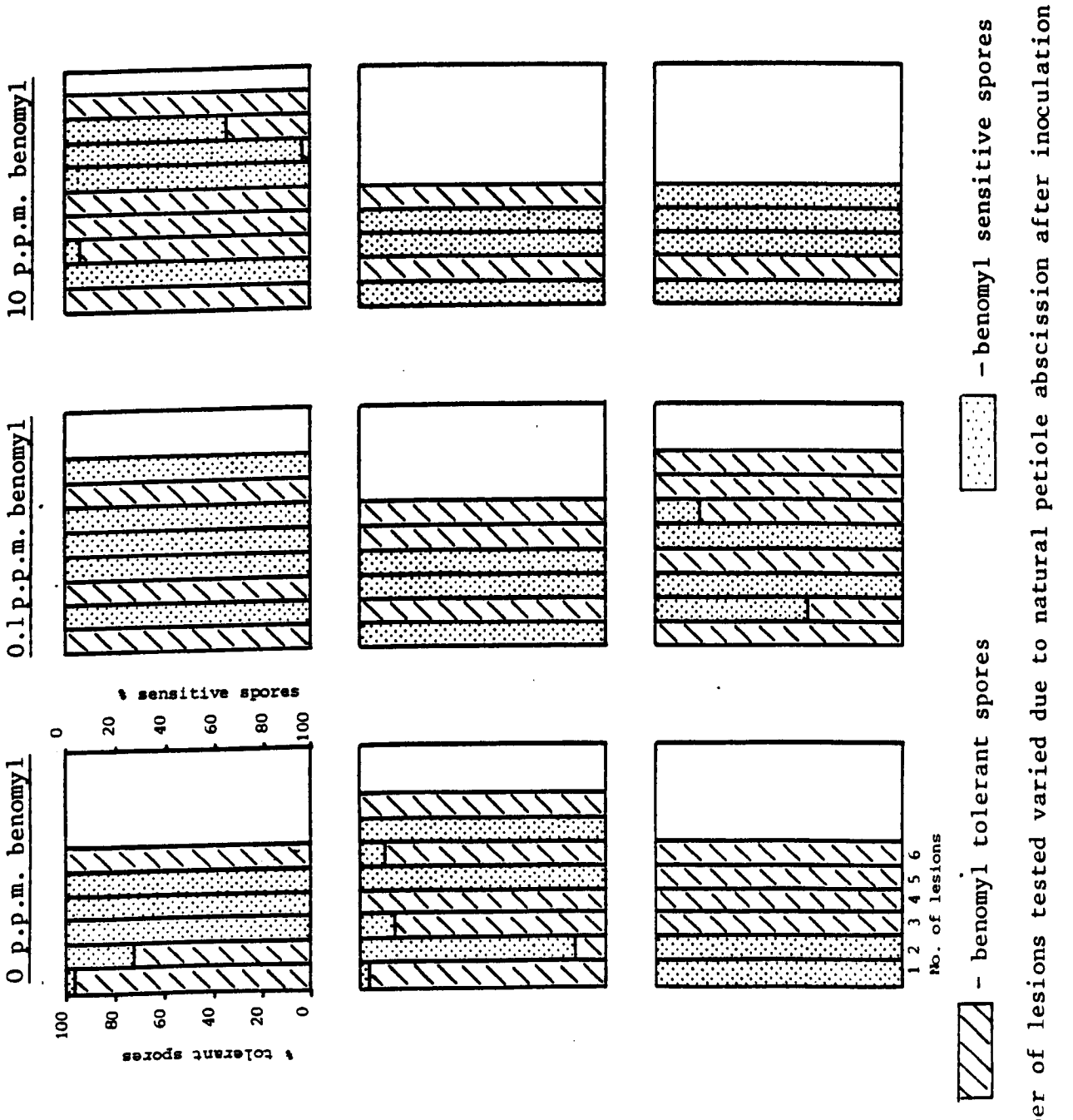
of histograms.

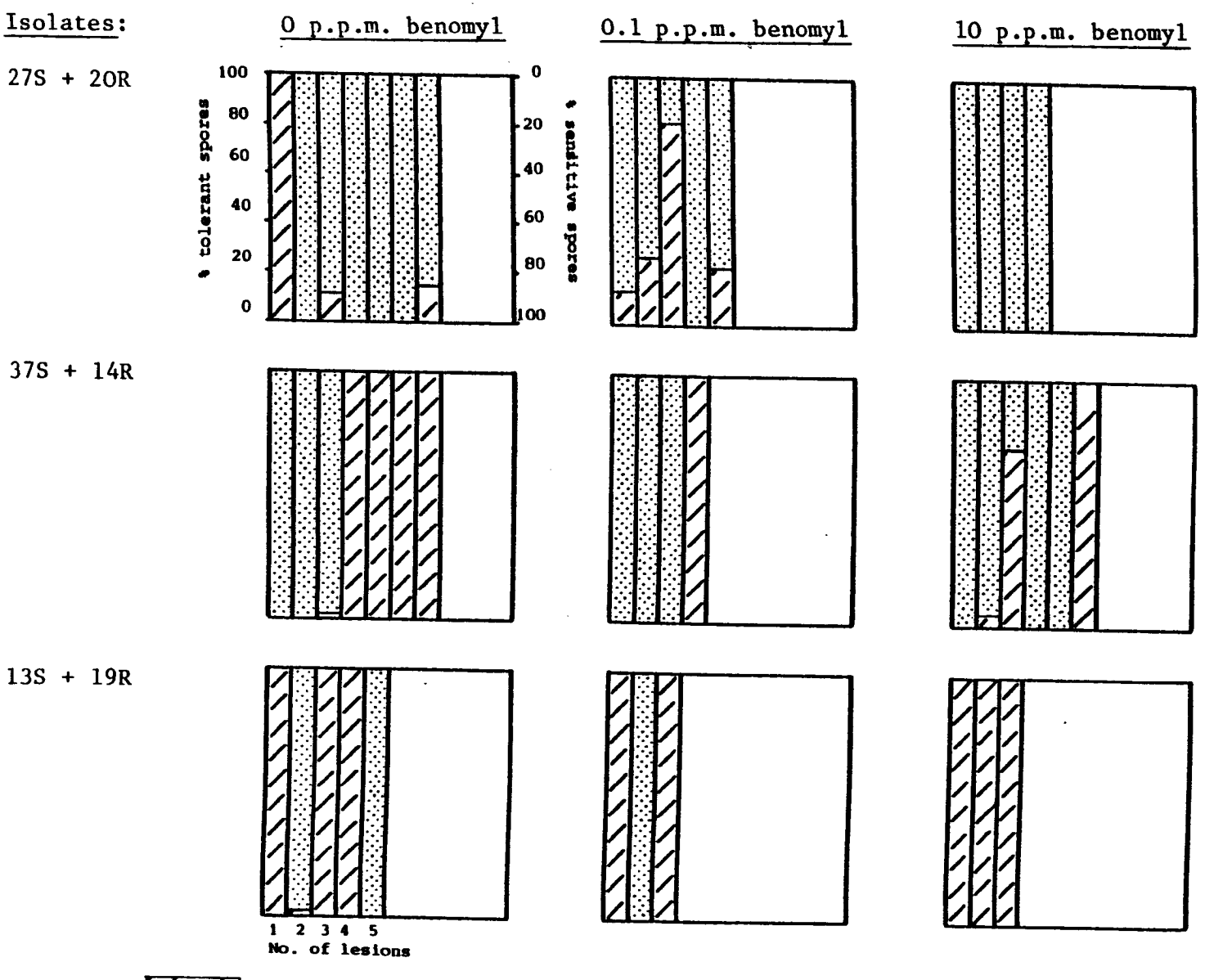
The majority of lesions were found to contain either tolerant or sensitive spores (Figure 31); only a few contained mixtures of both. Replicates of the same mixtures, however, differed considerably and overall no isolate was considered to have had any advantage over another. This was despite differences in pathogenicity between isolates within the same mixture. A single benomyl spray had no effect on the success of tolerant isolates. Pathogenicity tests of individual isolates revealed that the pathogenicity of some isolates had changed since the isolates were first tested 12 months earlier (Figure 32).

6.4.5 Competition between tolerant and sensitive isolates as parasites - 2

The previous experiment was inconclusive, indicating that the ability of an isolate to compete in mixed culture was subject to considerable variation. This was apparently not related to benomyl tolerance or pathogenicity. The experiment was repeated using two isolates, 33S and 17R, and two fungicidal treatments, 0 and 10 p.p.m. benomyl respectively, applied to run off. On this occasion, however, three consecutive sprays were applied. Petioles were inoculated as before with mixed or single isolates and the sprays applied at three day intervals after inoculation. Progress of the lesions were measured after seven days and the ratio of tolerant to sensitive spores determined after 12 days.

Figure 31: The percentage of tolerant and sensitive spores recovered from petiole lesions following inoculation with mixed benomyl tolerant and sensitive isolates (in the presence or absence of foliar sprays of benomyl)

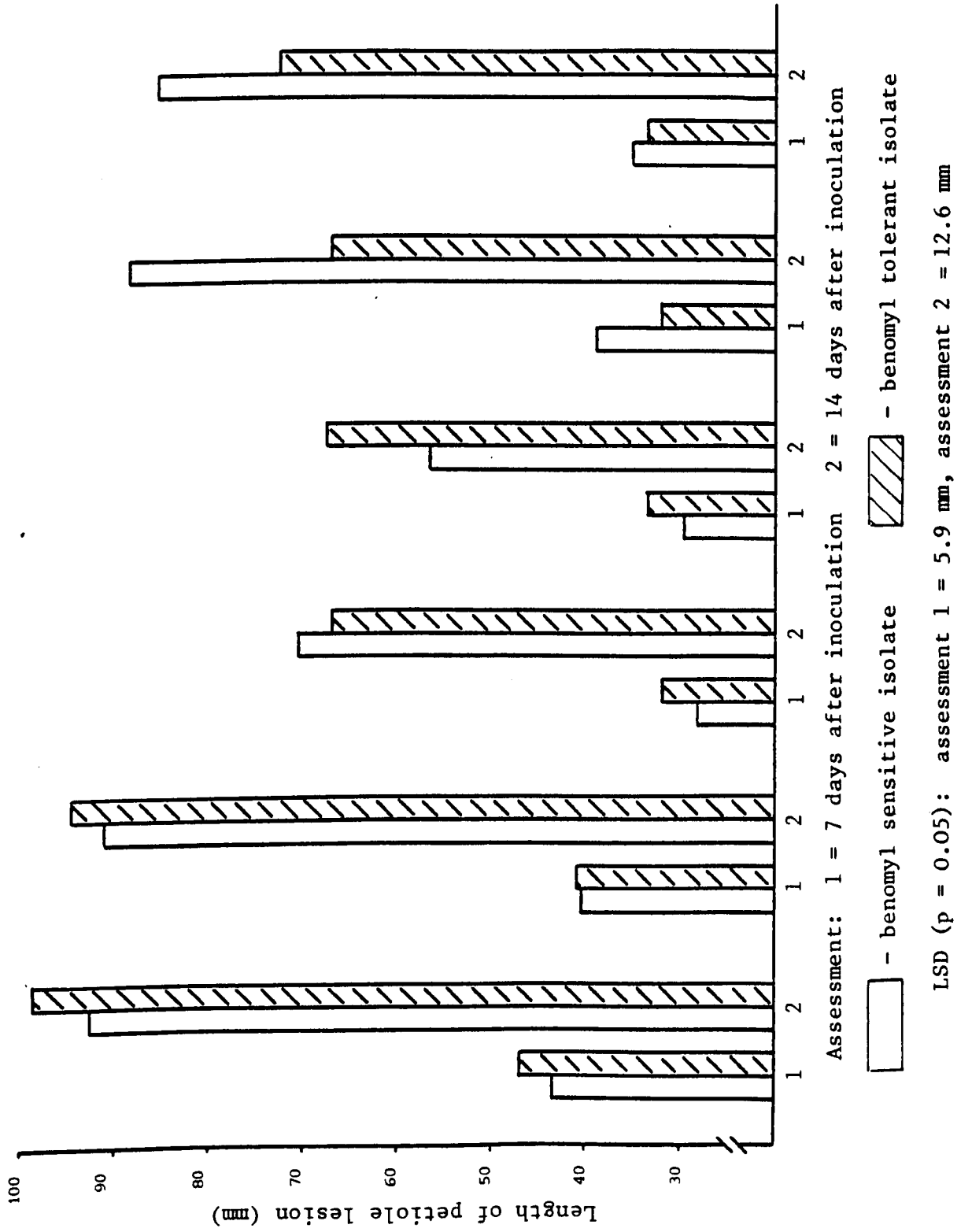




Number of lesions tested varied due to natural petiole abscission after inoculation

Figure 31: - continued

Figure 32: The pathogenicity of isolates of B. cinerea used in mixed inoculation experiments - tested on petioles of mature plants



33S and 17R did not differ in pathogenicity (Table 11) and when mixed, lesions tended to contain predominantly sensitive or tolerant spores (Table 12). Overall, neither isolate showed a tendency to dominate over the other. Three sprays of 10 p.p.m. benomyl slightly increased the ratio of tolerant to sensitive spores, but this was not significant ($p = 0.05$).

Table 11: Lesions spread on petioles inoculated with single or mixed isolates of *B. cinerea* in the presence or absence of benomyl

Inoculum	Mean lesion length mm (7 days after inoculation)	
	0 p.p.m. benomyl	10 p.p.m. benomyl
17R	48.3	47.1
33S	53.3	45.0
17R + 33S	47.5	49.3

L.S.D. (0.05) = 10.2. Means not significantly different (p = 0.05)

Table 12: Percentage of tolerant spores isolated from lesions resulting from a mixed inoculum of a sensitive and tolerant isolate of *B. cinerea*

Lesion	% tolerant spores	
	0 p.p.m. benomyl	10 p.p.m. benomyl
1	93	11
2	0	100
3	9	0
4	3	88
5	20	98
6	100	96
7	13	100
8	91	0
9	0	100
10	88	13
Mean	41.7	60.6

Means not significantly different (p = 0.05)

7. THE EPIDEMIOLOGY OF B. CINEREA INFECTING GLASSHOUSE TOMATO PLANTS

Botrytis stem lesions are very common on glasshouse tomato plants. Comparatively few of these lesions, however, seriously damage the plant. Past research has defined the conditions favourable for stem lesion development but has failed to explain why some lesions are aggressive and others not. This study was undertaken to investigate factors which might influence the successful colonization of tomato stems by B. cinerea.

7.1 Observations on B. cinerea stem and foliage infections on tomato plants

A study of tomato crops was undertaken to observe the development and determine the significance of various aspects of B. cinerea infections. Two tomato crops were examined at intervals throughout the growing season and records made of Botrytis infection:

Crop A: A half acre block (c.v. Sonato) planted out in the third week of April and grown without heat from the end of May. Watering was by a sprinkler system at ground level. One application of Benlate(R) was given at the end of May but treatment was discontinued because benomyl tolerant strains of B. cinerea were detected. Thiram dust was applied after the first deleafing, followed by applications of Elvaron(R) (dichlofluanid) and Rovral(R) (iprodione) at fortnightly intervals throughout the season. 100 plants with either stem or foliar B. cinerea infections were selected at random for study from 10 double rows comprising 2,200 plants. Four

assessments were made at intervals to record the position, size and number of Botrytis stem lesions and the presence of any infected petioles. The distance of the infection from the point of attachment to the stem was also recorded for infected petioles.

Assessment date	Extent of deleafing (height above ground, cm)	Height of crop (cm)
29/6/77	80 - 100	200
20/7/77	120 - 140	Crop stopped at 200
10/8/77	No further deleafing	-
31/8/77	" " "	-

A total of 30 lesions were recorded at leaf scars in the absence of any observed petiole infection. The majority of these consisted of light brown areas limited to the tissue immediately surrounding the leaf scar (Plate 13). These lesions were termed non-aggressive and they showed no further development throughout the study. Nine lesions produced longitudinal growth of more than 3 cm and some degree of penetration into the vascular system of the stem. These aggressive lesions tended to have a water soaked margin and profuse sporulation (Plate 14); two plants died as a result of these lesions. However, the remainder of the aggressive lesions, including one of 12 x 3 cm, subsequently dried up, causing no permanent damage to the plant. Aggressive lesions tended to be higher up the stem (mean height above ground = 67.5 cm) than the non-aggressive lesions (mean height above ground = 29.5 cm).

Plate 13

A non-aggressive stem lesion of *B. cinerea* on tomato

Plate 14

An aggressive stem lesion of *B. cinerea* on tomato

A further 30 stem lesions resulted directly from side shoot removal, the remaining stumps or scars becoming infected (Plate 15); of these, 20 were aggressive. These lesions resulted in the death of four plants. Three lesions were cut out by the grower and developed no further. The remaining aggressive lesions, including one of 11 x 3 cm, dried up naturally and caused no obvious permanent damage to the plant. Nearly all lesions resulting from side shoot removal developed at a height of 80 - 90 cm above the ground.

Four lesions were associated with infected fruit trusses, two of which became aggressive (Plate 16). These subsequently dried up and failed to kill the plant.

40 infected petioles were observed on the 20/7/77. 12 stem lesions subsequently developed at leaf scars associated with these infected petioles. Three of the 12 lesions became aggressive but all eventually dried up without further obvious damage to the plant. It was noted that infections on petioles resulted in a yellowing zone being formed in advance of the lesion. This appeared to stimulate the formation of an abscission layer so that the petiole was frequently shed before diseased tissue came in contact with the stem (Plate 17).

Crop B: consisted of a half acre block (c.v. Sonato), planted out at the end of April and grown without heat from early June. Plants were watered with an overhead sprinkler system when young and subsequently by drenching between rows with a hose pipe. Elvaron$^{(R)}$ was sprayed on at fortnightly intervals

Plate 15

Colonization of a tomato stem by B. cinerea following the removal of a side shoot:

(i) side shoot removed to leave a stump;

(ii) infection and colonization of the stump by B. cinerea;

(iii) colonization of the stem by B. cinerea resulting in an aggressive stem lesion.

Plate 16

Colonization of a tomato stem by B. cinerea following the infection of a fruit truss.

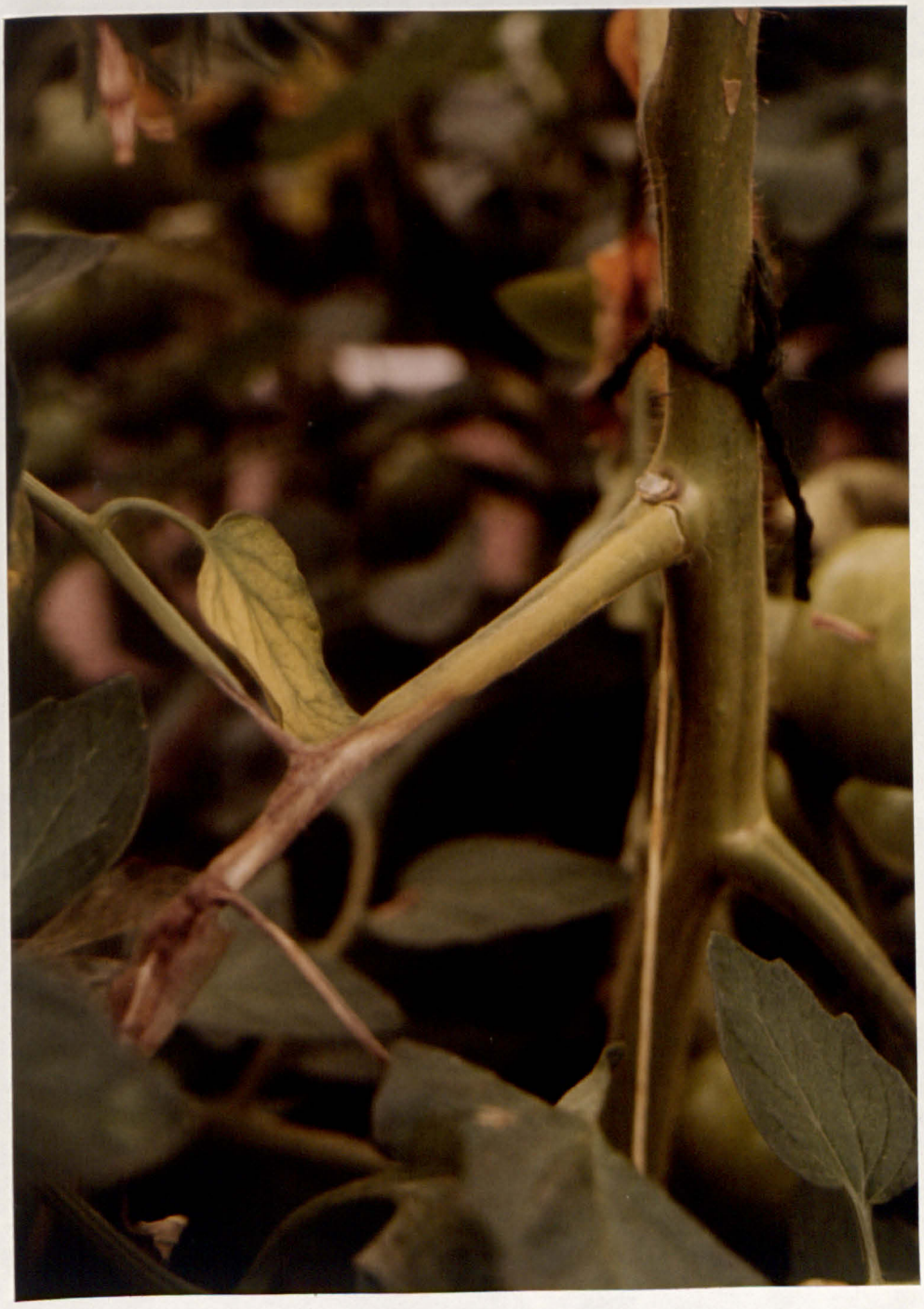
Plate 17

An infected tomato petiole showing characteristic yellowing in advance of the Botrytis lesion and subsequent abscission:

(i) yellowing in advance of the lesion;

(ii) petiole starting to absciss;

(iii) petiole shed before the fungus has reached the stem.



and plants were dusted with thiram after routine deleafing and side shoot removal. Any stem lesions noted by the grower at these times were treated with Daconil(R) (chlorothalonil) paste. 200 plants were selected at random for study from 10 double rows, comprising 2,000 plants. Four assessments were made at intervals and stem lesions and infected petioles recorded as with Crop A.

Assessment date	Extent of deleafing (height above ground, cm)	Height of crop (cm)
9/6/77	40 - 50	150
30/6/77	70 - 80	200
21/7/77	140 - 160	250
22/8/77	180 - 200	Crop stopped at 250

A total of 163 lesions were recorded at leaf scars in the absence of any observed petiole infection, 14 of these became aggressive resulting in the death of six plants. The great majority of lesions, however, were non-aggressive and subsequently dried out with no further development. Nearly all the lesions occurred between 0 and 60 cm above ground level despite deleafing having taken place up to a height of 200 cm.

20 stem lesions developed at scars or stumps resulting from side shoot removal, four of which became aggressive and killed the plants.

Three lesions developed from infected fruit trusses, one of

which became aggressive and killed the plant.

106 petiole infections were recorded, 30 of which gave rise to stem lesions. One of these became aggressive and killed the plant. The remaining 29, however, were non-aggressive and dried up with no further development. An additional 18 non-aggressive lesions developed from leaf scars to which pieces of necrotic petiole had been leaf attached.

Observations of stem lesion development in crops A and B have been summarized in the following table:

Area of stem lesion development	Number of lesions recorded	% aggressive lesions	% plants killed
leaf scar	193	12	4
leaf scar *	42	10	2
shoot scar/stump	50	48	16
fruit truss	7	43	14

* lesion directly attributed to an infected petiole.

7.2 An examination of the relationship between foliar Botrytis and the subsequent development of stem lesions

Although some information was obtained from observations on crops A and B (7.1), this study was undertaken particularly to determine the relationship between infected foliage (leaves and petioles) and stem lesions.

Crop C: This consisted of a three-quarter acre block (c.v. Verosa) planted out in the second week of May and grown

without heat; watering was by a sprinkler system at ground level. Fungicide application was identical to that of Crop A (7.1). Five adjacent rows (i.e. 2.5 double rows) comprising 518 plants were selected for study. On 1/7/77, four days before the first deleafing, plants were scored for the presence or absence of foliar Botrytis. Three more assessments were made during the season to record the position and number of stem lesions.

Assessment date	Deleafing date	Extent of deleafing (height above ground, cm)	Height of crop (cm)
1/7/77	-	-	120
21/7/77	7/7/77	60 - 80	200
10/8/77	26/7/77	80 - 100	250
6/9/77	17/8/77	160 - 180	Plants stopped at 250

On the 1/7/77 34.4% of the plants had foliage colonized by B. cinerea, although in all cases this was confined to the lower leaves. Following deleafing on the 7/7/77 the crop remained virtually free of foliar disease for the duration of the growing season. By the 21/7/77 10% of the plants had developed stem lesions, the majority of which (85.8%) were on plants which had a record of foliar disease on the 1/7/77. By the 6/9/77 56.4% of the plants had developed stem lesions, 40.8% of which had previously diseased foliage. Of the 43.6% of plants which had remained free of stem lesions up to this date, only 21.8% had a record of diseased foliage (Table 13). 32.8% of the plants with stem lesions recorded on the 10/8/77

Table 13: The relationship between stem lesion development and previous foliar colonization by B. cinerea

Row	Percentage of plants with foliar disease 1/7/77	Percentage of plants with stem lesions						Percentage of plants without stem lesions					
		21/7/77		10/8/77		6/9/77		21/7/77		10/8/77		6/9/77	
1	47	21	(86)	62	(53)	66	(51)	79	(36)	38	(33)	34	(31)
2	36	14	(57)	66	(42)	73	(42)	86	(33)	34	(18)	26	(12)
3	24	3	(100)	31	(35)	41	(35)	97	(20)	69	(15)	60	(14)
4	20	5	(100)	31	(33)	46	(26)	95	(15)	69	(13)	53	(13)
5	45	7	(86)	46	(55)	56	(50)	93	(42)	54	(37)	44	(39)
Mean	34.4	10	(85.8)	47.2	(43.6)	56.4	(40.8)	90	(29.2)	52.8	(23.2)	43.4	(21.8)

1

1

1

Figures in brackets are the percentage of the plants with a record of foliar Botrytis on 1/7/77.

1 Plants with stem lesions had a higher incidence of early foliar disease (recorded on 1/7/77) than plants without stem lesions (significant p = 0.01)

developed further stem lesions by 6.9.71. 17.4% of the plants without stem lesions on the 10/8/77 subsequently developed stem lesions by the 6/9/77.

Lesions were seen to develop at leaf scars up to 61 days after deleafing (Table 14). In the region of the stem 0 to 60 cm above the ground, 56 leaf scars became infected between 0 and 14 days after deleafing, 212 between 14 and 34 days and 70 between 34 and 61 days. During the period of 14 to 34 days after deleafing the 0 to 20 cm region of the stem above the ground was the least susceptible to infection (19 lesions recorded), with the 40 to 59 cm region being the most susceptible (109 lesions recorded).

The principal observations from crops A, B and C can be summarized as follows:

(1) The great majority of stem lesions formed at leaf scars and were non-aggressive.

(2) Aggressive lesions tended to form higher up the stem than non-aggressive lesions.

(3) Stem lesions were capable of developing at leaf scars up to 61 days after deleafing.

(4) Stem lesions tended to form on plants with a history of early foliar Botrytis.

(5) Plants with established stem lesions were more likely than healthy plants to develop subsequent stem lesions.

(6) Although diseased petioles were often associated with stem lesions, these lesions were usually non-

Table 14: The number of Botrytis stem lesions formed at leaf scars in relation to deleafing dates

Height above ground (cm) / Date	No. of new stem lesions recorded *					
	0 - 19	20 - 39	40 - 59	60 - 79	80 - 99	100+
21/7/77	26	28	2	0		
	Leaf scars exposed for 14 days →					
10/8/77	19	84	109	43	14	
	Leaf scars exposed for 34 days →				15 days →	
6/9/77	10	34	36	33	21	53
	Leaf scars exposed for 61 days →				42 days →	20 days →

* Total from five rows

aggressive. The yellow zone seen in advance of the petiole lesion appeared to stimulate the formation of an abscission layer with the consequent shedding of the infected petiole.

(7) Shoot scars were much more susceptible to B. cinerea infection than leaf scars.

7.3 The effect of de-leafing procedure on the susceptibility of leaf scars to infection by B. cinerea

Previous observations indicated that the majority of stem lesions occur at leaf scars. This experiment was designed to examine several husbandry factors which might influence the susceptibility of a leaf scar to infection. Treatments are listed below:

(1) Petioles removed by snapping and the freshly exposed leaf scar inoculated with a 6 mm mycelium/agar disc of B. cinerea isolate 2 AivS.

(2) Petioles removed by snapping and spores brushed onto the scar with a camel hair brush.

(3) Petioles cut off with a sharp knife flush to the stem and spores brushed onto the scar.

(4) As (3) above, except that the scar was covered with sellotape for 48 hours after inoculation to increase humidity.

(5) As (3) above, except that a necrotic area of tissue was induced before inoculation by stabbing the cut surface of the scar with a hot needle.

(6) Petioles cut off leaving a stump approximately 1 cm long. The stump was then brushed with spores.

Experiments were conducted in two commercial tomato crops c.v. Sonato (termed sites 1 and 2). Both crops were at a similar stage of development, having been planted out at the end of March and grown without additional heat from the end of May. Inoculations were made in June when the plants were approximately 2 m tall. Starting at a height of 30 - 40 cm from the base of each plant, petioles were removed at intervals of 20 - 50 cm to allow a maximum of six different treatments per plant. Each treatment, with the exception of treatment 1*, was replicated a minimum of 100 times and fully randomized along the length of a double row. Treatment 6 was only included at site 2. No fungicides were applied during the course of the experiment. The number and size of lesions that developed were recorded two weeks after treatment, subsequent assessments being made after a further four and ten weeks. Where lesions were considered to be a threat to the plant, fungal growth was stopped by painting the lesion with a slurry of Benlate(R).

All inoculations made with fungal mycelium gave rise to stem lesions (Tables 15 and 16). Out of a total of 100 inoculations from both sites, nine aggressive lesions had to be stopped with a slurry of Benlate(R) and four plants were killed. Despite rapid initial growth, however, the majority of lesions dried up within two to three weeks with no further

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* Treatment 1 was only replicated 40 times at site 1 and 60 times at site 2, since it was considered potentially dangerous to inoculate a large number of commercial plants in this way.

Table 15: The susceptibility of leaf scars to infection by *B. cinerea* - Site 1

Treatment (see p.128)	Method of petiole removal	Inoculum	Other factors	No. of replicates	No. of stem lesions	Percentage aggressive lesions	Percentage non-aggressive lesions
1	Hand	Mycelium	-	40	40	25	75
2	Hand	Spores	-	100	24	0	24
3	Knife	Spores	-	100	12	0	12
4	Knife	Spores	Sellotape - 48 hours	100	2	0	2
5	Knife	Spores	Necrotic spot	100	34	2	32

Table 16: The susceptibility of leaf scars to infection by *B. cinerea* - Site 2

Treatment (see p.128)	Method of petiole removal	Inoculum	Other factors	No. of replicates	No. of stem lesions	Percentage aggressive lesions	Percentage non-aggressive lesions
1	Hand	Mycelium	-	60	60	5	95
2	Hand	Spores	-	180	7	0	4
3	Knife	Spores	-	180	16	0	9
4	Knife	Spores	Sellotape - 48 hours	100	0	0	0
5	Knife	Spores	Necrotic spot	100	16	0	16
6	Knife	Spores	1 cm petiole stump	180	70	0	39

lesion growth being recorded. Inoculations with spores gave rise to few lesions, nearly all of which were non-aggressive (Tables 15 and 16). At site 1 the provision of a necrotic area of tissue led to the development of three times as many lesions as comparable treatments without this area. Two of these lesions became aggressive resulting in the death of the plants. At site 2, however, provision of a necrotic area had little effect on lesion development. Leaving a 1 cm petiole stump on the stem resulted in 39% of the inoculations giving rise to stem lesions (Table 16, treatment 6). The majority of these were, however, very small and none became aggressive. Petiole stumps at site 2 always became colonized with B. cinerea which sporulated profusely (Plate 18). Covering the leaf scar with sellotape after inoculation resulted in fewer lesions developing (Tables 15 and 16). It was considered possible that the sellotape itself may have inhibited spore germination. This was discounted, however, when in the laboratory spores in droplets of nutrient solution were found to germinate to the same degree on sellotape as on clean glass slides. At site 1, snapping the petiole by hand instead of using a knife resulted in the development of twice as many lesions, although none were aggressive (Table 15). The reverse was true at site 2, however, although few lesions developed at this site (Table 16). All the lesions resulting from artificial inoculation had developed by the time the first assessment was made (i.e. after two weeks). No lesions developed after this time and none of the non-aggressive lesions became aggressive in subsequent weeks.

Plate 18

Colonization of a petiole stump by B. cinerea following deleafing

7.4 The effect of two deleafing methods on the susceptibility of leaf scars to infection by B. cinerea

During routine deleafing petioles are usually pulled off the plants by hand. If insufficient care is taken, however, this can lead to the stem being damaged. Some growers consider that cutting the petioles with a sharp knife flush with the stem leaves a cleaner scar with less risk of damage and therefore a reduced chance of B. cinerea infection. A large scale comparison of the two deleafing methods was therefore undertaken. Throughout the season eight rows of tomatoes (336 plants) at site 2 (7.3) were deleafed by hand and eight rows with a knife. Fungicide sprays were applied as normal (Elvaron(R) applied at fortnightly intervals. Leaf scars were dusted with thiram after deleafing).

A total of 525 lesions were recorded on plants deleafed by hand and 472 on plants deleafed with a knife. No aggressive lesions developed, however, with all lesions being extremely small (10 - 15 mm in length).

7.5 The effect of prior infection of tomato stems on subsequent attack by B. cinerea

From observations of natural infection and inoculation experiments, it was clear that infections of leaf scars rarely resulted in the formation of aggressive lesions. Environmental conditions, particularly humidity and temperature, are known to be very important in predisposing plants to infection by B. cinerea. Less well understood, however, is the effect that changes in the physiological state of the plant may have on its susceptibility to B. cinerea infection. Previous

infection, for example, may induce changes in the plant. The following experiment was designed to investigate this possiblity. 12 week old pot grown tomato plants (c.v. Moneymaker), approximately 35 cm tall, were used. Stems were inoculated with a mycelium/agar disc of isolate 2 AivS, either 10 cm from soil level or 25 cm from soil level. These treatments corresponded to the 'lower' (L) and 'upper' (U) region of the stem respectively. A further series of plants were 'inoculated' in similar positions using sterile discs of PDA (control). Ten plants were used per treatment, all of which were randomized on a glasshouse bench. Three days after inoculation L plants were inoculated 15 cm above, and U plants 15 cm below the leading edge of the lesion which had arisen from the first inoculation (second inoculation). Control plants were similarly inoculated with 2 AivS mycelium/agar discs. The lengths of resulting lesions were measured after a further three days.

The lesions which developed on plants which had previously been inoculated with B. cinerea were significantly smaller than corresponding lesions on previously healthy plants (Table 17). Where the first inoculations had been in the lower region of the plant (L) lesions arising from the second inoculation were 27% smaller (significant $p = 0.01$). Where the first inoculation had been in the upper region of the plant, lesions from second inoculations were 19% smaller (significant $p = 0.05$).

During this experiment several observations were made concerning

Table 17: The effect of a previous stem lesion on the susceptibility of leaf scars to subsequent infection by B. cinerea.

Replicate	Length of lesion resulting from second inoculation (mm)					
	Above first inoculation			Below first inoculation		
	L		Control	U		Control
1	22	(25)	21	21	(38)	11
2	20	(18)	31	21	(25)	22
3	12	(32)	33	22	(43)	20
4	19	(30)	17	18	(28)	24
5	22	(23)	35	12	(40)	25
6	32	(22)	31	20	(30)	16
7	25	(18)	30	6	(21)	26
8	25	(30)	32	19	(30)	28
9	19	(35)	32	17	(29)	34
10	15	(28)	33	25	(37)	30
Mean	21.1 ++	(26.1)	29.5	18.1 +	(32.1)	23.6

L = first inoculation in the lower region of the plant.

U = first inoculation in the upper region of the plant.

Figures in brackets are the length of the lesion resulting from the first inoculation.

All lesions were measured four days after inoculation.

+ significantly different from control ($p = 0.05$)

++ significantly different from control ($p = 0.01$)

the effect of B. cinerea stem lesions on the plant. Leaves and petioles above and below the infection sites showed distinct epinasty and adventitous root formation became pronounced. When stems were examined it was found that there was considerable vascular browning in infected plants. This was particularly marked close to the lesion but was still visible at distances of up to 15 cm (above and below) the infection site.

7.6 The effect of prior infection of tomato stems on subsequent attack by B. cinerea in the field

This experiment was undertaken to see whether results obtained with pot grown plants in the glasshouse could be reproduced on mature plants in a commercial glasshouse. Mature tomato plants (c.v. Sonato) were used, approximately 2m tall when the experiment commenced at the end of June. A single petiole was removed from each of the 40 plants at a point approximately 60 cm above soil level. Twenty alternate plants were then inoculated with a mycelium/agar disc of isolate 2 AivS and the remaining 20 'inoculated' with a sterile disc of PDA (control plants). Four days after the first inoculation, petioles were removed from the fifth node above the inoculation site (approximately 45 cm above) and all the plants inoculated with isolate 2 AivS (second inoculation). The length of the resulting lesion was measured after a further four days.

Lesions which developed from second inoculations on plants previously inoculated with B. cinerea were 24.7% smaller than

corresponding lesions on control plants (significant p = 0.01) (Table 18). The experiment was discontinued after the four days to prevent serious damage to the plants. Lesions were stopped by painting with a slurry of Benlate.(R)

7.7 The effect of prior infection of petioles by B. cinerea on the susceptibility of the corresponding leaf scars to subsequent colonization by the fungus - I

The previous experiment demonstrated that an infection at one leaf scar on a plant could reduce the extent of lesions formed at other leaf scars following inoculation with B. cinerea. This experiment was designed to examine the effect of petiole infection on subsequent leaf scar susceptibility. The experiment was undertaken at site 2 (see 7.3) at the end of July, the plants had been stopped at a height of approximately 2.5 m. Four petioles per plant were used, all more than 100 cm from the stem base; two petioles were inoculated with isolate 2 AivS, 10 - 12 cm from the stem and two 'inoculated' with 'sterile' PDA. After intervals of three, five and nine days respectively, twenty infected petioles together with twenty uninfected controls were snapped off and the resulting leaf scars inoculated with isolate 2 AivS. In addition, twenty infected petioles were allowed to remain attached to determine whether or not stem lesions would arise.

Stem lesions formed following the removal of infected petioles, were always smaller (average length = 12.5 mm) than those formed following the removal of healthy (control) petioles (average length = 42.4 mm) (Table 19). The effect of increasing the time period between inoculations of the petiole and petiole removal further reduced stem lesion size (Table 19). The aggressiveness of stem

Table 18: The effect of a previous stem lesion on the susceptibility of subsequent leaf scars to colonization by *B. cinerea* - a field experiment

Replicate	Length of lesion resulting from second inoculation (mm)	
	Previously infected	Control
1	0 (23)	15
2	12 (20)	27
3	20 (25)	14
4	12 (18)	20
5	30 (22)	25
6	12 (19)	15
7	19 (21)	20
8	15 (20)	16
9	12 (16)	20
10	10 (24)	16
11	10 (23)	18
12	14 (20)	25
13	12 (17)	15
14	15 (18)	24
15	16 (22)	16
16	14 (21)	22
17	22 (17)	20
18	16 (23)	23
19	16 (19)	16
20	12 (24)	17
Mean	14.5* (20.6)	19.2

Figures in brackets are the length (mm) of the lesion resulting from the first inoculation.

All lesions measured four days after inoculation.

* significantly different from the control ($p = 0.01$)

Table 19: The effect of petiole infection on the subsequent susceptibility of the corresponding leaf scar to colonization by *B. cinerea*

No. of days following petiole inoculation prior to removal	Mean lesion spread on petiole at removal (mm)	Mean stem lesion length (mm) after 4 days		Percentage reduction in stem lesion length
		Infected petiole	Healthy petiole (control)	
3	20.3	16.8* (2)	41.3 (17)	59.4
5	34.5	9.8* (0)	37.8 (14)	74.2
9	99.3	11.3* (1)	48.3 (17)	76.7

Figures in brackets are the number of aggressive lesions out of 20.

* significantly different from the control ($p = 0.01$)

lesions was also greatly reduced when petioles were infected (Table 19). Three days after inoculation petioles showed characteristic yellowing and were slightly easier to break off than healthy petioles. This was especially noticeable with petioles on the lower part of the stem. Of the 20 infected petioles which had been allowed to remain attached to the stem, nine gave rise to stem lesions (average length = 9.4 mm), none of which were aggressive. The remainder abscissed before the fungus had reached the stem. Abscission was more common with older petioles lower down the stem.

7.8 The effect of prior infection of petioles by B. cinerea on the susceptibility of the corresponding leaf scars to subsequent colonization by the fungus - II

In order to verify the results obtained from the previous experiment the work was repeated the following season. The experiment was undertaken on a commercial glasshouse crop (c.v. Estrella) planted out at the beginning of April, approximately 2 m tall. Two petioles, 30 - 40 cm apart, mid-way up the stem, were inoculated with isolate 2 AivS 10 - 12 cm from the stem. The petioles were pulled from the plant 0, 2, 4 or 8 days after inoculation respectively. Resulting leaf scars were inoculated with 2 AivS. All treatments were replicated 10 times and fully randomized. Stem lesions resulting from the leaf scar inoculations were measured after six days' development. The degree to which the lesion had penetrated the stem was also assessed using a 0 - 4 scale (0 = no penetration, 4 = severe penetration, likely to result in plant death).

A petiole colonized for four or more days duration significantly reduced lesion size at the corresponding leaf scar following inoculation ($p = 0.01$). Both the extent of the lesion and penetration into the stem were reduced (Table 20). Inoculated petioles became easier to remove from the stem as petiole colonization progressed. After eight days an abscission layer at the junction of the infected petiole and stem was almost complete; a number of these petioles abscissed unaided.

7.9 The effect of infecting petioles with B. cinerea isolates of differing pathogenicity on the susceptibility of the corresponding leaf scars to subsequent colonisation by a highly pathogenic isolate

It was considered that the susceptibility of a leaf scar to infection could be influenced by the rate of colonization of the corresponding petiole. To investigate this possibility the previous experiment was extended to include ten petioles infected with a weak pathogen, isolate 9 BiS. These petioles were removed after four days' colonization and the corresponding leaf scars inoculated with isolate 2 AivS (a strong pathogen) as before.

When inoculated with the weaker pathogen, 9 BiS, lesion spread on the petiole was reduced by 46.3% compared to inoculations with the stronger pathogen 2 AivS. The degree of inhibition of subsequent lesions at the leaf scar, remained the same (Table 20).

7.10 The role of the abscission layer, formed between an infected petiole and the stem, on inhibiting subsequent colonization of the leaf scar by B. cinerea

The following experiment was designed to see whether the abscission layer provided a mechanical barrier to subsequent colonization of the leaf scar. Petioles were inoculated with isolates 2 AivS and 9 BiS and removed 0, 2, 4 or 8 days after inoculation as in experiments 7.8 and 7.9. A 2 - 3 mm thick slice was cut from the freshly exposed leaf scar with a sterile scalpel. Preliminary experiments had shown that this was sufficient to remove any abscission layers which may have formed in response to infection. The leaf scars were then inoculated with a mycelium/agar disc of isolate 2 AivS.

Where petioles had been colonized for 0, 1, 2 or 4 days the removal of a 2 - 3 mm thick slice from the leaf scar had no significant effect on subsequent lesion spread. Where petioles had been colonized for 8 days, however, removal of a 2 - 3 mm thick slice resulted in increased lesion spread (significant $p = 0.01$) at the leaf scar. Lesions were still significantly ($p = 0.01$) smaller than on leaf scars where the petioles were not infected prior to removal (0 days infection) and penetration of the fungus into the stem was reduced (Table 20).

7.11 The effect of extracts from B. cinerea infected tomato stems on germination of B. cinerea conidia in vitro

Previous experiments clearly demonstrated that infections of stems and petioles by B. cinerea make plants less susceptible to further colonization by the fungus. Preliminary experiments

Table 20: Lesion development at leaf scars inoculated with *B. cinerea* after the removal of infected petioles

No. of days following petiole inoculation prior to removal	Mean lesion spread on petiole (mm) prior to removal	Mean stem lesion length (mm) after 4 days	
		Direct inoculation	2 - 3 mm slice removed from scar prior to inoculation
2 AivS			
0	-	52.5 (2.0)	50.0 (2.5)
1	0	54.5 (2.0)	46.8 (1.8)
2	13.3	43.5 (1.5)	38.0 (1.5)
4	30.8	34.5 (1.2)	27.0 (0.6)
8	62.7	15.0 (0.5)	31.5 (0.4)
9 BiS			
4	14.3	34.5 (1.3)	37.5 (0.9)

Figures in brackets are the degree of stem penetration measured on a 0 (no penetration) to 4 (severe, likely to kill plant) scale.

LSD between treatments ($p = 0.05$) = 11.28

LSD between treatments ($p = 0.01$) = 15.08

suggested that infection could stimulate the production of fungal inhibitors in the plant. The following experiment was designed to test this hypothesis. The mid point of the stems of 10, 12 week old pot grown tomato plants (C.V. Moneymaker) were inoculated with a mycelium/agar disc of isolate 2 AivS. Four days after inoculation, 5 cm long segments were cut from the plants from a point 5 cm above the leading edge of the resulting stem lesion. The stem segments were bulked and an extract prepared. An extract was also prepared from healthy plants 'inoculated' with a sterile PDA disc using similar lengths of stem. A spore suspension of isolate 2 AivS (10^5 spores/ml) was prepared in phosphate buffer (pH6) and 0.2 ml of the suspension placed into each of seven cavity slides. Germination of the spores on the slide was then examined in the presence of the following additions to the spore suspension:

(1) 0.2 ml phosphate buffer (pH6).

(2) 0.2 ml 0.1% malt extract in buffer.

(3) 0.2 ml healthy stem extract in buffer.

(4) 0.2 ml infected stem extract in buffer.

(5) 0.2 ml infected stem extract in buffer x 2 dilution*.

(6) 0.2 ml infected stem extract in buffer x 10 dilution*.

(7) 0.2 ml infected stem extract in buffer boiled for 10 minutes and immediately cooled to room temperature.

* Dilutions were achieved by the addition of phosphate buffer (pH6) as required.

Each experiment was replicated twice. The mean percentage spore germination was established after two, four and six hours respectively, by examining 100 spores from each replicate. After six hours the average germ tube length was calculated by

measuring 50 germ tubes from each replicate.

After two hours, germination of spores was considerably reduced in extracts from infected plants when compared to healthy stem extracts (Table 21). However, the number of spores germinating in both extracts reached 100% after four hours. The delay in germination resulted in shorter germ tubes being produced after six hours. Diluting the infected stem extract two-fold had no effect on germination rate. A 10-fold dilution, however, substantially increased germination after two hours, to a level approaching germination in healthy stem extract. Boiling the infected stem extract also increased germination. Germ tubes grew faster in both these treatments and their length after six hours was greater than those formed in healthy stem extract. Germination of spores in 0.1% malt extract was equal to that in healthy stem extract. Germ tubes, however, were almost twice the length of those formed in healthy stem extract after six hours. The percentage germination was lowest in phosphate buffer, only 26.4% of the spores germinated after six hours compared to 100% for all other treatments.

7.12 The germination of B. cinerea conidia in extracts from stem segments taken from various points above and below the site of B. cinerea infection

The previous experiment (7.11) demonstrated the presence of a substance, inhibitory to spore germination, in tomato stems following B. cinerea infection. This experiment was undertaken in an attempt to determine the extent to which such an inhibitor, or inhibitors, are disseminated through the plant

Table 21: Spore germination in extracts from healthy stems and stems infected with B. cinerea

Treatment	Percentage germination after			Germ tube length (μ) after 6 hours*
	2 hours	4 hours	6 hours	
Phosphate buffer (pH6)	7.0	10.5	26.5	-
0.1% malt extract	85.0	100	100	83 ± 6.0
Healthy stem extract	81.0	100	100	46 ± 4.1
Infected stem extract	21.5	100	100	24 ± 2.5
Infected stem extract x 2 dilution	22.5	100	100	28 ± 2.9
Infected stem extract x 10 dilution	73.5	100	100	59 ± 5.7
Infected stem extract boiled	62.5	100	100	64 ± 5.4

* limits of the means significant, p = 0.05.

from the point of infection. Stems were inoculated with a mycelium/agar disc of isolate 2 AivS at a point 30 cm from the base of the plant. Four days after inoculation extracts were prepared from 5 cm sections of the stem removed from the following positions:

(1) 5 cm above the leading edge of the stem lesion.

(2) 15 cm above the leading edge of the stem lesion.

(3) 5 cm below the leading edge of the stem lesion.

(4) 15 cm below the leading edge of the stem lesion.

An extract was also prepared from petioles removed from the second, third and fourth nodes above the lesion. Two control extracts were prepared from healthy stems, one from 10 cm above and one from 10 cm below the point corresponding to the stem lesion on infected plants. A third control extract was prepared from petioles on healthy plants. Extracts were tested for their effect on spore germination as in the previous experiment (7.11); two replicate tests were made for each extract.

Spore germination was considerably delayed in the presence of extracts prepared from stem segments taken from above and below established stem lesions (Table 22). Germination delay was more pronounced, however, in extracts prepared from segments taken 5 cm away from the stem lesion than in extracts from segments 15 cm away. This delay was also more obvious in extracts from segments taken from above the stem lesion. Germ tube length after six hours was directly related to the rate of germination. Extracts prepared from petioles removed

Table 22: Spore germination in extracts from stem segments taken from different positions on stems relative to the point of infection with B. cinerea

Extract origin	Percentage germination after			Germ tube length (μ) after 6 hours*
	2 hours	4 hours	6 hours	
Control (upper stem)	72.0	100	100	27 ± 2.4
Infected - 5 cm above lesion	3.5	39.5	70.0	10 ± 1.2
Infected - 15 cm above lesion	10.5	58.0	91.5	13 ± 1.1
Control (lower stem)	70.5	100	100	25 ± 1.8
Infected - 5 cm below lesion	12.5	63.0	98.5	13 ± 1.4
Infected - 15 cm below lesion	30.5	86.5	100	15 ± 1.3
Control (petioles)	90.5	100	100	47 ± 4.6
Infected petioles	83.0	100	100	35 ± 3.2

* limits of the means significant, p = 0.05

from infected plants did not significantly delay germination compared to controls, although germ tube length after six hours was slightly reduced.

7.13 The germination of B. cinerea conidia in extracts from stems previously colonized for different periods of time with the fungus

The previous experiment (7.12) indicated that a spore germination inhibitor, produced in response to infection, was more concentrated in the region of the stem closest to the infection site. The following experiment was designed to determine whether the duration of colonization following infection influenced the levels of such inhibitors within the stem. The mid points of the stems of 12 week old tomato plants were inoculated with a mycelium/agar disc of isolate 2 AivS as before using 10 plants per treatment. Plants were inoculated sequentially to give, after eight days, lesions of 0, 1, 2, 4 and 8 days duration. 5 cm stem pieces were cut from all plants at a point 5 cm above the leading edge of the lesions resulting from inoculation and extracts prepared as in the previous experiment (7.12). Spore germination was examined in the presence of the extract; two replicates were made for each extract examined.

As the time between inoculation and extraction increased from 0 to 4 days there was a corresponding decrease in the percentage spore germination in the extracts after six hours incubation. After a period of 8 days between inoculation and extract preparation, spore germination in the extract after six hours incubation increased, although was still less than

recorded when extracts were prepared 0 days after inoculation. Germ tube length again appeared to be directly related to the rate of spore germination (Table 23).

7.14 The effect of B. cinerea culture filtrates on the germination of B. cinerea conidia - I

The previous experiments (7.11 to 7.13) suggested the presence of a fungal inhibitor in infected stems. They did not indicate, however, whether it was of fungal or plant origin. The following experiment was undertaken to investigate whether an inhibitor was produced by B. cinerea growing in vitro. 100 ml of tomato stem broth was placed into each of five 250 ml conical flasks and autoclaved at 121°C for 15 minutes. After cooling, each flask was inoculated with a 6 mm mycelium/agar disc of isolate 2 AivS and the flasks incubated at 23°C in the dark with continuous agitation. After 0, 1, 2, 4 and 8 days incubation culture filtrates were prepared by passing the culture through four layers of muslin and then through Whatman No. 2 filter paper under vacuum. Filtrates were stored frozen at -18°C until required. Spore germination after 2, 4, 6 and 24 hour incubation was examined as in previous experiments (7.11 to 7.13).

Increasing culture age from 0 to 4 days progressively increased the rate of spore germination in the culture filtrate (Table 24). A culture filtrate obtained from an 8 day old culture, however, almost completely inhibited spore germination; only 12% of spores germinated after 24 hours compared to 100% for all other culture filtrates.

Table 23: The inhibitory effect of extracts from plants with *B. cinerea* lesions of different ages

Days after inoculation	Mean length of stem lesion (mm)	Percentage germination after			Germ tube length (μ) after 6 hours*
		2 hours	4 hours	6 hours	
0	-	26	90	100	82 ± 8.7
1	8.5	6	80	85	68 ± 6.8
2	25.0	2	36	85	41 ± 6.1
4	47.5	0	16	70	34 ± 5.6
8	75.5	2	38	90	44 ± 6.1

* limits of the means significant, p = 0.05

7.15 The effect of B. cinerea culture filtrates on the germination of B. cinerea conidia - II

Experiment 7.14 was repeated with two additional treatments:

(i) 8 day old culture filtrate was autoclaved at 121°C for 15 minutes and then cooled to 20°C before testing spore germination.

(ii) 0.1% malt extract was added to 8 day old culture filtrate before testing spore germination.

The results proved very similar to the previous experiment (7.14). Autoclaving 8 day old culture filtrate had no effect on its inhibition of spore germination. The addition of 0.1% malt extract, however, completely removed the inhibitory effect (Table 24).

Table 24: The inhibitory effects of filtrates from cultures of *B. cinerea* grown in tomato stem broth

Age of culture (days after inoculation)	Percentage germination after							
	2 hours	2 * hours	4 hours	4 * hours	6 hours	6 * hours	24 hours	24 * hours
0	0	0	19	9	100	100	100	100
1	0	0	64	71	100	100	100	100
2	17	29	100	100	100	100	100	100
4	10	7	100	100	100	100	100	100
8	0	0	0	0	0	0	12	23
8 (autoclaved)	N.T.	0	N.T.	0	N.T.	0	N.T.	20
8 (0.1% malt extract)	N.T.	33	N.T.	72	N.T.	100	N.T.	100

* repeated experiment

N.T. Not tested

8. DISCUSSION

8.1 Variability

Experiments confirmed that single spore isolates of B. cinerea can differ sharply in morphology from their parent isolates. The characteristic production of two distinct morphological types, T_1 and T_2, by the majority of isolates could be considered similar to the "dual-phenomenon" reported by Hansen (1938). Hansen concluded that the production of a mycelial and conidial type was a feature of a large number of fungi imperfecti, including B. cinerea. Menzinger (1966) endorsed these findings reporting that 70% of the B. cinerea isolates he tested had the potential to produce conidial or mycelial types. The "duality" expressed by the isolates in this investigation, however, is unrelated to sporulation. Both T_1 and T_2 types were classified according to the growth habit of the mycelium; sporulation, though variable, was not a distinguishing feature. Non sporulating "mycelial" types were only very occasionally observed during the course of the investigation. It could be argued that the basis for type classification in this investigation was too narrow, thus allowing mycelial and conidial types to be overlooked. The segregation into the T_1 and T_2 types was so marked, however, that this must be considered unlikely. The morphological expression of B. cinerea isolates is known to be greatly influenced by cultural conditions, particularly growth media and temperature (Menzinger, 1966, Vanev, 1972). The fact that Hansen and Smith (1932) grew their isolates at room temperature, presumably subject to considerable fluctuations, and that Menzinger (1966) used Bio-Malt agar and

not PDA may have contributed in part to the morphological differences they observed. Differences in culture techniques may have also directly influenced variant segregation; although it is significant that the same proportion of isolates displayed intra-isolate variation in this investigation (70%) as reported by Menzinger (1966).

Considerable uniformity was seen within the T_1 and T_2 types from this investigation. Of the principal isolates studied 24 AiiS was the only exception with three distinct T_2 types being recognized. These types were unstable; T_4, for example, reverted back to the vigorously growing T_1 type after ten generations. This fits in well with the observations of Brierley (1931) that "variants may be consistent for a larger or smaller number of transfers and then revert partially or wholly". Menzinger (1966), who accepted heterokaryosis as an explanation for the occurrence of variant types, concluded that the heterokaryotic condition can be firmly secured within the mycelium, and that it can be maintained over long stretches of fungal growth without morphological indication of its presence. The experiments with isolate 23 AiR would support this view. A T_2 type was only revealed after a much more stringent testing procedure was adopted.

The majority of workers assume that dual segregation is an indication of the heterokaryotic nature of the fungi involved (Parameter et al, 1963). Although heterokaryosis has been demonstrated in B. cinerea (Hansen and Smith, 1932, 1935; Menzinger, 1966) and other fungi (Hansen, 1938; Jinks, 1952),

rigorous proof that the "dual phoenomenon" is a result of heterokaryotic segregation is lacking in most cases (Caten and Jinks, 1966). This has been emphasized by Burnett (1976) who considers it difficult to see how the dual phenomenon can occur in fungi with uninucleate conidia. Heteroplasmic dissociation has, however, been demonstrated as the cause of dual phenomenon in two isolates of Penicillia of the Asymetric group (Jinks, 1959) and has also been demonstrated in Aspergillus nidulans (Jinks, 1956; Croft, 1964), Aspergillus glaucus (Sharpe, 1958) and Aspergillus versicolor (Caten, 1965). Burnett (1976) suggests that many cases of sectoring in otherwise homogenous cultures may be a result of the assortment of extra-chromosomal elements. Jinks (1956) considers that each spore consists of a small sample of the cytoplasm of the fungus and unless cytoplasmic elements are evenly distributed some of these samples could be deficient and therefore unbalanced. This seems to relate directly to the dual segregation observed in this study. Whereas T_1 types stabilized quickly and were predominant over T_2 types, the latter were unstable and appeared to be growing under stress. T_2 types were slow growing and distorted, suggesting that the mycelium was "deficient" in some respect. Owing to the multinucleate nature of B. cinerea conidia, however, it can only be a matter of conjecture as to whether this deficiency was of cytoplasmic or nuclear origin. It may be significant that cultures of the T_2 type could give rise to the more vigorous T_1 type in subsequent generations indicating that the deficiency was not necessarily a permanent feature. This deficiency may also have been related to the cultural

conditions since the distortion observed in T_2 type cultures was greatly reduced when the incubation temperature was lowered.

Although there is now considerable evidence for the existence of heteroplasmons and heterokaryons, the frequency with which they occur and their significance in the field is open to question. It is likely that only a few of the cytoplasmic/nuclear variants present in a sample of asexual spores will survive competition in the field. Colonies derived from single spores in the laboratory, however, will escape the consequences of natural selection. In addition, single spore isolation would prevent anastomosis during spore germination and so prevent the spores from complementing each other's cytoplasm and nuclei. Variability demonstrated by single spore cultures in the laboratory cannot, therefore, be considered a direct reflection of the variability of the fungus in the field.

An integral part of the variability study was an investigation of the reaction to benomyl through successive generations of single spore cultures. Benomyl tolerant isolates displayed a similar degree of inherent variability as the sensitive isolates. There was no evidence to suggest that the acquisition of benomyl tolerance had influenced a strains inherent variability making it less, or better able to adapt. Experiments showed that the degree of benomyl tolerance was not influenced by successive subculturing onto fungicide free agar using the single spore isolation technique. This

stability of benomyl tolerance in B. cinerea has previously been demonstrated by Jordan and Richmond (1974) and Geeson (1976) who found that tolerance was unchanged after repeated mycelial disc subculture onto fungicide free agar. Pourtois et al (1976), however, were able to obtain both sensitive and tolerant strains from single isolates of B. cinerea (either tolerant or sensitive) using hyphal tip isolation. They suggested that this was a result of isolating homokaryotic strains from a heterokaryotic isolate. No evidence of isolates heterokaryotic for benomyl tolerance was found in this study; either the isolates tested were homokaryotic for benomyl tolerance or heterokaryons were missed because of the relatively small number of single spore isolations made.

The degree of in vitro variability shown by the isolates of B. cinerea used in this study emphasizes the need for caution in all research with this fungus. Steps must be taken to minimize the influence of inherent in vitro variability in all experiments. Where practical, the following points should be considered:

(1) As many isolates as possible should be examined to minimize the influence of inter and intra-isolate variation.

(2) If it is necessary to restrict the number of isolates used, the degree of inherent variability of each isolate should be tested using single spore isolation techniques. Isolates displaying the least tendency for variation should then be selected for study so that

reproducible results can be obtained.

(3) Ideally experiments should be undertaken with fresh field isolates. It is undesirable to store cultures for any length of time since segregation and degenerative changes can occur (Jinks, 1956; Burnett, 1976).

(4) Isolates should be sub-cultured by mass spore transfer to minimize possible nuclear and cytoplasmic segregation.

(5) Careful consideration should be given before drawing any conclusions from *in vitro* results.

8.2 Benomyl tolerance

B. cinerea isolates collected from tomato nurseries in Yorkshire and Lancashire differed in their gross morphology. These morphological differences could not be correlated with the isolates' reaction to benomyl; a finding in agreement with the observations of Geeson (1976). It is possible, however, that the large range of morphological types seen among the isolates examined may have masked any morphological changes associated with the development of benomyl tolerance. The diversity of morphological types observed was greatly reduced when isolates were collected from a single glasshouse. At Chartwell Growers, for example, 40 isolates collected were all morphologically similar and all benomyl tolerant. This suggests that within the confines of a single glasshouse a morphologically distinct population may develop. At N.W. Growers, both tolerant and sensitive strains were present in a single glasshouse. The sensitive isolates were morphologically similar but differed from the majority of

tolerant isolates which in turn displayed a variety of morphological types. When benomyl was in use and the development of sensitive strains suppressed, tolerant strains may have assumed a morphological diversity of their own through possible anastomosis and heteroplasmic/heterokaryotic dissociation. The extent of this dissociation may have been influenced by the environmental conditions prevailing in the glasshouse at that time. The morphological differences observed in this investigation were probably of little significance in themselves since they related only to the *in vitro* appearance of the colony. It became clear, for example, that isolates, including those which were morphologically similar, displayed great variability in growth rate on agar. This was exemplified at Chartwell Growers where the variable growth rate on PDA of 40 morphologically similar benomyl tolerant isolates could not be correlated with their growth rate on PDA amended with 100 p.p.m. benomyl.

Significant differences in growth rates on PDA were observed between isolates collected over a wide area, but initially it was difficult to relate these differences to the isolates' reaction to benomyl. However, when the growth rates of isolates from 10 different glasshouses were examined it became apparent that tolerant isolates tended to be slower growing than sensitive isolates within any one glasshouse. A similar trend was observed with isolate pathogenicity, tolerant isolates tending to be less pathogenic. The majority of reports concerning *B. cinerea* isolates, however, suggest that growth rate and pathogenicity are unaffected by benomyl

tolerance in the absence of benomyl (Jordan and Richmond, 1974; Bolten, 1976; Dennis and Cohen, 1976; Geeson, 1976; Cho, 1977). It may be significant, however, that in the majority of these reports, very few isolates have been compared. This is especially relevant to B. cinerea where the extent of natural variation existing between isolates is very large. In addition this variation is probably increased when isolates are collected from a variety of host plants in different geographical localities as in the study by Geeson (1976).

Experiments with mixtures of tolerant and sensitive isolates were interesting since they gave some insight into the behaviour of strains in competition. In vitro experimentation on agar demonstrated that one isolate usually survived at the expense of the other when spores of the two isolates were mixed. Coexistence of the two isolates in vitro was only maintained for a few generations. This suggested that in vivo, tolerant and sensitive strains are more likely to exist in separate lesions than as mixtures. Support for this hypothesis was obtained from the field when it was found that in glasshouses containing both tolerant and sensitive strains 84% of the Botrytis lesions containing tolerant strains produced 100% tolerant spores. The addition of 1 p.p.m. benomyl to the agar totally inhibited the sensitive isolate in the mixture; a dose of 0.1 p.p.m. (sub-lethal to sensitive strains), however, had no effect. This lower dose should have slightly inhibited mycelial growth of sensitive isolates (Bollen and Scholten, 1971; Geeson, 1976) but this may have been compensated for by a slight increase in spore

production. Although such a response has not been reported in the literature it may be significant that tolerant isolates in this study showed a reduced growth rate but increased sporulation in the presence of 100 p.p.m. benomyl. Altering the ratio of tolerant to sensitive spores influenced which isolate became dominant in only two of the six cases examined, suggesting that an isolate's capacity for sporulation was a more important criterion for survival. This, however, was not always the case, although the rate of spore germination (not examined) may have been an important factor. The possibility that isolates behave differently in mixtures cannot be ignored since any interactions may affect sporulation. Certain discrepancies in the results of repeated experiments, especially the inconsistency in sporulation displayed by the majority of isolates, only serve to illustrate once again the variable nature of B. cinerea in vitro.

Investigation into the behaviour of mixed isolates as parasites reflected a similar trend to studies of their behaviour as saprophytes. Lesions resulting from inoculations of mixed isolates contained predominantly either tolerant or sensitive spores. The success of an isolate also seemed unrelated to its previously determined pathogenicity. It is possible that the growth of a weaker pathogen can be enhanced by the presence of a stronger pathogen. B. cinerea is considered a weak parasite and readily takes advantage of damaged or senescent tissue (Thomas, 1921; Smith, 1970). The inconsistency between replicates of the same mixture is difficult to understand but suggests that differences between the isolates

are not great enough to allow one isolate to predominate throughout. Surprisingly 10 p.p.m. benomyl sprays did not increase the success rate of tolerant isolates. Although this dose is well below the manufacturers' recommended rate (500 p.p.m.) it should have limited the growth of sensitive isolates. Benomyl was not applied, however, until three days after inoculation, by which time it is possible that dominance may have already become established. B. cinerea mycelium also tends to grow within the vascular system of the petiole and this may have provided initial protection from the chemical.

Although there were no indications that benomyl tolerance had influenced an isolate's ability to compete in mixed culture, either as a saprophyte or parasite, too few isolates were examined to allow any conclusions to be made regarding the relative fitness of tolerant strains. The ability of a strain to adapt to a constantly changing environment must be of great importance, since natural selection is likely to eliminate strains with even the smallest defect. Experiments demonstrated that an isolate's capacity for sporulation was influenced by changes in cultural conditions and, more importantly, showed how different isolates responded in different ways. Any defect in the speed or degree of response to environmental change, easily missed in vitro, may result in a strain being disadvantaged in the field.

The only effective way of determining the relative fitness of tolerant and sensitive strains is by detailed survey work

in the field. Observations from 17 glasshouses emphasized how widespread benomyl tolerance had become in B. cinerea infecting tomato crops. Of 680 isolates collected in this study during 1977: 64.3% were tolerant compared with 49.6% in a survey in 1974 (Fletcher and Scholefield, 1976). All the glasshouses sampled in this study contained tolerant strains of B. cinerea although, in some cases, benzimidazole fungicides had not been used for the previous three years. Samples taken from two glasshouses over three consecutive years showed little decline in the incidence of tolerant strains, even in the absence of the fungicide. A similar situation exists with other fungi: Dovas et al (1976) found that three years after the cessation of benomyl application the frequency with which tolerant strains of Cercospora beticola were isolated from sugar beet had not changed. Washington (1977) reported that tolerant strains of Venturia inaqualis had persisted for three years since the use of benzimidazoles in apple orchards had been discontinued. It would seem, therefore, that benomyl tolerant strains of B. cinerea and certain other fungi are able to survive in the fungal population long after the use of the fungicide has been discontinued. However, Ruppel et al (1980) reported that although 100% of C. beticola isolates obtained from Arizona sugar beet fields in 1978 (three years after benomyl had been discontinued) were tolerant, the degree of tolerance had sharply declined since the withdrawal of the fungicide. Whereas in 1976, 100% of tolerant isolates had grown on PDA amended with 100 p.p.m. benomyl, none grew at this concentration in 1978, only showing growth at 10 p.p.m. The authors suggested that

this apparent increase in sensitivity may be academic since isolates capable of tolerating 10 p.p.m. *in vitro* can induce severe leaf spot in sugar beet sprayed with normal field rates of benomyl (Ruppel, unpublished). It may indicate, however, that strains exhibiting the highest levels of tolerance have a reduced fitness or that tolerant strains will gradually revert to complete sensitivity. It is therefore important that in any future survey work, *B. cinerea* isolates are screened for tolerance on agar containing a range of benomyl concentrations.

Although the majority of *Botrytis* lesions did not contain mixtures of tolerant and sensitive spores, it is important that research workers are aware of this possibility. A false impression of the prevalence of benomyl tolerance within a crop could be obtained if lesions were only screened for the presence or absence of tolerant strains. The mixed lesions may have resulted from the movement of spores within the same glasshouse, from two or more distinct strains growing in close association or from a single heterokaryotic strain. Strains of *B. cinerea* heterokaryotic for benomyl tolerance have been observed (Pourtois *et al*, 1976) and could be important in maintaining a small pool of tolerant nuclei in the population in the absence of the selection pressure. The fact that the majority of lesions with mixed strains produced less than one per cent tolerant spores may reflect the dissociation of a heterokaryon containing a small number of tolerant nuclei.

Observations highlighted the variation in benomyl tolerance between *B. cinerea* populations in different glasshouses. In one

glasshouse, where benomyl was used regularly, tolerance was uncommon (nine per cent of isolates), whereas in another, where benomyl had not been used for the previous three years, 100% of isolates were tolerant. This suggests that strains of B. cinerea within the confines of a single glasshouse may form a distinct population, the characteristics of which cannot be assumed to be the same as that in any other glasshouse. Thus, in the absence of the fungicide, it seems possible that some populations may revert to complete sensitivity, whereas others may retain their tolerance for much longer.

Where benomyl is failing to control disease and tolerant strains have been identified, continued use of the chemical is futile. In addition it may increase disease incidence (Bollen and Scholten, 1971; Jordan and Richmond, 1974; Paulus et al, 1976), by eliminating the naturally occurring phylloplane microflora which can be antagonistic to pathogens (Fokkema, 1976). Growers have been forced, therefore, to look for alternative methods of control for grey mould. There has been renewed interest in protective chemicals for high volume spraying such as the traditional alternatives to benomyl, chlorothalonil and diclofluanid (Fletcher and Harris, 1979; Morgan, 1979). Recently introduced protective fungicides for use against B. cinerea include the dicarboximide fungicides iprodione (Rovral$^{(R)}$) and vinclozolin (Ronilan$^{(R)}$) (Buchenauer, 1976; Leroux et al, 1977). These chemicals are all effective against benomyl tolerant strains of B. cinerea (Jordan and Richmond, 1974; Fletcher and

Scholefield, 1976; Leroux et al, 1977; Griffin and Smith, 1980). Morgan (1979), in fact, reported better control of a benomyl tolerant isolate than a sensitive isolate with these chemicals. Fungicides acting as multisite inhibitors are to be preferred, since tolerance is less likely to develop (Dekker, 1976). This has been demonstrated in apple orchards in the U.S.A. where tolerance of Venturia inaequalis to captan has not occurred despite 20 years of intensive use. Strains tolerant to dodine, which by disrupting the permeability of the cell membrane has a slightly more specific mode of action than captan, were identified after 10 years. Tolerance to benomyl, however, occurred after only three years' use (Szkolnik and Gilpatrick, 1973; Jones and Walker, 1976). The dicarboximide fungicides, iprodione and vinclozolin, are highly effective against B. cinerea suggesting a specific mode of action. Disruption of lipid biosynthesis or cell wall synthesis is thought to be involved (Fritz et al, 1977; Albert, 1978). It is not surprising, therefore, that strains of B. cinerea tolerant to these fungicides have been identified in the laboratory (Leroux et al, 1977) and, more importantly, isolated from strawberries in the field (Dennis and Davies, 1978; Pappas et al, 1979). Some of these strains also exhibited cross tolerance to benomyl. A break down of Botrytis control by dicarboximide fungicides in strawberries has recently been reported in Belgium (Maraite et al, 1981). The dangers of continued use of these fungicides are therefore clear.

It is interesting to consider what action can be taken to

reduce the chance of tolerance developing. In the absence of tolerance to a fungicide or where the number of tolerant strains are very small the use of mixtures of fungicides with different modes of action should be considered. Lack of tolerance in B. cinerea infecting strawberries in Australia has been attributed to the use of mixtures (Delp, 1980). In addition to B. cinerea infection, Colletotrichum acutatum (strawberry black spot) is common in Australia and as it is not controlled by benomyl, mixtures of benomyl plus captan are applied to control strawberry diseases. This mixture programme was started in 1972 and no tolerance to benomyl has been reported during the subsequent eight years. In countries where benomyl has been used exclusively for strawberry disease control, however, tolerance problems have developed. Another example of the successful use of fungicide mixtures relates to the control of Phytophthora infestans with metalaxyl. This fungicide was introduced for the control of potato late blight in Eire, Holland and Switzerland in 1979. In 1980 control failed and metalaxyl tolerant strains of P. infestans were isolated (Sozzi, personal communication). In the United Kingdom, however, where metalaxyl has not been used exclusively but only as a metalaxyl/mancozeb mixture, no such breakdown of control occurred and, despite extensive monitoring, no tolerant strains were found (Miller, unpublished). Although these circumstantial examples are interesting, experimental evidence to show the effectiveness of using mixtures to combat the development of tolerance is lacking. Working with a mathematical model, which assumed the initial (year one) frequency of tolerant spores in any fungal population to be

1.5×10^{-8}, Delp (1980) produced a theoretical example concerning Cercospora leaf spot on peanuts. In the model, when benomyl was used alone, tolerance reached an unacceptable level within three years. When a benomyl/maneb mixture was used, however, with maneb providing 80% control of the tolerant strain, development of a significant level of tolerance (10^7 lesions on 3,750 acres) was delayed indefinitely. The model also showed that development of tolerance was delayed for a lesser, but still significant, period when use of the mixture was not started until after the first or even second year. Since action to avoid the development of tolerance to new fungicides must be taken quickly, it would be very imprudent to ignore such circumstantial and theoretical evidence. Once tolerance becomes established in a population the use of mixtures is probably futile. Bolay et al (1976) found that benzimidazoles combined with non-systemic fungicides were no more effective against grey mould on grapes than the non-systemic alone. Similarly the incidence of benomyl tolerance in grey mould of tomatoes was unaffected by sprays of benomyl and dichlofluanid mixtures (Fletcher and Scholefield, 1976). Morgan (1979) also reported that a carbendazim/maneb mixture was ineffective against a carbendazim tolerant strain of B. cinerea from tomato. Kable and Jeffery (1980) concluded from a mathematical model that mixtures were ineffective when tolerant strains exceeded one per cent of the population. Until mixed products are readily available it may be useful to alternate between fungicides with different modes of action (Dekker, 1976; Delp, 1980). Using their mathematical model, Kable and Jeffery (1980) suggest that when spray coverage is

complete, selection for tolerance proceeds at the same rate whether the "at-risk" fungicide is used on its own or in mixture. If complete cover can be guaranteed, it would be possible to use two fungicides in rotation. With reduced spray cover (< 99%), however, only the use of mixtures will delay the development of tolerance.

Whether benomyl could ever be used again for tomato grey mould control following the development of tolerant strains is open to question. It is now clear that the tolerant strains persist for a considerable time in the absence of the selection pressure, benomyl. Until tolerance disappears, therefore, the use of benomyl alone, in mixture or rotation, is probably ill advised. Some glasshouses, however, seem to lose their tolerant populations faster than others. If a grower can in some way monitor the level of tolerance on his nursery, it may be possible for him to reintroduce benomyl in a mixture or rotation. However, tolerance may develop much faster than before if experiences with insecticide resistance can be used as a guide (Keiding, 1967). It is likely, therefore, that the use of one of the newer fungicides iprodione or vinclozolin in mixture (according to manufacturers' recommendation) or rotation with, for example, dichlofluanid may provide the best chemical solution for B. cinerea control. When using a fungicide, however, he should ensure complete coverage at the correct dose, thereby preventing the fungus coming into contact with sublethal doses. He should treat only when necessary. Routine treatment may be superfluous and could be actively selecting tolerant strains from the

population; an accurate method of disease forecasting would be of great value here.

8.3 Epidemiology

Observations on commercial crops made during the three years of this investigation indicate that the majority of Botrytis stem lesions on tomatoes are non-aggressive. The low incidence of aggressive lesions made it difficult to determine their cause, although they were rarely seen to be associated with infected petioles. This is in contrast to the findings of Smith (1970) who considered that the vast majority of B. cinerea stem lesions were associated with petioles infected prior to deleafing. In situations where cultural and environmental conditions are conducive to rapid colonization infected petioles must, therefore, still be considered a major threat to the health of the plant. In this study some infected petioles did cause stem lesions but these usually only consisted of a superficial browning of the leaf scar. In most cases the petioles abscissed well before the petiole lesion reached the stem. A similar reaction to B. cinerea has been reported in Begonia (Kamoen, 1976) where infected leaves usually break off before the fungus reaches the stem. This is known by growers as "the cleansing" of the begonia. The stimulation of petiole abscission by B. cinerea in tomatoes was demonstrated by Verhoeff (1967). He found that petiole stumps remaining after deleafing abscissed more quickly when infected. Materials responsible for the characteristic yellowing zone observed in advance of petiole infections may have a direct influence on abscission. Kamoen

(1976) reported a similar yellowing zone surrounding B. cinerea lesions on Begonia leaves and found it to be rich in citric acid. This he described as a vivo-toxin secreted by the fungus. Ilieva (1971) similarly related the formation of an abscission layer in tomatoes to B. cinerea toxins.

Although petioles were not directly implicated, stem lesions did nevertheless tend to develop on plants with a history of early foliar Botrytis. It seems likely that the infected foliage provided a dense spore inoculum close to the freshly exposed leaf scars. Later, in the absence of observed foliar disease, leaf scars were probably infected from inoculum provided by sporulating stem lesions. Interestingly plants with stem lesions were more susceptible to further infection than correspondingly healthy plants. However, some late developing stem lesions may still have been a direct consequence of foliar disease through the establishment of latent infections at leaf scars. The implication of spores as a major source of inoculum is in direct contrast to the observation of Thomas (1921) and Smith (1970), who claimed that Botrytis spores are not able to infect leaf scars. Wilson (1963, 1964) and Verhoeff (1967), however, have shown that spore inoculum often initiates stem lesions. Although few lesions developed at leaf scars artifically inoculated with spores in this study, this does not necessarily suggest that spores are not a major source of inoculum in the field. A survey of 146 tomato crops in 1976 (Fletcher and Harris, 1979) suggests that few leaf scars become infected, whatever the source of infection. The low success rate of B. cinerea inoculations under experimental

conditions may merely reflect the relatively low success rate of B. cinerea inoculum in the field.

Whether the non-aggressive stem lesions represent a potential threat to the infected plant is not clear. In this investigation these lesions were observed to remain virtually unchanged throughout the growing period of the crop, confirming the findings of Smith (1970). It is interesting, however, that lesions often developed at leaf scars long (up to 61 days) after deleafing. Wilson (1963) found that lesions could develop up to 11 weeks after inoculation with spores. He referred to this as latent infection and considered that subsequent lesion development was initiated when the plant entered a susceptible phase. Latent infection by B. cinerea has also been demonstrated in grape flowers (McClellan and Hewitt, 1973) where the fungus infects the stylar end of the flower and becomes latent in the necrotic stigma and style tissue. Later, as the berry develops, the fungus may become active and rot the fruit in mid-season. This may occur in the absence of rain and is normally associated with late-season grape rots. Similar infections have been observed in strawberry flowers (Powelson, 1960). Verhoeff (1970) regarded ghost spotting of tomato fruits by B. cinerea as a latent infection. Although latency arising from earlier spore inoculation was not demonstrated conclusively in this investigation, it would seem the most plausible explanation for late developing lesions. Infection of old leaf scars may occur, but Verhoeff (1967) reported that after deleafing a cambium forms, sealing the leaf scar. Infection by conidia

was then very unlikely. The ability of B. cinerea conidia to form a latent infection at deleafing would obviously enhance the success of the fungus, enabling it to survive on the plant until conditions were suitable for growth. It is well documented that the physiological state of the tomato stem influences its susceptibility to B. cinerea (Wall, 1940; Wilson, 1964; Stall et al, 1965; Verhoeff, 1965; Smith, 1970; Kamoen, 1976). Wilson (1963, 1964) has shown that the period of latency is reduced as the plant ages and that it is affected by the water content of the plant. Latent infections may, therefore, be activated when the plant enters a physiologically susceptible state.

The reason why the majority of B. cinerea stem lesions on tomatoes are non-aggressive was not obvious from this study. Clearly, however, the growth of the fungus must be sufficiently rapid to escape restriction by the host defence mechanisms if a lesion is to become aggressive. The rate of growth of the fungus and the rate at which the host defence mechanisms operate are likely to be subject to a complex interaction of genotype and environmental conditions. Such conditions would also apply to aggressive lesions which may cease to develop following some change in environmental conditions; such 'checking' of aggressive lesions was often observed in this study.

The provision of a saprophytic base in inoculation experiments was shown to make subsequent colonization more likely. Although a mycelium/agar disc was the most effective inoculum, the

presence of necrotic tissue or petiole stumps at the leaf scar also increased inoculation success. Bewely (1923) and Smith (1969, 1970) have stressed the importance of a saprophytic base for B. cinerea infection, although they placed emphasis on the role of infected petioles. As the extent of nutrient supply available to a fungus will influence its growth rate it is possible to speculate that aggressive lesions will only develop at leaf scars that provide a suitable saprophytic base. Physical injury caused by tearing during deleafing may provide necrotic tissue for such a base. This has prompted some growers into cutting petioles with a knife at deleafing rather than snapping the petioles by hand. When compared experimentally, the two deleafing methods resulted in the leaf scars being equally susceptible to B. cinerea infection. Used incorrectly, however, a knife may encourage the development of stem lesions; if the petiole is not cut flush with the stem the remaining stump will rapidly become colonized by B. cinerea.

Little reference is made in the literature to the role of side shoot stumps and scars in B. cinerea infection of tomatoes. Side shoot removal is a routine cultural practice generally done on a weekly cycle (Kingham, 1973). Unlike the leaves removed during deleafing, a large proportion of the shoots removed are from the upper, more succulent part of the stem, a region known to be particularly susceptible to B. cinerea (Wilson, 1962; Verhoeff, 1965; Smith, 1970). This investigation also showed that aggressive lesions tended to form higher up the stem than non-aggressive lesions. The

resulting stumps and scars were often seen to be infected by B. cinerea and a large number of stem lesions resulted. Infection of side shoot scars would appear to arise directly from spore inoculum as side shoots were never seen to be infected prior to removal. It is possible that some infections originated from infected flower petals adhering to side shoot scars; this was rarely observed, however. A much greater proportion of these lesions became aggressive compared to lesions formed at leaf scars lower down the stem (48% compared to 12%). During routine observations it was noticed that many of the aggressive stem lesions seen with infected petioles still attached could have originated from infected shoot scars and not from the petiole. It should be noted that the majority of nodes have shoot scars; their significance in initiating stem lesions has probably been greatly underestimated in the past.

The laboratory observation that previous Botrytis infection of stems and petioles makes the stem less susceptible to subsequent attack by the fungus is previously unreported. Such induced resistance in plants, however, is not a new phenomenon. Matta (1971) has reviewed numerous instances where prior inoculation with bacteria, viruses and fungi can confer reduced susceptibility to plants. Commonly the resistance is induced by mildly virulent or avirulent strains of pathogens (Rahe et al, 1969; Sinha and Das, 1972; Sinha and Trivedi, 1978) but can also result from prior infection by microorganisms unrelated to the test species (Mandryk, 1963; Jenns and Kuć, 1977). Occasionally, as in this study, protection is elicited

by and effective against the same virulent pathogen. For example, Richmond et al (1979) found that penetration of cucumber leaves by Colletotrichum lagenarium was reduced in plants systemically protected by previous infection of lower leaves with the pathogen.

At first it was thought that the resistance to infection conferred on leaf scars as a result of prior infection of the petiole resulted from the formation of a mechanical barrier, the abscission layer. This layer, when well formed, was found to confer a small degree of resistance but its mechanical removal failed to significantly increase the susceptibility of the leaf scar. It seems likely, therefore, that the resistance is attributed to a chemical resistance factor (RF) formed in response to B. cinerea infection. Since it was detected at some distance from the infection site, the RF or RF precursors must diffuse or be transported within the host. RF was found above and below infection sites on the stem suggesting that both the phloem and xylem could be involved in its movement.

The inhibition of spore germination by crude extracts from infected plants was attributed to the presence of RF. The dilution incurred during extraction and the fact that activity was only lost after a further 10 fold dilution, suggested that the material reached a high concentration within the plant. Plant extracts containing RF were only bioassayed against B. cinerea spores, the effect of RF on mycelium is not known. Germ tube growth seemed to be unaffected by the RF, however

it is possible that the RF was metabolized, degraded or broken down during the course of the bioassay. The main effect of the RF was to reduce the rate rather than the final level of germination. This may also be an indication of the loss of RF activity with time. Fungitoxic extracts have previously been demonstrated in rice plants infected with Drechslera oryzae (Trivedi and Sinha, 1976) and carrot slices infected with B. cinerea (Harding and Heale, 1978). In both examples, infection led to the development of induced resistance in the host.

Although it is now known that cultures of some isolates of B. cinerea can produce antibiotics active against fungi and bacteria, these are reported to have little inhibitory effect against B. cinerea itself (Blakeman, 1980). In vitro studies of the fungus grown in tomato stem broth in this investigation revealed that culture filtrates taken after up to four days growth stimulated spore germination. The almost total inhibition of spore germination in culture filtrates from eight day old cultures was probably a fungistatic response to lack of nutrients, since germination occurred in these filtrates after the addition of 0.1% malt extract. The observation that autoclaving culture filtrates from eight day old cultures did not diminish their inhibitory effect on spore germination would support the view that spore germination was restricted by nutrient availability. It should be noted that the RF formed in vivo was inactivated by boiling. The formation of the RF, therefore, was almost certainly stimulated by a fungus/host interaction.

The chemical nature of the RF formed *in vivo* was not investigated. One chemical resistance mechanism found in plants is phytoalexin production. Phytoalexins are broad spectrum antimicrobial chemicals formed in plants, particularly as a result of fungal attack (Kuć, 1972). Generally, however, phytoalexins are considered to be restricted to the immediate neighbourhood of the infection site (Tarr, 1972). There is, however, increasing evidence, although largely circumstantial, for the movement of phytoalexins within the plant (Cruickshank and Mandryk, 1960; Chamberlain and Paxton, 1968; Caruso and Kuć, 1979; Staub and Kuć, 1980). The RF seen in this study was present in tissue at some distance from the site of infection. This does not, however, necessarily imply the movement of the RF. It could be synthesized at any location in the plant in response to fungal metabolites which had in turn diffused or been transported in the plant. Circumstantial evidence of such metabolites was indicated by the vascular browning, epinasty of the petioles, adventitious root formation and petiole yellowing seen at distances from the infection site. Culture filtrates from *B. cinerea* have previously been shown to induce necrosis, wilting, vascular plugging and browning of the vessels in cut shoots of *Vicia fabae* (Purkayastha, 1969). If fungal metabolites can induce disease symptoms, it is quite possible that they can also induce the formation of RF. This hypothesis is supported by the observations of Heale and Sharman (1977) who found that fungal invasion was not necessary to reduce the susceptibility of fresh cut carrot slices to *B. cinerea*. They demonstrated that heat killed conidia of *B. cinerea* or a cell free fluid

in which spores had germinated would induce partial resistance to infection. Harding and Heale (1980) have recently found that the RF in this instance is associated with the production of 6-methoxymellein, p-hydroxybenzoic acid and the acetylenic compound falcarinol. It is perhaps significant that the furanoacetates wyerone, wyerone acid and wyerone epoxide are considered as major components of the phytoalexin response of V. fabae to B. cinerea (Hargreaves et al, 1977). Rishitin is the only phytoalexin so far identified in tomatoes (de Whit and Flach, 1979), but it has been reported that this is rapidly metabolized by B. cinerea (Lyon, 1976). A large number of other phytoalexins, mostly sequiterpenes, have, however, been identified in closely related members of the Solanaceae, particularly potatoes (Stoessl et al, 1976).

The degree of induced resistance displayed at any particular site on a tomato stem seemed to depend on the duration of colonization following infection and the distance of the second inoculation from the first infection site. This is uncharacteristic of induced resistance which is often associated with weak or avirulent pathogenes (Matta, 1971). It is perhaps significant, however, that infections of petioles with a weakly pathogenic isolate induced the same level of resistance at the corresponding leaf scars as a strongly pathogenic isolate.

Inhibition of subsequent Botrytis colonization of leaf scars, where previously attached petioles had been diseased, was very

marked. As little as three days prior colonization of the petiole not only reduced subsequent lesion growth at the leaf scar but also greatly reduced the number of aggressive lesions that developed. These experiments would suggest that induced resistance may have considerable influence on the incidence of stem lesions in the field. However, this was not apparently supported by observations in commercial crops. Plants with a history of early foliar infection tended to have more stem lesions and those plants with established stem lesions were more likely to develop further stem lesions than correspondingly healthy plants. It is interesting, however, that the majority of these lesions were non-aggressive and so the field observations may be reflecting the success of an induced resistance mechanism. Although a very speculative hypothesis, further work on induced resistance in tomato plants is clearly justified.

It is in the growers' own interest to take another look at disease control in their crops. Highly specific fungicides are extremely useful, but every effort must be made to prolong their life. If tolerance continues to be a problem commercial companies may consider that further development of fungicides is futile, especially for horticultural use. The horticultural market is very small compared to agriculture and tolerance is considered more likely to develop because of the protected environment (Dekker, 1976). In addition, the high value crops carry a financial risk to the manufacturer, in terms of possible compensation for crop loss, which is far in excess

of the likely returns from the market. More reliance should, therefore, be placed on routine cultural practices, such as removal of crop debris and avoidance of high humidity, currently often neglected in favour of routine chemical sprays. An awareness, for example, of the significance of shoot scars in allowing entry of B. cinerea into the stem may encourage some growers to remove side shoots at times of low humidity. It may also be possible in the future to manipulate plants in some way so as to maximize any induced resistance response to Botrytis infection.

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APPENDIX I

A comprehensive description of B. cinerea based solely on morphological characters (Groves and Loveland, 1953)

"Sclerotia hard, black, plano-convex and firmly attached to the substrate, very variable in size and shape, rounded to elongated and more or less curved, sometimes convoluted and very irregular, 1 - 15 mm in length, sometimes forming confluent masses; in section composed of clearly interwoven, hyaline, thick-walled hyphae with a thin, pseudoparenchymatous rind composed of dark brown to black, almost isodiametric cells about 5 - 10 μ in diameter.

Conidiophores arising from the mycelium or from the sclerotia, erect, brown, septate, simple or usually branched towards the tip, the top rounded or slightly swollen, 5 - 22 μ in diameter, 1 - 3 mm in height; conidia greyish-brown in mass, nearly hyaline under the microscope, ellipsoid to ovoid, one-celled, borne on small stalks arising from the tips of the conidiophores, and forming botryose clusters, 8 - 14 - (18) x (5) - 6 - 9 μ.

Spermatia hyaline, globose, one-celled, 2 - 2.5 μ in diameter, produced endogenously from hyaline, flask-shaped phialides which may arise simply from the mycelium or more commonly are aggregated into clusters forming spermadochia."

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APPENDIX II

The development of a rapid pathogenicity test

Although the inoculation of mature plants grown under commercial conditions and the measurement of subsequent lesions represents an ideal pathogenicity test, there are clear disadvantages. The number of available plants and the time taken for each test would severely limit the number of isolates that could be examined. A simpler and faster test was therefore developed using excised tomato cotyledons, a source of plant material quickly and easily produced in bulk. The validity of this test was examined by comparing it with a conventional pathogenicity test using mature plants. Each test was set up as follows:

(i) Mature plant test: A double row of plants (C.V. Sonato), approximately 2 m high, growing in a polythene tunnel, were used. Four petioles were inoculated per plant, each with a mycelium/agar disc of a different isolate; six replicate randomized inoculations were made for each isolate. Resulting petiole lesions were measured after 7 days (total lesion spread measured) and 14 days (spread from point of inoculation to the stem measured). After 34 days any stem lesion corresponding to the previously inoculated petiole was also measured.

(ii) Excised cotyledon test: Excised cotyledons were inoculated with a modified mycelium/agar disc (see 4.15). 10 replicates were prepared for each isolate and incubated in moist chambers at 20°C under 16 hour

day illumination. Lesion spread was measured after four days. Isolates were arranged in order of pathogenicity, according to the test (Table I), and the coefficients of rank correlation calculated.

A high degree of correlation (significant $p = 0.01$) was obtained when growth on excised cotyledons was compared to the severity of stem lesions on mature plants. Significant ($p = 0.05$) correlation was also obtained between growth on excised cotyledons and lesion spread on mature plant petioles after 7 and 14 days.

Table I A comparison of isolate pathogenicity of B. cinerea using different pathogenicity tests

Lesion spread on petiole after 7 days	Rank No.*	Lesion spread on petiole after 14 days	Rank No.*	Severity of stem lesions after 34 days	Rank No.*	Growth on excised cotyledons after 4 days	Rank No.*
24 AiiiS	1	24 AiiiS	1	5 AiiiS	1	33 AiiS	1
5 AiiiS	2	5 AiiiS	2	24 AiiS	2	5 AiiiS	2.5
33 AiiS	3	37 BiR	3	45 AvS	3	24 AiiiS	2.5
27 AiS	4	33 AiiS	4	32 AiR	4	2 AivS	4
24 AiiS	6.5	27 AiS	5	33 AiiS	5	23 AiR	5
23 AiR	6.5	45 AvS	6	2 AivS	6	32 AiR	6
4 BiiR	6.5	9 AiiiS	7	23 AiR	7	4 BiiR	7
37 BiR	6.5	36 AvR	8	4 BiiR	9	44 BiiR	8
15 BvR	9	4 BiiR	9	9 AiiiS	9	27 AiS	9
2 AivS	10	32 Air	10	15 AivS	9	7 AiiiS	10
45 AvS	11	2 AivS	11	27 AiS	12	21 AvR	11
9 AiiiS	13	34 BiR	12	15 BvR	13	15 AivS	12.5
15 AivS	13	15 AivS	13	7 AiiiS	13	37 BiR	12.5
25 AiR	13	24 AiiS	14.5	21 AvR	13	36 AvR	14
7 AiiiS	15.5	12 AvR	14.5	12 AvR	15	15 BvR	15
12 AvR	15.5	21 AvR	16	37 BiR	17	9 AiiiS	16
36 AvR	17	23 Air	17	34 BiR	17	45 AvS	17.5
44 AiiR	18	44 BiiR	18	18 AiiR	17	24 AiiS	17.5
21 AvR	20	15 BvR	19	36 AvR	19	34 BiR	19.5
43 AiR	20	7 AiiiS	20	24 AiiS	20	12 AvR	19.5
34 BiR	20	25 AiR	21	44 BiiR	21	24 BivS	21
46 AiS	22	46 AiS	22	25 AiR	22	19 BiS	22
24 BivS	23	43 AiR	23	46 AiS	23	18 AiiR	23
18 AiiR	24	18 AiiR	24	43 AiR	24	43 AiR	24
32 AiR	25	19 BiS	25	19 BiS	25	25 AiR	25
9 BiS	26	24 BivS	26	24 BivS	26	9 BiS	26
19 BiS	27	9 Bis	27	9 BiS	27	46 AiR	27

* 1 = most pathogenic; 27 = least pathogenic.

Calculation of the Coefficient of Rank Correlation (r)

(1) Lesion spread on petiole after 7 days / Growth on excised cotyledons after 4 days $r_{27} = 0.70^{+}$

(2) Lesion spread on petiole after 14 days / Growth on excised cotyledons after 4 days $r_{27} = 0.64^{+}$

(3) Severity of stem lesions after 34 days / Growth on excised cotyledons after 4 days $r_{27} = 0.79^{++}$

\+ Significant positive correlation ($p = 0.05$)
++ Significant positive correlation ($p = 0.01$)

APPENDIX III

Morphological types exhibited by 20 isolates over two generations

ISOLATE	TYPE	MYCELIUM	SPORES				SCLEROTIA		
		Growth/Form	Colour	Quantity	Position	Colour	Quantity	Position	Diameter
2 AivS	T_1	Rapid, regular, fluffy at edge	Greyish white	Dense	Edge	Umber	0	-	-
3 AvR	T_1^1	Rapid, regular, fluffy	Greyish white	Slight	Through-out	Ochreous	0	-	-
	T_1^2	Rapid, regular, felt like becoming fluffy at edge	Yellowish white	Absent	-	-	0	-	-
	T_2	Slow, sparse, distorted flat	Greyish white	Sparse	Through-out	Umber	++++	Through-out	≤ 1 mm
5 AiiiS	T_1	Rapid, regular, fluffy, prone to floculation	Greyish white	Dense	Edge	Ochreous	+	Edge	≤ 1 mm
	T_2	Slow, sparse, distorted, flat	Yellowish white	Dense	Centre	Ochreous	+++	Centre	≤ 1 mm
9 AiR	T_1	Rapid, regular, fluffy	Greyish white	Moderate	Through-out	Ochreous	0	-	-
	T_2	Slow, sparse, distorted, fluffy	Greyish white	Moderate	Through-out	Ochreous	0	-	-
9 BiS	T_1	Rapid, regular, flat becoming fluffy at edge	Greyish white	Sparse	Through-out	Umber	+	Through-out	≤ 2 mm

ISOLATE	TYPE	MYCELIUM	SPORES				SCLEROTIA		
		Growth/Form	Colour	Quantity	Position	Colour	Quantity	Position	Diameter
9 BiiR	T_1^1	Rapid, regular, felt like becoming dense at edge	Greyish white	Moderate	Edge	Umber	0	-	-
	T_1^2	Rapid, regular, felt like becoming dense at edge	Greyish white	Moderate	Through-out	Umber	0	-	-
	T_1^3	Rapid, regular, fluffy prone to floculation	Greyish white	Slight	Through-out	Ochreous	0	-	-
9 AiiiR	T_1	Rapid, regular, flat becoming fluffy at edge	Greyish white	Dense	Edge	Umber	++	Edge	≤ 2 mm
	T_2	Slow, sparse, distorted, flat	Ochreous centre	Moderate	Centre	Ochreous	++++	Through-out	≤ 1 mm
10 AiS	T_1^1	Rapid, regular, flat becoming dense at edge	Greyish white	Dense	Edge	Umber	0	-	-
	T_1^2	Rapid, regular, flat with fluffy outer ring	Ochreous	Sparse	Through-out	Ochreous	0	-	-
10 BiS	T_1	Rapid, regular, flat becoming fluffy at edge	Greyish white	Dense	Edge	Umber	0	-	-
	T_2	Slow, sparse, distorted, flat	Ochreous centre	Dense	Centre	Ochreous	0	-	-

ISOLATE	TYPE	MYCELIUM	SPORES				SCLEROTIA		
		Growth/Form	Colour	Quantity	Position	Colour	Quantity	Position	Diameter
15 AivS	T_1	Rapid, regular, flat becoming fluffy at edge	Greyish white	Dense	Edge	Umber	0	-	-
15 BvR	T_1	Rapid, regular, flat becoming fluffy at edge	Greyish white	Dense	Edge	Umber	+++	Edge	≤ 1 mm
	T_2	Slow, sparse, distorted flat	Ochreous centre	Very slight	Centre	Ochreous	0	-	-
23 BiR	T_1	Rapid, regular, flat becoming dense at edge	Greyish white	Dense	Edge	Umber	0	-	-
	T_2	Slow, sparse, distorted, flat	Greyish white	Moderate	Centre	Umber	++++	Through-out	≤ 1 mm
24 AiiiS	T_1	Rapid, regular, flat becoming fluffy at edge	Yellowish white	Dense	Edge	Ochreous	0	-	-
	T_2	Slow, sparse, distorted, flat	Yellowish white centre	Moderate	Centre	Ochreous	++++	Through-out	≤ 1 mm
27 BiS	T_1	Rapid, regular, flat central zone, becoming fluffy	Greyish white	Dense	Edge	Ochreous	0	-	-

ISOLATE	TYPE	MYCELIUM	SPORES				SCLEROTIA		
		Growth/Form	Colour	Quantity	Position	Colour	Quantity	Position	Diameter
33 AiiS	T_1	Rapid, regular, fluffy	Yellowish white	Moderate	Through-out	Ochreous	0	-	-
	T_2	Slow, spare, distorted, flat	Yellowish white centre	Moderate	Centre	Ochreous	++++	Through-out	≤ 1 mm
34 BiR	T_1	Rapid, regular, flat	Greyish white	Dense	Edge	Umber	++	Through-out	≤ 2 mm
	T_2	Slow, sparse, distorted, flat	Ochreous centre	Dense	Centre	Ochreous	++++	Through-out	≤ 1 mm
36 AvR	T_1	Rapid, regular, felt like	Ochreous	Sparse	Through-out	Ochreous	++	Edge	2 - 4 mm
	T_2	Slow, sparse, distorted, flat	Yellowish white	Sparse	Centre	Ochreous	+++	Centre	≤ 1 mm
37 BiR	T_1	Rapid, regular, flat becoming fluffy at edge	Greyish white	Dense	Edge	Umber	++	Through-out	≤ 1 mm
	T_2	Slow, spare, distorted, flat	Ochreous centre	Sparse	Through-out	Ochreous	+	Through-out	≤ 1 mm

ISOLATE	TYPE	MYCELIUM	SPORES				SCLEROTIA		
		Growth/Form	Colour	Quantity	Position	Colour	Quantity	Position	Diameter
43 AiR	T_1	Rapid, regular, flat becoming fluffy at edge	Greyish/ white	Dense	Edge	Umber	0	-	-
	T_2	Slow, sparse, distorted, flat	Ochreous centre	Sparse	Through-out	Ochreous	++++	Through-out	≤ 1 mm
43 AvR	T_1	Rapid, regular, slightly fluffy	Yellowish white	Moderate	Through-out	Ochreous	0	-	-
	T_2	Slow, sparse, distorted, flat	Yellowish white	Sparse	Through-out	Ochreous	0	-	-