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Studies On The Survival Of Microsclerotia Of Verticillium Dahliae

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STUDIES ON THE SURVIVAL OF MICROSCLEROTIA OF *VERTICILLIUM DAHLIAE*

by

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Abstract

The soilborne plant pathogen *Verticillium dahliae* Kleb. produces numerous multicellular, melanized resting structures called microsclerotia (MS) inside senescing host tissues. Melanin has been implicated in the long-term survival of fungal resting structures in soil. A method for the laboratory production, isolation, harvest and storage of stable populations of MS was developed and a procedure for plating individual MS was used to quantitatively measure both lethal and sub-lethal treatment effects. MS of 75-106 μm size exhibited faster and more synchronous germination, produced larger colonies and survived long term storage at 24 C better than MS of 53-75 μm or <53 μm. Addition of 1 or 10 mg/L of tricyclazole (TCZ) to the medium inhibited melanin production but did not reduce MS germination, growth rate or survival when stored for >35 weeks at 24 C. UV irradiation of albino MS for 2 h severely inhibited germination (a lethal effect) and reduced colony size (a sublethal effect). Melanized MS survived better than melanin deficient MS when buried in soil. TCZ treated MS buried in various soils often gave rise to colonies which were not *V. dahliae*, implicating soil microorganisms in the reduction of their survival. Inoculation of soil with *Talaromyces flavus* or *Trichoderma aureo viride* reduced the survival of melanized and TCZ treated MS to 51% and <10% or 79% and <14% respectively after 5 weeks burial. A possible mycoparasite isolated from MS (*Penicillium* sp.) reduced the survival of melanized and TCZ treated MS to 73% and <20% respectively. The addition of bloodmeal (BM) to soil at 1% w/w eradicated melanized MS and reduced the infection of eggplants with *V. dahliae* from 90% to 20%. Within 15 days of adding BM the soil pH rose from 5.5 to 8.2 and returned to original levels after 25 days. Since MS suspended above BM amended soil were killed within 6 days, a volatile fungitoxic factor (possibly ammonia) was implicated. Burial of MS in soil amended with 1% w/w urea or 0.5% ammonium acetate completely inhibited germination but 1% ammonium sulphate had no effect.

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For my Father,

Berton Murray Hawke

(1913-1991)

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List of Abbreviations

BM	Bloodmeal
CDX	Czapek Dox growth medium
FBK	Fish, Bone and Kelp meal
MCDX	Modified, semi solid, Czapek Dox growth medium
MS	Microsclerotia
PDA	Potato Dextrose Agar growth medium
SPT	Soil Pectate Tergitol medium
TCZ	Tricyclazole

Chapter One

General Introduction

Verticillium spp. are found worldwide and cause diseases which affect as many as 45 plant families. In addition, several species are parasites of other fungi and of insects (DeVay & Pullman, 1981). The genus is composed of hyphomycete fungi having verticillately branched conidiophores with phialides producing conidia terminally in mucilaginous heads (Heale, 1988). *Verticillium* spp. belong to the Class Deuteromycetes (also called the Fungi Imperfecti) (Table 1.1), since they have no known sexual stage of reproduction (Horst, 1990). *Verticillium* was first described by Reinke and Berthold in 1879 as a wilt disease of potato which produced dark brown cells and dark thickened hyphal masses and which they named *Verticillium albo-atrum.* According to Isaac (1949), Reinke and Berthold described tissue in diseased potato that became black or dark brown due to a blackening of the hyphae, which also became septate and spheroid. Some of the original drawings of Reinke and Berthold are reproduced in Smith (1965) and Berkeley et. al. (1931). Reinke and Berthold decided that the darkened cells constituted an overwintering resting mycelium and called it "dauermycelien" or "sklerotien". In 1913, however, Klebahn isolated a form of *Verticillium* from dahlia which formed sclerotia from the budding of the cells of a single hypha rather than from the anastomosis of several hyphae as described by Reinke and Berthold (Isaac, 1949). He considered this isolate sufficiently different from *V. albo-atrum* to identify it as a separate species which he called *V. dahliae*. Over the eighty years since then, there has been considerable debate about the validity of separating *V. albo-atrum* and *V. dahliae* into distinct species (Berkeley et. al., 1931; Isaac, 1949; Smith, 1965), and much of the literature contains references to *V. albo-atrum* DM form (for dark mycelial variant) or *V. albo-atrum* MS (microsclerotial variant) however, the distinct species are now widely accepted. The distinction between *V. dahliae* and *V. albo-atrum* is made morphologically by the resting structures produced. *V. dahliae*

Table 1.1

Classification of *Verticillium* spp. [a]

Kingdom: Mycota

Phylum: Ascomycota

Class: Deuteromycetes

Order: Moniliales

Family: Moniliaceae

Genus: *Verticillium*

Species: *dahliae, albo-atrum, tricorpus*

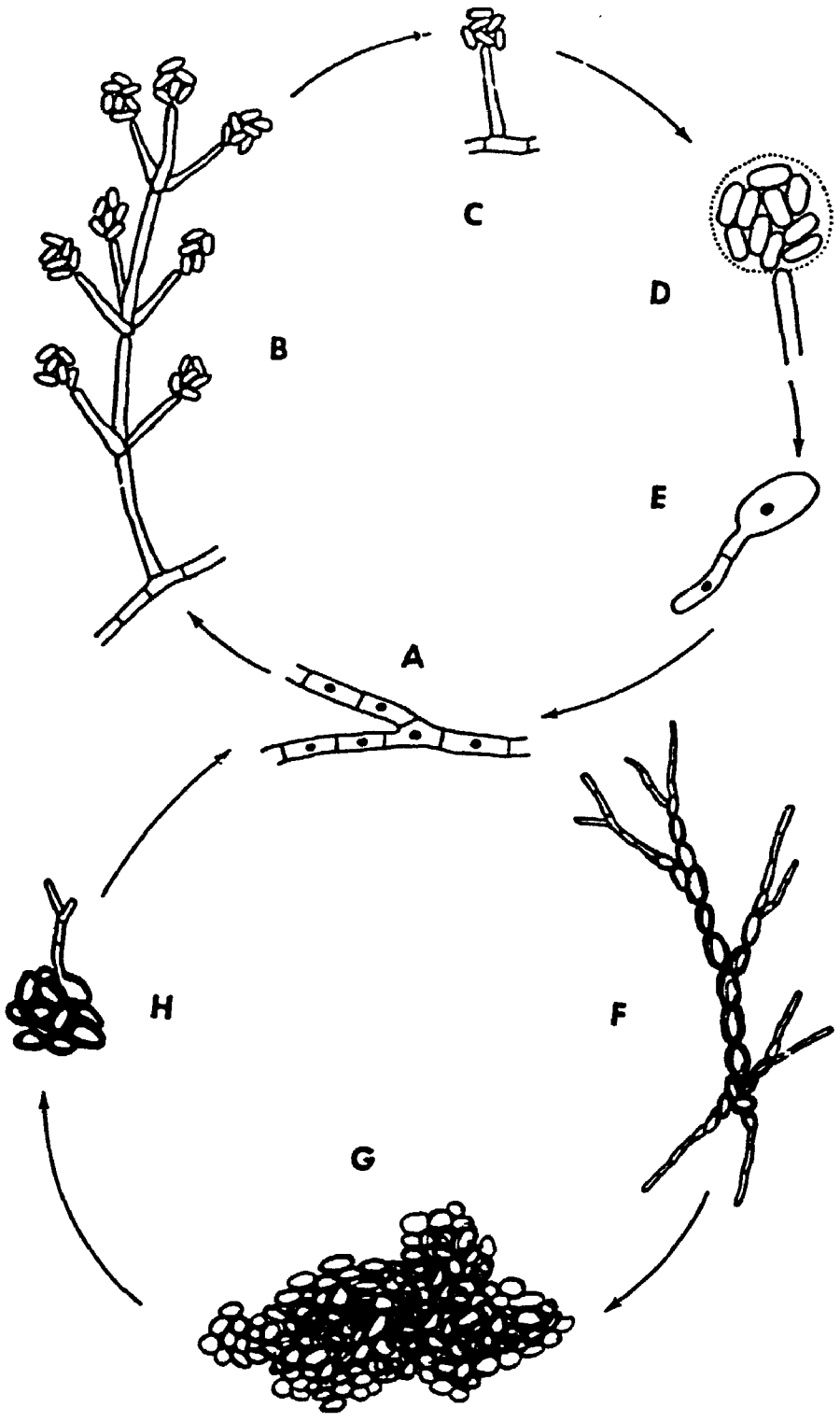
[a] according to the classification in Horst, 1990.

produces microsclerotia (multicellular, melanized structures formed by the budding of hyphal cells) and *V. albo-atrum* produces dark mycelium (the melanized hyphae described by Reinke and Berthold). Another species, *V. nigrescens*, forms melanized chlamydospores and *V. tricorpus* forms all three types of resting structure (dark mycelium, microsclerotia and chlamydospores, Isaac, 1949). Physiological factors such as growth temperature range, sensitivity to UV irradiation, pH and use of C or N sources (Isaac, 1949; Puhalla, 1973) as well as protein bands on polyacrylamide gels (Hall, 1969) or isozymes separated by isoelectric focussing (Carder, 1989) have also been used to define the different species.

The life cycle of *V. dahliae* is presented in Fig. 1.1. The vegetative hyphae (Fig. 1A) are 2-4 μm in diameter, septate, hyaline, and thin walled while the conidiophores (Fig. 1B) are verticilliately branched, septate, hyaline, 80-160 μm long, with one to three whorls consisting of one to five phialides per whorl (Smith, 1965). The hyaline, elliptical conidia (3-5.5 by 1.5-2 μm) are borne singly at the apices of the phialides, often aggregating into heads (Fig. 1D). The microsclerotia (Fig. 1G) are multicellular, melanized structures consisting of both thick walled and thin-walled cells (Gordee & Porter, 1961; Nadakavukaren, 1963; Griffiths, 1970; Brown & Wyllie, 1970; Hall & Ly, 1972). Hall and Ly (1972) described a three stage process for the development of MS of *V. dahliae*, including initiation (the swelling of hyphal cells and the development of numerous septa), enlargement (the vegetative budding of the initial cells into lipid-packed cells forming a three dimensional structure) and maturation (the development of a black pigment in the inner cells of the structure). The structure of MS in *V. dahliae*, therefore, is not similar to the sclerotia found in *Sclerotium rolfsii*, which are differentiated into a rind (composed of thick-walled, melanized but empty cells), a medulla (non-uniform cells, some with thick, multilayered walls) and cortex (composed of large cells containing vesicles which probably contain nutrient reserves) (Chet, 1975; Willetts & Bullock, 1992).

Figure 1.1

The life cycle of *Verticillium dahliae* including: A: vegetative hyphae B: verticilliate conidiophores showing whorls of phialides C: a single phialide, with conidia borne at the tip D: a single head of hyaline elliptical conidia E: germination of a conidium F: initiation of microsclerotia formation showing the swelling and rounding up of hyphal cells G: a mature microsclerotium, showing multicellular structure and melanization of the cells H: the formation of a germ tube from a germinating microsclerotium. Source: Barnes, E.H. 1979. Atlas and Manual of Plant Pathology, Second Edition. New York, Plenum Press.


A
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Pegg (1974) reported that in one study of 1770 *Verticillium* isolates in Ontario, 84.3% were of *V. dahliae*, 5.5% were of *V. albo-atrum* and 10.2% were of *V. nigrescens*. In southern and central Ontario, *V. dahliae* is involved in Potato Early Dying Syndrome (PED) with 80% of the potato plants collected in the Alliston potato-growing region being infected with *V. dahliae* and only 3 sites exhibiting <20% disease incidence (Lazarovits, 1988). Powelson and Rowe (1993) reported that although estimates of yield reduction due to PED are unreliable, reductions of up to 30-50% in yield of potatoes have been reported. Visual symptoms of Verticillium wilt in potato include the inward curling of the leaflet margins, the chlorosis of leaflets along the midrib and the subsequent necrosis and abscission of the leaves (Perry & Evert, 1983). General physiological symptoms of wilt include tissue necrosis, accelerated respiration, ethylene and auxin production and accumulation of phenols and other aromatic compounds (Goodman, Kiraly & Wood, 1986). Wilt pathogens are considered to increase resistance to water flow in the plant by their physical presence as well as the liberation of gums, gels and phenolic compounds from the host cell walls through the activity of pectic and cellulolytic enzymes (Goodman, Kiraly & Wood, 1986). The plants themselves may also contribute to wilting through non-specific pathogen induced wound responses which include the obstruction of vessels by the formation of gums or tyloses (Goodman, Kiraly & Wood, 1986). Work with Verticillium wilt of cotton has shown that a phytotoxic protein-lipopolysaccharide complex isolated from *V. dahliae* induces all the symptoms characteristic of the disease (Keen, Long & Erwin, 1972; Nachmias, Buchner & Krikun, 1982).

V. dahliae is considered a monocyclic pathogen since it only produces one disease cycle in a year (Agrios, 1988). In the disease cycle of *V. dahliae*, MS present in the soil are stimulated to germinate by root exudates produced by the host plant and the hyphae penetrate and colonize the root cortex, systemically colonizing the vascular system by entering the xylem vessels and producing conidia. At the end of the life of the host plant, the foliage senesces and MS are formed inside the vascular tissue (Plate 1.1), becoming

Plate 1.1

Microsclerotia of *Verticillium dahliae* inside the stems of potato plants grown in the field. Each plant was inoculated with 10 mL of a suspension of 1.3 X 10^6 conidia/mL of *V. dahliae* before transplantation into the field.

distributed into the soil along with the dead plant material (Rowe, 1985). The level of MS in the soil therefore climbs from year to year, forming an ever increasing "inoculum bank". MS are extremely long-lived, surviving 13 years in culture in the laboratory at room temperature and successfully causing infection in tomato plants after 14 years in field soil (Wilhelm, 1955). The presence of these highly persistent MS in the soil makes *Verticillium* very difficult to control. The three most important methods for controlling soilborne diseases of field crops are generally considered to be crop rotation, the use of fumigants and/or the use of resistant varieties (Coley-Smith, 1990). Crop rotation is obviously not a very useful strategy since the MS can survive in the field for so long and *Verticillium* has such a wide host range, including naturally occurring weeds (Johnson et al., 1980; Slattery, 1983). Powelson and Rowe (1993) comment that although long-term rotations may lead to lower populations of viable MS, the literature is contradictory about the effectiveness of crop rotation in preventing significant yield loss due to *V. dahliae*. Soil fumigation with chlorpicrin, metham sodium (Ben-Yephet, Siti & Frank, 1983), 1,2-dibromoethane and 1,3-dichloropropene (Ben-Yephet, Letham & Evans, 1981) is effective in controlling Verticillium wilt, however, it is expensive and raises concerns about the correct application of the chemical, the indiscriminate elimination of both harmful and beneficial soil inhabitants and the potential negative effects on the environment (Powelson & Rowe, 1993). At the present time, there are potato cultivars available which confer some resistance to *Verticillium*, however complete protection has not been realized (Powelson & Rowe, 1993).

Clearly, any method that would hope to control a soilborne disease such as *V. dahliae* would aim to reduce the populations of the survival structures (Baker & Cook, 1974). One of the features of MS of *V. dahliae* which enables them to survive in the soil so effectively is the presence of melanin. Melanin has been implicated in the protection of fungal resting structures from a number of environmental stresses, including degradation from hydrolytic enzymes and attack from microbial antagonists (Bell & Wheeler, 1986). A

chemical (tricyclazole) which inhibits pentaketide melanin synthesis has been used successfully to control rice blast disease (*Pyricularia oryzae*) and to prevent penetration of plant cells by *Colletotrichum* (Kubo et al., 1985; Howard & Ferrari, 1989). In these fungi, pentaketide melanin plays a direct role in the infection process. Indirect effects of melanin inhibition may also may offer a potentially important target for the control of pathogenic melanized fungi. For instance, the inhibition of melanin formation is already being investigated as a method for controlling dematiaceous fungi, a group of melanized, medically important fungal pathogens of humans (Dixon, Szaniszlo & Polak, 1991). Previous work (Butler, Hawke and Lazarovits, unpublished) has illustrated that tricyclazole (TCZ) applied as a soil drench to the root region of potato plants in the field inhibited the melanization of MS produced in the plant tissue at the end of the growing season (Plate 1.2 and 1.3). This suggested that if melanin was important for the survival of MS, melanin deficient MS should have a much shorter life span than fully melanized when reintroduced to the soil. If non-melanized MS did indeed survive less well than melanized MS, then the disease incidence could be reduced in the subsequent cropping year. The main objective of the research described in this thesis, therefore, was to examine the role of melanin in the survival of MS of *V. dahliae* by exposing melanized and non-melanized MS to both abiotic and biotic factors (Chapter Four). Currently the role of melanin in the survival of MS of *V. dahliae* is a matter of speculation since there is no definitive experimental evidence to demonstrate its importance as an adaptation for survival in soil. This may be due, in part, to the lack of a suitable procedure for the accurate detection and quantitative measurement of MS survival. Considerable preliminary work for this thesis was carried out to try to determine the survival of melanized and non-melanized MS in the field. However, all procedures used produced inconclusive results because of the high variability in the survival data collected (Butler, Hawke and Lazarovits, unpublished). Clearly, it was necessary to develop a method for the accurate measurement of quantitative differences in MS survival before differences between melanized and non-

Plate 1.2

The appearance of normal microsclerotia of *Verticillium dahliae* inside the stems of potato plants grown in the field. Magnification 400X.

Plate 1.3

The appearance of non-melanized microsclerotia of *Verticillium dahliae* inside the stems of potato plants grown in the field and treated with a soil drench of 100 mg/L tricyclazole. Magnification 400X.

melanized MS could be compared. The most important task was the establishment of a procedure for the laboratory production of stable populations of MS of *V. dahliae*. The work involving the development of such procedures is presented in Chapters Two and Three. Finally, it was hypothesized that enhancement of populations of saprophytic microorganisms by incorporation of organic amendments into soil would enhance the mortality of MS, particularly those which were melanin deficient. Experiments involving the burial of MS in soil to which organic amendments were added revealed that some amendments were extremely effective at reducing the survival of all MS, regardless of the extent of melanization. Research examining the impact of these amendments on MS survival is described in Chapter Five.

Chapter Two

Production and Isolation of Microsclerotia

2.1 Introduction

Although *V. dahliae* is an economically important pathogen world wide (Powelson & Rowe, 1993), few studies have examined the survival characteristics of *V. dahliae* microsclerotia (MS), most likely because the MS are very small (38-150 µm in diameter) and therefore difficult to manipulate. As a result, there is little published work on the survival of individual MS of *V. dahliae* (Huisman & Ashworth, 1974; Congly & Hall, 1976; Ben-Yephet & Pinkas, 1977; Ben-Yephet, Siti & Frank, 1983) compared to larger sclerotia of fungi such as *Sclerotium cepivorum* (Coley-Smith, 1959; Leggett & Rahe, 1985; Gerbrandy 1989), *S. rolfsii* (Beaute & Rodriguez-Kabana, 1981; Henis & Papavizas, 1983) or *Sclerotinia sclerotiorum* (Merriman, 1976; Papavizas, 1977; Huang & Kokko, 1989). In previous studies where survival of *V. dahliae* was investigated, viability tests usually involved spreading a known quantity of inoculum (often soil or plant tissue) on a semi-selective medium and counting the number of colonies formed (Pullman, DeVay & Garber, 1981; Pullman et al., 1981; Stapleton & DeVay, 1982; Menzies & Griebel, 1967; Gilbert & Griebel, 1969; Green & Papavizas, 1968; Green, 1969; Ausher, Katan & Ovadia, 1975). Since such inoculum often contained an undefined mixture of hyphae, conidia and MS, it was impossible to know whether the colonies produced originated from single MS or aggregates of all these structures. Even when attempts were made to isolate MS specifically (Lazarovits et al., 1991a b), they frequently clumped together (Isaac, Fletcher & Harrison, 1971; Huisman & Ashworth, 1974) or became damaged during processing (Ashworth Harper & Andris, 1974). Use of such preparations generally resulted in unacceptably high within-treatment variability (Ashworth, Waters, George & McCutcheon, 1972), which could potentially mask important differences between treatments. Viability between experiments was even more problematic because

MS produced in the laboratory were often short-lived and their survival differed from preparation to preparation. Since many phenomena of interest in biological control experiments take weeks or months to manifest themselves, this rapid loss of viability made it impossible to monitor long-term survival of MS.

Initially, three different methods of producing MS under controlled conditions and monitoring their survival after exposure to experimental conditions were examined. While the harvesting procedure was the same for each method, two different growth media were used and the procedure used to evaluate survival of the MS was different. Once the best method for producing MS in the laboratory was determined, the number of MS produced per Petri plate was estimated and the survival of MS in long-term storage was monitored.

2.2 Methods

A culture of *Verticillium dahliae* (designated Vd Bean) was isolated from stem cross-sections of eggplants which had been planted in naturally infested field soil collected from a farm in Alliston, in Central Ontario. The cultures were maintained on Czapek-Dox agar (CDX, see Appendix 1) at a constant 24 C in continuous darkness. A sample of the fungus cut from the edge of the colony was transferred to fresh CDX media every 3 to 4 weeks. Periodically, the virulence of the fungus was checked by inoculating potato seedlings with the pathogen. The roots of the seedlings were soaked 15 mins in inoculum prepared by blending a culture of the fungus with sterile distilled water (SDW). Once the plants exhibited visual symptoms of disease, the fungus was reisolated from 0.5 cm cross sections of the surface-sterilized stems after incubation on fresh media at 24 C.

The procedure for harvesting MS from the growth med ım and isolating MS from conidia and hyphae was adapted from the Homogenizer-Screen Method of Gordee (1961). After several weeks growth, the contents of up to five Petri dishes were blended in a Waring blender with 200 mL tap water for three to four 10 sec pulses, poured into a nested set of Tyler standard sieves of mesh size 150, 200, 270 and 400 (corresponding to openings of 106, 75, 53 and 38 μm). After rinsing the homogenate with tap water, the MS

were washed back into the blender and blended again for three to four 10 sec pulses. This procedure was repeated until the MS on each sieve were singular and free of hyphal fragments. This was monitored by observing a sample of MS on a microscope slide under a stereo dissecting microscope between each rinse. The final MS preparations were washed off the sieves in a minimal volume of water (2-10 mL) and mixed with approx. 50 g dry, acid-washed quartz sand to prevent the MS from drying into clumps. The sand was pretreated by sieving into particles of 300 μm or larger, soaking overnight in concentrated HCl and rinsing in tap water until the pH was no longer acidic. The sand/MS mixtures were distributed into 15 cm diameter watch glasses and air dried overnight in the laminar flow hood. Difficulties with the MS adhering to the watch glass during drying were alleviated by pre-treating the glass with silicon (Sigmacote®, Sigma Chemical Co. St. Louis, Mo.).

Initially, Czapek-Dox agar (CDX, see Appendix 1) was used as the growth medium to produce MS in the laboratory. After sterilization by autoclaving, CDX agar was dispensed into Petri dishes, cooled and inoculated with a single plug from an actively growing culture of *V. dahliae*. Inoculated CDX agar dishes were incubated inverted, in a plastic sleeve, in the dark at 24C.

In an initial attempt to monitor the survival of MS, samples of the dry sand/MS mixtures were stored in a light-proof plastic bottle at 24 C for up to 56 days. Periodically, 5 samples of approx. 50 mg each were weighed out and distributed onto Petri plates containing an agar medium (SPT, see Appendix 3) which was semi-selective for *V. dahliae*. The sand/MS mixtures were distributed evenly over the plate with the aid of an Andersen Air Sampler (Harrison & Livingston, 1966; Butterfield & DeVay, 1977). After 2 weeks incubation in the dark at 24 C, the sand was removed from the agar surface by rubbing gently with a rubber policeman under running tap water. The plates were then examined using a compound dissecting microscope and colonies of *V. dahliae* were counted and recorded.

To ensure that colonies were der ɔd from individual MS the feasibility of directly transferring MS to agar plates was examined. The fungus was also grown on CDX agar and harvested by the blending and wet-sieving process but the MS were separated from the sand after drying by placing the sand/MS mixture on a 100 mesh sieve (150 µm opening) and gently rubbing the mixture with a rubber policeman. The sand remained in the sieve, the MS fell through the sieve and were collected on a piece of paper. Once collected, the MS were further sorted into size fractions by returning them to the appropriate sieve and gently tapping or rubbing them with an artist's brush. MS were stored dry, in small plastic tubes with lids (Eppendorf microcentrifuge tubes). MS were easily manipulated by sprinkling them onto small pieces of paper and moving them around with an artists brush. MS were picked up individually on the tip of a sterile hypodermic needle under a stereo dissecting microscope (see Plate 2.1) according to the method of Ben-Yephet and Pinkas (1977). Petri dishes containing SPT agar medium (Appendix 3) were placed on a grid of 25 squares and one MS was placed on the surface of the agar within each square (a process termed "plating"). One or two plates were made for each treatment (25 or 50 MS). Transfer of MS was done on the open lab bench because the antibiotics in the SPT medium prevented any airborne contamination. The needle was flamed often to reduce the risk of transferring contamination between treatments. The dishes were stacked, inverted, in a plastic sleeve and incubated at a constant temperature of 24 C in continuous dark. After a minimum of 2 weeks incubation, a count of the number of MS which germinated and formed colonies could be made.

The method of producing, isolating and plating MS just described was optimized by replacing CDX agar with a modified, semisolid CDX growth medium (MCDX, see Appendix 2). After sterilization by autoclaving, MCDX was cooled, inoculated with several large plugs of agar cut from actively growing colonies of *V. dahliae*, shaken well, and 30 mL aliquots were immediately dispensed into Petri dishes. The dishes were stacked inside clear, rigid plastic utility boxes and incubated in the dark at 24 C.

To determine the number of MS produced per plate, 12 dishes of MCDX were inoculated with *V. dahliae* and 2 dishes were harvested each week for 6 weeks after inoculation. The total mass of MS harvested each week was recorded. The MS were then dry sieved into 3 different size classes: >106 μm, 75-106 μm and <75 μm and the mass of MS in each size class recorded. To determine the approximate number of MS (>75 μm) per mg, 1 mg dry MS was added to 5 mL of sterile distilled water and 2 drops of Tween-20 and stirred for 15 minutes on a magnetic stirrer. A 100 μl drop was pipetted onto a microscope slide and the number of MS was counted under a binocular microscope. The mean of ten separate counts was calculated and multiplied by 50 to get the number of MS per mg.

2.3 Results

MS germination fell to half the original level in 8 weeks when MS were grown in CDX agar and plated as sand/MS mixtures with the Andersen Sampler (Fig. 2.1). The variability in the data was very high, although the number of colonies was calculated from a mean of 5 values. Using the sand/MS mixtures, it was also impossible to tell whether a colony was produced by a single MS or an aggregation of several MS.

When MS were grown in CDX agar but transferred individually to plates using a needle (Plate 2.1), the percent germination was low (approx. 50%) immediately after harvesting, or declined rapidly (from 90% to as low as 15%) in the first week after harvesting (data not shown). While the MS produced in CDX and MCDX were morphologically similar, separating MS from solid (1.5%) agar generally required extensive blending and rinsing. In contrast, MS recovered from the semi-solid (0.25% agar) MCDX growth medium required much less processing and exhibited 98% and 94% germination after 46 and 59 weeks respectively, of dry storage at 24 C.

Over a 6 week growth period, *V. dahliae* produced an average of 119.7 mg of MS (dry weight, S.E.=9.9, N=5) in a single plate containing 30 mL of MCDX media (Table 2.1). Since the average number of MS per mg of dry MS was calculated to be 11,865

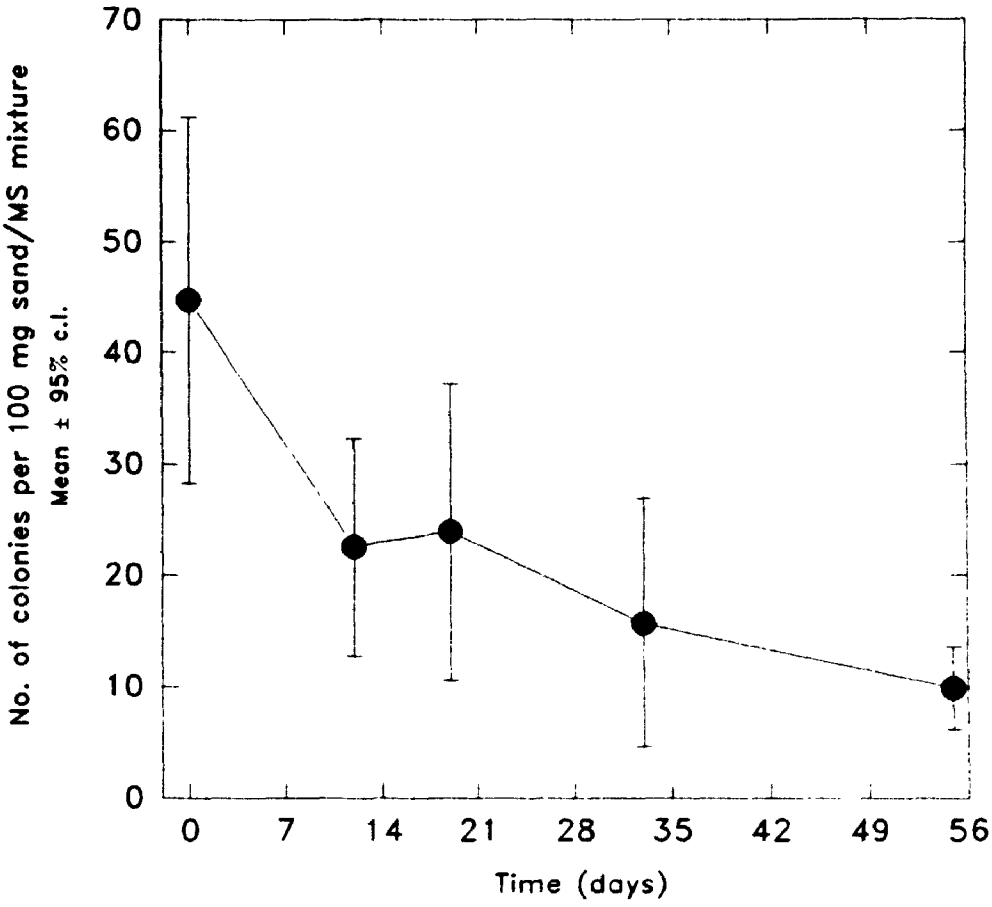


Figure 2.1
Mean number of colonies produced per 100 mg of sand/MS mixture plated with the Andersen Sampler over an 8 week period (N=5, ± 95% C.L.)

Plate 2.1

Illustration of the procedure for plating individual microsclerotia (MS) of *V. dahliae* onto plates of agar media. The MS were sprinkled onto paper and placed under the microscope. Single MS were picked up on the tip of the needle and transferred, one at a time, onto the surface of the agar in the centre of each grid square on the plate.

Table 2.1 Dry weight of MS (mg) recovered per plate of MCDX media.

	Time of Harvest (weeks)[a]				
Size of MS	2	3	4	5	6
>106 μm	6.3	9.2	53.8	34.4	21.2
75-106 μm	26.5	45.8	39.0	44.6	30.9
53-75 μm	26.9	32.3	26.6	25.5	25.1
<53 μm	35.6	43.1	22.1	30.6	18.9
Total	95.3	130.4	141.5	135.1	96.1

[a] Two plates (60 mL media) were harvested each week over a 6 week period.

(S.E.=1171.6, N=10), the total number of MS produced per plate was approx. $1.4X10^6$. The distribution (by weight) of MS in each of the 4 size classes harvested weekly over 5 successive weeks is shown in Table 2.1. Two weeks after inoculation, the smallest MS were most numerous and there were few MS of the largest size. After 2 weeks, however, the proportion of larger MS (>75 μm) increased and the proportion of smaller MS decreased.

2.4 Discussion

The medium chosen as the most suitable for producing and isolating MS was MCDX. The low agar content permitted the separation of MS from the culture more quickly and with little damage to the MS. More than a million MS were recoverable from each 30 mL plate of MCDX, providing enough MS for the same lot to be used in several experiments. Also, the survival of these MS stored at 24 C was still 94% after 14 months. These features of this method of MS production and isolation are extremely important because they mean that a uniform population of MS can be used in many experiments over long periods of time. Since many biological control experiments (especially those done under field conditions) require much time and effort, it is critical to have a population of MS which will not decline in vigour in the middle of the experiment as a result of damaging harvesting procedures.

The method of spreading sand/MS mixtures over the plate with the Andersen Sampler resulted in high variability in estimates of MS survival because the MS did not remain uniformly distributed in the sand mixtures. This meant that there was variability in the number of MS within the 5 replicate samples plated. Nicot and Rouse (1987) also found that the Andersen Sampler method was the least precise (i.e. exhibited the least agreement of repeated measurements of the same quantity) of three different methods tested for quantitatively assessing the level of *V. dahliae* present in soil. Within each plate, it was also impossible to determine whether each colony arose from a single MS or several MS clumped together. This method was time-consuming because of the number of samples of

the sand/MS mixture which had to be weighed out and then plated and it also required a large number of plates of media.

The method which was chosen as the most accurate for measuring MS survival was the plating of individual MS with the needle. Manipulation of the dry, individual MS with the use of the needle was quite simple with the assistance of a good microscope and a steady hand. Once proficiency at handling the MS was gained, a plate of 25 MS could be made in 1 to 2 minutes. This method had the important advantage of permitting the fate of each individual MS to be followed and eliminated the problem of clumping of the MS. The variability in the results of the individual plating method was much lower than in the method of spreading sand/MS mixtures on the plate with the Andersen Sampler.

The medium chosen for assaying survival of MS after experimentation was SPT. It was the preferred choice since it contained antibiotics which permitted the plating to occur on the lab bench and it contained tergitol which slowed down fast-growing soil fungi which might compete with *V. dahliae*. This was particularly useful for experiments when MS were buried in soil before being transferred to plates. *V. dahliae* was easily identified on this medium because it grew down into the agar and produced distinctive MS after about a week of incubation at 24 C. The plates were also transparent which permitted daily measurement of germination and colony diameter with the use of a dissecting microscope. Thus, in addition to simply knowing the number of MS which were alive or dead at the end of an experiment, one could also evaluate the rate of germination and growth.

The procedures outlined here for the production, isolation and harvesting of individual MS (which were free from conidia and hyphae) produced a large number of MS which maintained high, uniform viability for more than a year and permitted the same lot of MS to be used in many experiments over a long period of time. The method of plating individual MS on SPT media after exposure to experimental conditions allowed the germination and colony diameter of each individual MS to be measured accurately.

Chapter Three

The Effect of Size on the Growth and Survival of Microsclerotia

3.1 Introduction

While microsclerotia of *V. dahliae* are reported to range in size from 15 or 50 μm to 200 μm (Isaac, 1949; Hall & Ly, 1972), the effect of MS size on germination and colony growth has not been thoroughly investigated. Huisman & Ashworth (1974) mentioned that smaller, immature MS of *V. dahliae* isolated from soil exhibited only 7% germination while larger, more mature MS had 80% germination. Congly & Hall (1975) sieved MS of *V. dahliae* and worked with the 44 μm size, although they gave no explanation why they chose that particular size. Hoes and Huang (1975) reported that sclerotia of *Sclerotinia sclerotiorum* collected from soil ranged in size from <3 to >6 mm and remarked that size and shape might affect sclerotial viability but provided no data to support this. In some plant species, seeds of differing size or morphology are produced (sometimes by the same plant) and may exhibit distinct patterns of dormancy, germination or vigour (Cavers & Harper, 1966). In order to determine whether MS size was of importance, the germination and growth of colonies produced by MS of various size classes was investigated. In addition, the effect of storage temperature on the viability of MS was examined. Since it was desirable to keep one lot of MS for a long time so it could be used in long-term experiments, it was important to determine the optimal conditions for MS storage.

3.1 Methods

MS were produced on MCDX media, isolated, harvested and dried on quartz sand as described in Chapter 2. The dry sand/MS mixtures were then placed in a 100 mesh sieve (150 μm opening) which was set on top of a set of nested sieves of 150, 200, 270 and 400 mesh size (106, 75, 53 and 38 μm openings) and gently rubbed with a rubber policeman. The MS fell through onto the sieves below, leaving the sand in the top sieve. The MS

were gently tapped and rubbed with an artist's brush until they were sorted into three size classes: 75-106, 53-75 and <53 μm. The MS were collected and stored in Eppendorf tubes at 24 C until needed.

One hundred MS of each of the 3 size classes were transferred onto 4 plates of SPT media and 50 MS of each size were plated onto 2 plates of either CDX agar or Potato-Dextrose agar (PDA). PDA was prepared by adding 39 g of Difco Bacto Potato Dextrose agar to 1 L distilled water (DW). Both CDX and PDA received 1 ml/L of the same antibiotic mixture added to SPT (see Appendix 3). The plates were examined daily for a week under a dissecting microscope and the number of MS which had germinated was recorded. Beginning on the 2nd or 3rd day after plating, the diameter of each colony present on the plate was measured daily with a micrometer to the nearest 0.5 mm and recorded.

Two different experiments were performed to investigate the effect of storage temperature on survival of MS. In the first, MS of 3 size classes (75-106, 53-75 or <53 μ m) were placed into 1.5 ml capped plastic tubes and placed into a container which had desiccant in the bottom to control humidity. The containers were sealed and stored at 24, 4, -25 and -70 C. A refrigerator freezer was used to provide -25 C. One sample of 25 MS was removed from each tube weekly for 10 weeks and transferred onto SPT. The number of MS which had germinated to form a colony was counted and recorded after a minimum of 2 weeks incubation. In the second experiment, only MS of 75-106 μm were used and a -25 C chest freezer was used instead of the refrigerator freezer. To avoid repeatedly exposing the MS to rapid temperature changes during sampling, a small sub-sample of sand/MS mixture was placed into each of 64 plastic tubes and a set of 16 tubes was placed at each temperature in a sealed container with desiccant. At 1, 2, 3, 4, 6, 8, 10 and 36 weeks, 2 tubes were removed from each temperature and 25 MS from each tube were transferred onto SPT.

The long term survival of MS of different size classes was monitored during storage at 24 C. Four different harvests of MS were tested. In two experiments, MS of 75-106 and 53-75 μm were monitored for up to 14 weeks. In the other two experiments, MS of 75-106, 53-75 and <53 μm were monitored for up to 35 weeks. In all cases, the MS were stored in Eppendorf tubes at 24 C and 50 MS were plated onto 2 plates of SPT at each sampling time. The number of MS which germinated and formed colonies was recorded after the SPT plates were incubated for at least 2 weeks at 24 C.

3.2 Results

The mean number of MS of <53 μm, 53-75 μm or 75-106 μm size which germinated on SPT plates over a 7 day period is shown in Fig. 3.1. While there was no difference in the final number of MS which germinated, the rate of germination between MS of different size classes differed significantly (χ^2= 146.1, d.f.=10, $p<0.001$). The 75-106 μm MS germinated faster than the 53-75 or <53 μm MS, which showed similar germination rates (χ^2=7.05, d.f=5, p=0.217). Virtually all 75-106 μm MS germinated within 24 hours after plating on SPT, while the germination of smaller MS extended over 2 to 3 days (Fig. 3.2). When plated on CDX or PDA, there was no detectable difference in rate of germination between MS of different sizes, primarily because the germination reached 90% or more in the first 24 hours. On all three media, the larger MS (75-106 μm) produced larger colonies than those formed by the smaller MS (Fig. 3.3 A-C). Analysis of colony diameter distribution (divided into 6 categories ranging from 1 to 6 mm) revealed that of 4 day old colonies produced on SPT from MS of 75-106 μm size, 70% fell into the 5 mm category (Fig. 3.4 C). In contrast, only about 50% of MS 53-75 μm and 25% of MS <53 μm produced colonies on SPT which were 5 mm after 4 days growth (Fig. 3.4 A, B). A greater proportion of smaller colonies was produced by the smaller MS. A significant relationship between MS size and colony diameter after 4 days growth on SPT was confirmed by a chi-square test (χ^2=39.3, d.f.=8, $p<0.001$). In similar experiments done with different harvests of MS, there was always a significant reduction in the rate of both

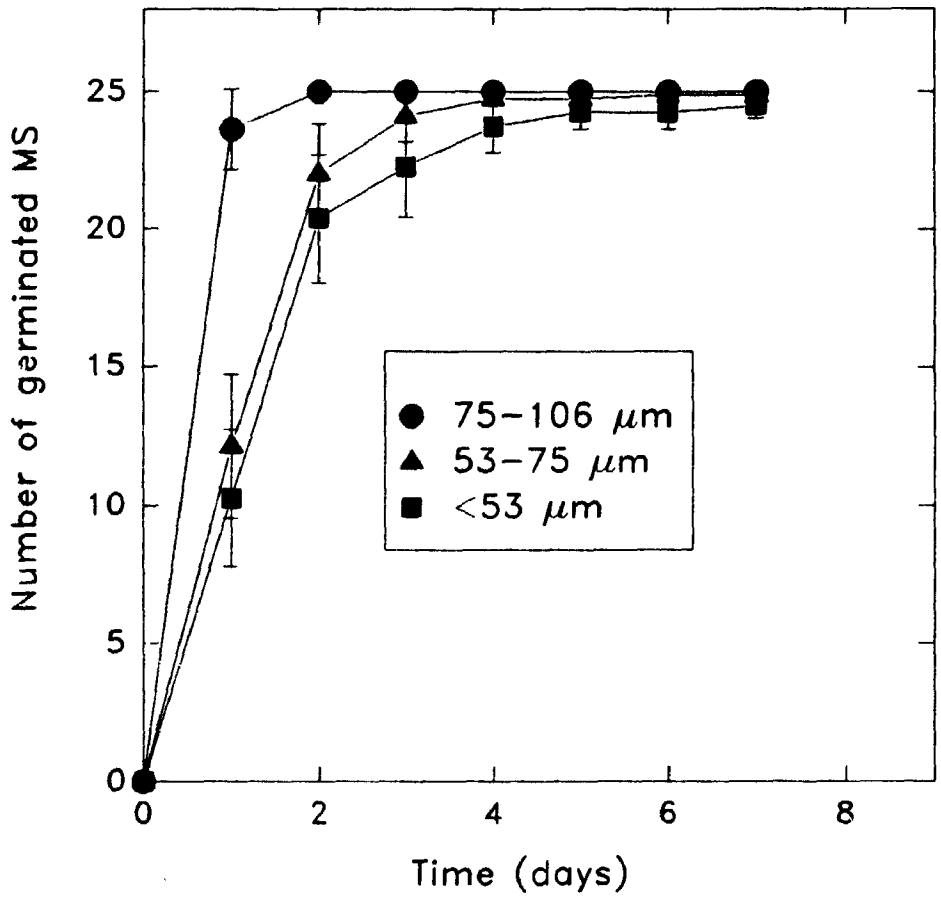


Figure 3.1
Mean number of germinated microsclerotia (MS) on SPT media (±95% C.L., N=8). Legend refers to MS size classes measured.

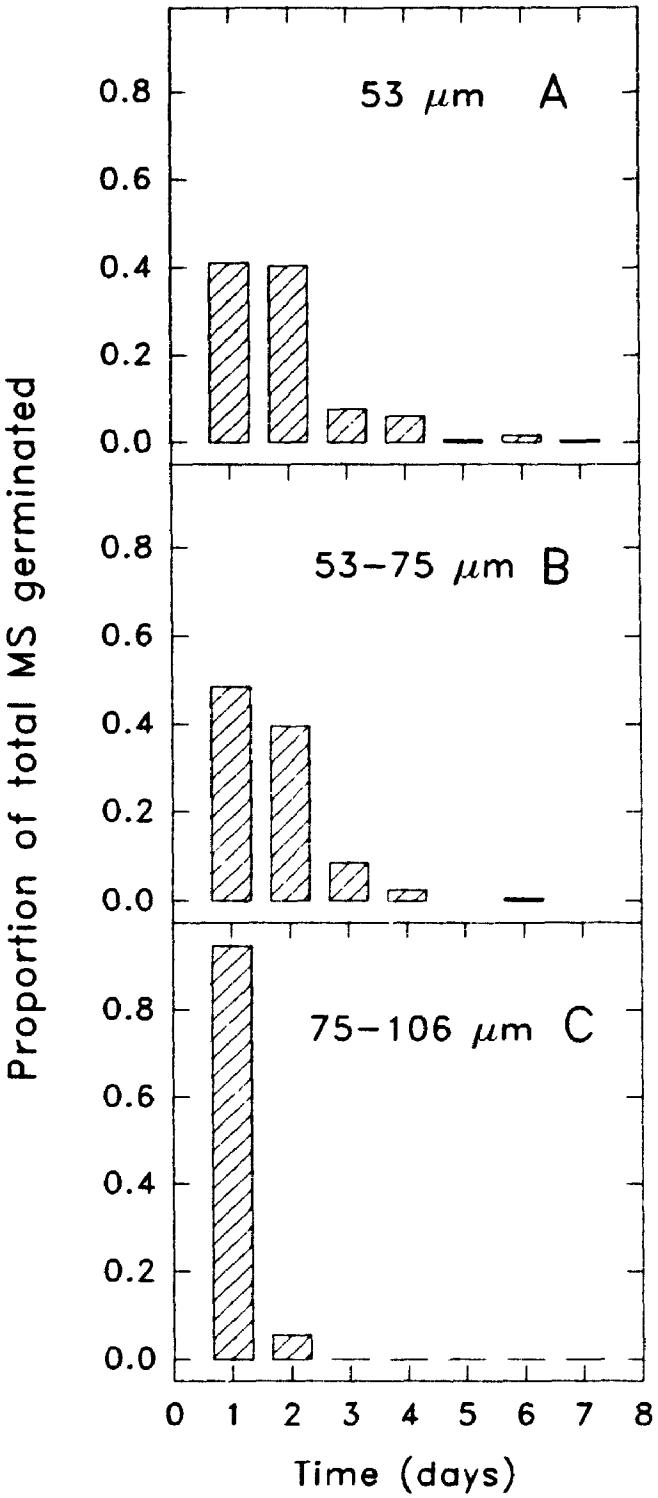


Figure 3.2
The proportion of the total number of microsclerotia (MS) in each of 3 size classes which germinated on each day after transferring onto SPT media.

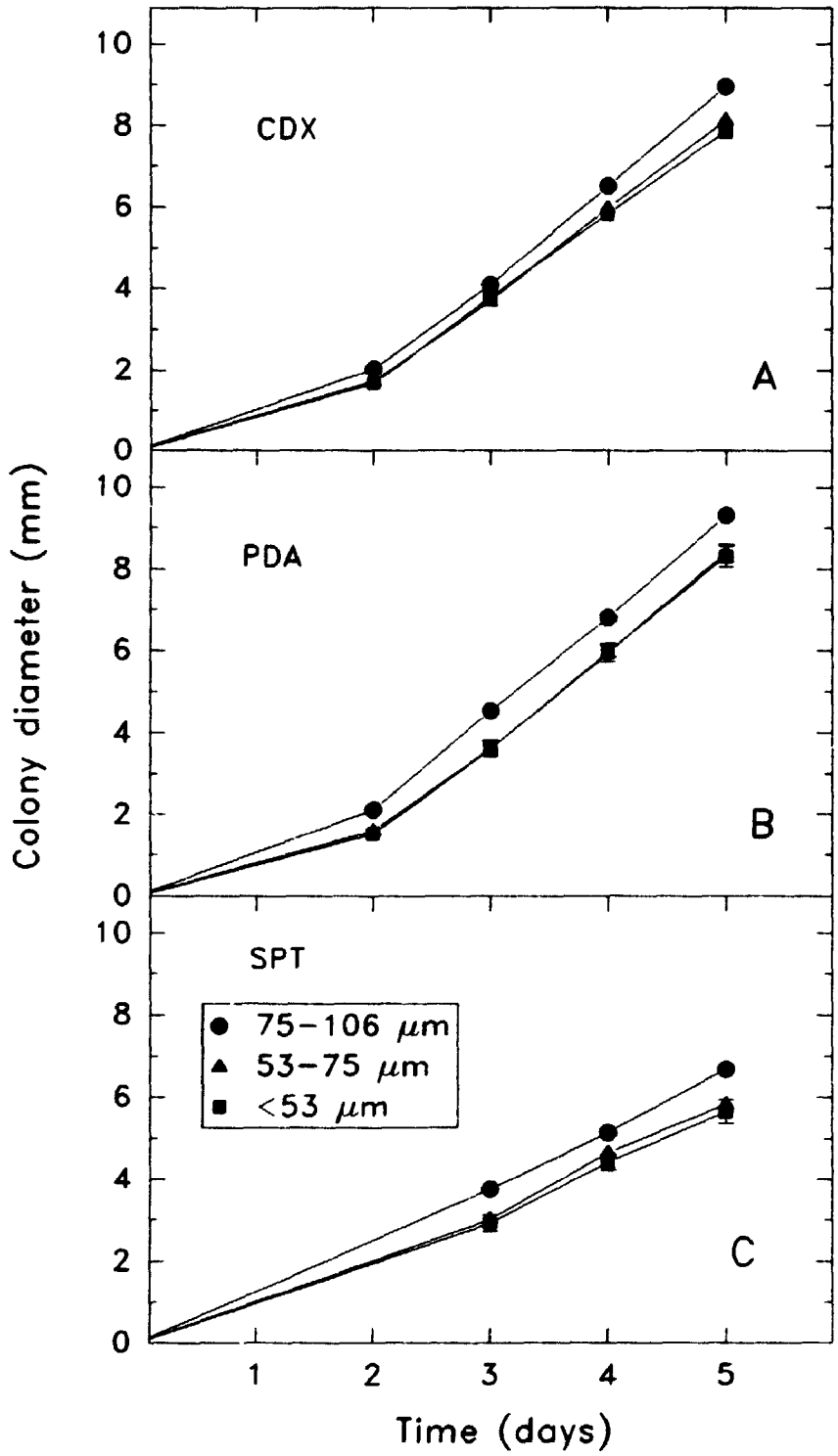


Figure 3.3
Mean diameter of colonies (±95% C.L.) produced by MS of three size classes on different media. A, CDX media (N=50); B, PDA media (N=50); C, SPT media (N=100).

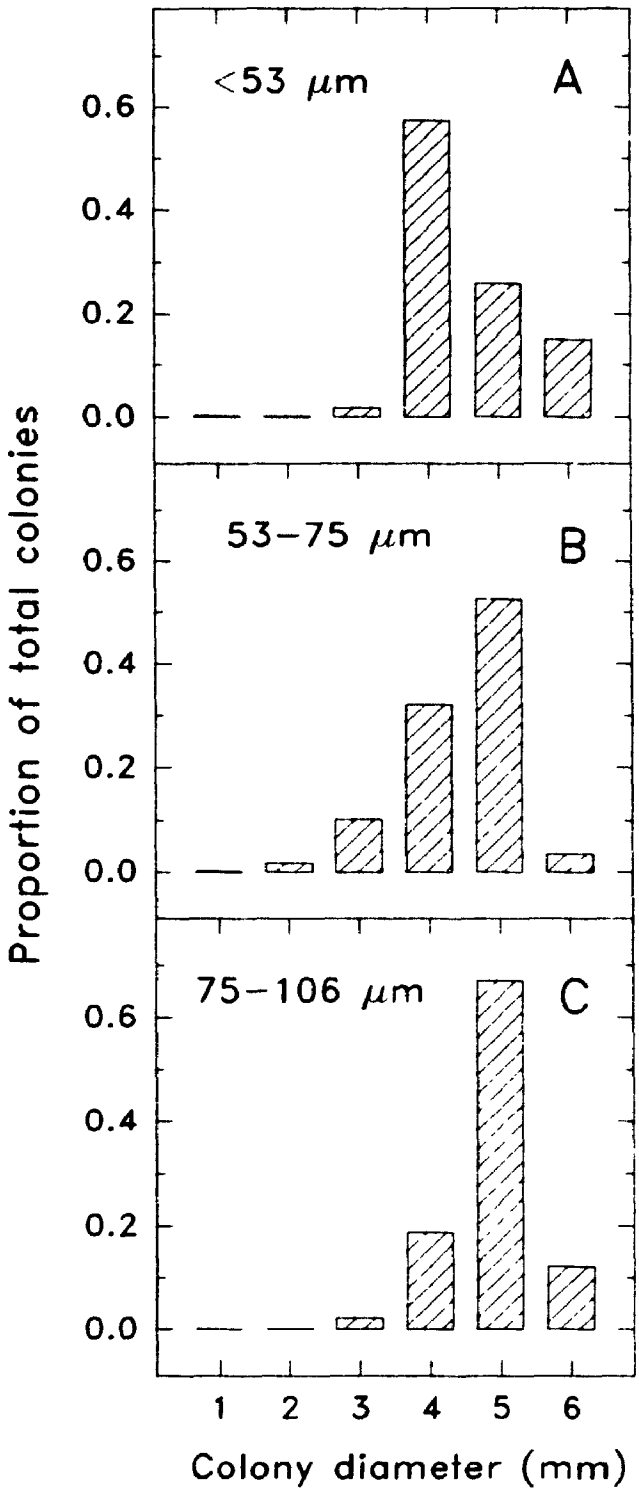


Figure 3.4
The proportion of colonies produced by MS of three size classes which fell into each colony size class after 4 days growth on SPT.

germination and colony growth in MS <53 μm, however, the MS of intermediate size (53-75 μm) occasionally performed more like the larger MS (>75 μm) than the smaller MS (data not shown).

When MS of 3 size classes were stored at various temperatures in 2 different experiments, larger MS (>106 μm) survived better than smaller MS over the 10 week period and exhibited less variability in germination over time. There was little difference in the survival of MS stored at 24, 4 or -70 C. In experiment 1, MS were stored at -25 C in a refrigerator freezer, which reduced survival in every size class to < 20% after 4 to 5 weeks (Fig. 3.5 A-C). No reduction in survival occurred in experiment 2, when MS were stored in a chest type freezer at -25 C (Fig. 3.5 D). The variability in germination of 75-106 μm MS in the second experiment (Fig. 3.5 D) was very low compared to the first experiment (Fig. 3.5 A-C) perhaps because the MS were destructively sampled instead of being repeatedly taken out, sampled and then returned to the storage site.

Larger MS (75-106 μm) generally survived better than smaller MS when stored at 24 C (Fig. 3.6 and 3.7). The smaller MS shown in Fig. 3.6A lost viability at a faster rate than the larger MS when stored up to 14 weeks. In another experiment (Fig. 3.6B), there was no difference in survival of MS of different sizes after 12 weeks. The monitoring of survival was terminated because the experiments in which the MS were being used were concluded.

When MS were stored for longer time periods (over 8 months), the larger MS also tended to survive better than smaller MS, although the length of time that the small MS maintained high germination varied with the different harvests of MS. In one experiment, the smaller MS (<75 μm) began to lose viability after 8 to 12 weeks and by 35 weeks, their germination dropped below 40% (Fig. 3.7A). In an experiment where MS from a different harvest were sampled, the survival of MS < 75 μm showed only a slight reduction on the 35th week of monitoring (Fig. 3.7B). Results from another experiment not included in Figs. 3.6 or 3.7 showed that MS of 75-106 μm and 53-75 μm exhibited

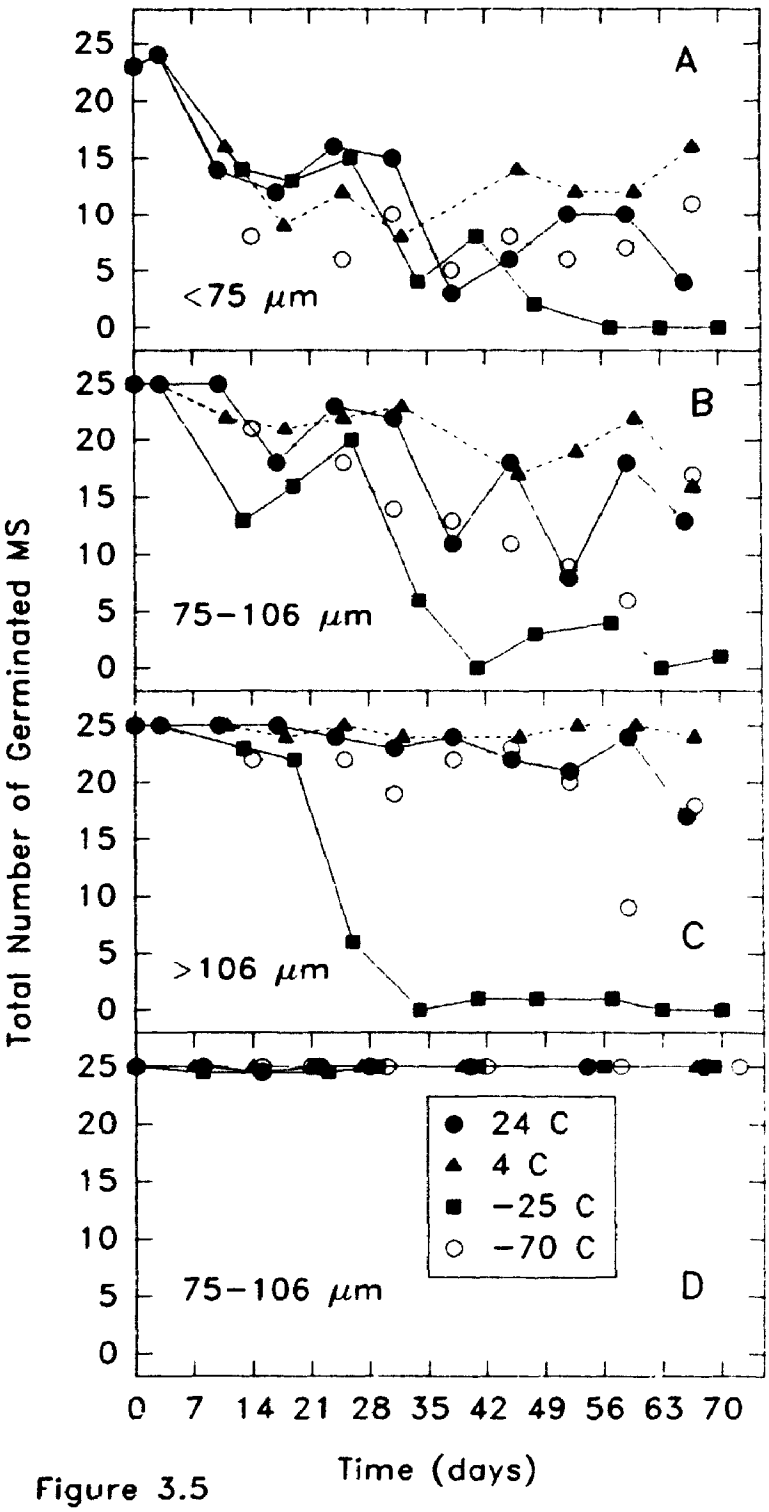


Figure 3.5
The survival of MS stored at 4 different temperatures for up to 70 days. Values represent the total number germinated (out of 25) at each sampling time. A, B, and C represent MS from the first experiment. D represents MS from the second experiment.

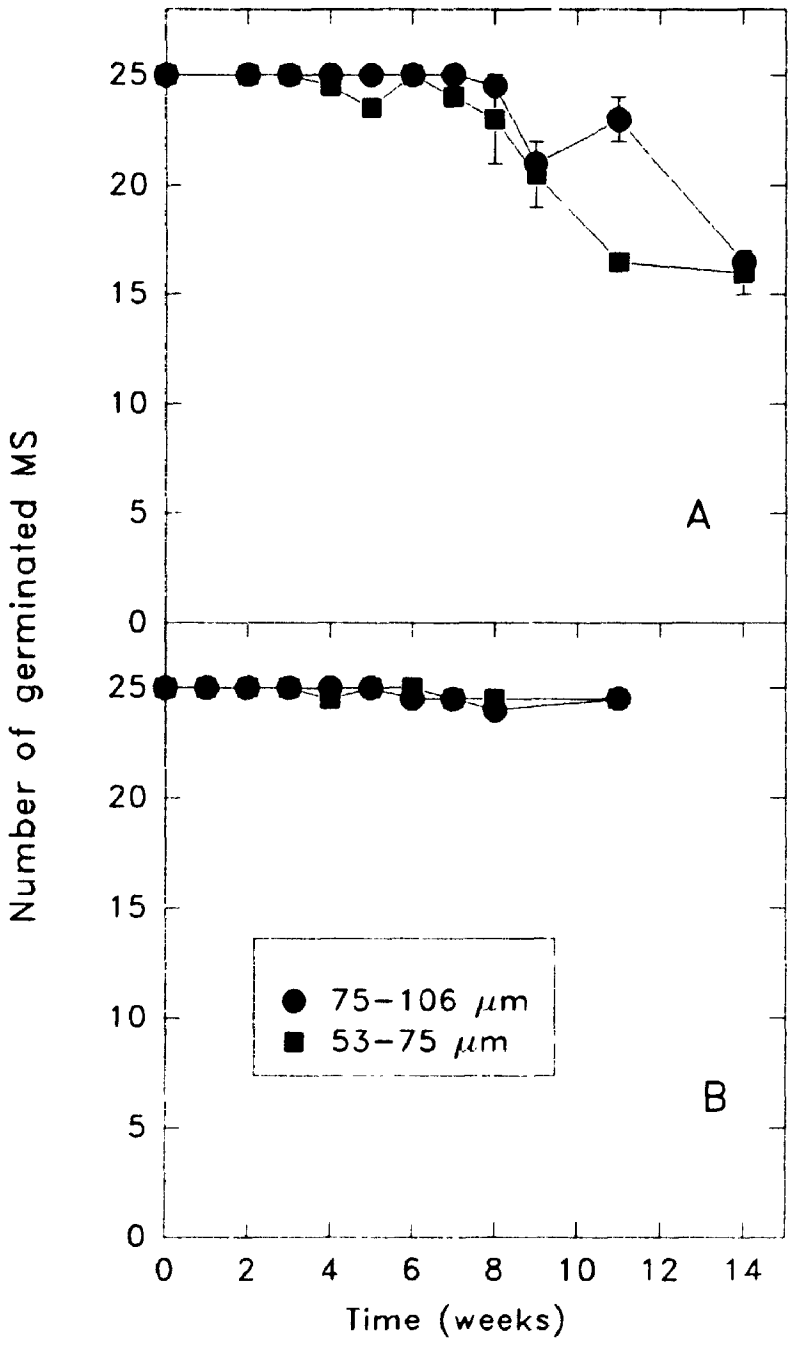


Figure 3.6
The mean number of microsclerotia (MS) of two size classes which germinated over a period of 14 weeks (N=2, ±1 S.E.). A and B represent two different harvests of MS.

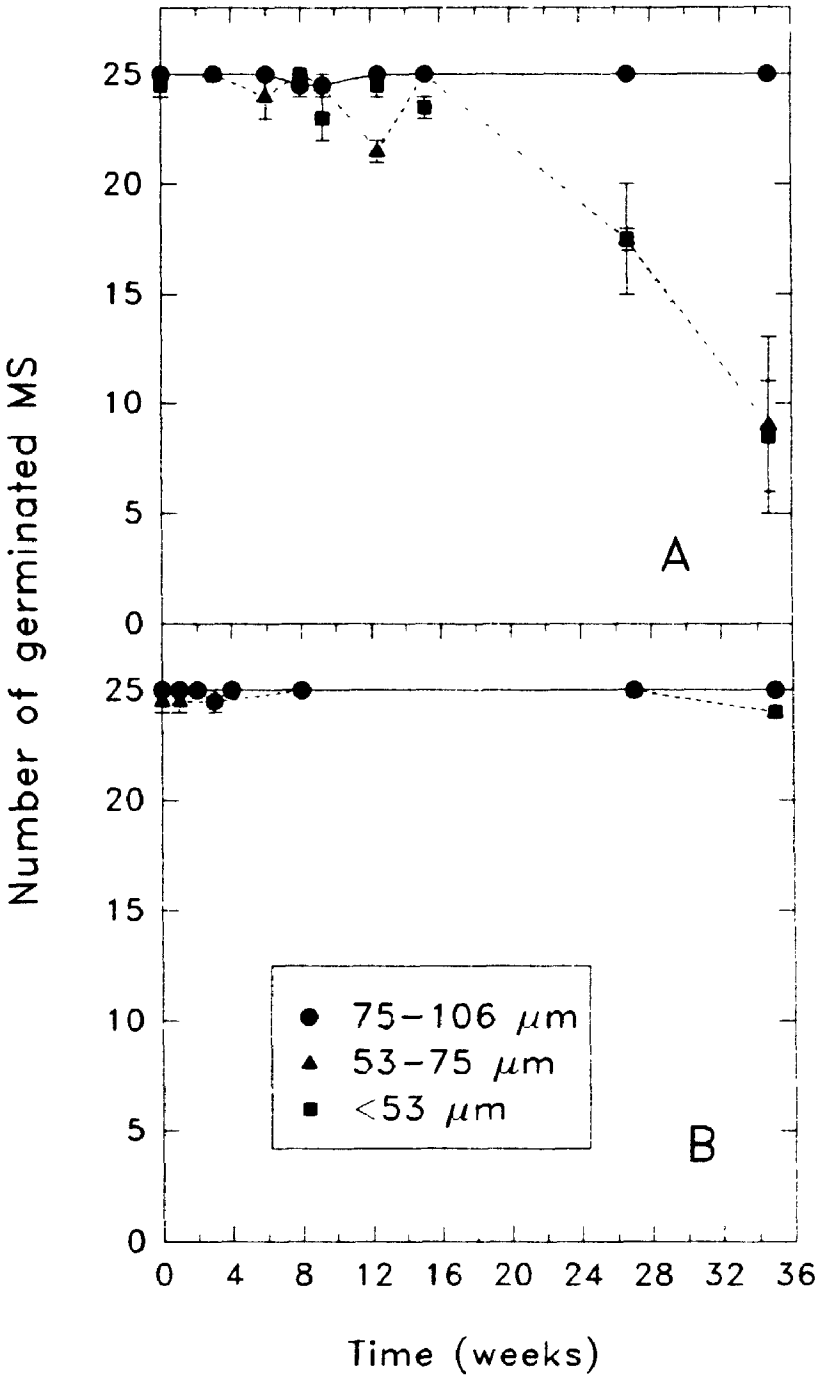


Figure 3.7
The mean number of microsclerotia (MS) of three size classes which germinated over a nine month period (N=2, ±1 S.E.). A and B represent two different harvests of MS.

98% and 90% germination, respectively after storage at 24 C for 46 weeks and 94% and 78% germination after 59 weeks.

3.4 Discussion

One of the difficulties in studying the survival of fungal resting structures is the presence of a large within-treatment variability which often masks the treatment effects. Considering that such studies often require long time periods and labour intensive preparations, it is very frustrating to find at the end of the experiment that no statistically significant treatment effect can be detected. Finding ways to control within-treatment variability is therefore highly desirable.

The results presented show that the size of the MS has an impact on the rate of both germination and colony growth. MS >75 μm in size exhibited faster and more synchronous germination than any other size class. They also produced colonies which grew faster than those produced by smaller MS. This is supported by a study of the viability of individual MS of *V. dahliae* of different sizes (75-125, 37-75 and <37 μm), which reported that the level of germination and the number of germinating hyphae were positively correlated to MS size (Ben-Yephet & Pinkas, 1977). The other characteristic of larger MS (>75 μm) was their ability to survive long-term storage at 24 C better than smaller MS. Increased MS size may represent greater maturity of the MS. In Chapter Two, it was shown that the proportion of smaller MS (<53 μm) decreased while that of larger MS (>75 μm) increased as cultures in MCDX matured from 2 to 6 weeks of age (Table 2.1). Perry and Evert (1984) also found that MS produced within the first few weeks after inoculation were generally smaller and consisted of fewer cells than those observed later.

The germination and growth characteristics displayed by MS of different sizes have several important consequences. First, by using MS of uniform size in experiments studying MS survival, variability in germination and colony diameter can be better controlled and the within-treatment variance subsequently reduced. Since MS of 75 μm or

more survive long-term storage better than smaller MS and are also produced in the most abundance (see Table 2.1), they are the best candidates for experimental use. Second, if MS >75 μm are used in experiments, then reductions in the rate of growth and germination of treated MS compared with untreated MS will be a reflection of the sub-lethal effect of that treatment. Since MS are multicellular structures, a treatment which is effective in killing 90% of the cells within an MS may go undetected if success is measured simply by the production of a colony. If there is a reduction in the rate of germination or colony growth, then the treated MS are essentially responding like smaller structures, which contain fewer cells.

There was little difference between survival of MS of 75-106 μm stored dry at 24, 4, -25 or -70 C. Storage in a refrigerator freezer was detrimental to the MS while storage at -25 C in a chest freezer did not reduce survival. An investigation of the two freezers revealed that while the chest freezer maintained a constant -25 C, the temperature in the refrigerator freezer fluctuated. The frost-free cycle of the refrigerator freezer was most likely responsible for this phenomenon. It is recommended, therefore, that when storing MS for long term experiments, they not be kept in refrigerator freezers. During all of the experiments described in this thesis, a sample of the MS being used were kept in storage at 24 C nd their survival was checked by plating 50 MS every 2 to 4 weeks. If the baseline survival of the MS at 24 C fell below 75%, the experiment was terminated.

Chapter Four

The Role of Melanin in Survival of Microsclerotia

4.1 Introduction

Melanins are dark brown to black pigments which are found in organisms as diverse as microbes, plants, insects and larger animals such as humans but are not essential for growth and development (Swan, 1963; Bell & Wheeler, 1986). Melanin has not been isolated as a single chemical compound of definite composition but rather it exists as a polymer composed of phenol or indole based subunits which are sometimes complexed with peptides, carbohydrates and fatty acids (Swan, 1963; Gadd & deRome, 1988). Melanins are generally insoluble in water, aqueous acid and organic solvents and absorb all visible wavelengths, particularly in the UV region (Bell & Wheeler, 1986). In ultrastructural examinations of *V. dahliae*, melanin was found as distinct granules within the walls of the MS as well as outside the cell walls in the intercellular spaces (Brown & Wyllie, 1970; Ellis & Griffiths, 1974; Griffiths, 1970; Wright & Abrahamson, 1970; Traquair, Gaudet & Kokko, 1987). While the exact mechanism of deposition of extracellular melanin is not understood, it has been suggested that the melanin is not formed in the cytoplasm but is formed extracellularly, in association with the fibrillar network (Wheeler, Tolmsoff & Meola, 1976).

Melanins present in fungi can result from one of several distinct biosynthetic pathways (Bell & Wheeler, 1986). The melanin biosynthetic pathway in *V. dahliae* has been well characterized using mutants which were deficient for normal production of melanin. Most of the intermediates in the biosynthesis of melanin in *V. dahliae* have been identified (Fig. 4.1A) and the pathway confirmed by feeding experiments (Bell et. al, 1976; Bell, Stipanovic & Puhalla, 1976; Wheeler et. al., 1978). *V. dahliae* produces DHN melanin which is derived from pentaketides produced via the acetate-malonate pathway (Goodman, Kiraly & Wood, 1986). In the biosynthetic pathway of DHN melanin

Figure 4.1

A: The pentaketide pathway of melanin biosynthesis and some of the associated shunt pathways resulting from inhibition by the chemical tricyclazole (TCZ).

1,3,6,8-THN = 1,3,6,8-tetrahydroxynaphthalene

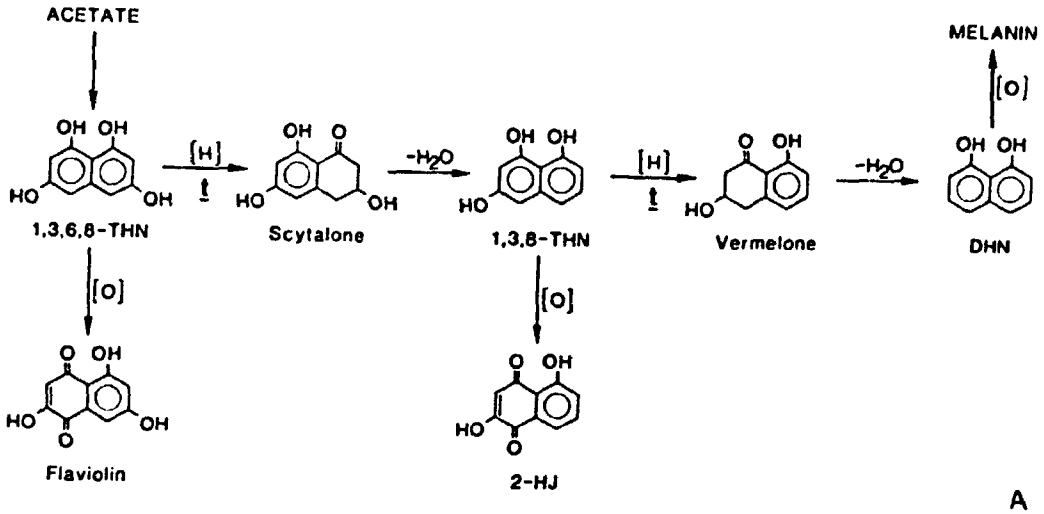
1,3,8-THN = 1,3,8-trihydroxynaphthalene

DHN = dihydroxynaphthalene

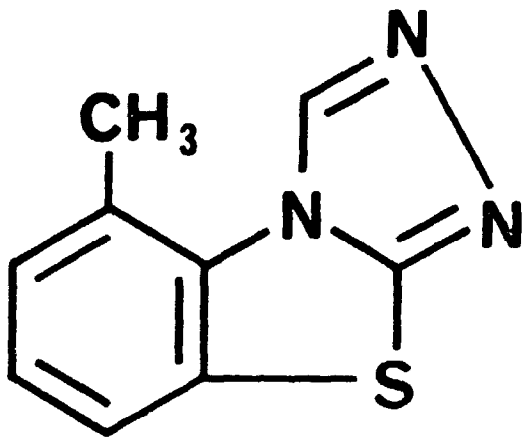
2-HJ = 2-hydroxyjuglone

The site of activity of reductase enzymes is indicated by the symbol [H] and the site of dehydratase enzymes is indicated by the symbol $-H_2O$. Addition of 1 mg/L TCZ to the growth medium of *V. dahliae* results in a blockage of the pathway between 1,3,6,8-THN and scytalone at the position marked t. Similarly, addition of 10 mg/L blocks the pathway between 1,3,8-THN and vermelone at the position marked t. Source: Bell, A.A., and Wheeler, M.H. 1986. Ann. Rev. Phytopathol. 24:411-451.

B: The chemical structure of tricyclazole (TCZ).



A



Tricyclazole

B

(Fig. 4.1A) the first identified intermediate, 1,3,6,8-THN, is reduced to scytalone, dehydrated to 1,3,8-THN, reduced to vermelone and dehydrated to form DHN. DHN undergoes auto-oxidation to the final product, melanin (Bell & Wheeler, 1986). A number of mutants deficient in the activity of certain enzymes present in the pathway were created by Bell et al. (1976). The albino mutant (Alm-1) was thought to be blocked in the pathway at an unknown step between acetate and 1,3,6,8-THN and therefore produced hyaline MS. Melanization was restored to the albino mutant when it was fed (+)-scytalone (Bell et. al., 1976). The various brown mutants (Brm 1,2,3 or 4) were blocked in the pathway between scytalone and melanin due to the elimination of the activity of either dehydratase or reductase, which resulted in the accumulation of brown-coloured shunt products (flaviolin or 2-hydroxyjuglone) in the growth medium (Bell & Wheeler, 1986).

Information on the DHN melanin pathway has also been obtained through the use of a chemical which inhibits the pathway. Tricyclazole (5-methyl-1,2,4-triazolo[3,4b]-bensothiazole or $C_9H_7N_3S$, M.W.=189.24) was first registered as a systemic fungicide used to control rice blast disease, *Pyricularia oryzae* (Froyd et. al., 1976). The structure of TCZ is presented in Fig. 4.1B. Subsequently, it was shown that, in *V. dahliae* and *P. oryzae*, TCZ at concentrations of 1 or 10 μg/mL interfered with the reductive step between 1,3,8-THN and vermelone without inhibiting vegetative growth (Tokousbalides & Sisler, 1978, 1979). In *P. oryzae*, TCZ specifically blocked the production of melanin in the appressoria and prevented the appressoria from achieving the high internal hydrostatic pressure required for penetration (Howard & Ferrari, 1989). When TCZ was applied at a concentration of 1 μg/mL, the pathway was blocked between 1,3,8-THN and vermelone and 2-HJ accumulated in the medium, while at 10 μg/mL, the pathway was blocked between 1,3,6,8-THN and scytalone and flaviolin accumulated in the media (Bell & Wheeler, 1986).

Melanins have been implicated in the survival and longevity of fungal reproductive structures (Lockwood, 1960; Bell & Wheeler, 1986). One of the functions attributed to

melanin is the protection of microorganisms from the damaging effects of UV light. As early as the 1930's, experiments on the effect of UV irradiation on the survival of pigmented fungal propagules were conducted. Weston (1931) found that white and orange forms of urediniospores of *Puccinia graminis tritici* were killed more easily by UV light than red or grey spores and concluded that the pigment in the cell wall acts to protect the cell from UV light. He also reported that if the pigmented spores were allowed to germinate prior to irradiation, the fungus was much more susceptible to injury by UV light (Weston, 1932). Durrell (1964) reported that non-pigmented spores of 56 species of fungi were killed by UV light and that when the dark pigment in spores was blocked by the addition of hexachloracetone, the pale spores were killed within 4 minutes. Bell & Wheeler (1986) cite several Russian studies which show that damage by UV light is greater in melanin-deficient mutants and in younger, less heavily pigmented colonies. It has also been demonstrated that the melanized wild-type strain of the human pathogenic fungus *Wangiella dermatitidis* was more resistant to the lethal effects of UV irradiation than was an albino strain of the same fungus (Dixon, Szaniszlo & Polak, 1991).

Melanins have also been suggested to play a role in the protection of fungi from degradation by enzymes (Bell & Wheeler, 1986). The dark mycelium produced by *Helminthosporium sativum* and *Alternaria solani* was more resistant to lysis by soil than hyaline mycelium (Lockwood, 1960). *Fusarium solani* was converted to spheroplasts by chitinase and glucanase but *Rhizoctonia solani*, which contained melanin in its walls, was not affected (Potgieter & Alexander, 1966). Microorganisms which decomposed the hyphal walls of *Sclerotium rolfsii* did not affect the melanized sclerotia and spores of *Aspergillus phoenicis* were not digested by chitinase and glucanase until after the melanized surface spicules were removed from the spore walls (Bloomfield & Alexander, 1967). The hyphal walls of 10 day old melanized, wild type *Aspergillus nidulans* were 18.7% digested by a mixture of glucanase and chitinase while the walls of a non-melanized mutant were 86.1% digested (Kuo & Alexander, 1967b). The pigmented conidia of

Cochliobolus sativus were also resistant to cell-wall lysing enzymes whereas hyaline isolates only survived 2 weeks in soil (Old & Robertson, 1970). Bull (1970) reported that non-melanized walls of *Aspergillus nidulans* were 82% digested by a crude lytic enzyme complex and that the sensitivity of pigmented cell walls to lysis was correlated to their level of melanization. The susceptibility of laboratory prepared walls of *S. rolfsii* to glucanase and chitinase was shown to be dependent upon both melanin content and the physical structure of the walls (Chet & Henis, 1969). Melanized cells of *Phaeococcomyces* sp. were resistant to enzymatic cell wall digestion and did not form spheroplasts whereas non-melanized cells did form spheroplasts (Butler & Lachance, 1987).

One possible mechanism by which sclerotia are protected from degradation in soil may be their resistance to penetration by soil microorganisms (Henis, Adams, Lewis & Papavizas, 1983). Since some biocontrol agents act as parasites by using enzymes to degrade the cell walls of the host, it has been suggested that melanin plays a role in protection of sclerotia. The ability of melanized and non-melanized MS to survive when buried in different soils or soil amended with microbial antagonists has not been extensively investigated. *Talaromyces flavus* has received attention as a potential biocontrol agent for *V. dahliae* (Marois, Fravel & Papavizas, 1984; Fravel, Kim & Papavizas, 1987) and has been demonstrated to reduce Verticillium Wilt of eggplant (Marois, Johnston, Dunn & Papavizas, 1982). The viability of MS of *V. dahliae* was reduced by 32-41% after 2 weeks burial in soil amended with 10^3 ascospores of *T. flavus* per g of soil (Marois, Fravel & Papavizas, 1984). *T. flavus* was shown to produce an enzyme which killed MS of *V. dahliae* (Fravel, Kim & Papavizas, 1987) and which was subsequently identified as glucose oxidase (Kim, Fravel & Papavizas, 1988). *T. flavus* was shown to hyperparasitize *Sclerotinia sclerotiorum* (McLaren, Huang & Rimmer, 1986) and *Rhizoctonia solani* (Boosalis, 1956) by coiling around the host cells and directly penetrating the cell wall. However, *T. flavus* has not been shown to parasitize *V. dahliae* (Kim, Fravel & Papavizas, 1988).

Trichoderma spp. have been shown to act as biocontrol agents against sclerotia of *Sclerotium rolfsii* (Henis, Adams, Lewis & Papavizas, 1983; Elad, Barak & Chet, 1984) and *Sclerotinia sclerotiorum* (Huang, 1980) and hyphae of *Rhizoctonia solani* (Elad, Chet & Katan, 1980). The number of sclerotia was reduced to 42% when sclerotia of *S. sclerotiorum* were buried in soil infested with *T. viridae* (Huang, 1980). Sclerotia of *S. rolfsii* were penetrated by hyphae of *Trichoderma*, which lysed the internal tissue and produced chlamydospores inside the sclerotia but not all of the penetrated sclerotia were completely degraded and some isolates of *Trichoderma* were more adept at br. ching the sclerotia walls than others (Henis, Adams, Lewis & Papavizas, 1983). Disease caused by *R. solani* was significantly decreased and bean yield significantly increased in field studies where naturally infested soils were inoculated with *T. harzianum* (Elad, Barak & Chet, 1984). Hyphae of *R. solani* were penetrated by hyphae of *Trichoderma* which had coiled around them and removal of the coiling hyperparasite revealed a pattern of partial lysis on the host hyphae (Elad, Chet, Boyle & Henis, 1983). It has been suggested that cell-wall degrading enzymes β-(1,3)-glucanase and chitinase are involved in the mycoparasitic activity of *Trichoderma* and that a differential level of activity of these hydrolytic enzymes may be related to the variability in antagonistic activity between different isolates (Elad, Barak & Chet, 1984).

While there is evidence to support the relationship between melanin and survival of fungi in soil (Lockwood, 1960; Hurst & Wagner, 1969; Old & Wong, 1976; Old & Robertson, 1970; Huang, 1983), there is no direct evidence to support this relationship in *V. dahliae*. There is only circumstantial evidence implicating melanin in the survival of *V. dahliae* because it is the melanized MS which are responsible for long-term survival and overwintering, not the hyaline mycelia and conidia (Schreiber & Green, 1962, McKeen & Thorpe, 1981). The objective of the work presented in this chapter was to investigate the role of melanin in the survival of MS of *V. dahliae* through the use of an albino mutant or cultures of *V. dahliae* in which the formation of melanin was inhibited by TCZ. The

survival of melanized and non-melanized MS was compared when the MS were exposed to various abiotic (temperature, UV light and cell-wall degrading enzymes) or biotic (different soil types, organic amendments or antagonistic microorganisms) factors.

4.2 Methods

4.2.1 Culture Methods

The medium used to produce the MS was MCDX. Production of melanin in the MS was blocked by the addition of tricyclazole (TCZ) to the media to give a final concentration of 1 or 10 mg TCZ per L media. A TCZ stock solution of 10 mg TCZ per mL of distilled 95% ethanol was prepared and stored at -25 C. To make medium containing 10 mg TCZ/L, 100 µl of the stock solution was added to 100 mL of medium. To produce 1 mg TCZ/mL medium, 0.1 mL of stock solution was added to 0.9 mL of 95% ethanol and 100 µl was added per 100 mL medium. The medium containing no TCZ (0 mg TCZ/L) was amended with 100 µl of 95% ethanol per 100 mL of medium in order to maintain a uniform concentration of ethanol in each treatment. The cultures were inoculated, incubated in the dark at 24 C for 2 to 4 weeks, and harvested according to the methods outlined in Chapter 2. The albino mutant of *V. dahliae* (Alm-1) used in some experiments was provided by Dr. M. Wheeler (USDA, College Station, Texas) and was grown in the same medium and under the same conditions as described above.

4.2.2 Electron Microscopy

Cultures of *V. dahliae* were grown on CDX agar (Appendix 1) amended with either 0 or 20 mg/L TCZ for 4 weeks at 24 C. Samples of each culture were taken by cutting the agar into 5 mm squares and then fixed with OsO_4. The samples were soaked for 15 mins in a solution of 2% gluteraldehyde, 2% formaldehyde, 0.05M sucrose, and 0.05 M cacodylate buffer (pH 7.2) at room temperature. The fixation was continued for another 15 minutes on ice and then an equal volume of cold 2% OsO_4 in 0.05 M cacodylate buffer (pH 7.2) was added. After 1.5 h, the samples were washed 5 times with cold distilled water and placed in cold 0.5% aqueous uranyl acetate overnight. The samples were then

dehydrated in an ethanol series followed by 100% acetone and embedded in Spurr's low-viscosity resin which was polymerized at 70 C overnight. Thin sections were cut with a diamond knife, collected on uncoated copper grids and stained with 2% aqueous uranyl acetate and lead citrate (Reynolds, 1963). The micrographs were taken with a Jeol JEM-100S transmission electron microscope operated at 60 kV.

4.2.3 Effect of Melanin on MS Growth and Survival

The germination and growth rate of MS receiving either 0, 1 or 10 mg/L TCZ was evaluated. Three size classes (75-106, 53-75 or <53 μm) of MS were tested for each TCZ treatment by transferring 50 MS of each type onto SPT media. The plates were incubated at 24 C and the germination and colony diameter were measured daily with the aid of a dissecting microscope.

The long-term survival of MS receiving either 0, 1 or 10 mg/L TCZ was monitored for 35 weeks by storing 75-106 μm MS in Eppendorf tubes at 24 C and plating 50 MS per treatment on SPT at each sampling time. After 2 weeks incubation at 24 C, the number of colonies produced was recorded.

4.2.4 Storage Temperature

Two different experiments were performed to investigate the effect of temperature on the survival of melanized and non-melanized MS. The MS were produced in MCDX with the addition of either 0, 1 or 10 mg TCZ per mL of media and were grown and harvested as outlined above. In the first experiment, MS of 75-106 μm were placed into small, capped plastic tubes and placed into a sealed container with desiccant in the bottom to control humidity. The containers were stored at 24, 4, or -70 C. One sample of 25 MS was removed from each tube weekly for 10 weeks and transferred onto SPT. The number of MS which had germinated to form a colony was counted and recorded after a minimum of 2 weeks incubation at 24 C. In the second experiment, small sub-samples of MS were mixed with sand and placed into plastic tubes and a set of 48 tubes (16 for each of the 0, 1 or 10 mg/L MS) was placed at either 24, 4, -25 or -70 C. At 1, 2, 3, 4, 6, 8, 10 and 36

weeks, 2 tubes were removed from each temperature and 25 MS from each tube were transferred onto SPT.

4.2.5 Irradiation of MS with UV light

The effect of short wavelength UV light (approximately 254 nm) on the survival of normally melanized MS was compared with that of MS in which the formation of melanin was blocked either by the addition of TCZ or by genetic mutation. The 4 types of MS tested were either produced from our own strain of *V. dahliae* grown with the addition of either 0, 1 or 10 mg TCZ per litre of media or from the albino mutant, Alm-1. All the MS were produced in modified, semisolid CDX and harvested as previously described. The MS tested were 75-106 μm in diameter. A minimum of 25 MS were placed on a single 1.5 mm plastic square with each type of MS on a separate square. The squares were arranged on a tray and placed in a light-proof box, 15 cm away from the UV light source (Camag UV Cabinet II). Irradiance was 140 $\mu W/cm^2$ as measured by a J-225 meter from Ultra-Violet Products, Inc. San Gabriel, CA. Temperature was controlled by placing the tray on a layer of crushed ice. Another set of MS of each type was placed inside small plastic tubes covered with foil to prohibit exposure to UV irradiation but permitting exposure to all other experimental conditions. Two squares of melanized or albino MS were removed after 2, 4, 6, 8, 12, 24, 36 or 48 hours. Two squares of MS grown with 1 or 10 mg/L TCZ were removed after 2, 6, 12, 24, 36 or 48 hours. The MS inside the foil-wrapped tubes were removed after 6, 12, 24, 36 or 48 hours. The MS were immediately transferred to plates of SPT agar, placing 25 MS on each plate. After 4 days of incubation in the dark at 24 C, the diameter of each colony on every plate was measured to the nearest mm and recorded. After a minimum of 2 weeks incubation, the final number of MS which had germinated was counted and recorded.

4.2.6 Exposure of MS to Enzymes

The survival of melanized MS and MS in which the formation of melanin was blocked by TCZ was tested after exposure to a cell-wall degrading enzyme. Cultures were

produced on MCDX media amended with either 0 or 10 mg/L TCZ. After 3 weeks growth, one half of a plate was blended and wet-sieved using a 200 mesh (74 μm) sieve. Once the MS were singular and free of hyphae, they were recovered from the sieve, resuspended in 5 mL DW and 1 mL was pipetted into each of 3 Eppendorf tubes. This was done for both melanized and TCZ-treated MS. The basic protocol used was developed by Dickinson and Isenberg (1982) as a means of producing spheroplasts from fungal cells by digesting their cell walls with Novozyme 234. The MS were pelleted by microcentrifugation for 3 min at 13,000 rpm, the supernatant was discarded and the MS washed twice by resuspending them in 1 mL DW and subsequent centrifugation. A pretreatment solution of 100 mM ethylene diamine tetra acetic acid (EDTA) and 100 mM 2-(N-morpholino)ethanesulphonic acid (MES), was adjusted to pH to 6.0 with KOH. Each tube received 1 mL of a mixture of 6 mL pretreatment solution and dithiothreitol (DTT) was added just before use, to a final concentration of 5 mM. After vortexing to resuspend the MS in the pretreatment mixture, the tubes were incubated at 24 C for 1.5 hours. The tubes were then spun down, the supernatant discarded and the MS washed twice with 0.6 M sorbitol and 20 mM MES (pH 6.0). A treatment solution was prepared (0.6 M KCl, 0.01 M citrate/phosphate, pH 5.85) and DTT was added just before use to a final concentration of 5 mM. Each tube of MS received 1 mL of the treatment solution. The enzyme was prepared by adding 6 mg Novozyme 234 (Novo Nordisk, Denmark) to 200 μl treatment solution and pipetting 100 μl into each of 2 Eppendorf tubes. One tube of enzyme was suspended in boiling water for 30 minutes to denature the enzyme. The control treatment received no enzyme, the 2nd treatment received 50 μl enzyme and the 3rd treatment received 50 μl boiled enzyme. After vortexing well, the tubes of MS were incubated overnight at 30 C. The tubes were then spun down, the supernatant discarded and the MS rinsed in DW. The MS were resuspended and 250 μl was pipetted into a nylon packet containing 2 g acid washed quartz sand. The packets were sealed shut with a bag sealer, rinsed well in tap water, blotted dry and air dried in the laminar flow hood for 30

mins. The MS were then plated individually onto SPT media, with 2 plates of 25 MS each per treatment. The plates were incubated at 24 C and germination was measured daily.

4.2.7 Burial in Various Types of Soil

Five different soils were usedincluding: 1) "Alliston", a sandy loam soil from a potato field 2) "Lundy", a sandy loam soil from a potato field which had been amended yearly with poultry manure 3) "Simcoe", a fox sandy loam 4) "Sand", a coarse sand and 5) "GH Mix", a greenhouse potting mixture consisting of black muck, peatmoss and sand in a 5:3:2 ratio. Soil 1 and 2 were collected from Alliston in Central Ontario and soil 3 from Simcoe in Southwestern Ontario. The soils were stored at 8 C until used. Survival of MS in each soil was evaluated under two different sets of conditions. In both cases, the MS were grown with the addition of either 0, 1 or 10 mg TCZ per L of media and harvested as described above. Nylon mesh packets (2X3 cm) were prepared and filled with a small amount of acid-washed quartz sand and some MS (75-106 μm in size) from one of the TCZ-amended cultures. The packets were heat sealed shut. In one experiment, a 1200 g batch of each soil was divided between 4 plastic pots (55X80X80 mm) for a total of 20 pots. Two packets of each type of MS were buried 2.5 cm deep in the soil. The pots were placed in a walk-in growth room with 24 C day and 20 C night temperatures and 15 hours per day of fluorescent lighting. The pots were watered thoroughly with tap water every few days so that the packets were infiltrated with soil water. After 3 weeks, one packet of each type of MS was recovered and twenty-five MS from each packet were transferred onto SPT agar. After 17 weeks, the second set of packets were recovered and plated. In some of the soils, the packets were infiltrated by soil particles and organic material. Since such particles made identification of the MS difficult and could carry fungal comtaminants, it was determined whether rinsing the packets free of such debris altered the outcome of the viability determinations. After transferring 25 MS from each packet to SPT plates, the remaining sand/MS mixtures from each soil treatment were pooled into one sample and sealed inside new mesh packets. The new packets were placed in a beaker with a

cheesecloth cover and rinsed under running tap water for 3 h. After blotting and air-drying overnight, 50 MS from each packet were transferred onto SPT.

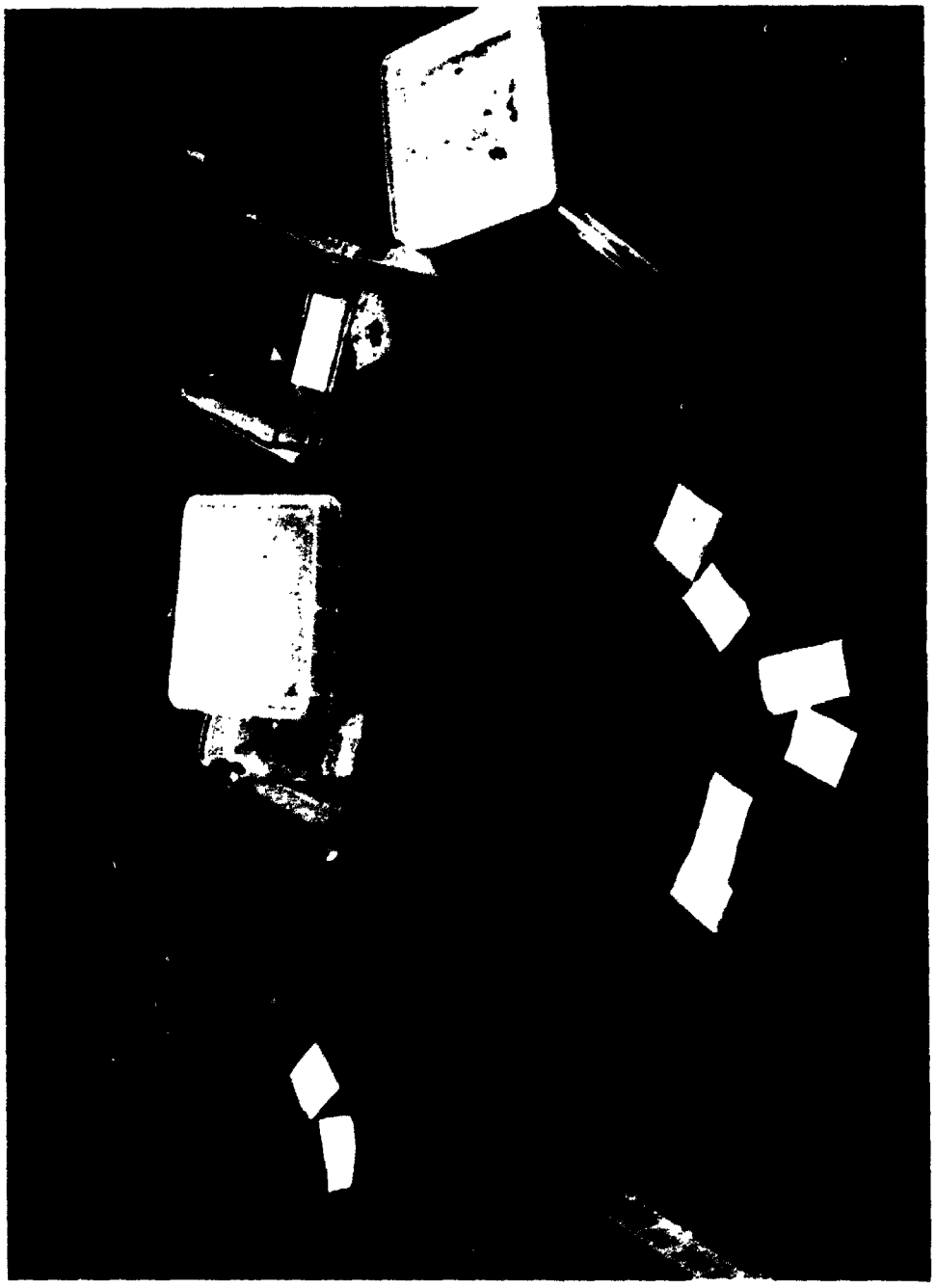
In a second experiment, an 800 g batch of each soil was distributed into four Magenta™ boxes (95X65 mm rigid plastic boxes with lids which permitted air circulation) such that each Magenta box received 200 g of one soil type. The water content of each soil was determined by measuring the weight of 5, 10 g samples of soil before and after oven-drying at 70 C for 12 hours. The moisture capacity was determined in the same manner except the soil was saturated with water at the beginning of the procedure. Soil moisture was adjusted to achieve 50% of the soil moisture capacity by adding the appropriate amount of sterile distilled water. Two packets of each type of MS were buried 5 cm deep in the soil (see Plate 4.1). The Magentas were incubated at 24 C in darkness. One set of packets was recovered after 4 weeks and the second set after 20 weeks of burial in the soil. The packets were air-dried for an hour after which any soil adhering to the packets was removed by brushing with an artists paintbrush. The packets were opened and 25 MS from each packet were transferred to SPT agar. All the SPT agar plates were incubated in the dark at 24 C and the number of MS which germinated and formed colonies was recorded 3 weeks after plating onto SPT. Many of the colonies formed were contaminated with other fungi. Each colony was therefore categorized as: 1) *V. dahliae*, 2) *V. dahliae* mixed wtih another fungus inhibitory to its growth 3) *V. dahliae* coexisting with another fungus or 4) a fungus other than *V. dahliae*. In addition, the number of MS which failed to produce a colony of any kind was recorded. The number of MS which germinated to produce colonies of pure *V. dahliae* in each experiment was analyzed by a two-way analysis of variance using the SigmaStat statisitical package.

4.2.8 Burial in Soil with Organic Amendments

Each of 20 Magenta jars was filled with 200 g of dry, sandy loam field soil (Alliston soil). One of 4 organic amendments was added to each of 4 Magentas at 1% w/w and mixed in completely with the dry soil. The amendments used were: bloodmeal (BM),

Plate 4.1

The apparatus used for the experiments involving the burial of microsclerotia (MS) of *Verticillium dahliae* in soil. The Magenta boxes (™ Magenta Corp. Chicago, Il.) were filled with 200 g of air-dry soil and the nylon mesh packets filled with acid-washed quartz sand and MS of 75-106 μm size were buried in the soil at a depth of 5 cm. The green plastic mesh nets were used in experiments where it was necessary to suspend the packets of sand/MS mixtures in the headspace above the soil.



fishmeal/bonemeal/kelp (FBK) mixture, chitosan (deacetylated chitin) and a fish emulsion (Muskie). The control received only SDW. The bloodmeal was Granular Bloodmeal 12-0-0 (provided by Canagro Elmira, Ontario N3B 3A2) and the FBK was Green Earth Granular Organic Fish/Bone & Kelp Meal 5-6-1 (by Wilson Laboratories Inc., Dundas, Ontario L9H 3H3). Muskie™ was a 5-1-1 fish emulsion fertilizer (also by Wilson Laboratories Inc.) and was prepared by adding 5 mL of emulsion per litre of SDW. The flakes of chitosan were pulverized in a ball grinder to the consistency of a fine powder to aid in dispersal in the soil. The 4 boxes in the Muskie treatment each received 30 mL of the diluted emulsion. All other boxes received 30 mL SDW to bring the soil up to 50% moisture capacity. Lost moisture was replaced during the experiment by the additon of SDW if required. MS were prepared from cultures amended with either 0, 1 or 10 mg TCZ/mL media and added to sand inside nylon packets as previously described. Three days after the water and amendments were added to the boxes, one set of packets was buried in every Magenta at 5 cm depth. The Magentas were incubated in the dark at 24 C. After 32 days of burial, the packets were recovered and the survival of the MS assayed as described above. The number of MS which germinated to produce colonies of pure *V. dahliae* was analyzed by a two-way analysis of variance using the SigmaStat statisitical package.

In a related experiment, 16 Magenta boxes were prepared by adding 200 g Alliston soil per box. There were 4 replicate boxes per treatment. The treatments were: 1% chitosan, 1% BM or 1% FBK. One set of boxes received no amendment. The amendments were added to the dry soil, mixed thoroughly and then 30 mL of SDW was added to each box to bring the soil moisture level up to 50% of the soil moisture capacity. The boxes were incubated at 24 C. Moisture level was maintained by addition of SDW when necessary. Four months after the amendments were added, one set of packets containing MS which had been treated with either 0, 1 or 10 mg/L TCZ was buried in each box. The packets remained buried for 4 weeks at which time 25 MS from each packet were plated

onto SPT media. The number of MS which germinated to produce colonies of pure *V. dahliae* was analyzed by a two-way analysis of variance using the SigmaStat statisitical package.

4.2.9 Burial in Soil Infested with Antagonists

Each of 12 Magenta jars was filled with 200 g of dry, sandy loam field soil and repeatedly autoclaved until the soil was sterile. The treated soils were inoculated with either *Talaromyces flavus* (provided by Dr. D. Fravel, USDA, Beltsville, USA) or *Trichoderma aureo viride* (provided by Dr. R. Brammal, OMAF, Simcoe, Ontario). The third treatment consisted of soil inoculated with a *Penicillium* sp. which was isolated from MS recovered in the experiments described in Section 4.2.8 above. The *Penicillium* was isolated from MS buried in soil amended with either 1% bloodmeal or fish, bone and kelp meal and can be seen as the pinkish-red pigmented colonies in Plate 4.7. The final treatment was soil inoculated with a bacterium identified as *Bacillus polymixa.* It was recovered from plates of PDA where it was causing noticeable inhibition of the growth of colonies of *V. dahliae*. The Magentas containing the control soils received 30 mL SDW each, to achieve 50% of the soil moisture capacity. There were 4 Magenta jars per treatment. The fungal inocula were prepared by adding half an actively growing agar culture of either fungus to approximately 200 mL of SDW and vigorously shaking. The bacterial inocula was prepared by flooding a plate of the bacteria with SDW and diluting the cell suspension to 200 mL. After adding 30 mL of the appropriate suspension to each Magenta, the boxes were weighed so that any moisture lost during the course of the experiment could be replenished by the addition of SDW. The Magentas were incubated at 24 C in the dark for 10 weeks so that the organisms would colonize the entire soil column. Nylon packets containing acid-washed quartz sand were autoclaved and afterward, MS from cultures amended with either 0, 1 or 10 mg TCZ/mL of media were sealed inside the packets. Two packets of each type of MS were buried in every Magenta. To preserve the sterility of the system, burial and recovery was done in a laminar flow hood and forceps

were flamed during use. The packets were recovered after either 20 or 35 days of burial. The survival of the MS was assessed by transferring 25 MS from each packet onto SPT agar as described above. After incubation at 24 C for more than 3 weeks, the number of colonies produced the MS was recorded. Since many of the colonies were composed of *V. dahliae* mixed with another fungus, each colony was categorized as to the fraction of the colony that *V. dahliae* occupied, namely: 1) *V. dahliae* 2) 3/4 *V. dahliae* 3) 1/2 *V. dahliae* 4) 1/4 *V. dahliae* or 5) a fungus other than *V. dahliae*. In addition, the number of MS which failed to germinate and form a colony were recorded as being dead. The number of MS which germinated to produce colonies of pure *V. dahliae* was analyzed by a two-way analysis of variance using the SigmaStat statisitical package.

4.3 Results

4.3.2 Electron Microscopy

Electron micrographs illustrating the appearance (at 5000X) of MS which were normally melanized is presented in Plate 4.2A. The melanin granules were visible densely packed in between the cells of the MS. In contrast, when MS were treated with 20 mg/L TCZ, no melanin granules were visible (at 4000X) in the intercellular spaces (Plate 4.2B). Some cells within the MS appeared empty, however, many other cells contained large lipid-filled vacuoles. At higher magnification (15000X), the absence of the melanin granules between the cells of MS treated with 20 mg/L TCZ (Plate 4.3B) was conspicuous in comparison to the normally melanized MS in Plate 4.3A).

4.3.3 Effect of Melanin on MS Growth and Survival

The rate of germination of three size classes of MS receiving either 0, 1 or 10 mg/L TCZ is presented in Fig. 4.2. In all cases, the germination rate and the level of germination of the smallest MS (<53 μm) was lower than that of larger MS. The MS >53 μm all reached 100% germination within 3 to 6 days after plating. When the diameter of each colony was measured daily, the growth of the smallest MS (<53 μm) was reduced compared with larger MS (Fig. 4.3 A-C). There was more of a difference in MS

Plate 4.2

A: Electron micrograph (magnification 5000X) showing the appearance of normally melanized cells of a 4 week old microsclerotium of *Verticillium dahliae*. The granules of melanin (marked with arrow) can be seen packed in to the intercellular spaces.

B: Electron micrograph (magnification 4000X) showing the appearance of cells of a 4 week old microsclerotium grown in the presence of 20 mg/L of the melanin inhibiting chemical, tricyclazole. There are no melanin granules visible in the intercellular spaces.

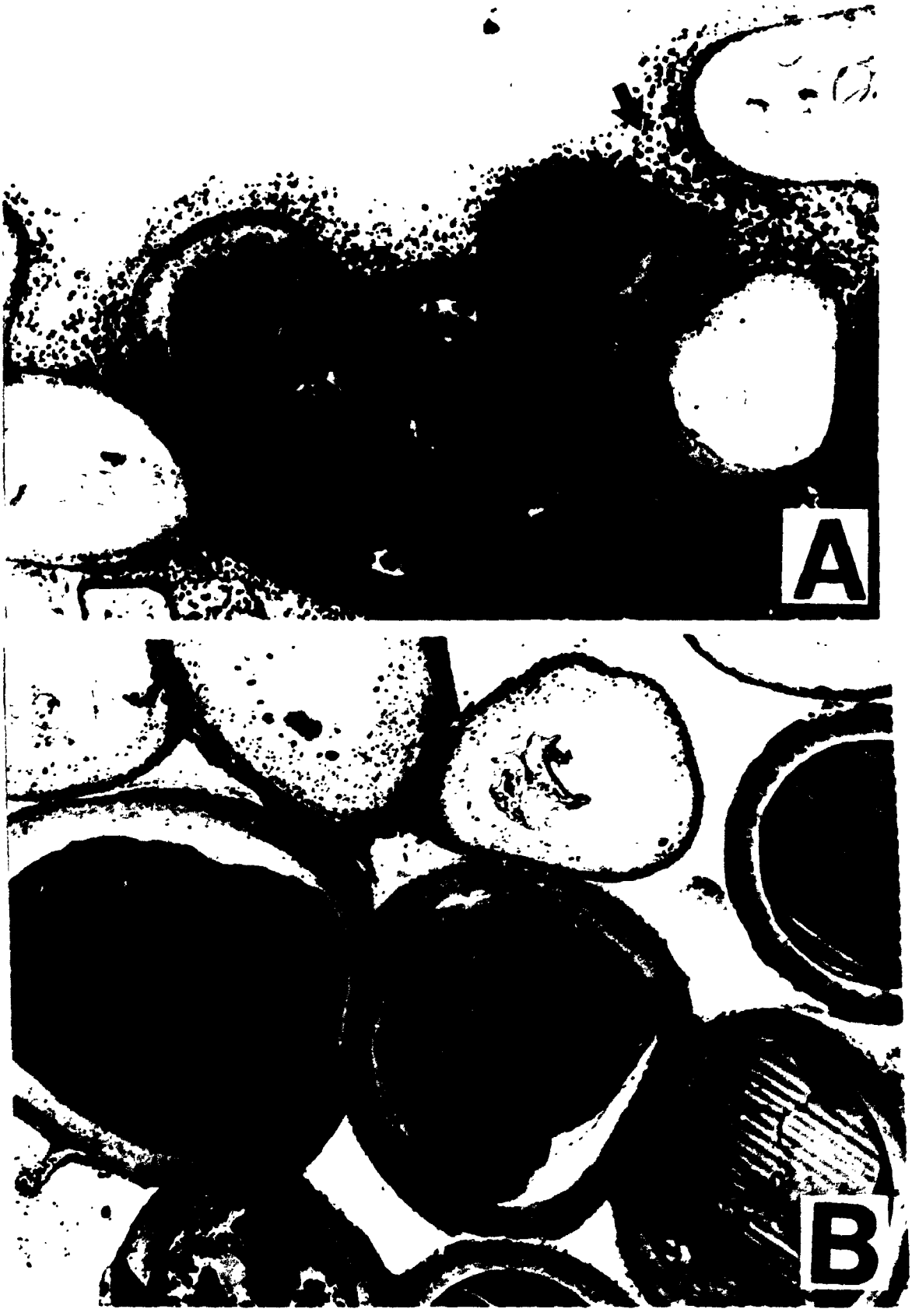
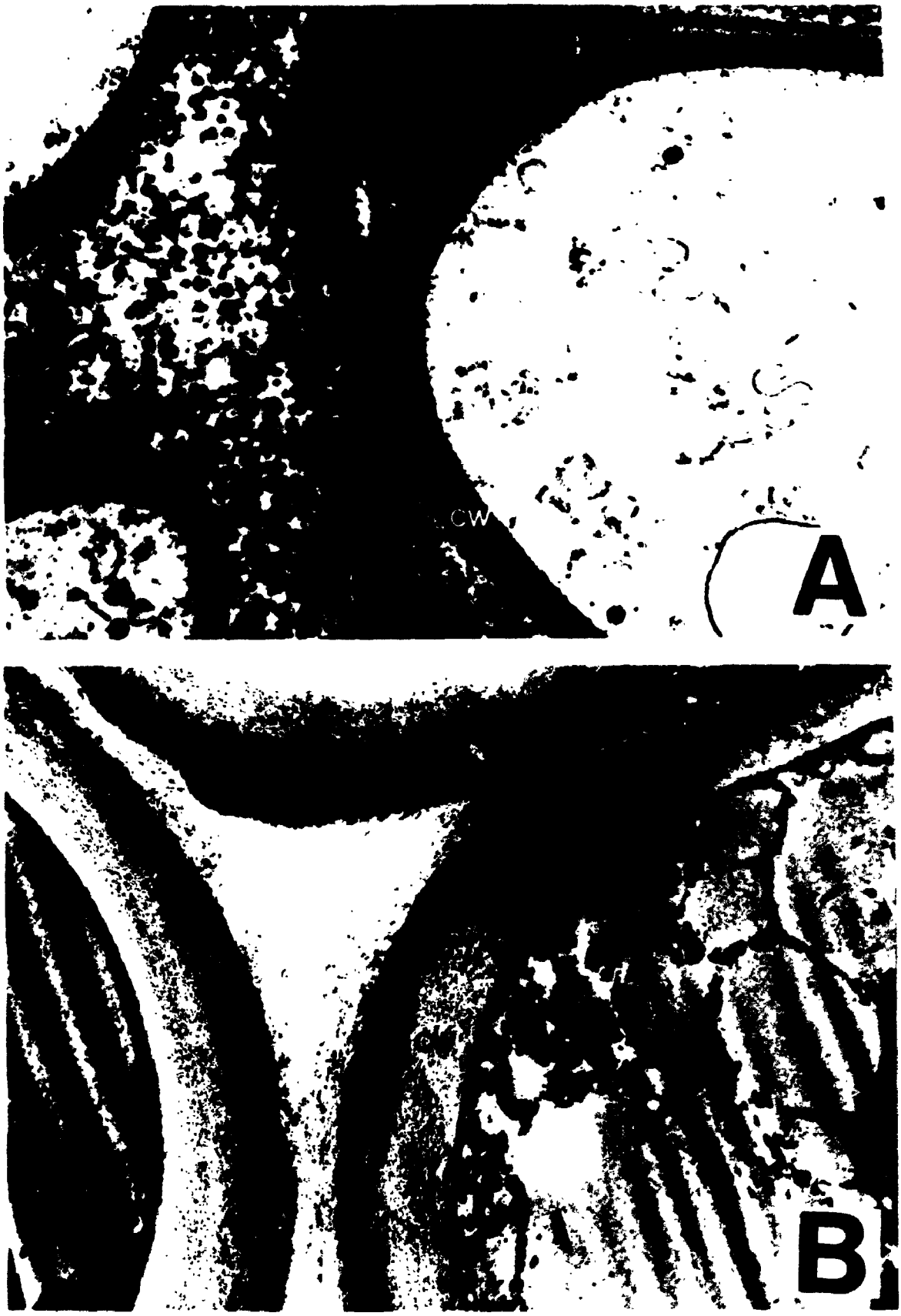

A
B

Plate 4.3

A: Electron micrograph (magnification 15000X) showing the appearance of normally melanized cells of a 4 week old microsclerotium of *Verticillium dahliae*. The granules of melanin (marked with arrow) can be seen packed in to the intercellular spaces. CW=cell wall L=lipid-filled vacuole

B: Electron micrograph (magnification 15000X) showing the appearance of cells of a 4 week old microsclerotium of *Verticillium dahliae* grown with the addition of 20 mg/L of the melanin inhibiting chemical, tricyclazole to the growth medium. There are no melanin granules visible in the intercellular spaces.


CW
A
B

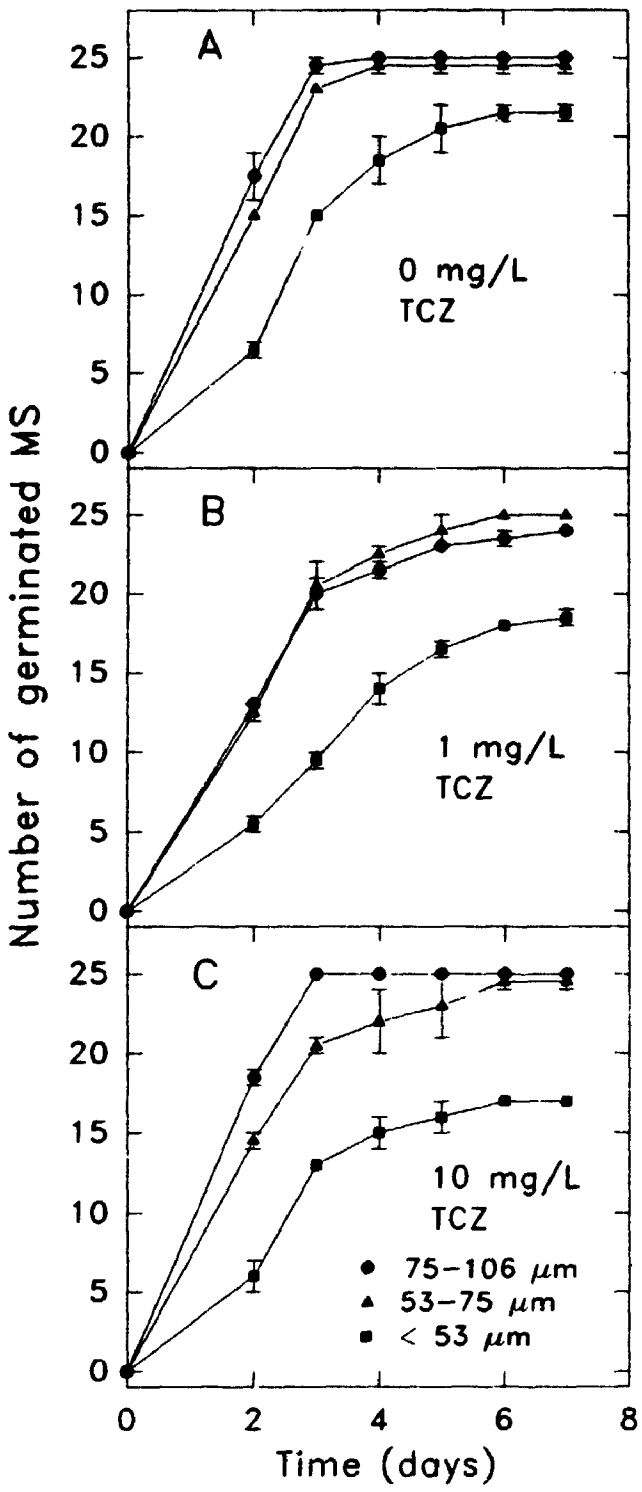


Figure 4.2
Mean number of germinated microsclerotia (MS) which were treated with 0, 1 or 10 mg/L tricyclazole (TCZ), sorted into 3 size classes and plated onto SPT (±1 S.E. N=2).

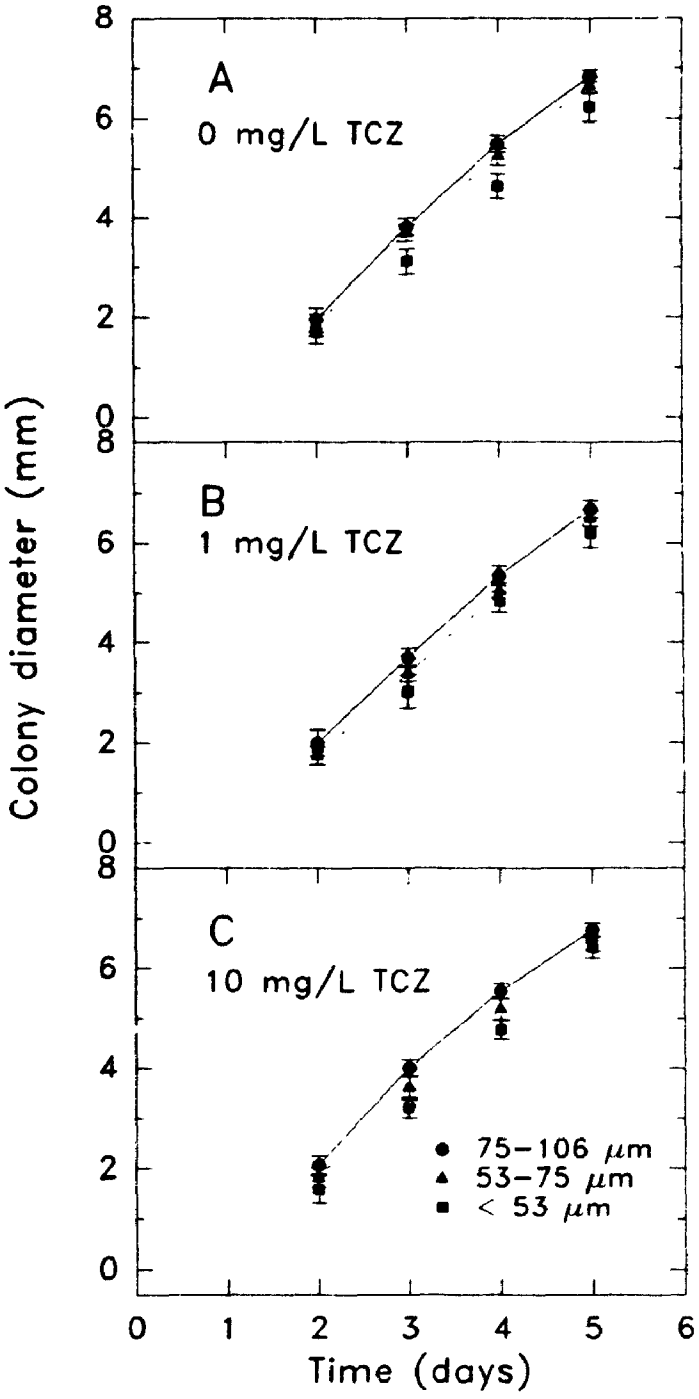


Figure 4.3
Mean diameter of colonies (±95% C.L., N=50) produced by microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) and sorted into 3 size classes.

germination and colony growth between MS of different size classes than there was between melanized MS and those which had been treated with TCZ.

In one experiment on long-term storage at 24 C, the survival of 75-106 μm-sized MS receiving 1 and 10 mg/L TCZ was reduced after 35 weeks by 24% and 58%, respectively, compared with melanized MS which still showed 100% viability (Fig 4.4 A). In two other experiments, there was no difference in the survival of melanized and non-melanized MS after 35 weeks (The data for 1 experiment shown in Fig. 4.4 B and the data for the other experiment not shown). In another experiment, survival after 42 weeks storage at 24 C was 98%, 96% and 90% for 0, 1 or 10 mg/L TCZ treated MS, respectively (Fig. 4.4 C). By 59 weeks, the survival was 94% for melanized MS while MS receiving 1 or 10 mg/L TCZ exhibited 78% or 82% survival, respectively (data not shown in Fig 4.4 C). The experiments were terminated at this stage because all of the MS had been used up in these experiments.

4.3.4 Temperature

There was little difference in the survival of MS receiving either 0, 1 or 10 mg/L TCZ when stored at 24, 4 and -70 C in 2 different experiments. MS from the 0 and 10 mg/L TCZ treatments, when stored at -25 C in a refrigerator freezer, showed <20% survival after 6 weeks (Fig 4.5 A-C). Under the same conditions, MS from the 1 mg/L TCZ treatment were unaffected. In the second experiment, where -25 C was provided by a chest freezer rather than a refrigerator freezer, 100% survival of MS was found at all temperatures after 10 weeks of storage, regardless of TCZ treatment. By 36 weeks, all MS stored at 24, 4 -25 and -70 C exhibited >92% survival (data not shown). The data for the melanized controls in these experiments are the same as that already presented in Chapter Two. As previously mentioned, the variability in the second experiment was greatly reduced by destructively sampling the MS compared with the first experiment where the same tube was repeatedly removed, sampled and returned to the storage site.

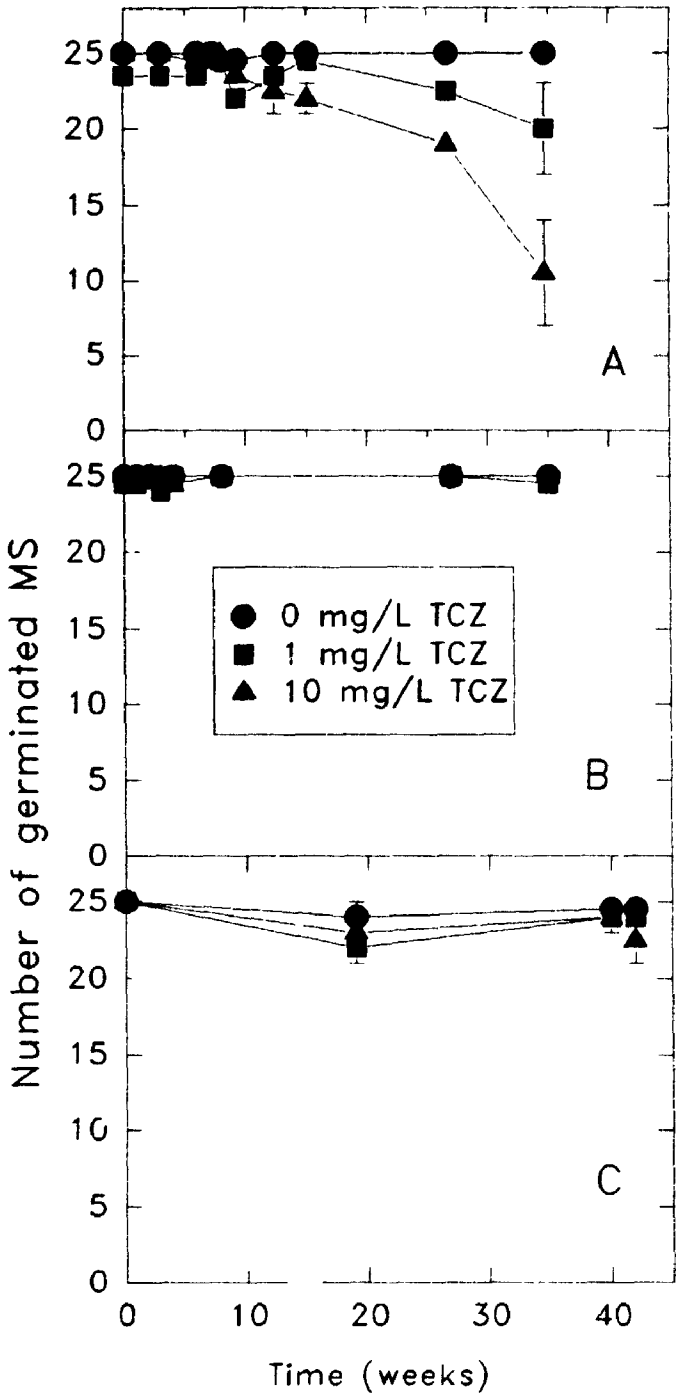


Figure 4.4
Mean number of microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) which germinated after long-term storage at 24 C. A, B and C represent the results of 3 different experiments (±1 S.E. N=2).

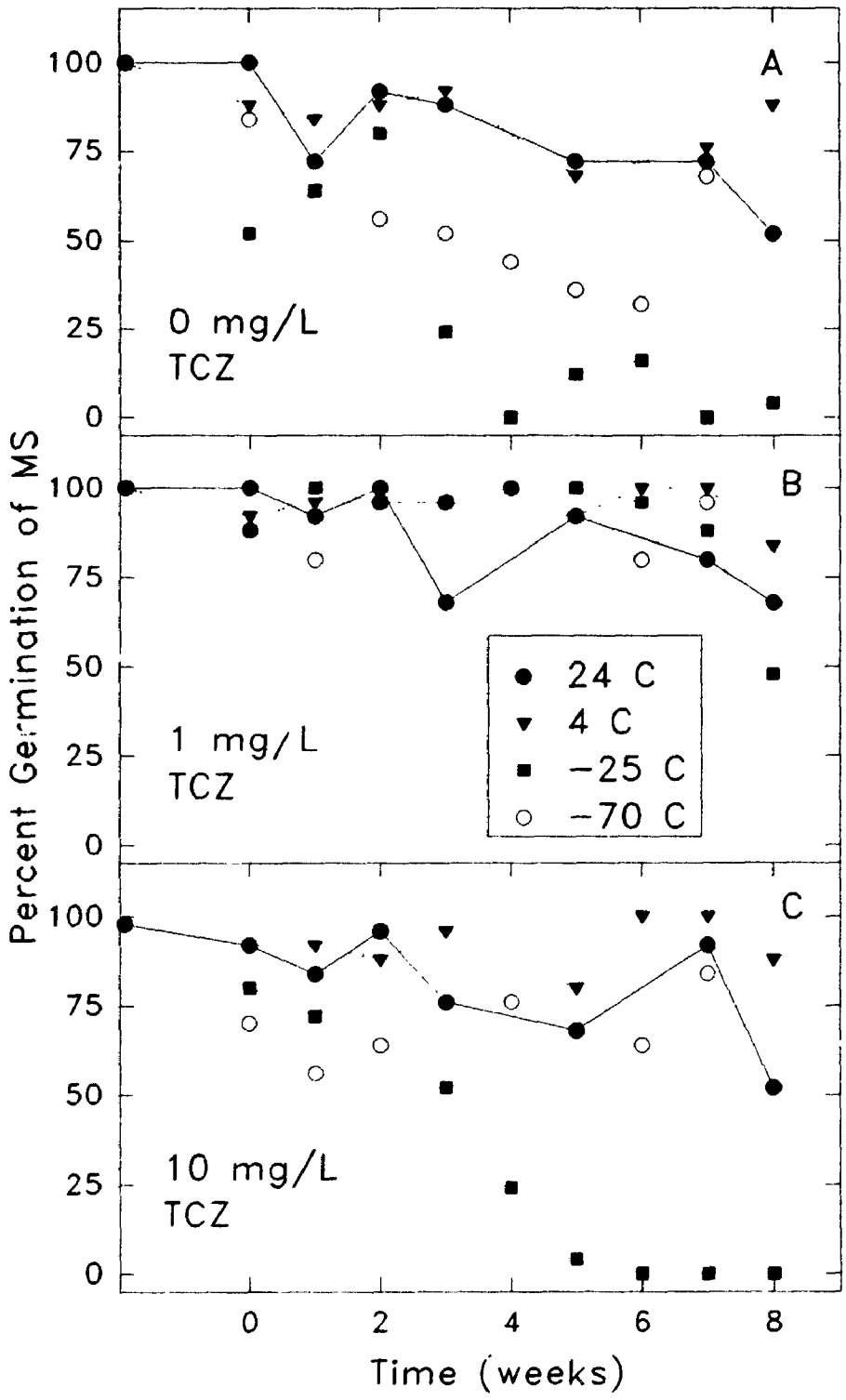


Figure 4.5
The survival of microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) when stored for 8 weeks at 4 different temperatures.

4.3.5 Irradiation of MS with UV light

Wild type melanized MS showed no reductions in viability after 48 h of exposure to UV light (Fig. 4.6A). MS from the 1 mg/L TCZ treatment showed reduced viability only after more than 36 h exposure (Fig. 4.6B). When MS from the 10 mg/L TCZ treatment were exposed to more than 12 hours of UV light, their survival was reduced compared to the control MS (Fig. 4.6C). In contrast, exposure of albino MS to UV irradiation for more than 6 h caused complete kill (Fig. 4.6D and Plate 4.4).

Measurement of colony diameters after 4 days growth revealed that after 48 h of UV exposure, melanized MS produced colonies which were less than half of that produced by non-irradiated controls (Fig. 4.7A). Irradiated MS in the 1 mg/L TCZ treatment also produced smaller colonies than non-irradiated MS and after 48 h of irradiation, colony diameter was less than half that seen in the non-irradiated controls (Fig. 4.7B). There was no difference in the diameter of colonies produced by the irradiated or control MS from the 10 mg/L TCZ treatment (Fig. 4.7C). Exposure of albino MS to UV for more than 2 h also resulted in smaller colonies after 4 days growth (Fig. 4.7D).

4.3.6 Enzymes

The percent germination of MS exposed to the cell wall degrading enzyme Novozyme for 24 hours was different over a number of experiments. The results of 2 typical experiments are shown in Fig. 4.8 A and B. MS which were treated with 10 mg/L TCZ were inhibited more by enzymatic digestion than were melanized MS (Fig. 4.8). In the experiment shown in Fig. 4.8 A, MS plated immediately after harvesting reached 100% germination in the first 24 hours and MS which were incubated with no enzyme reached 100% germination within 48 hours. The level of germination of melanized MS and TCZ-treated MS which were digested with enzyme reached 80% and 36%, respectively. In another experiment (Fig. 4.8 B), MS with melanin production blocked by TCZ achieved less than 30% germination when digested 24 h with Novozyme while MS in all other treatments reached >80% germination When the MS were digested in enzyme which had

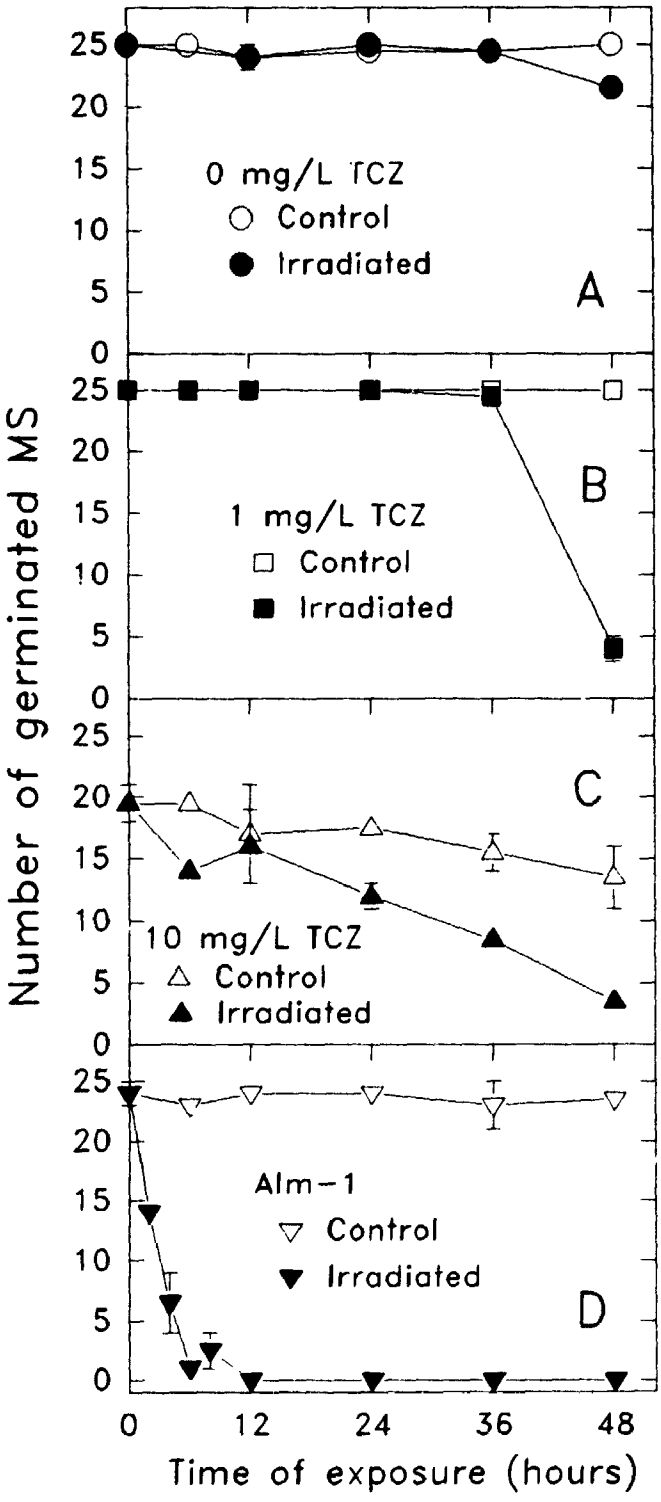
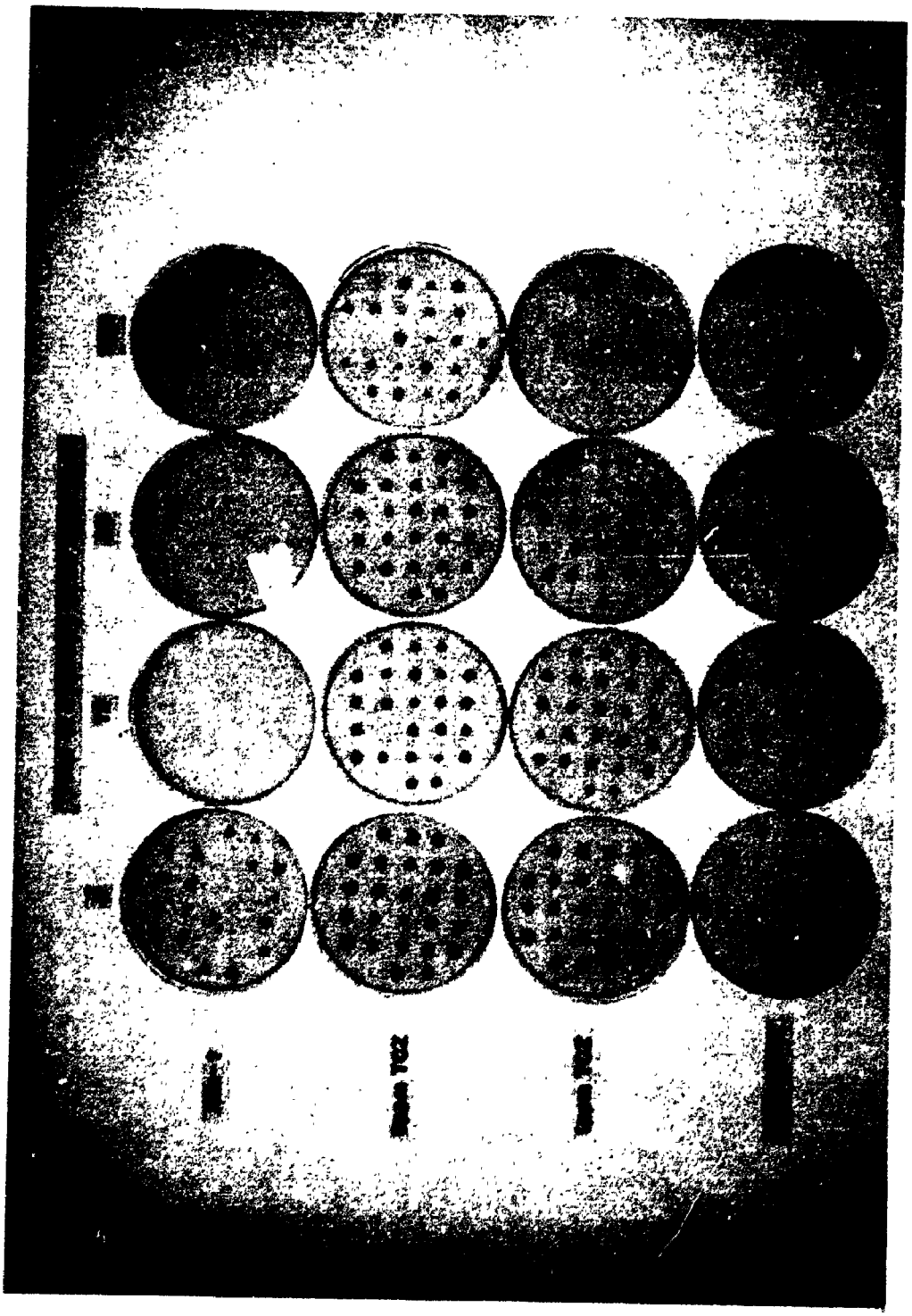


Figure 4.6
Mean number of microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) which germinated after exposure to short wavelength UV light for a maximum of 48 hours (±1 S.E. N=2).

Plate 4.4

A representative sample of plates of soil-pectate-tergitol (SPT) medium illustrating the results of plating microsclerotia (MS) of *Verticillium dahliae* which had been irradiated with short wavelength (254 nm) ultraviolet light for 0, 12, 24 or 48 hours. The MS tested were from an albino mutant culture of *V. dahliae* (Alm-1) or from cultures of *V. dahliae* which received either 0, 1 or 10 mg/L of the melanin inhibiting chemical tricyclazole (TCZ) in the growth medium. No MS from the albino culture survived 12 hours or more of exposure to UV, in contrast to normally melanized MS in the 0 mg/L TCZ treatment. MS treated with 1 mg/L TCZ showed reductions in survival after 48 h of exposure to UV light. MS treated with 10 mg/L showed reductions in survival after 12 h of UV irradiation. The photo was taken after the plates had incubated at 24 C for 3 weeks.



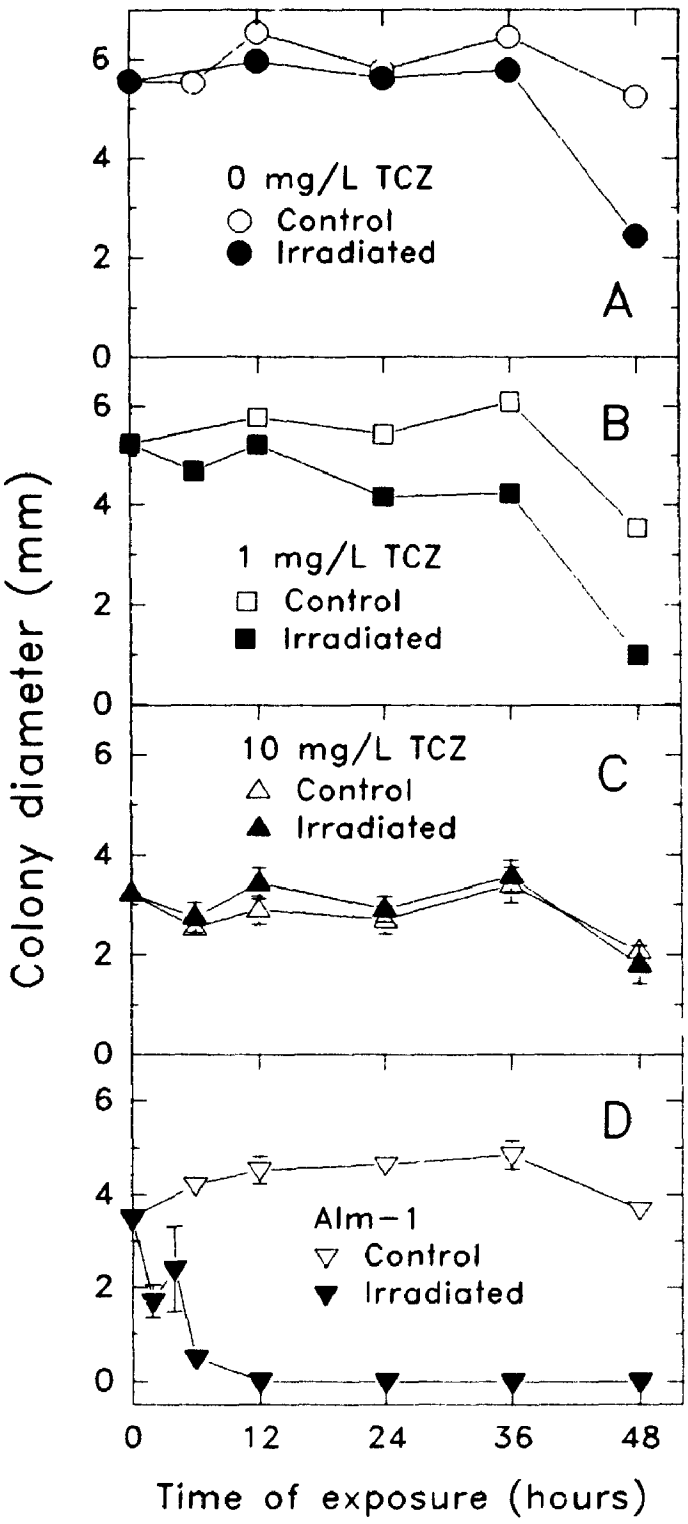


Figure 4.7
Mean diameter of colonies (±1 S.E., N=50) after 4 days growth on SPT agar which were produced by microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) and irradiated for various lengths of time with UV light.

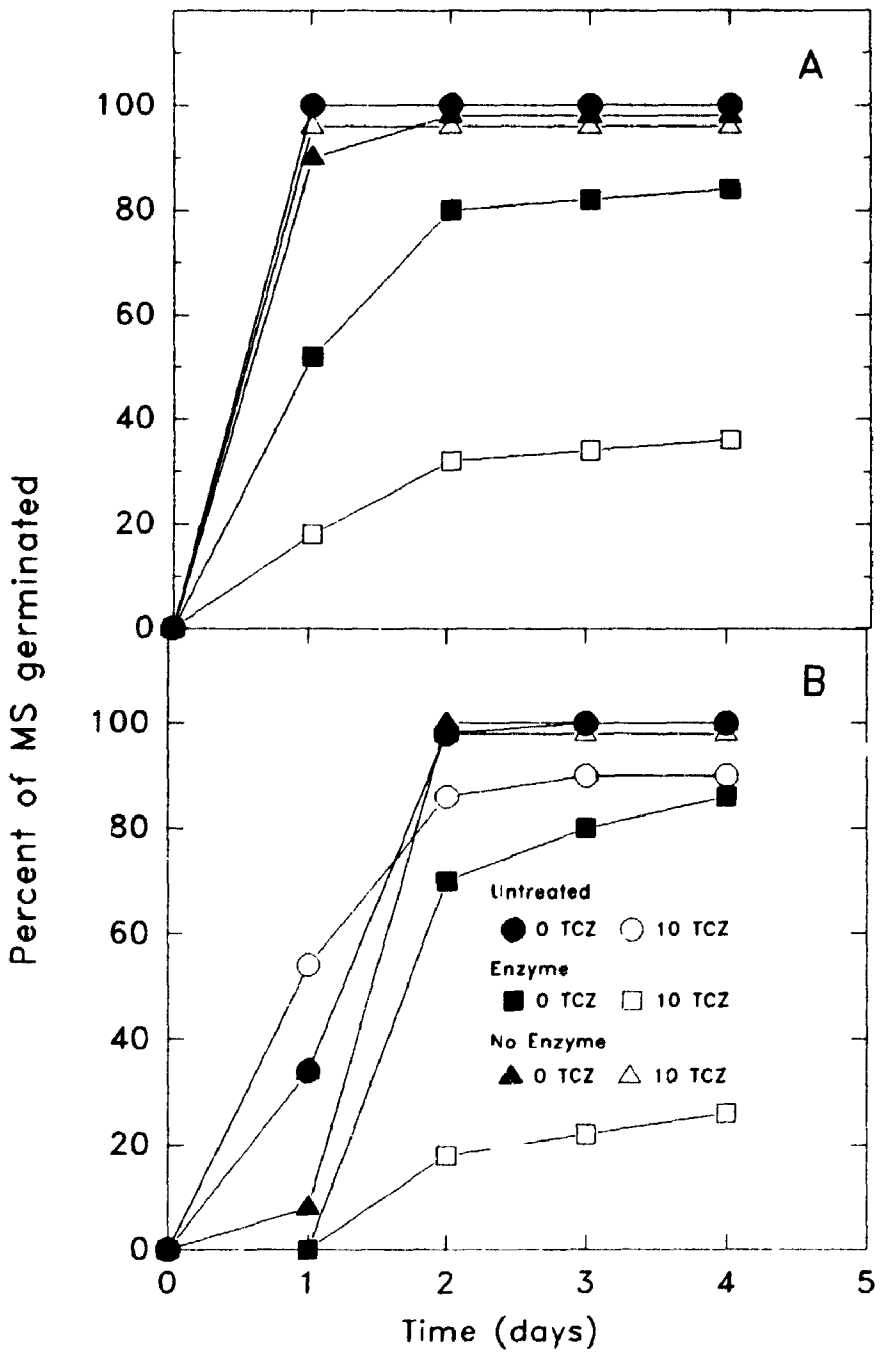


Figure 4.8
The percentage of microsclerotia which germinated after harvesting (circles), after 24 h digestion with Novozyme (squares) or after 24 h digestion without Novozyme (triangles). Fig. A and B represent the results of 2 different experiments.

been denatured by boiling for 30 mins, germination of both melanized and non-melanized MS was 94 and 92%, respectively. In one experiment, the digestion of melanized or TCZ treated MS in Novozyme for 24 h was found to have no effect on the survival of either type of MS. However, the rate of germination of the 10 mg/L TCZ treated MS was reduced compared and only 4% of the MS had germinated in the first 24 h after plating onto SPT, whereas 50% of the melanized MS had germinated by that time. By three days after plating, however, both TCZ treated and melanized MS reached 98% germination (data not shown).

4.3.7 Burial in Various Soils

The number of colonies produced by MS recovered from soil in Magenta boxes after 4 weeks of burial was determined after 5 days and results are shown in Table 4.1. The colony diameters were measured at this time but as will become obvious, this did not yield the information desired and the data are not shown. The number of colonies produced by MS from TCZ treated cultures was only slightly reduced following burial in several of the soil types in comparison to melanized MS (Table 4.1). Evaluation of these plates at 14 days revealed that not all the colonies counted at 5 days were composed of *V. dahliae*. The pattern of colony development from each MS could be readily categorized as: a) colony composed of *V. dahliae* only b) colony composed of *V. dahliae* mixed with an inhibitory fungus, c) colony composed of *V. dahliae* coexisting with another fungus, d) colony composed of a fungus other than *V. dahliae* or e) no colony was formed. When analyzed in this manner, considerably more information on the behaviour of the MS during burial was gained (Table 4.2). After 4 weeks of burial, MS recovered from the GH mix were found to produce an unusually low number of colonies composed of *V. dahliae* alone and an unexpectedly high number of colonies of *V. dahliae* associated with other fungi (Table 4.2). In every soil type except for Sand, the melanin deficient MS were more frequently associated with other fungi than were the melanized MS. MS buried in the GH mix and Lundy soil were less likely to produce pure colonies of *V. dahliae* than MS buried

Table 4.1 The percentage of colonies which arose from MS treated with either 0, 1 or 10 mg/L tricyclazole (TCZ) and buried for 4 weeks in Magenta boxes containing either sand, greenhouse potting mix (GH Mix), Simcoe soil, Alliston soil or Lundy soil. The colonies were counted 5 days after plating onto SPT media.

Soil Type	[TCZ] mg/L	Percent of MS which Produced a colony
Sand	0	100
	1	95
	10	99
GH Mix	0	100
	1	91
	10	98
Simcoe	0	93
	1	92
	10	95
Alliston	0	100
	1	94
	10	98
Lundy	0	98
	1	96
	10	93

Table 4.2 The percentage of colonies of different types produced by MS treated with either 0, 1 or 10 mg/L tricyclazole (TCZ) and buried for 4 weeks in Magenta boxes containing either sand, greenhouse potting mix (GH Mix), Simcoe soil, Alliston soil or Lundy soil. The colonies were measured after more than 14 days after plating onto SPT.

		Percent of MS giving rise to colonies composed of:				
Soil Type	**[TCZ]**	*V. dahliae* [a]	Mixed [b]	Coexist [c]	Other [d]	Dead [e]
Sand	0 mg/L	100	0	0	0	0
	1	95	0	0	0	5
	10	99	0	0	0	1
GH Mix	0	56	0	44	0	0
	1	36	0	53	2	9
	10	14	2	70	12	2
Simcoe	0	87	1	0	5	7
	1	88	3	0	1	8
	10	60	16	0	19	5
Alliston	0	100	0	0	0	0
	1	87	4	1	2	6
	10	72	10	3	13	2
Lundy	0	67	7	0	24	2
	1	39	11	22	24	4
	10	24	21	6	42	7

[a] colonies were composed of *V. dahliae* only
[b] colonies were composed of *V. dahliae* mixed with an inhibitory fungus
[c] colonies were composed of *V. dahliae* coexisting with another fungus
[d] colonies were composed of a fungus other than *V. dahliae*
[e] no colony was formed

in the other soils. MS buried in the Lundy soil produced a larger proportion of colonies which contained no *V. dahliae* at all.

The measure of survival which was used in this and all subsequent experiments was the number of MS which successfully germinated to form colonies which were entirely composed of *V. dahliae*. Several lines of evidence led to the conclusion that colonies which were mixtures of *V. dahliae* and other fungi were produced by MS that had been weakened or damaged by sub-lethal treatment effects. First, extensive washing of the MS after recovery from the soil did not reduce the number of mixed colonies obtained. In addition, the number of mixed colonies increased with increasing duration of burial, suggesting that soil microbes were intimately associated with the MS and may have been colonizing those cells which were damaged. Also, the same few fungal species were repeatedly isolated from buried MS, indicating that their association with the MS was not random. Several of these isolates have been placed in our culture collection and one was shown to reduce the survival of MS buried in soil to which it had been added, indicating that MS which give rise to mixed colonies have likely experienced sublethal damage.

When the number of colonies of *V. dahliae* formed from MS buried for 4 weeks in Magenta boxes (Table 4.2) was analyzed by a two-way ANOVA, the soil type and TCZ treatment had a significant ($p<0.001$) effect on MS survival, but there was no significant interaction ($p=0.53$). The data for the percentage of MS which produced pure colonies of *V. dahliae* was summarized graphically according to the method recommended by Hassard (1991) for visualizing interactions in a 2 way analysis of variance. The graph presents the experimental results on the vertical axis (percent survival in this case), the levels of one of the factors (TCZ treatment) on the horizontal axis and the second factor (soil type) is plotted on the graph. In general, when the curves are parallel, no interaction between the treatments in the second factor (soil type) is indicated. When the curves are not parallel, then the differences between treatments in the second factor (soil type) are dependant

upon the levels of the first factor (TCZ treatment) and an interaction exists. The data from Table 4.2 is presented in Fig. 4.9.

After 20 weeks of burial in Magenta boxes containing GH mix or Lundy soil, many MS (particularly from the 10 mg/L TCZ treatment) were found to give rise to colonies which were obviously not *V. dahliae* (Table 4.3 and Plate 4.5). None of the MS buried in sand, however, formed colonies which became associated with other fungi. All the soils (except Alliston soil) exhibited some capacity to reduce the survival of non-melanized MS especially those MS receiving the higher concentration of TCZ (Table 4.3). At 20 weeks, the greatest reduction in MS survival occurred in the Lundy and GH mix soils, with Simcoe soil being the next most active soil type. Figure 4.10 illustrates the results of a 2-way ANOVA which showed that soil type and TCZ treatment were significant ($p<0.001$ and $p=0.002$, respectively) in reducing MS survival but there was no significant interaction ($p=0.492$).

Burial of MS for 3 weeks in the growth room, in pots containing the various soils, resulted in minor reductions in survival of non-melanized MS in all soils (Table 4.4). The only obvious reduction in survival occurred in the 10 mg/L TCZ treated MS buried in Lundy soil. The soil type and TCZ treatment significantly reduced MS survival ($p=0.003$ and $p=0.015$, respectively) but there was not a significant interaction ($p=0.264$) according to the results of a 2-way ANOVA (Fig. 4.11). After burial in the Simcoe, Alliston and Lundy soils for 17 weeks, very dramatic reductions were obtained in the survival of MS from the 10 mg/L TCZ treatment, such that only 54, 73 and 48%, respectively produced pure colonies of *V. dahliae* (Table 4.5). The survival of MS which were treated with 1 mg/L TCZ was also significantly reduced compared to melanized MS when buried in Simcoe, Alliston or Lundy soils for 17 weeks. In the Simcoe soil, MS treated with 10 mg/L TCZ produced no colonies at all more frequently than they produced colonies of another fungus (Table 4.5) In contrast, the 10 mg/L TCZ treated MS buried in the Lundy soil were frequently associated with other fungi (Plate 4.6). Burial in sand, once again, did

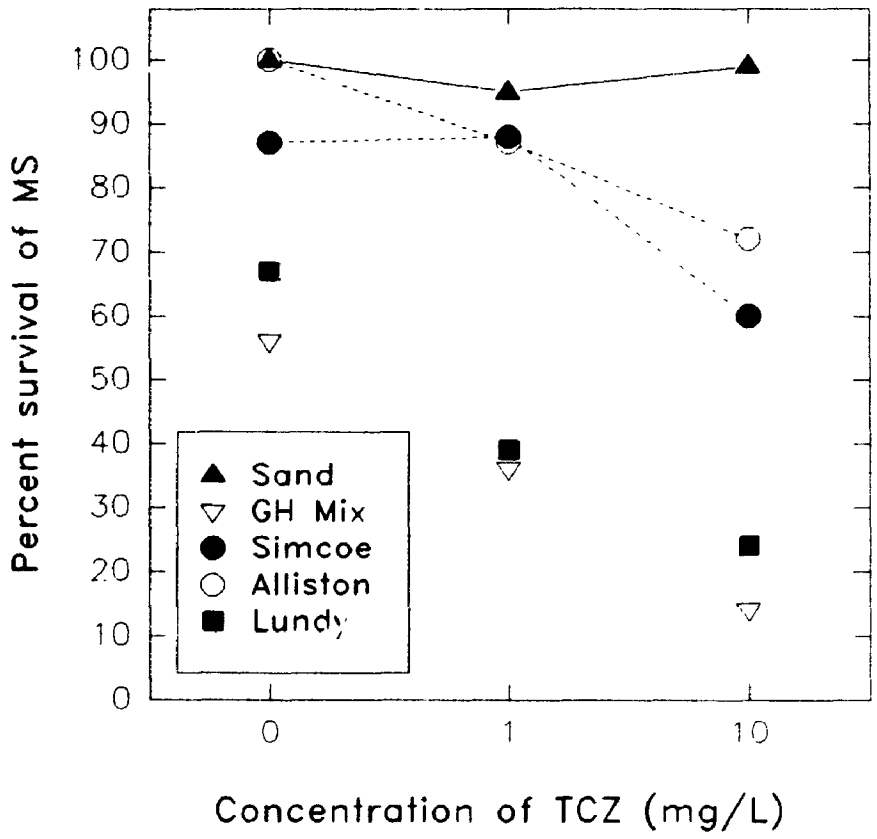


Figure 4.9
The percent survival of microsclerotia (MS) treated with 0, 1 or 10mg/L tricyclazole (TCZ) when buried for 4 weeks in Magenta boxes filled with various soil types.

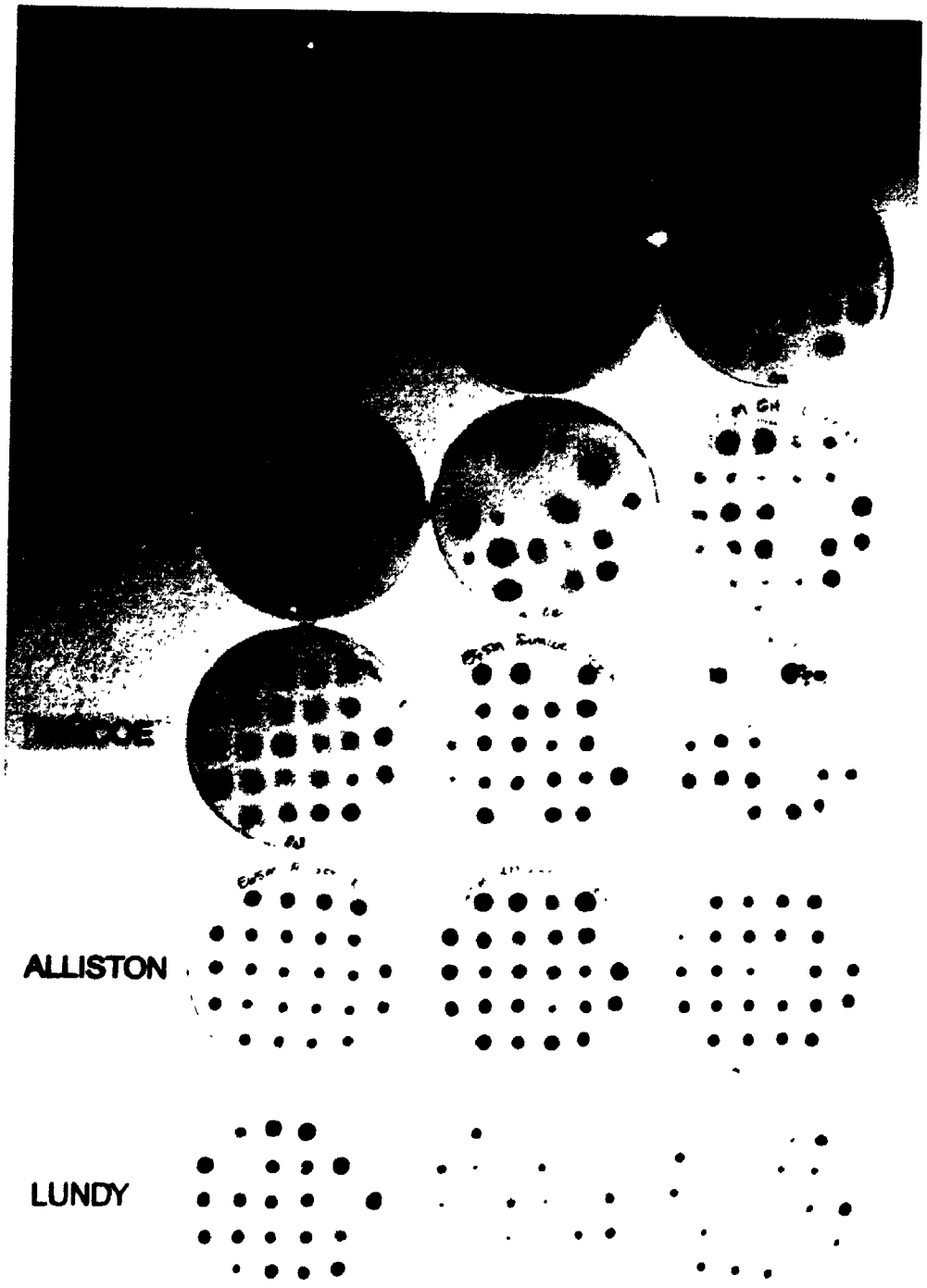
Table 4.3 The percentage of colonies of different types produced by MS treated with either 0, 1 or 10 mg/L tricyclazole (TCZ) and buried for 20 weeks in Magenta boxes containing either sand, greenhouse potting mix (GH Mix), Simcoe soil, Alliston soil, or Lundy soil.

		Percent of MS giving rise to colonies composed of:				
Soil Type	**[TCZ]**	*V. dahliae* [a]	Mixed [b]	Coexist [c]	Other [d]	Dead [e]
Sand	0 mg/L	100	0	0	0	0
	1	100	0	0	0	0
	10	84	0	0	0	16
GH Mix	0	65	7	11	6	11
	1	34	14	8	21	23
	10	18	15	12	46	9
Simcoe	0	92	2	3	1	3
	1	72	4	0	2	22
	10	63	2	1	12	22
Alliston	0	99	0	0	0	1
	1	98	1	0	0	1
	10	96	2	0	0	2
Lundy	0	66	3	0	28	3
	1	59	6	1	28	6
	10	50	17	1	28	4

[a] colonies were composed of *V. dahliae* only
[b] colonies were composed of *V. dahliae* mixed with an inhibitory fungus
[c] colonies were composed of *V. dahliae* coexisting with another fungus
[d] colonies were composed of a fungus other than *V. dahliae*
[e] no colony was formed

Plate 4.5

A representative sample of plates of soil-pectate-tergitol (SPT) medium illustrating the results of plating microsclerotia (MS) of *Verticillium dahliae* which had been buried for 20 weeks in Magenta boxes filled with 200 g of either sand, greenhouse potting mix (GH mix) or sandy loam soil collected from fields in Simcoe (Simcoe) or Alliston, Ontario (Alliston and Lundy). The Lundy soil was collected from a potato field which had been amended yearly with poultry manure and had a history of being free of *Verticillium* disease. The MS tested were from cultures of *V. dahliae* which received either 0, 1 or 10 mg/L of the melanin inhibiting chemical tricyclazole (TCZ) in the growth medium. Colonies which were not *V. dahliae* could be detected on this medium either by the production of distinctive aerial structures or by the production of pigments, as shown in the case of the red colonies on the plates with MS treated with 0 and 1 mg/L TCZ. The MS treated with 1 or 10 mg/L TCZ which were buried in the GH mix, Simcoe or Lundy soils formed a larger number of colonies which were not *V. dahliae* than did melanized MS or MS buried in other soils.


ALLISTON
LUNDY

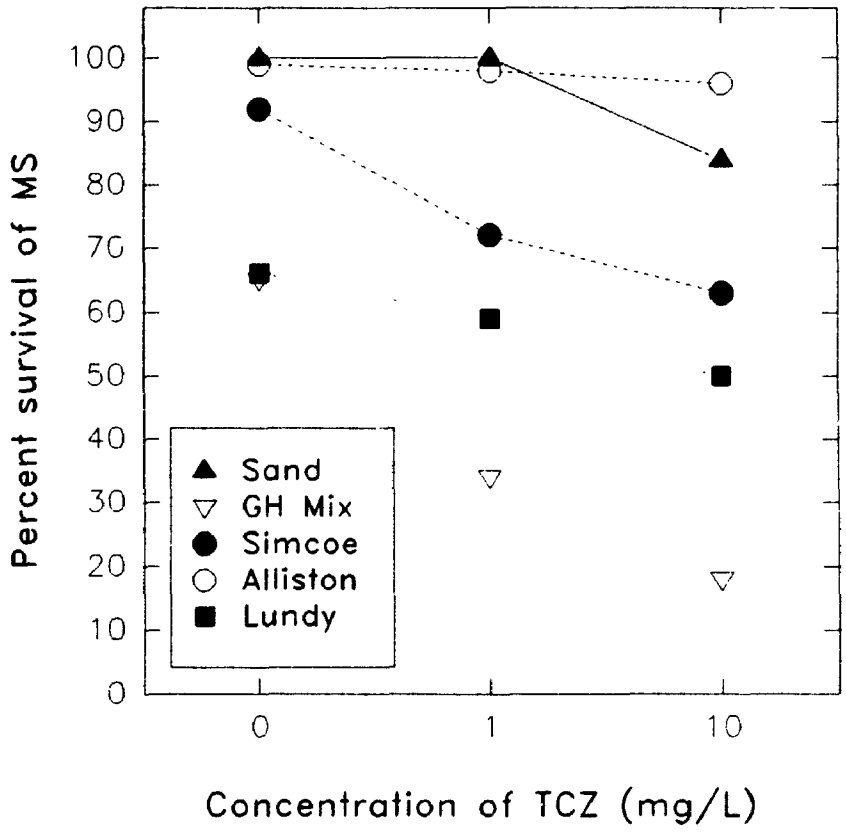


Figure 4.10
The percent survival of microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) when buried for 20 weeks in Magenta boxes filled with various soil types.

Table 4.4 The percentage of colonies of different types produced by MS treated with either 0, 1 or 10 mg/L tricyclazole (TCZ) and buried for 3 weeks in the growth room in pots containing either sand, greenhouse potting mix (GH Mix), Simcoe soil, Alliston soil, or Lundy soil.

		Percent of MS giving rise to colonies composed of:			
Soil Type	**[TCZ]**	*V. dahliae* [a]	Mixed [b]	Other [c]	Dead [d]
Sand	0 mg/L	98	1	0	1
	1	99	0	1	0
	10	94	6	0	0
GH Mix	0	97	1	1	1
	1	87	8	5	0
	10	89	2	8	3
Simcoe	0	99	0	0	1
	1	84	2	9	5
	10	94	3	1	2
Alliston	0	100	0	0	0
	1	99	1	0	0
	10	95	3	2	0
Lundy	0	96	4	0	0
	1	79	8	10	3
	10	68	18	11	3

[a] colonies were composed of *V. dahliae* only
[b] colonies were composed of *V. dahliae* mixed with another fungus
[c] colonies were composed of a fungus other than *V. dahliae*
[d] no colony was formed

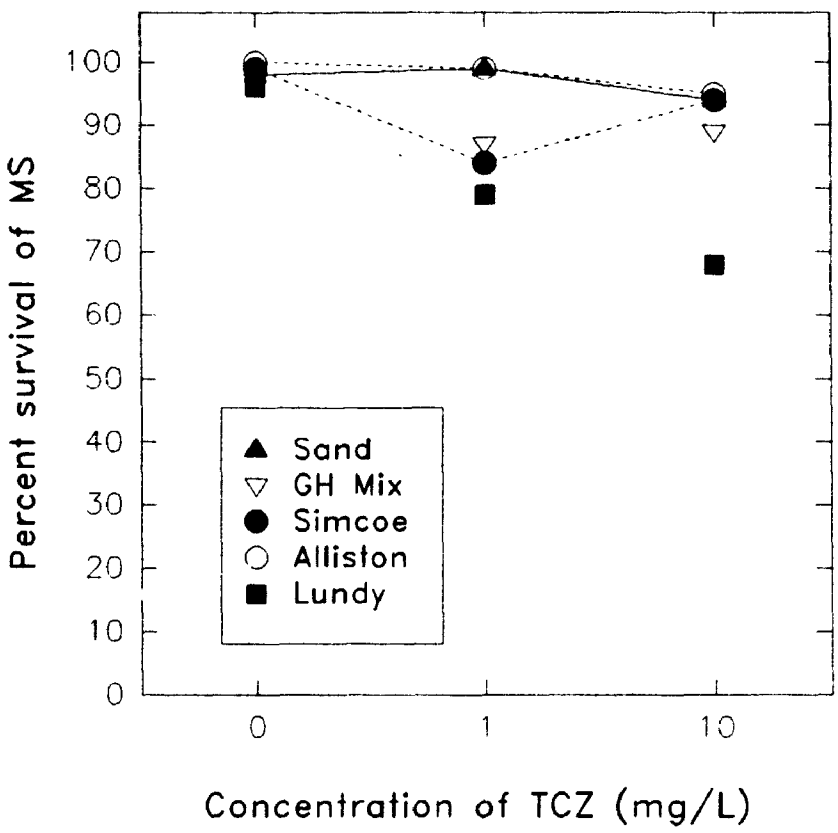


Figure 4.11
The percent survival of microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) when buried for 3 weeks in pots filled with various soil types in the growth room.

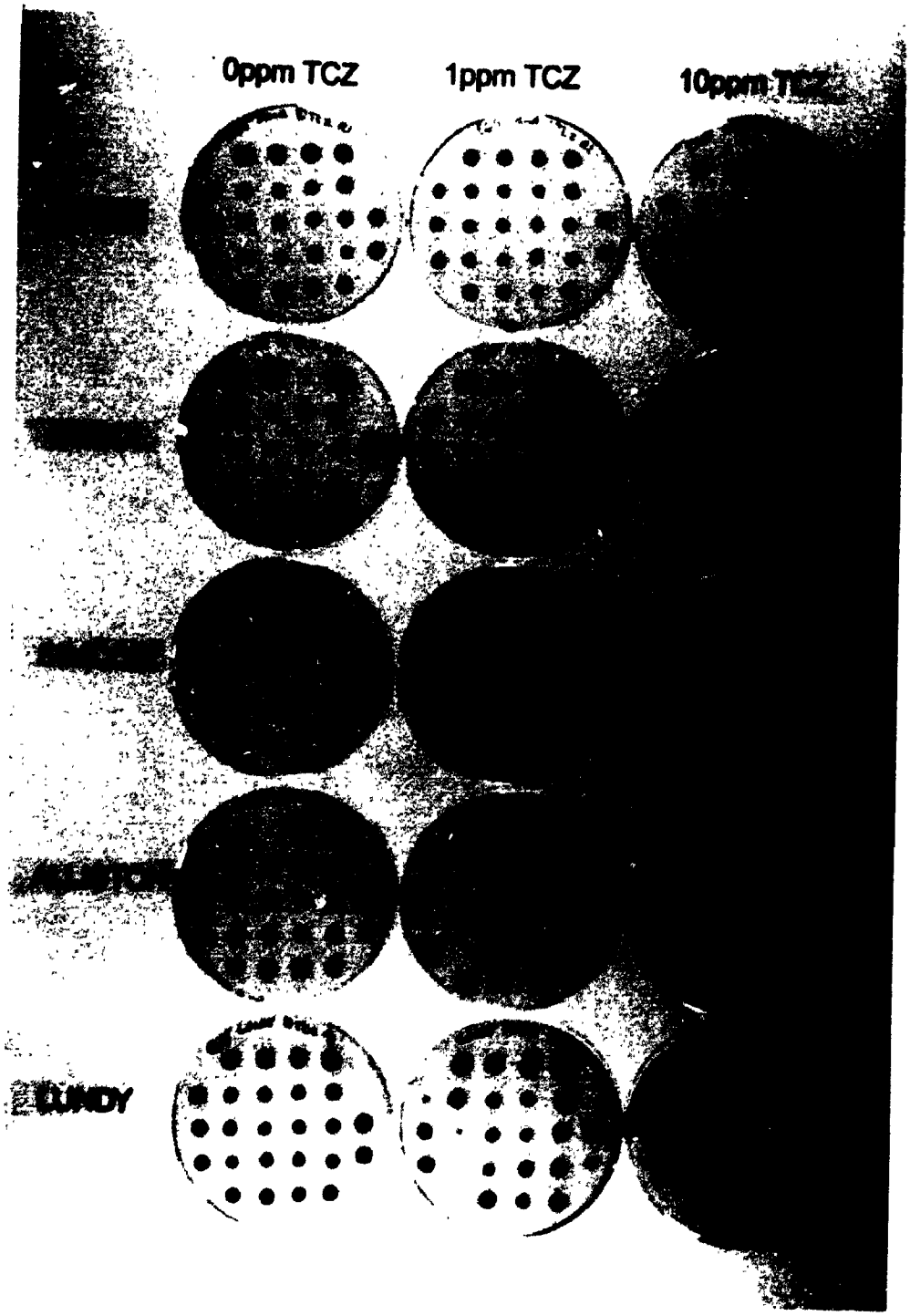
Table 4.5 The percentage of colonies of different types produced by MS treated with either 0, 1 or 10 mg/L tricyclazole (TCZ) and buried for 17 weeks in the growth room in pots containing either sand, greenhouse potting mix (GH Mix), Simcoe soil, Alliston soil, or Lundy soil.

		Percent of MS giving rise to colonies composed of:			
Soil Type	**[TCZ]**	*V. dahliae* [a]	Mixed [b]	Other [c]	Dead [d]
Sand	0 mg/L	98	1	0	1
	1	100	0	0	0
	10	100	0	0	0
GH Mix	0	86	1	9	4
	1	98	1	0	1
	10	91	1	3	5
Simcoe	0	100	0	0	0
	1	53	0	5	42
	10	54	0	16	30
Alliston	0	90	0	1	9
	1	69	0	2	29
	10	73	2	4	21
Lundy	0	100	0	0	0
	1	63	5	28	4
	10	48	17	31	4

[a] colonies were composed of *V. dahliae* only
[b] colonies were composed of *V. dahliae* mixed with another fungus
[c] colonies were composed of a fungus other than *V. dahliae*
[d] no colony was formed

Plate 4.6

A representative sample of plates of soil-pectate-tergitol (SPT) medium illustrating the results of plating microsclerotia (MS) of *Verticillium dahliae* which had been buried for 17 weeks in pots filled with 200 g of either sand, greenhouse potting mix (GH mix) or sandy loam soil collected from fields in Simcoe (Simcoe) or Alliston, Ontario (Alliston and Lundy). The MS tested were from cultures of *V. dahliae* which received either 0, 1 or 10 mg/L of the melanin inhibiting chemical tricyclazole (TCZ) in the growth medium. Many of the MS treated with 10 mg/L TCZ and buried in the Simcoe soil did not form any colonies at all, whereas those buried in Lundy soil formed many colonies which were not *V. dahliae.*


0ppm TCZ
1ppm TCZ
10ppm TCZ
LUNDY

not significantly affect the survival of either melanized or non-melanized MS. Overall, the reduction in survival of TCZ treated MS buried 17 weeks in pots depended on the soil type and the results of a 2-way ANOVA revealed a significant interaction ($p=0.02$) between soil type and TCZ treatment (Fig. 4.12). Unlike the response in the Magenta boxes, GH mix did not have a significant effect upon the survival of either melanized or non-melanized MS, even after 17 weeks (Table 4.5 and Fig. 4.12).

Exhaustive rinsing of the MS that had been buried for 17 weeks in pots (under running tap water for 3 h) did not significantly affect either the frequency of MS which produced colonies of pure *V. dahliae* or the frequency of MS which gave rise to colonies other than *V. dahliae* ($p>0.999$) compared to those MS which had not been washed. The packets of sand/MS mixture buried in the pots contained soil particles and bits of plant material which had infiltrated the packets during the course of the experiment. Rinsing the packets removed such debris, making it easier to find the MS and transfer them without carrying the debris over on the end of the needle. The fact that the frequency of MS which produced colonies of fungi other than *V. dahliae* was the same in washed and unwashed packets showed that these other fungi were not merely contaminants, but were actually in intimate contact with the MS.

4.3.8 Burial in Soil with Organic Amendments

Burial of melanized and TCZ treated MS for 32 days in soil alone or soil amended with 1% w/w of either fish, bone and kelp (FBK), seaweed, Muskie fish emulsion or blood meal (BM) had a significant effect ($p<0.001$) on MS survival (Table 4.6). The survival of all MS, regardless of melanization, was reduced to zero when they were buried in soil amended with 1% BM. The survival of MS treated with TCZ was reduced to <2% and the survival of melanized MS was reduced to <50% when MS were buried in soil amended with FBK. The water, seaweed or Muskie treatments were the least effective at reducing MS survival. Analysis by a 2-way ANOVA indicated that both the amendment and TCZ treatment had a significant effect and there was also a significant interaction

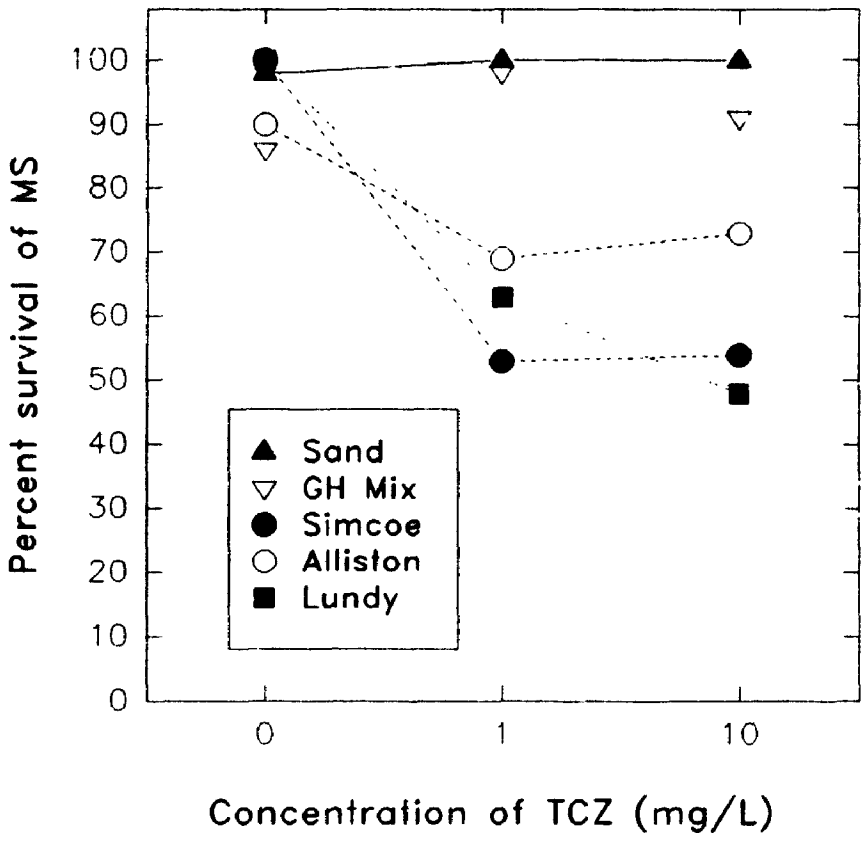


Figure 4.12
The percent survival of microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) when buried for 17 weeks in pots filled with various soil types in the growth room.

Table 4.6 The percentage of colonies of different types produced by MS treated with either 0, 1 or 10 mg/L tricyclazole (TCZ) and buried for 32 days in soil amended with either no amendment or 1% fish, bone and kelp meal (FBK), 1% bloodmeal (BM), 1% seaweed or 1% Muskie fish emulsion. The packets of MS were buried 3 days after the amendments were added to the soil.

		Percent of MS giving rise to colonies composed of:			
Amendment	[TCZ]	*V. dahliae* [a]	Mix [b]	Other [c]	Dead [d]
Water	0 mg/L	100	0	0	0
	1	100	0	0	0
	10	23	5	51	21
1% FBK	0	41	6	43	10
	1	2	6	67	25
	10	1	1	92	1
1% BM	0	0	1	60	39
	1	0	0	81	19
	10	0	0	82	18
1% Seaweed	0	98	0	0	2
	1	97	0	0	3
	10	63	6	10	21
1% Muskie	0	96	0	0	4
	1	97	0	0	3
	10	31	13	31	25

[a] colonies were composed of *V. dahliae* only
[b] colonies were composed of *V. dahliae* mixed with another fungus
[c] colonies were composed of a fungus other than *V. dahliae*
[d] no colony was formed

($p<0.001$). In general, MS treated with 10 mg/L TCZ exhibited significantly reduced survival in all treatments, including the control, in comparison with MS treated with either 0 or 1 mg/L TCZ (Fig. 4.13). The appearance of the SPT plates after more than 2 weeks incubation at 24 C is presented in Plate 4.7. MS buried in the BM or FBK treatments produced colonies with distinctive red pigmentation and this fungus was isolated and identified as a member of the genus *Penicillium* with the assistance of Dr. Jim Traquair (Department of Plant Sciences, U. of Western Ontario).

In contrast to the results when MS were buried 3 days after the amendments were added to soil, burial of melanized and TCZ treated MS for 4 weeks in soil that had been pre-incubated for 4 months with either no amendment or 1% chitosan, BM or FBK, resulted in no significant reduction of survival of melanized MS in any treatment (Table 4.7). MS treated with either 1 or 10 mg/L TCZ exhibited a significant reduction in survival when buried in soil amended with either BM or chitosan (Fig. 4.14). Both soil amendment and TCZ treatment had a statistically significant effect on MS survival ($p<0.001$) and there was a significant interaction ($p=0.03$).

4.3.9 Burial in Soil Infested with Antagonists

Over 75% of the melanized MS recovered after 3 weeks of burial in soil containing SDW or microbial antagonists gave rise to colonies of pure *V. dahliae* (Table 4.8). Only in the presence of *Trichoderma* was the survival of melanized MS reduced to 57%. In contrast, all the TCZ treated MS showed significant reductions in survival. Of all the treatments, *Bacillus polymixa* had the least effect although less than 40% of the MS were able to give rise to pure colonies of *V. dahliae*. Burial in soil infested with *Penicillium* sp. was the most damaging since 64% of the MS treated with 10 mg/L TCZ and 45% of the MS treated with 1 mg/L MS gave rise to colonies which were obviously not *V. dahliae*. In general, treatment of MS with TCZ significantly reduced the survival of MS in soils amended with various antagonists ($p<0.001$) as did the addition of microbial antagonists

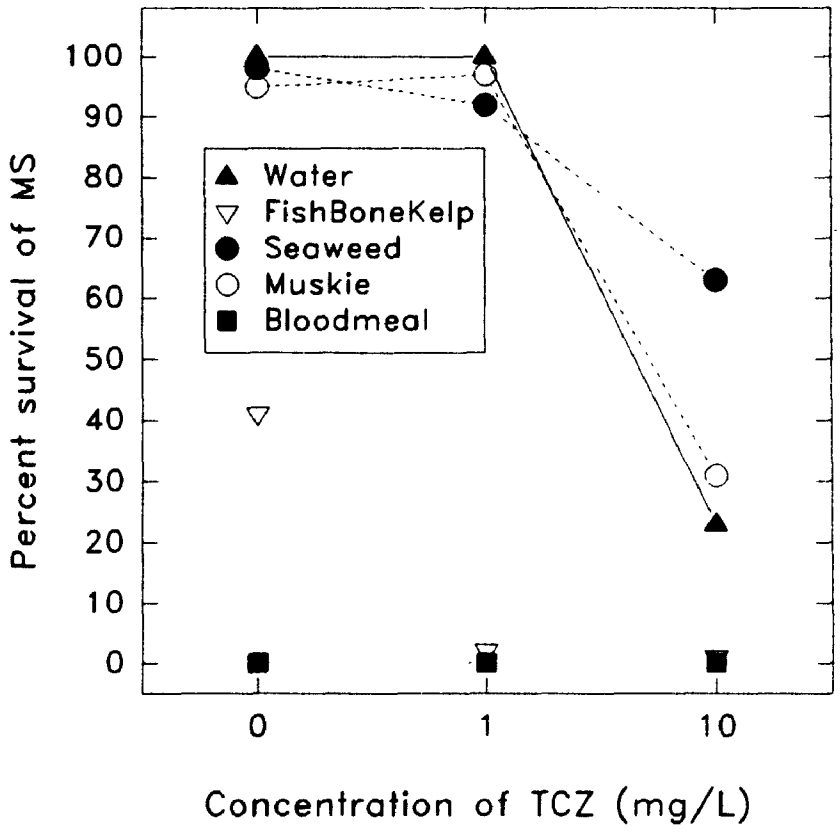


Figure 4.13
The percent survival of microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) when buried for 32 days in Magenta boxes containing soil amended with either 1% fish, bone and kelp meal, 1% seaweed, 1% Muskie fish emulsion, 0.5% bloodmeal or soil receiving no amendment.

Plate 4.7

A representative sample of plates of soil-pectate-tergitol (SPT) medium illustrating the results of plating microsclerotia (MS) of *Verticillium dahliae* which had been buried for 32 days in Magenta boxes containing 200 g sandy loam soil which had been amended with either 1% bloodmeal, 1% fish, bone and kelp meal, 1% seaweed or received no amendment (Distilled water). The MS tested were from cultures of *V. dahliae* which received either 0, 1 or 10 mg/L of the melanin inhibiting chemical tricyclazole (TCZ) in the growth medium. None of the MS, regardless of melanization, germinated to form colonies of *V. dahliae* when they were buried in soil amended with bloodmeal, however, many of the MS formed the red colonies shown. Many of the MS buried in soil amended with fish, bone and kelp meal also produced the red colonies, especially those MS treated with TCZ. The red fungus was subsequently isolated and identified as a member of the genus *Penicillium*.

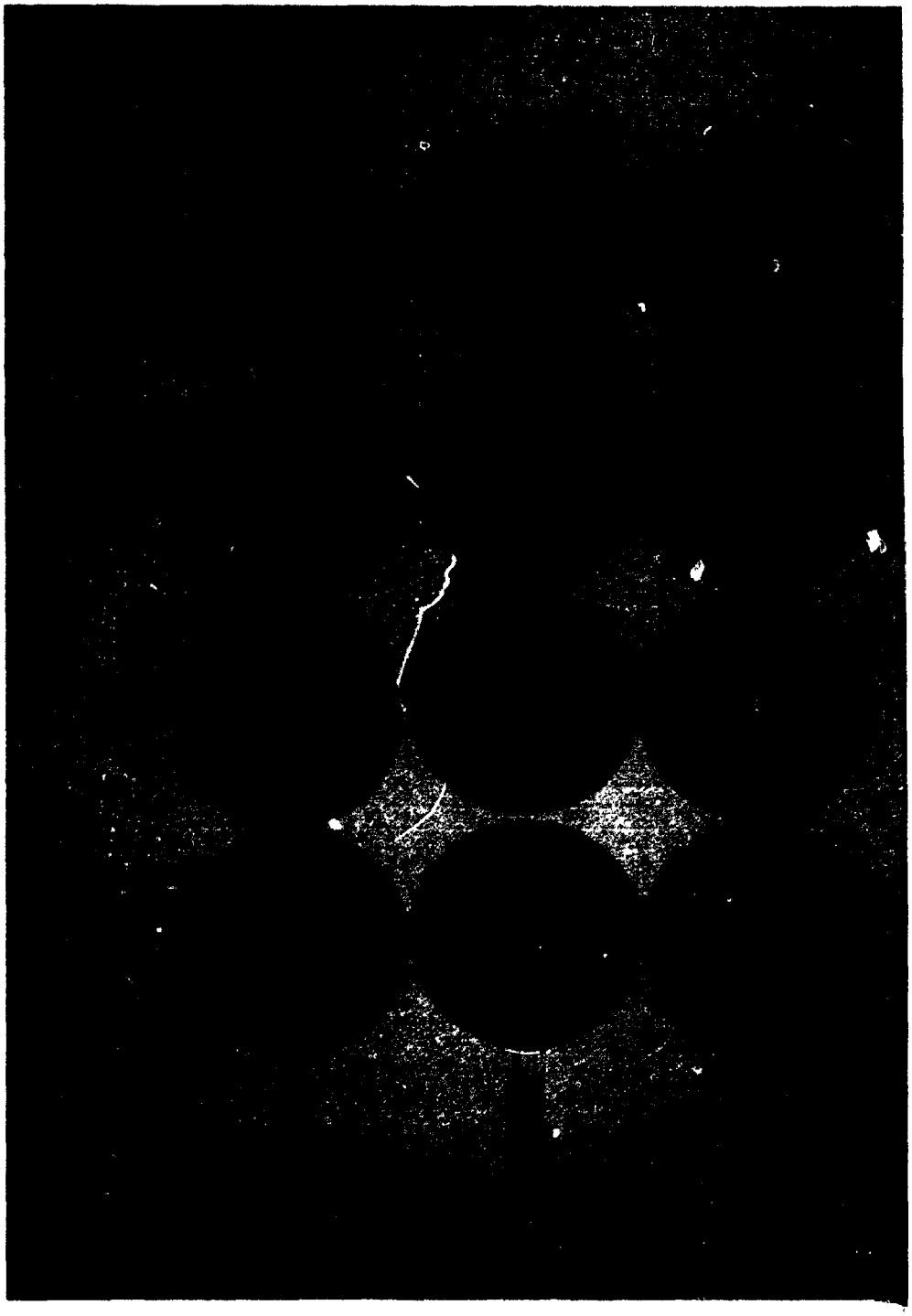


Table 4.7 The total number of colonies of different types produced by MS treated with either 0, 1 or 10 mg/L tricyclazole (TCZ) and buried for 4 weeks in soil amended with either fish, bone and kelp meal (FBK), bloodmeal (BM) or chitosan. The amendments were added to the soil and incubated at 24 C for 4 months before the packets containing MS were buried.

		Percent of MS giving rise to colonies composed of:			
Amendment	[TCZ]	*V. dahliae* [a]	Mix [b]	Other [c]	Dead [d]
Water	0 mg/L	98	1	0	1
	1	94	1	0	5
	10	95	1	0	4
1% FBK	0	99	0	0	1
	1	93	2	1	4
	10	92	7	0	1
1% BM	0	96	4	0	0
	1	84	6	0	10
	10	77	15	4	4
1% Chitosan	0	99	0	0	1
	1	64	28	8	0
	10	49	38	13	0

[a] colonies were composed of *V. dahliae* only
[b] colonies were composed of *V. dahliae* mixed with another fungus
[c] colonies were composed of a fungus other than *V. dahliae*
d no colony was formed

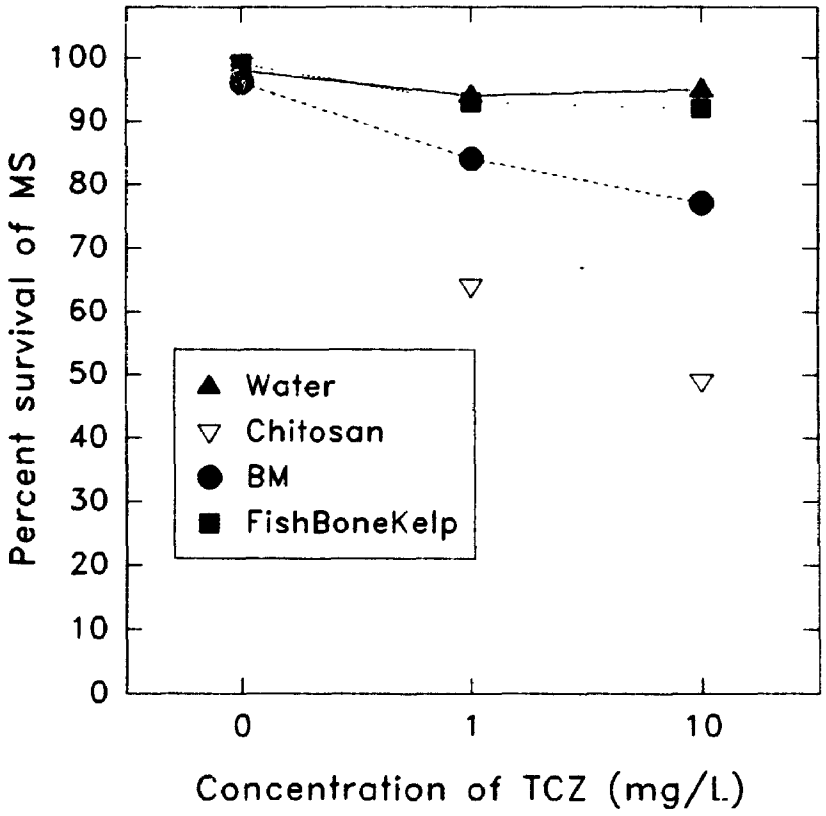


Figure 4.14
The percent survival of microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) when buried for 4 weeks in Magenta boxes filled with soil amended with 1% (w/w) of chitosan, bloodmeal (BM), Fish Bone and Kelp meal (FBK) or soil with no amendment (Water). The amendments were added 4 months prior to burial of the MS.

Table 4.8 The percentage of colonies of different types produced by MS treated with either 0, 1 or 10 mg/L tricyclazole (TCZ) and buried for 3 weeks in Magenta boxes containing soil amended with either no amendment (Water) or *Bacillus*, *Trichoderma*, *Talaromyces* or *Penicillium*.

		Percent of MS giving rise to colonies composed of:					
Amendment	[TCZ]	1 [a]	2 [b]	3 [c]	4 [d]	5 [e]	Dead [f]
Water	0 mg/L	86	10	2	0	1	1
	1	25	28	12	3	32	0
	10	13	37	25	9	15	1
Bacillus	0	93	4	0	2	1	0
	1	40	26	10	0	23	1
	10	37	29	10	6	18	0
Trichoderma	0	57	8	13	3	6	13
	1	22	16	12	15	33	2
	10	9	5	27	26	29	4
Talaromyces	0	95	0	0	0	5	0
	1	24	22	14	7	29	4
	10	11	25	9	13	41	1
Penicillium	0	77	15	1	0	6	1
	1	18	25	9	2	45	1
	10	2	15	12	6	64	1

[a] colonies were composed of *V. dahliae* only
[b] colonies were 3/4 composed of *V. dahliae* and 1/4 composed of another fungus
[c] colonies were 1/2 composed of *V. dahliae* and 1/2 composed of another fungus
[d] colonies were 1/4 composed of *V. dahliae* and 3/4 composed of another fungus
[e] colonies were composed of a fungus other than *V. dahliae*
[f] no colony was formed

($p<0.001$), however, there was not a statistically significant interaction ($p=0.086$) (Fig. 4.15).

After 5 weeks burial, a dramatic reduction in the survival of MS buried in soil amended with *Talaromyces* was observed (Table 4.9) as the survival of melanized MS was only 51% and that of MS receiving TCZ was <10%. After 5 weeks of burial, the *Trichoderma* and *Talaromyces* treatments were the most effective at reducing the survival of non-melanized MS (Table 4.9). Both TCZ treatment and the antagonist added to the soil were significant factors in reducing the survival of MS buried for 5 weeks according to the 2-way ANOVA ($p<0.001$). There was no significant interaction ($p=0.092$). The control treatment (which was supposed to be completely sterile) also reduced survival of non-melanized MS likely because the soil did not remain sterile throughout the experiment. Overall, the bacteria treatment was the least effective in reducing MS survival (Fig. 4.16). The appearance of the colonies formed by MS which were buried for 5 weeks is presented in Plate 4.8. Based upon visual observations and the production of the characteristic pinkish red pigment on SPT media, the colonies growing out of the MS arose from the *Penicillium* sp. Both *Talaromyces* and *Trichoderma* were less readily identifiable on SPT media.

4.4 Discussion

The presence of melanin in microorganisms has been correlated with environmentally stressful habitats such as the desert, alpine regions or the upper biosphere (Bell & Wheeler, 1986). Melanin also plays a very important role in biological colouration and defense in many organisms, including humans, other vertebrates, invertebrates and possibly plants (Bell & Wheeler, 1986). Albino mutants of fungal propagules are rarely found in nature. For instance, Huang (1981) discovered only one tan coloured sclerotium of *Sclerotinia sclerotiorum* in a sample of 4388. Although it is repeatedly stated that the role of melanin in the survival of fungal propagules is well-established, 25 years after Kuo

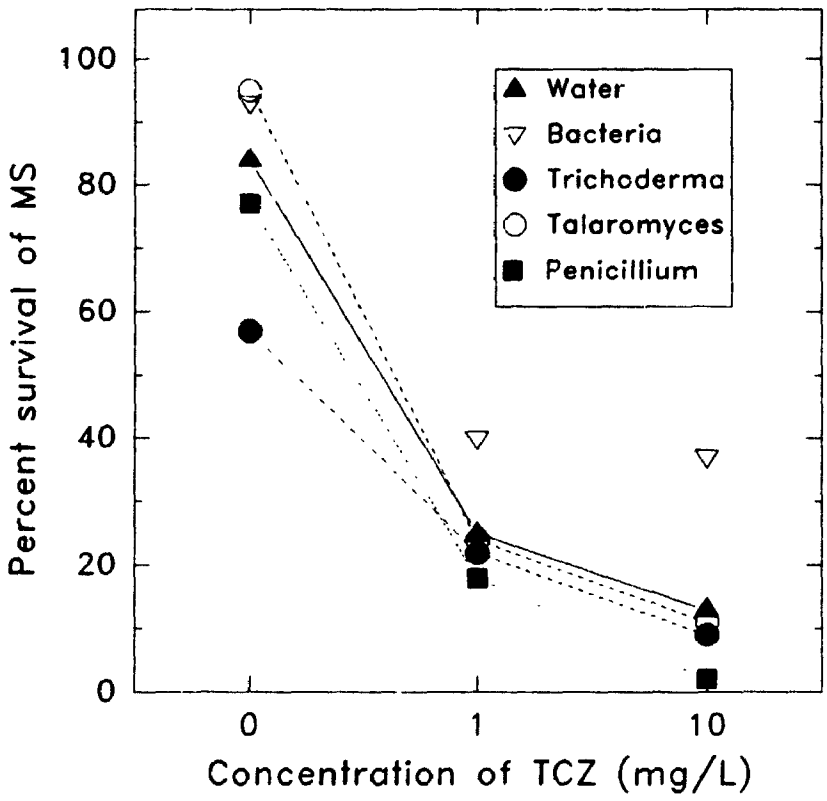


Figure 4.15
The percent survival of microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) when buried for 3 weeks in Magenta boxes filled with soil amended with various microorganisms.

Table 4.9 The percentage of colonies of different types produced by MS treated with either 0, 1 or 10 mg/L tricyclazole (TCZ) and buried for 5 weeks in Magenta boxes containing soil amended with either no amendment (Water) or *Bacillus*, *Trichoderma*, *Talaromyces* or *Penicillium*.

		Percent of MS giving rise to colonies composed of:					
Amendment	[TCZ]	1 [a]	2 [b]	3 [c]	4 [d]	5 [e]	Dead [f]
Water	0 mg/L	86	2	2	0	10	0
	1	16	40	6	3	35	0
	10	5	28	18	8	42	0
Bacillus	0	95	0	1	0	4	0
	1	70	1	9	6	15	0
	10	66	5	8	7	14	0
Trichoderma	0	79	2	10	0	6	3
	1	14	7	21	33	22	3
	10	4	2	4	38	49	3
Talaromyces	0	51	32	3	2	12	0
	1	6	30	12	16	36	0
	10	5	48	14	10	22	1
Penicillium	0	73	10	3	2	11	1
	1	20	27	18	9	26	0
	10	16	25	17	10	32	0

[a] colonies were composed of *V. dahliae* only
[b] colonies were 3/4 composed of *V. dahliae* and 1/4 composed of another fungus
[c] colonies were 1/2 composed of *V. dahliae* and 1/2 composed of another fungus
[d] colonies were 1/4 composed of *V. dahliae* and 3/4 composed of another fungus
[e] colonies were composed of a fungus other than *V. dahliae*
[f] no colony was formed

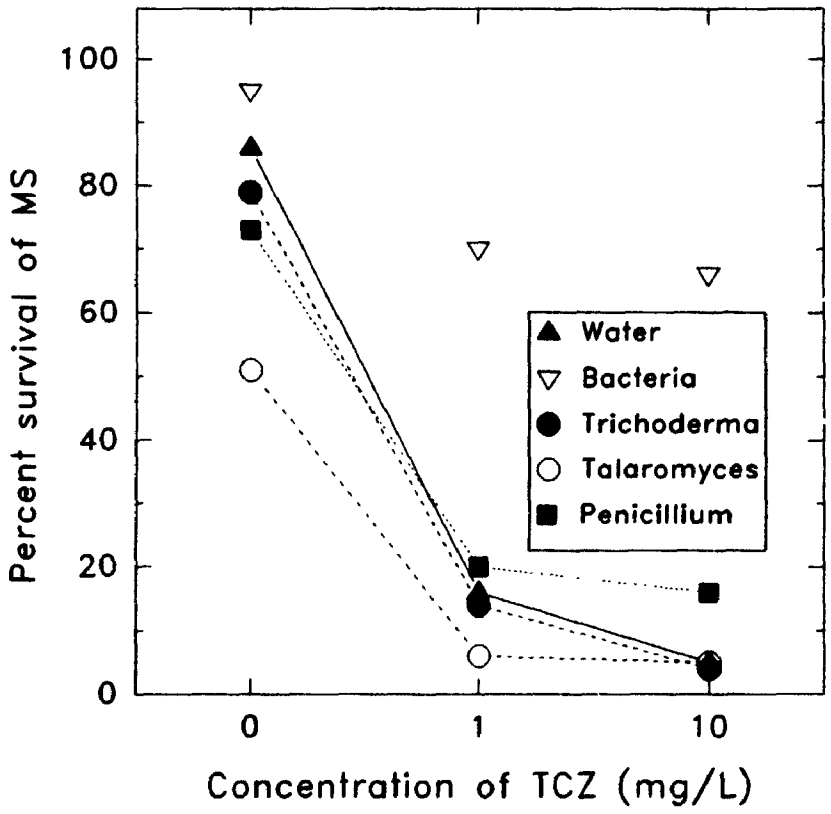
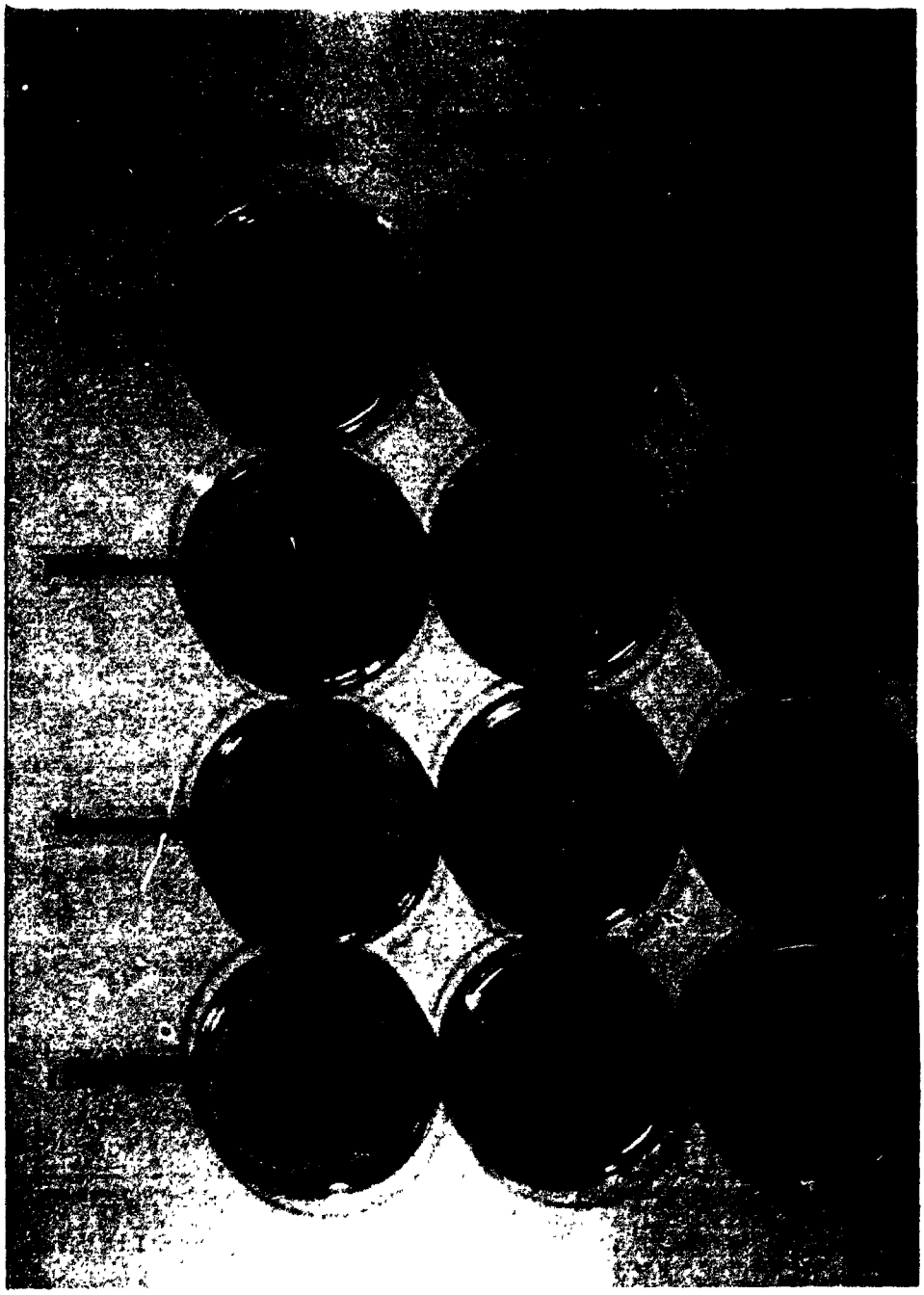


Figure 4.16
The percent survival of microsclerotia (MS) treated with 0, 1 or 10 mg/L tricyclazole (TCZ) when buried for 5 weeks in Magenta boxes filled with soil amended with various microorganisms.

Plate 4.8

A representative sample of plates of soil-pectate-tergitol (SPT) medium illustrating the results of plating microsclerotia (MS) of *Verticillium dahliae* which had been buried for 5 weeks in Magenta boxes containing 200 g of sandy loam soil which had been infested with either *Trichoderma aureo viride*, *Talaromyces flavus*, *Penicillium* spp. or which received no inoculant (Water). The MS tested were from cultures of *V. dahliae* which received either 0, 1 or 10 mg/L of the melanin inhibiting chemical tricyclazole (TCZ) in the growth medium. MS treated with 10 mg/L TCZ tended to produce colonies which were not *V. dahliae* when they were buried in soil inoculated with *Trichoderma*, *Talaromyces* or *Penicillium*. TCZ treated MS buried in Penicillium inoculated soils often produced colonies of *Penicillium*, as can be seen by the distinctive red colouration of the colonies on the plates. While the identity of the colonies produced on the plates of TCZ treated MS buried with *Trichoderma* or *Talaromyces* was not visually distinctive, subsequent isolation of the fungi showed that they were primarily *Trichoderma* or *Talaromyces*.



and Alexander (1967) wrote that evidence favouring a role for melanin in fungal persistence in nature was circumstantial, the statement is still largely true.

There is accumulating experimental evidence, however, to support the concept that melanin is an important protective barrier against damaging UV light (see references cited in 4.1 Introduction) Results from my experiments carried out with melanin deficient MS of *V. dahliae* also demonstrated the vital role of melanin as a protectant against UV induced damage. Exposure of albino MS to even 2 h of UV irradiation severely inhibited germination (a lethal effect) and reduced colony size (a sub-lethal effect). MS which were melanin deficient because of treatment with TCZ were also affected by UV but not as acutely as albino MS. Whereas albino MS were completely hyaline, TCZ treated MS contained some pigment in their walls due to the presence of such oxidative products of shunt metabolites as flaviolin and 2-HJ. The capacity for protection from UV exhibited by melanin and other polymeric condensed ring structures arises from their ability to act as stable free radicals which sequester damaging photon-generated unpaired electrons in an inactive configuration (Commoner, Townsend & Pake, 1954). It is not clear, however, whether UV radiation is actually a significant factor in the survival of soilborne fungi under natural conditions. Short wavelength UV (250-270 nm), which is the most damaging in laboratory experiments, is virtually absent from solar radiation in the field (Rotem, Wooding & Aylor, 1985). In any case, exposure to light is more likely to play an important role in the survival of fungi inhabiting the phyllosphere than in soilborne pathogens like *V. dahliae*. The objective of including a study on the effect of short wavelength UV irradiation on the survival of MS in *V. dahliae* was to test the capacity of the procedures outlined in Chapters Two and Three to accurately and quantitatively measure the difference in survival between melanized and non melanized MS. If the assay procedure was unable to reflect the damaging effects of a potent treatment like UV light, then it would never be useful for detecting and quantifying the effects of more subtle treatments.

Melanin did not appear to play a major role in the survival of individual MS when stored and handled under laboratory conditions. The inhibition of melanin in MS by treatment with 1 or 10 mg/L TCZ did not reduce either the germination or growth rate compared to melanized MS. In fact, MS size had more impact upon germination and colony growth than did TCZ treatment. In one experiment, a reduction in the viability of TCZ treated MS (75-106 µm) was seen after only 10 to 12 weeks storage at 24 C. In two other experiments testing long-term survival, however, no difference in viability between melanized MS and those treated with either 1 or 10 mg/L TCZ was evident after more than 35 weeks storage at 24 C.

It was hypothesized that dehydration of non-melanized MS stored under desiccated conditions over a range of temperatures might reduce their survival compared to melanized MS. There has been evidence to support the theory that melanin acts as a regulator of osmotic potential in melanized fungal cells. Howard and Ferrari (1989) showed that appressoria of *Magnaporthe grisea* are lined with a layer of melanin which acts as a barrier to solute efflux but permits water influx. Inhibition of melanin resulted in plasmolysis of these appressoria by low solute concentrations, while normally melanized appressoria were not plasmolyzed. However, treatment with TCZ did not affect the survival of 75-106 µm MS of *V. dahliae* stored for either 8 or 36 weeks at various temperatures. MS receiving 1 mg/L TCZ, for some unexplained reason, did not display the loss of viability in the refrigerator freezer at -25 C that was encountered with either melanized MS or those treated with 10 mg/L TCZ (Fig. 4.5).

Melanin has been clearly implicated as a mechanism by which microorganisms protect themselves from damage and degradation by cell wall degrading enzymes (see 4.1 Introduction). Melanin is notoriously intractable and is insoluble in water, aqueous acid and most solvents (Swan, 1963; Kuo & Alexander, 1967b; Bell & Wheeler, 1986). It was found in this study that the survival of melanized MS exposed for 24 hours in Novozyme was greater than that of MS treated with TCZ. The procedures used in this study for

digestion of cells with Novozyme have been found to produce spheroplasts successfully from *Schizosaccharomyces pombe* (Dickinson & Isenberg, 1982), the hyaline conidia of *V. dahliae* (Dr. K. Dobinson, personal communication) and hyaline spores of *Ustilago maydis* (M. Latosek-Green, personal communication). In excess of 90% of spores were shown to be converted to spheroplasts after 2 h of digestion. Loss of germinability of MS after digestion in Novozyme was not due to lysis of MS, however, because they appeared structurally intact when examined under a light microscope. Normally melanized strains of the human pathogen *Wangiella dermatitidis* were also found to be more resistant to lysis by cell wall degrading enzymes (Zymolase) than were strains which had melanin synthesis inhibited by the addition of TCZ (Dixon, Szaniszlo & Polak, 1991).

According to this study, the most important role which melanin plays in the survival of MS is protection from microbial populations in soil. Normally melanized MS clearly had an advantage over melanin deficient MS when they were buried in soils, including those which had been amended with either organic materials or certain specific microorganisms. The results of all the burial experiments consistently showed that the inhibition of melanin by 10 mg/L TCZ was a significant factor in the reduction of survival of MS. When MS were buried for 20 weeks in Magenta boxes filled with various soils, the survival of MS treated with 1 or 10 mg/L TCZ was significantly reduced compared to melanized MS, irregardless of the soil type. The reduction in survival of MS treated with 10 mg/L TCZ was consistently lowest in the sand, where very little biological activity could be expected. When MS were buried for 17 weeks in pots in the growth room, however, the survival of melanized and non-melanized MS was dependent upon the type of soil they were buried in. Melanization had no effect on survival of MS buried in sand or GH mix, but the survival of MS treated with 1 or 10 mg/L was significantly reduced in Alliston, Simcoe and Lundy soils. It is clear that the activity of the various soils towards MS was related to the experimental conditions. Alliston soil was effective in reducing MS survival in pots but not in Magenta boxes and conversely, GH mix was effective when in

Magenta boxes but not in pots. The Magenta boxes provided the ideal environment for microbial growth in GH mix since the numbers of MS associated with other fungi was so much higher in this environment than they were in GH mix in the pots. The reduction of MS survival in Alliston soil in pots was due to the failure of the MS to produce a colony of any kind and was not associated with enhanced microbial activity. Despite these differences in the ability of the various soils to reduce survival, the trend observed was that melanized MS survived better than TCZ treated MS when buried in soil. It was also observed that after burial for 17 or 20 weeks in pots or Magenta boxes, respectively, MS treated with either 1 or 10 mg/L TCZ were more likely to give rise to colonies that were not *V. dahliae* than melanized MS, which implicated soil microorganisms, primarily fungi, in the reduction of survival of non-melanized MS. Other researchers have also reported the contamination of sclerotia buried in soil with other fungal or bacterial species. The decrease in germinability of sclerotia of *Sclerotium rolfsii* when buried in amended soil was correlated to an increase in the number of actinomycetes and bacteria which were associated with the recovered sclerotia (Henis & Chet, 1968). Six bacteria and 1 fungus were isolated from sclerotia of *Sclerotium cepivorum* in muck soil and were shown to produce diffusable antibiotics which were antagonistic to growth of the fungus in culture (Utkhede and Rahe, 1980). In the experiments carried out here, the role of bacteria in MS survival was not examined as the SPT media used precludes the growth of these organisms. Dried sclerotia of *S. cepivorum* were reportedly colonized by *Trichoderma* when they were buried in soil (Leggett & Rahe, 1985). Burial of sclerotia of *Sclerotinia minor* for 41 days in solarized field plots resulted in 86-94% of the recovered sclerotia being colonized by microorganisms (Vannacci, Triolo & Materazzi, 1988). None of these experiments, however, examined the differential survival of melanized and non-melanized sclerotia. Only the work by Huang has reported the results of the burial of melanized and non-melanized sclerotia in soil. Huang (1983) showed that tan-coloured sclerotia of *Sclerotinia sclerotiorum* survived poorly under greenhouse and field conditions, with less

than 3% recovery of tan sclerotia buried 4 months in the field or 1 month in the greenhouse, compared to 83% recovery for black sclerotia. Huang (1983) also reported that 60% of tan sclerotia of *Sclerotinia sclerotiorum* isolated from artificially infected sunflower plants were parasitized by *Coniothyrium minitans* compared with only 14% of black sclerotia and concluded that the poor survival of tan sclerotia in soil could be attributed to their invasion by various hyperparasites, including *C. minitans*. In another study Huang (1985) also found that the frequency of contamination of incompletely melanized sclerotia of *S. sclerotiorum* with *Rhizopus*, *Fusarium* and *Alternaria* was significantly higher than that of melanized sclerotia.

The inhibition of melanin by treatment with 10 mg/L TCZ had a significant impact on the survival of MS buried in soil to which certain organic amendments were added. The survival of MS treated with 10 mg/L TCZ was significantly reduced when buried for 32 days in soil alone or in soil amended with seaweed and Muskie fish emulsion. The survival of melanized MS or those treated with 1 mg/L TCZ was unaffected by those same treatments. In contrast, melanin was less important in MS survival when soil was amended with either fish, bone and kelp meal (FBK) or bloodmeal (BM). The survival of melanized MS buried for 32 days in soil amended with FBK was reduced to 41% and the survival of TCZ treated MS to zero. The addition of BM to soil completely eradicated the MS, regardless of the presence of melanization. Much of the ability of BM and FBK to reduce the survival of MS was lost when the MS were not buried until 4 months after the amendment was added. The complete loss of viability which was achieved when both melanized and non-melanized MS were buried in BM led to many questions regarding the efficacy of BM as a natural method for the control of *V. dahliae*. As a result, further experiments investigating the effect of organic amendments on the survival of MS buried in soil were carried out. Since the role of melanin was minimized in the survival of MS buried in FBK amended soil and was not a factor in the survival of MS buried in BM amended soil, only the melanized MS were tested in subsequent experiments. The role of

organic amendments in the survival of soilborne fungi in general and *V. dahliae* in particular, is discussed in Chapter Five.

Melanin protected MS when they were buried in Magenta boxes filled with soil that had been infested with various microorganisms. In every treatment, the survival of melanized MS buried for 3 or 5 weeks was significantly higher than the survival of TCZ treated MS. Overall, MS survival was highest in the soil inoculated with *Bacillus polymixa* and after 5 weeks burial, MS survival was the lowest in soil inoculated with *Talaromyces flavus*. Even the survival of melanized MS was reduced to 51% in the presence of *T. flavus* and the survival of TCZ treated MS was reduced to <10%. *Trichoderma* reduced the survival of melanized MS to 79% and the survival of 1 and 10 mg/L TCZ treated MS to 14 and 4%, respectively after 5 weeks burial. The fungi which were reisolated from MS buried in the *Trichoderma*, *Talaromyces* and *Penicillium* treatments were almost exclusively these same fungi. As discussed in the Introduction (Section 4.1), both *Trichoderma* and *Talaromyces* have been shown to act as biocontrol agents against soilborne fungi and *Talaromyces* can inhibit *V. dahliae* in particular. It has been demonstrated that *T. flavus* produces glucose oxidase, which catalyzes a reaction that produces hydrogen peroxide as one of the products (Kim, Fravel & Papavizas, 1988). The action of hydrogen peroxide has therefore been linked to the antibiotic activity of *T. flavus*. It is possible that the melanin present in the MS acts to control the damage caused by free radicals which might be liberated by the action of the hydrogen peroxide.

The *Penicillium* which had been isolated from MS buried in soil amended with bloodmeal and fish, bone and kelp meal was also effective at reducing the survival of TCZ treated MS. It is possible that this fungus could act as a biocontrol agent against *V. dahliae* and further work should be done to test its efficacy both in the laboratory and in the field.

The poor survival of MS in the control treatment (sterilized soil and SDW) was due to fungal contamination of the soils in that treatment. Table 4.9 illustrated that the low

number of MS which produced colonies of pure *V. dahliae* in the control treatment was due to the association of the MS with another fungus. The autoclaved soil became contaminated, even though the sand and packets were sterilized before burial and the packets were buried and recovered with flamed forceps in the laminar flow hood.

Chapter Five

The Effect of Organic Amendments on MS Survival

5.1 Introduction

The "green revolution", which led to significant increases in crop productivity, was in part fueled by the availability of chemical fumigants which protected crop plants from fungal parasites. The achievements obtained in the control of foliar pathogens, however, were not realized in the protection of the root system. As a result, diseases caused by such soilborne plant pathogenic fungi as *Rhizoctonia, Sclerotinia, Fusarium* and *Verticillium* continue to extract heavy losses in the production of numerous crops. Chemical control is often expensive and can indiscriminately eliminate a range of microorganisms creating a "microbial vacuum" that can result in the rebounding of the population of the target or other pathogenic species. Leaching of chemicals away from the target site can lead to chemical contamination of groundwater supplies which have implications on human and animal health. These concerns have stimulated research which is directed towards the use of an integrated approach to the control of soilborne plant diseases. The soil is a complex environment in which no one component works in isolation and synergistic relationships are common. *Verticillium*, for example, has been shown to interact with nematodes in the development of potato early dying disease (Rowe, Riedel & Martin, 1985; Riedel, Rowe & Martin, 1985). When nematodes were successfully controlled by soil amendment, a concomitant reduction in severity of Verticillium wilt in tomatoes was found although the amendment had no direct effect on *Verticillium* itself (Miller & Edgington, 1962). In another example, *Enterobacter cloacae* destroyed *Botrytis cinerea* when in the presence of chitinolytic enzymes produced by *Trichoderma harzianum* (Harman, Hayes & Lorito, 1993). Rather than sterilizing the soil, the trend must be toward the improvement of soil health and the management of soil populations by reducing the conditions favourable to the pathogen and enhancing the conditions favourable to beneficial organisms.

The concept of manipulating soil composition for the benefit of crop production is not new. For many generations, organic soil amendments such as animal manures, green manures and products such as bonemeal and bloodmeal have been used by farmers and gardeners worldwide as fertilizers (Thurston, 1992). The relationship between organic amendments and the control of soilborne diseases was being discussed in the scientific literature in the 1940's (West & Hildebrand, 1941; Sandford, 1946; Sandford, 1947; Wilhelm, 1951). Bloodmeal and fishmeal, for example, have long been used as sources of nitrogen in organic gardening and they, along with other amendments, have now been shown to exhibit disease controlling activity as well. The addition of bloodmeal or fishmeal to naturally infested field soil reduced the infection of tomato plants with *V. dahliae* to zero (Wilhelm, 1951). Addition of alfalfa and oat residues at 1% (w/w) reduced the number of viable propagules of *V. dahliae* in the soil by up to 60% (Green & Papavizas, 1968). When fine residues of oats or barley were incorporated into soil in a greenhouse study, suppression of Verticillium wilt of potatoes was correlated to increasing rates of the amendments (Tolmsoff & Young, 1959). Survival of MS was reduced by 33 and 40% with the addition of 0.2% (w/w) of laminarin or chitin, respectively, to soil (Jordan, Sneh & Eddy, 1972). Barley straw amendments significantly reduced the population of *V. dahliae* in naturally infested soil in greenhouse experiments and the level of control increased as the rate of amendment increased from 0.4 to 1.6% (Harrison, 1976). The addition of different forms of inorganic nitrogen to soil has also been shown to reduce the infection of tomato plants by *V. dahliae* (Wilhelm, 1951) as well as reducing the survival of *S. rolfsii* (Henis & Chet, 1968), *Phytophthora* (Tsao & Oster, 1981) and other soilborne fungal pathogens (Henis & Katan, 1975).

While there is ample literature reporting successful control of soilborne pathogens such as *V. dahliae* with soil amendments, it is clear that the effects are not attributable to any single mode of action. Soil amendments may act by affecting the inoculum density or controlling the saprophytic activities of the pathogen in the soil, by enhancing the activity

of biocontrol agents, by the production of volatile or nonvolatile substances which could be stimulatory or suppressive to the pathogen, by providing the pathogen with a food source, or by influencing soil fungistasis in some other fashion (Papavizas, 1975). Volatile substances have been implicated in the inhibition of soilborne fungi (Hora & Baker, 1974; Pavlica et al., 1978) and the addition of some amendments, such as chitin, has caused the formation of gaseous compound such as ammonia (Schippers & Palm, 1973).

In this chapter, the results of an investigation of the effect of various organic and inorganic amendments on the survival of MS of *V. dahliae* in soil are presented. Wilhelm (1951) previously reported that the infection of tomato plants by *V. dahliae* was completely inhibited by the addition of certain amendments to the soil. The mechanism by which disease suppression occurred has remained a mystery. In repeating the experiments of Wilhelm using MS as an indicator organism, it was observed that bloodmeal (BM) completely eradicated all MS regardless of melanin content (see Section 4.3.8). There are no known treatments which can achieve this level of control of *Verticillium*, and it was surprising that the use of such amendments was not investigated further in the years following. With the increasing interest in the control of pathogens like *V. dahliae* without the use of chemical fumigants, soil disinfestation by natural organic materials may be highly desirable.

5.2 Methods

5.2.1 Addition of Various Amendments to Soil

In addition to the bloodmeal recommended by Wilhelm (1951), several other amendments were tested for activity against MS of *V. dahliae*. The amendments were chosen for their potential to enhance microbial populations. They were applied at a rate of 1% w/w because applications of lower rates was shown to be ineffective (Wilhelm, 1951). Twenty Magenta boxes were filled with 200g air dry soil (sandy loam soil from Alliston, Ontario) and 4 sets of 4 boxes each were amended with 1% (w/w) of either forest soil, composted sheep manure, Lundy soil (see section 4.2.7) or bloodmeal (BM). One set of

boxes received no amendment. The forest soil was collected from the first 5 cm under the leaf litter of a beech-maple climax forest. Nylon mesh packets containing acid-washed quartz sand and 75-106 μm MS were prepared and a single packet was buried in each box at 5 cm depth. The boxes were incubated at 24 C. After 44 days, the packets were retrieved and the 25 MS from each packet was transferred onto SPT media. After 2 weeks incubation at 24 C, the plates were examined and the number of MS which germinated to form colonies was counted.

Bloodmeal was also tested in another experiment at a lower rate and was compared to several other organic amendments. Wilhelm (1951) reported that fish meal also controlled *V. dahliae*, however, he did not provide a description or a source for this material. As a result, 2 commercially available organic fertilizers, based on fish products, were purchased and tested. For this experiment, 24 Magenta boxes were filled with 200 g air dry soil (sandy loam soil from Alliston, Ontario). There were 6 treatments including bloodmeal (BM), fish, bone and kelp meal (FBK), chitosan, Muskie, seaweed, and no amendment. There were 4 replicate boxes per treatment. The BM was added at 0.5% w/w and the FBK, seaweed and chitosan at 1% w/w. (The BM, FBK, Muskie and chitosan amendments were previously described in an experiment presented in Section 4.2.8). The Muskie fish emulsion was prepared by adding 5 mL of emulsion per litre of distilled water. The 4 boxes in the Muskie treatment each received 30 mL of the diluted emulsion. All other boxes received 30 mL SDW to bring the soil moisture level up to 50% of the soil moisture capacity. Nylon mesh packets containing acid-washed quartz sand and MS of 75-106 μm size were prepared and 3 packets were buried at 5 cm depth inside each box. The boxes were incubated at 24 C and one packet was removed from each box after either 11, 18 or 32 days. After the packets were recovered, 25 MS from each packet was plated onto SPT. The plates were incubated at 24 C for at least 2 weeks after which the number of MS which germinated and formed colonies was recorded.

5.2.2 Amendment of Soil with Various Concentrations of Bloodmeal

An experiment was performed to test the effectiveness of bloodmeal over a range of concentrations similar to those reported in Wilhelm (1951). Twenty Magenta boxes were filled with 200 g air dry soil (sandy loam soil from Simcoe, Ontario) and there were 5 treatments: 1) no BM 2) 0.25% BM 3) 0.5% BM 4) 1% BM and 5) 1% sterilized BM. There were 4 Magenta boxes per treatment and the percentage of BM was added per weight of air dry soil. The amendments were mixed thoroughly into the soil and 8 mL of SDW was added to each box to bring the soil moisture level up to 50% of the soil moisture capacity. Packets containing acid-washed quartz sand and MS of 75-106 μm size were prepared and 5 packets were buried at 5 cm depth inside each box. In order to determine whether the survival of MS would be affected even when the MS were not in physical contact with the soil, 3 packets were suspended above the soil surface on nets made of plastic mesh (Plate 4.1). The boxes were incubated at 24 C and one packet was removed from each box after 2, 4, 6, 8, and 10 days of burial. One of the suspended packets was removed from each box after 2, 6, and 10 days. After the packets were recovered, 25 MS from each packet were plated onto SPT media as previously described.

5.2.3 Amendment of Soil with Sterilized Bloodmeal

A bloom of fungus was frequently observed on the soil surface in the boxes containing soil amended with BM. In order to investigate whether the toxic effect of BM might be due to microbial activity in the BM itself or in natural soil, the soil and BM were sterilized by autoclaving before use. In this experiment, 16 Magenta boxes were filled with 200 g of soil (Alliston soil) and 8 of the boxes were sterilized by autoclaving 4 times for 30 mins each over several days. Several 2 g samples of BM were also sterilized by autoclaving 3 times for 20 mins each. The sterility of the soil and BM was tested by plating a sample from each container onto PDA media and checking for any fungal or bacterial growth. There were 4 boxes per treatment and the 4 treatments used were: 1) soil plus BM 2) soil plus sterilized BM 3) sterilized soil plus BM and 4) sterilized soil plus sterilized BM. The

BM was added to dry soil at a rate of 1% (w/w) and mixed completely. Each box received 33 mL of SDW to bring the soil moisture up to 50% of the soil moisture capacity. Nylon mesh packets containing acid-washed quartz sand were sterilized by autoclaving and MS of 75-106 μm size were added to each packet with a flamed spatula inside the laminar flow hood. The packets were sealed with a soldering iron while still inside the hood. Immediately after adding the BM and water to the boxes, 1 packet was buried inside each box and the boxes were incubated at 24 C. After 24 h the packets were recovered (under sterile conditions) and 25 MS from each packet were plated onto SPT. Three days later, another set of packets was prepared in a similar manner and 1 packet was buried inside each box. After 4 days of incubation at 24 C, the packets were recovered and 25 MS from each packet were plated onto SPT. Seven days after the BM and water were added to the boxes, a third set of packets was prepared and buried, 1 packet per box. After 4 days of incubation at 24 C, the packets were recovered and 25 MS from each packet were plated onto SPT.

In the subsequent experiment, new boxes of soil, samples of BM and packets of sand/MS mixtures were prepared in exactly the same manner as described above. Immediately after adding the BM and SDW to the boxes, 2 packets were buried inside each box. The packets were removed from each box after 2 or 5 days of incubation at 24 C. Immediately after recovery, 25 MS from each packet were plated onto SPT.

5.2.4 Amendment of Soil with Urea, Ammonium Acetate or Ammonium Sulphate

As discussed in the Introduction, inorganic forms of nitrogen have been shown to inhibit the survival of soilborne fungi and ammonia has been suggested to be involved in this effect. The objective of this experiment was to investigate the effect of several forms of inorganic nitrogen on the survival of MS in soil. Twenty Magenta boxes were filled with 200g air dry soil (sandy loam soil from Alliston, Ontario). There were five treatments, with 4 Magenta boxes per treatment. Two treatments consisted of either ammonium sulphate ($(NH_4)_2SO_4$, M.W.=132.14) or urea (NH_2CONH_2, M.W.=60.06)

added to the soil at 0.5% by weight of air dry soil. Ammonium acetate (CH_3COONH_4, M.W.= 77.08) was added to two treatments at either 0.5 or 0.125% (w/w). The fifth treatment was the control and did not receive any amendment. Once the chemicals were well mixed into the dry soil, 30 mL of SDW was added to each Magenta box in order to bring the soil moisture level up to 50% of the soil moisture capacity. Nylon packets containing acid-washed quartz sand and MS of 75-106 μm size were prepared and 2 packets were buried at 5 cm depth inside each box. The boxes were incubated at 24 C and the packets were removed after 1 or 5 days burial. Once recovered from the soil, 25 MS from each packet were transferred to SPT media as previously described. Four days after plating onto SPT, the diameter of each colony was measured and recorded. The number of MS which germinated and formed a colony of *V. dahliae* was recorded after the plates were incubated for 2 weeks at 24 C. The data were analyzed by a Kruskal-Wallis Test and the multiple comparisons were done by a Student-Newman-Keuls test.

5.2.5 Eggplant Bioassay

The purpose of this experiment was to determine whether the addition of BM to *Verticillium*- infested soil could protect normally susceptible plants from becoming infected with the pathogen. Air dry soil (a sandy loam soil from Alliston, Ontario) was weighed out into 5 batches of 2 Kg each. The soil batches were amended with 20 g amounts of BM or 20 mg amounts of MS, according to the treatments listed below. The MS used were 75-106 μm in size and were cultured in MCDX media and harvested as previously described. The particular concentration of MS used was chosen because experiments in our labo.atory had shown that infesting soil with 100 MS/g of soil resulted in a minimum of 70% infection in eggplant or potato. The autoclaved BM was prepared by weighing out 20 g of BM into each of several Erlenmeyer flasks, covering, and autoclaving for 20 minutes, 3 times over 48 hours. The sterility of the BM was confirmed by sprinkling some of the BM from one of the flasks onto PDA agar media and incubating at 24 C. There were 5 soil treatments in the eggplant bioassay experiments: 1) soil alone

2) soil amended with 1% (w/w) BM 3) soil infested with MS 4) soil amended with 1% BM and MS and 5) soil amended with 1% autoclaved BM and MS. The soil plus the amendments were each placed into a plastic bag and mixed thoroughly. The soil in each treatment was then distributed between 20 plastic pots, watered, and placed in the walk-in growth room. Eggplant seeds (*Solanum melongena*, cv. Imperial Black Beauty) were sown in vermiculite and grown at 24/20 C day/night temperatures and 15 h per day fluorescent light for 2-3 weeks before transplantation into the soil treatments. The seedlings were separated from each other while floating them in tepid water and one seedling was transplanted into each pot of soil. In one set of experiments, the soil treatments were prepared and the eggplant seedlings transplanted into the soils on the same day. In subsequent experiments, the amended soils were left in the growth room (and watered daily) for 10 to 14 days before the eggplant seedlings were transplanted into the soils. No fertilization was provided during these experiments. After transplantation, the plants were allowed to grow for 5 to 6 weeks before they were harvested. The plants were harvested by cutting them off at soil level, immediately after which the fresh weight and leaf area were measured. The leaf area was measured twice for each plant with a portable leaf area meter (Li-Cor Model LI-3000, Lambda Instruments Corp., Lincoln, Nebraska) and the mean recorded. The stem of each plant was surface sterilized by soaking 5 minutes in a 1:5 Javex (5% sodium hypochlorite) solution and air dried in the laminar flow hood for several minutes. Several cross sections from each stem (2 cm thick) were transferred onto SPT media and the plates incubated at 24 C for one week, after which each plant was scored as being infected with *V. dahliae* or healthy. The experiment was repeated 3 times. The leaf area and fresh weight data were analyzed by a Kruskal-Wallis Test and the multiple comparisons were done by a Student-Newman-Keuls test. The plant infection data were analyzed by the χ^2 Test. The SigmaStat™ statistical program was used to do the analysis.

5.2.6 pH Determination during Eggplant Bioassay

To determine whether the addition of 1% BM to soil during the eggplant bioassay experiments caused any change in the pH of the soil, the bioassay described above was repeated, with the exception that only 1 Kg of soil (amended with 10 g BM and/or 10 mg MS) was prepared for each treatment, and there were 13 plants per treatment instead of 20. The pH of the soil was sampled by removing a 10 g sample of soil from one pot and placing 5 g into each of 2, 25 mL Erlenmeyer flasks. Each flask received 10 mL of DW and was shaken at 175 rpm for 2 hr at room temperature. The soil solutions were centrifuged at 2900 g for 10 min at room temperature and the pH of the supernatant was recorded. The first pH determinations were done on the same day that the soil and amendments were prepared, and subsequently at 4, 7, 10, 14, 19, 28, 45 and 67 days afterwards. The eggplant seedlings were transplanted into the pots 20 days after the soils were first prepared. The plants were harvested after 6 weeks of growth, as described above.

5.3 Results

5.3.1 Addition of Various Amendments to Soil

The survival of MS buried for 44 days was reduced to zero by the addition of 1% BM to the soil. In contrast, MS buried for the same time period in soil amended with forest soil or manure exhibited over 80% survival. Survival in the soil amended with Lundy soil was reduced to 69% and survival in the control treatment was 64%. The boxes which contained the BM amendment were observed to have a very strong ammonia-like smell and the soil was covered with fungal mycelium. The other treatments exhibited no such odour or visible fungal growth.

In a subsequent experiment using the same amendments, MS buried for 11 days survived equally well in all treatments and MS survival remained over 80%. After 18 days burial, however, the survival of MS buried in the boxes containing either FBK or BM had dropped to 68 and 30%, respectively. This was a significant reduction ($p=0.008$)

compared with the other treatments, in which survival was >94%. When the MS were buried in soil amended with 0.5% BM for 32 days, survival was reduced to zero. MS survival in the boxes receiving FBK and chitosan was significantly reduced compared to that of the boxes receiving Muskie, seaweed or no amendment (Fig. 5.1).

5.3.2 Amendment of Soil with Various Concentrations of Bloodmeal

Concentrations of BM less than 1% w/w were not successful in reducing the survival of MS over a 10 day period. When the soil was amended with 1% BM, however, MS survival dropped to zero by 6 days of burial while the survival of MS in all other treatments remained above 90% (Fig 5.2A). Reductions in MS survival in the 1% BM treatment were also observed when the MS were not in physical contact with the amended soil but were suspended in the headspace above the soil. The mortality of MS suspended in the headspace above the soil amended with 1% BM was delayed 4 days compared to those which were buried in the amended soil (Fig 5.2B).

5.3.3 Amendment of Soil with Sterilized Bloodmeal

Survival of MS was found to be 100% after burial for 24 h in soil plus BM, soil plus sterilized BM, sterilized soil plus BM and sterilized soil plus sterilized BM. A second set of packets were buried in the soils 3 days after incorporation of the amendments. After 4 days of burial in the soils, there was no reduction in the germination of the MS in any of the treatments however, there was a significant reduction ($p<0.001$) in the diameter of 4 day old colonies in the treatments which contained non-sterilized BM. The mean diameter of 4 day old colonies (in mm) was 4.3 and 4.2 respectively for the sterile soil plus sterile BM and soil plus sterile BM treatments and 3.5 and 3.2 respectively, for the soil plus BM and the sterile soil plus BM treatments. When the final set of packets were buried, the BM had been incubating in the soil for 10 days. MS from these packets were recovered after burial for 4 days. The survival of MS in treatments containing non-sterile BM was zero compared to >98% in the other 2 treatments.

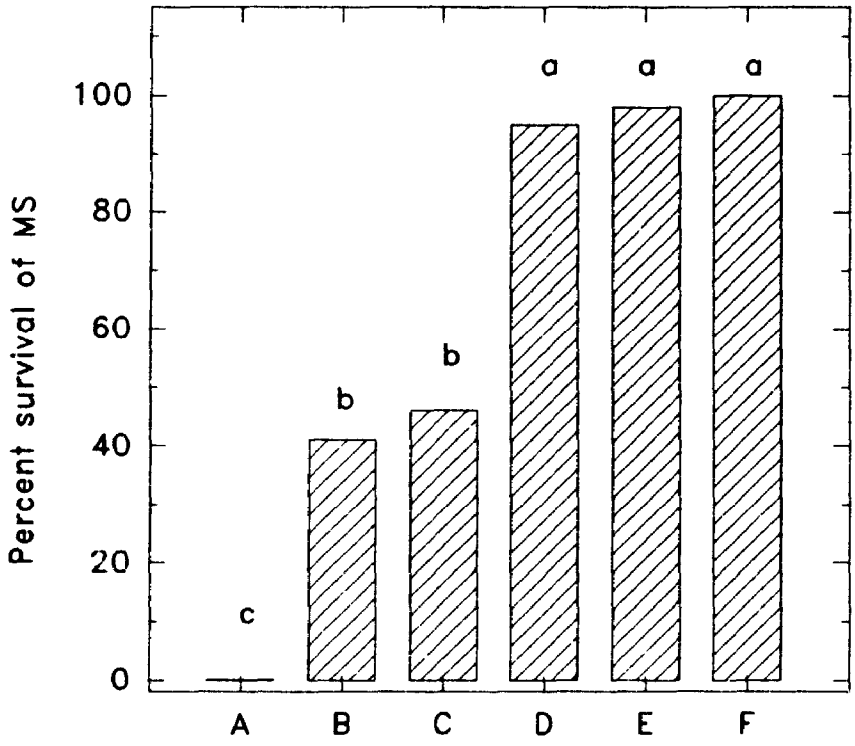


Figure 5.1
The percent survival of MS after burial for 32 days in soil amended with: A 0.5% Bloodmeal, B 1% fish Bone Kelp Meal, C 1% Chitosan, D 1% Muskie Fish Emulsion, E 1% Seaweed, F No Amendment. Bars followed by the same letter are not significantly different according to Student-Newman-Keuls test at p=0.001.

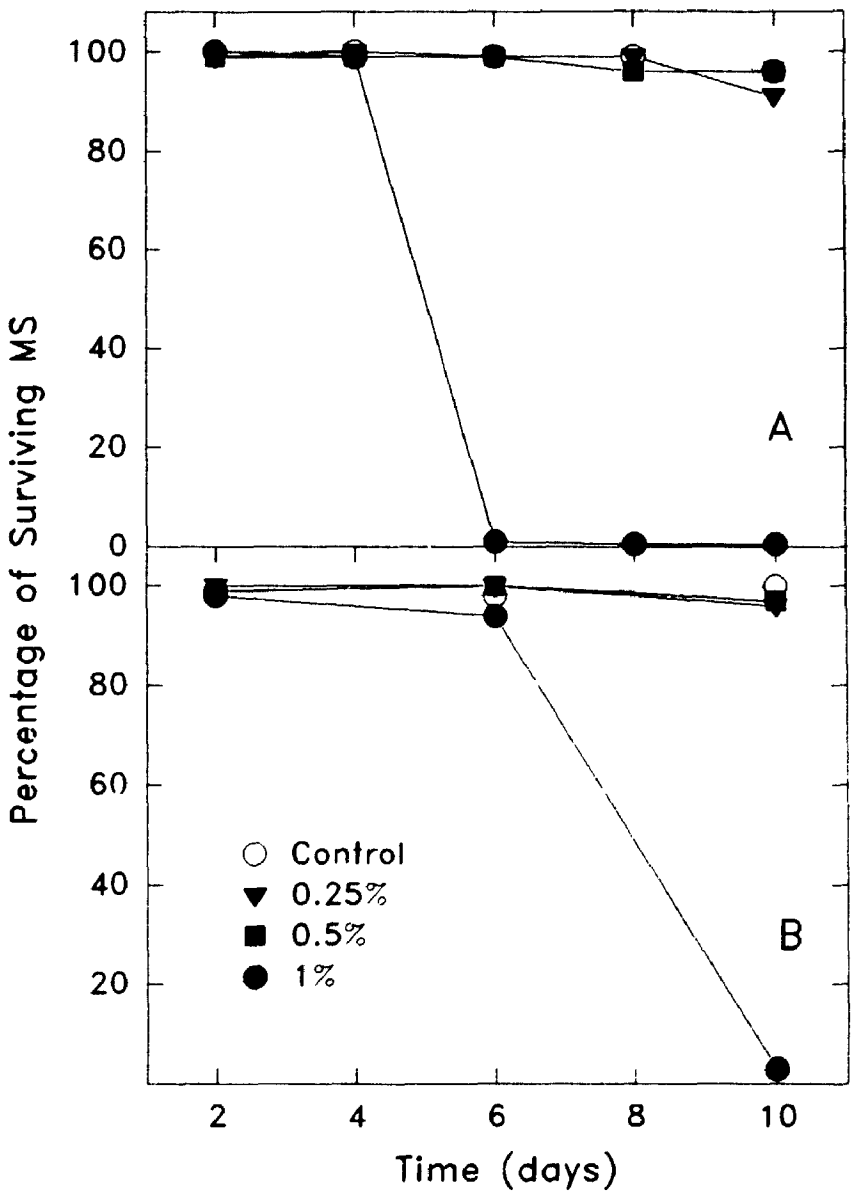


Figure 5.2
The percent survival of MS which were buried in (Fig. A) or suspended above (Fig. B) soil (Control) or soil amended with either 0.25%, 0.5% or 1% Bloodmeal for 2 to 10 days duration.

In a parallel experiment, the BM was allowed to incubate in the soil for a week prior to the burial of the packets. The survival of MS in treatments containing non-sterile BM fell to zero after 2 days burial compared with 94 and 93% after 2 and 5 days burial, respectively, in the sterile soil plus sterile BM treatment. When buried in soil plus sterile BM, survival of MS was reduced to <76%.

5.3.4 Amendment of Soil with Urea, Ammonium Acetate or Ammonium Sulphate

MS buried for 1 day in the 0.5% urea or 0.5% ammonium acetate treatment produced significantly smaller colonies compared with all the other treatments (Fig. 5.3). When the diameter of the 4 day old colonies was analyzed, the treatment had a significant effect upon colony diameter ($p<0.001$). The survival of MS buried for 1 day was 80% in the 0.5% ammonium acetate treatment and 90% in the urea treatment compared with >96% survival in the other treatments.

After 5 days burial in soil amended with either ammonium acetate (0.5%) or urea (1%) all MS recovered were found to be dead (Fig. 5.4). Ammonium sulphate (1%) had no effect upon MS survival whereas 0.25% and 0.125% ammonium acetate lowered MS survival to 74 and 52% respectively.

5.3.5 Eggplant Bioassay

Eggplant seedlings transplanted into the soils on the same day that the BM and MS were added to the soil began to wilt and rot and by 10 days, all the seedlings in the BM treatments were dead. The amended soils produced an ammonia-like odour. In subsequent experiments, the seedlings were not transplanted until more than 10 days after the soil treatments were prepared and phytotoxicity was not observed. There was a significant relationship between the infection of the plants with *V. dahliae* and the soil treatment ($\chi^2=35.83$, $p<0.001$). There was 90% infection in eggplants planted in soil which was infested with either MS or MS plus sterilized 1% BM compared to no infection in soil alone or soil plus 1% BM (Fig. 5.5). When soil plus MS was amended with 1% BM, however, the infection level was reduced to 20% but there was no reduction in infection

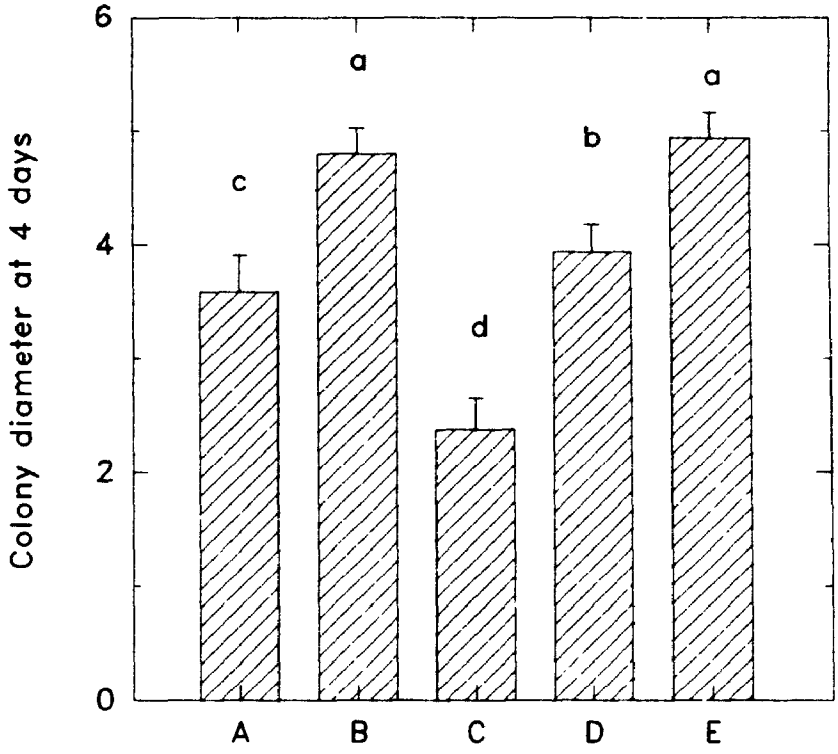


Figure 5.3
Mean diameter of colonies (± 95% C.L., N=62) produced by MS buried in soil amended with A 0.5% urea, B 0.5% ammonium sulphate, C 0.5% ammonium acetate, D 0.25% ammonium acetate, E 0.125% ammonium acetate for 1 day. Colony diameters were measured 4 days after plating. Bars with the same letter were not significantly different at p=0.002 by Student–Newman–Keuls test.

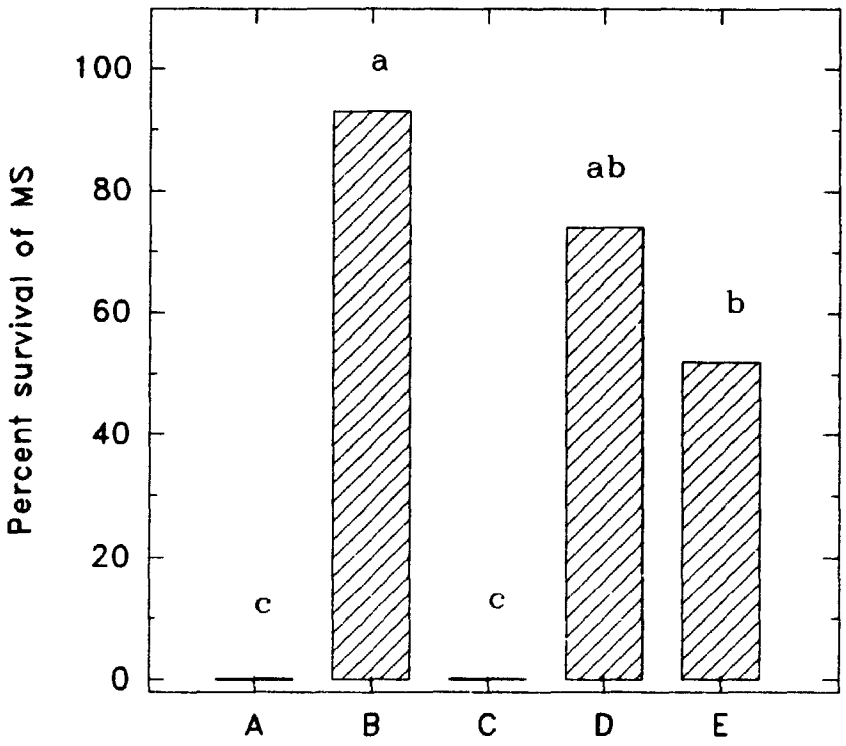


Figure 5.4
The percent survival of MS after burial for 5 days in soil amended with A 0.5% urea, B 0.5% ammonium sulphate, C 0.5% ammonium acetate, D 0.25% ammonium acetate, E 0.125% ammonium acetate. Bars with the same letter were not significantly different at p=0.002 by the Student–Newman–Keuls test.

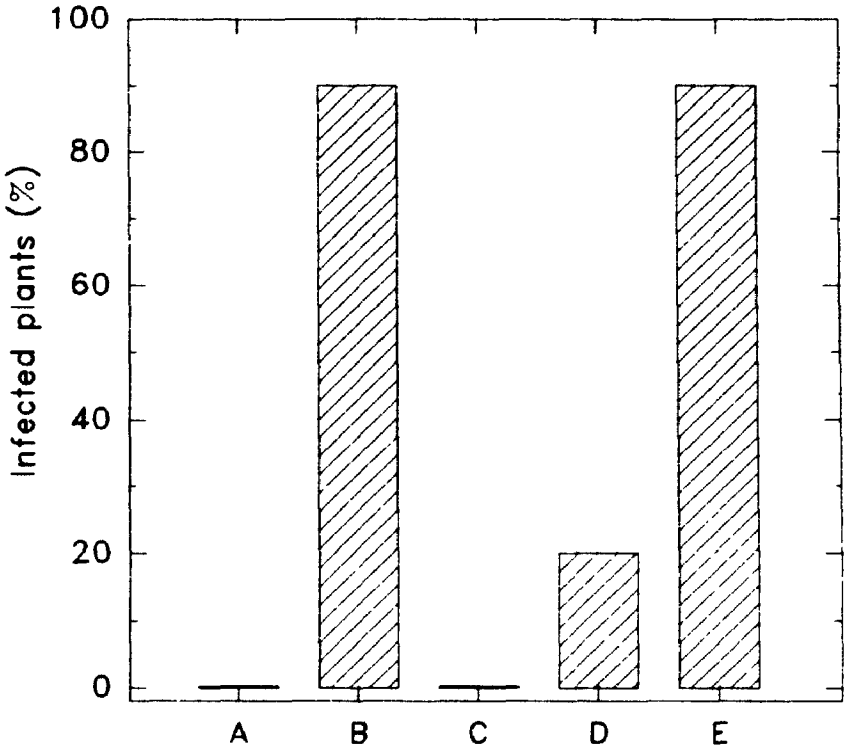


Figure 5.5
The percentage of plants which were infected with V. dahliae after 6 weeks growth in soil amended with: A No amendment, B microsclerotia, C 1% Bloodmeal, D microsclerotia + 1% Bloodmeal, E microsclerotia + 1% sterilized Bloodmeal.

when sterilized BM was used (Fig. 5.5). The addition of BM to the soil (whether sterilized or not) significantly increased both the fresh weight ($H=43.71$, $p<0.001$) and leaf area ($H=42.99$, $p<0.001$) of eggplants compared with soils receiving no BM (Fig. 5.6 and 5.7). Plants grown in soil amended with sterilized BM were also larger than those in soils without BM but they had significantly lower fresh weights and leaf areas than plants in soil receiving non-sterilized BM. The lowest plant fresh weight and leaf area was found in the plants which were grown in soil infested with MS. The appearance of the plants at harvest is shown in Plate 5.1. In both the treatment containing MS and that containing MS plus 1% sterile BM, chlorosis and the curling and browning of leaf margins characteristic of Verticillium wilt was expressed. Plants growing in the 1% BM and 1% BM plus MS treatments were extremely vigorous as indicated by their size and the dark green colouration of the leaves. In contrast, the control plants (grown in soil alone) appeared chlorotic and stunted.

5.3.6 pH Determination during Eggplant Bioassay

The result of the pH determinations is presented in Fig. 5.8. The soil pH was quite uniform at the start of the experiment, ranging from 5.3 to 5.6 in the various treatments on the day the soils were first prepared. The pH of soil amended with 1% BM, however, rose from 5.5 to a maximum of 8.2 within the next 14 days and then fell steeply and levelled off at approximately 5.0 after 28 days. This rise and fall of pH was also seen in the other soil treatments which contained BM, although the maximum pH was reached later and was not as high. In the soil amended with BM and MS, the pH reached a maximum of 7.3 after 19 days and levelled out at approximately 5.0 after 45 days. When the soil received MS and 1% autoclaved BM, the maximum pH of 7.0 was reached at 19 days and the pH levelled out at 5.6 after 45 days. In the soil alone and soil plus MS treatments, the pH remained near 5.5 to 6.0 for the first 30 days, after which it rose to near 7.0 and 6.5, respectively. There was a significant relationship between the infection of the eggplants and the soil treatment ($\chi^2 = 27.14$, $p<0.001$). The percent infection of eggplants in the soil plus MS

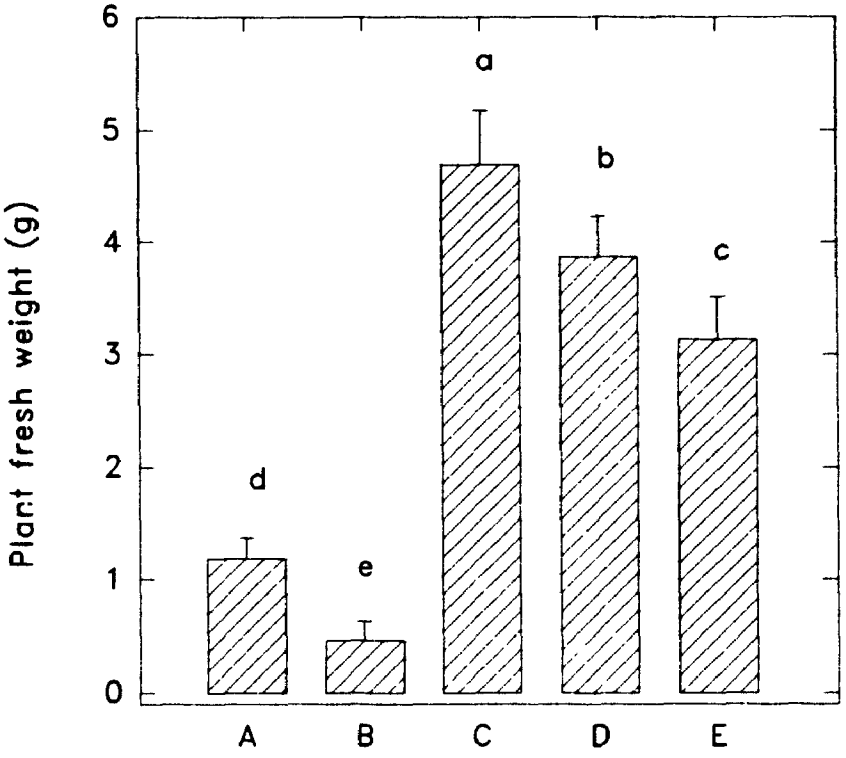


Figure 5.6
The mean fresh weight of eggplants (± 95% C.L., N=10) which were grown for 6 weeks in soil amended with: A No amendment, B microsclerotia, C 1% Bloodmeal D microsclerotia + 1% Bloodmeal, E microsclerotia + 1% sterilized Bloodmeal. Bars with the same letter were not significantly different at $p<0.001$ by Student -Newman-Keuls test.

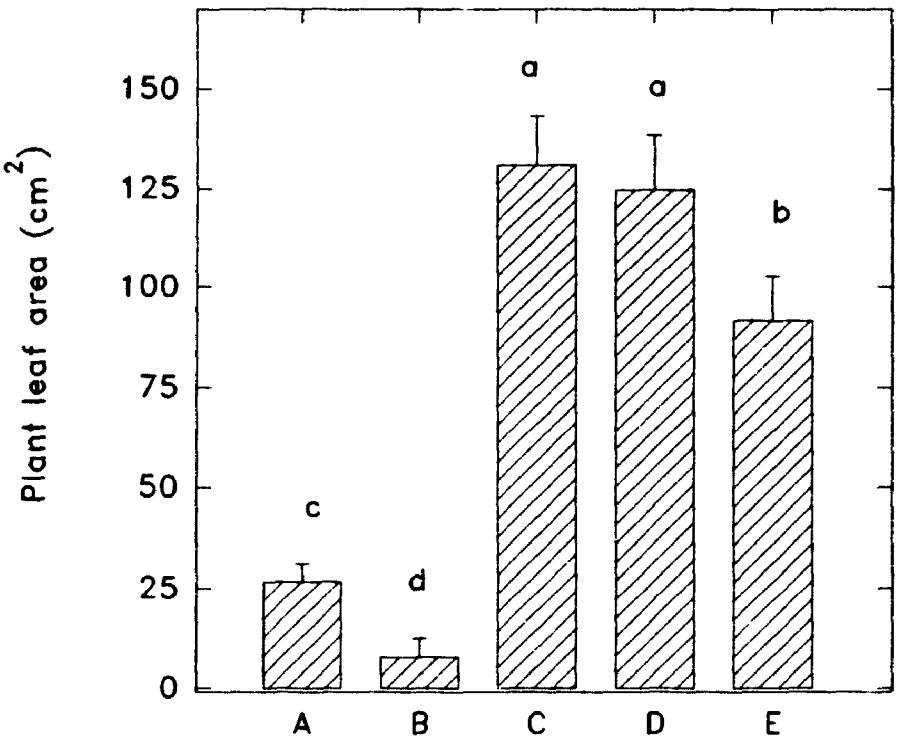


Figure 5.7
The mean leaf area (cm^2) of eggplants (± 95% C.L., N=10) which were grown for 6 weeks in soil amended with: A No amendment, B microsclerotia, C 1% Bloodmeal, D microsclerotia + 1% Bloodmeal, E microsclerotia + 1% sterilized Bloodmeal. Bars with the same letter were not significantly different at $p<0.001$ by the Student–Newman–Keuls test.

Plate 5.1

The appearance of eggplants (*Solanum melongena* cv. Imperial Black Beauty) after 6 weeks of growth in soil alone or soil amended with microsclerotia of *Verticillium dahliae* (+MS), 1% bloodmeal (+ 1% BM), microsclerotia plus 1% bloodmeal (+1% BM + MS) or microsclerotia plus 1% sterilized bloodmeal (+ 1% sterile BM + MS). The 3 week old seedlings were transplanted into the soils 14 days after the amendments had been added to the soil.

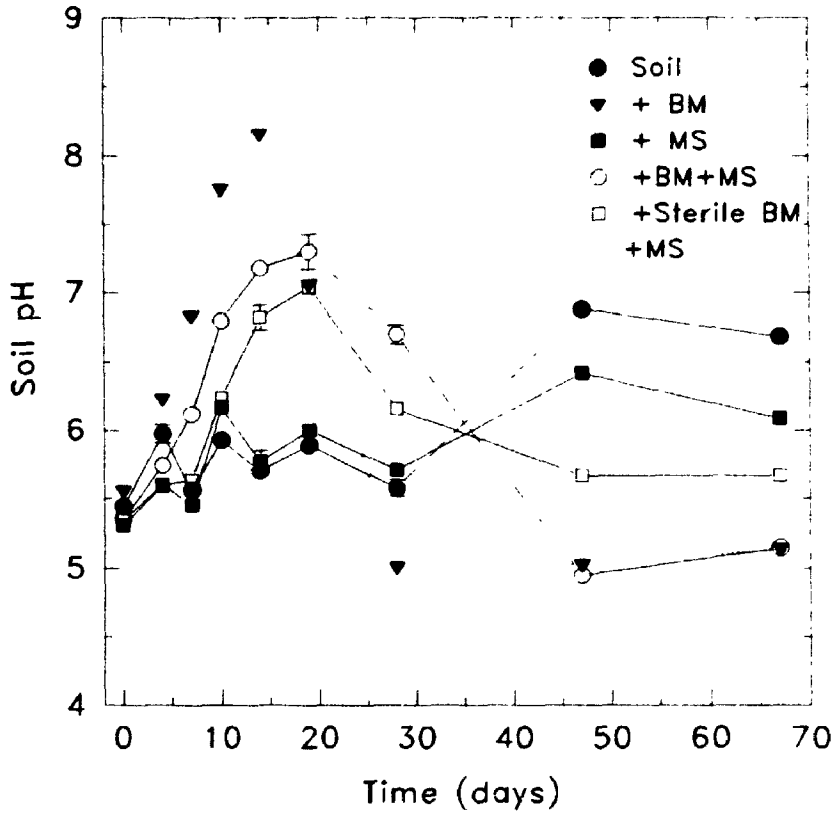


Figure 5.8
The change in pH measured in soil alone or soil which was amended with either 1% Bloodmeal, microsclerotia, 1% Bloodmeal + microsclerotia or 1% sterilized Bloodmeal + microsclerotia. Points represent the mean pH (± 1 S.E., N=2).

and the soil plus MS and 1% sterilized BM treatments was the highest and that of plants in the soil alone or soil plus 1% BM treatments was the lowest (Fig 5.9). As was seen in section 5.3.5 the infection of eggplants grown in soil infested with MS was reduced from 71% to 35% by the incorporation of 1% BM into the soil (Fig. 5.9). The fresh weight (Fig. 5.10) and leaf area (Fig. 5.11) were significantly higher ($p<0.001$) in treatments containing either BM or sterilized BM than in treatments not receiving BM.

5.4 Discussion

These experiments confirmed the results of Wilhelm (1951) regarding the efficacy of BM as an inhibitor of *V. dahliae* when administered to soil at a rate of 1% w/w. The addition of BM to the soil at this rate reduced the survival of MS buried in the soil to zero within 4-6 days. Concentrations of BM which were less than 1% were not effective in reducing the survival of MS buried for 10 days although 0.5% BM reduced MS viability after 35 days. Wilhelm (1951) reported that a rate of 0.1% BM resulted in 82% infection of tomato plants, whereas all the plants were healthy when there was 1% BM in the soil. The killing effect of 1% BM may have been attributable to a volatile fungitoxic factor, since MS which were suspended above the same soil were also killed within 6 days. Attempts to determine whether the killing effect was due to microorganisms present in the BM were inconclusive. Although the results presented in Section 4.3.3 showed that substantial reduction in MS survival occurred only in soils amended with non-sterile BM, subsequent experiments being carried out in our laboratory involving the amendment of soil with BM as well as fish meal, feather meal or bone and meat meal have shown that sterilization of the amendments does not prevent the killing effect but only delays it by several days. It seems probable that the initial experiments were terminated too early to detect the decline in survival of MS exposed to sterilized BM. The results of the latter experiments did confirm, however, that when both the soil and the BM are sterilized, there is no reduction in MS viability. This is a clear indication that there is microbial involvement in the release of the toxic volatile.

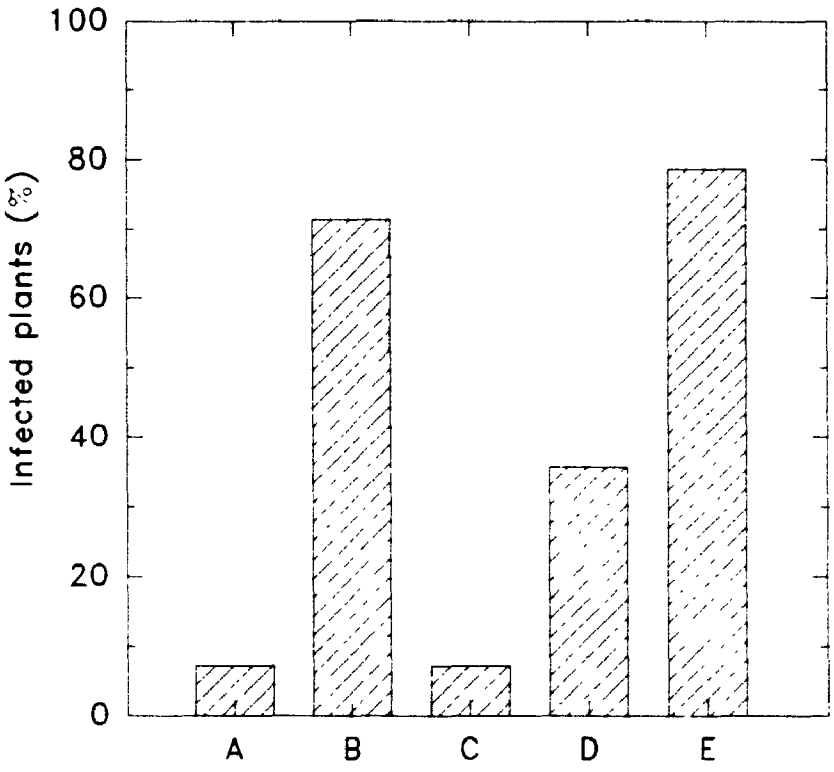


Figure 5.9

The percentage of plants which were infected with V. dahliae after 6 weeks growth in soil amended with: A No amendment, B microsclerotia C 1% Bloodmeal, D microsclerotia + 1% Bloodmeal E microsclerotia + 1% sterilized Bloodmeal.

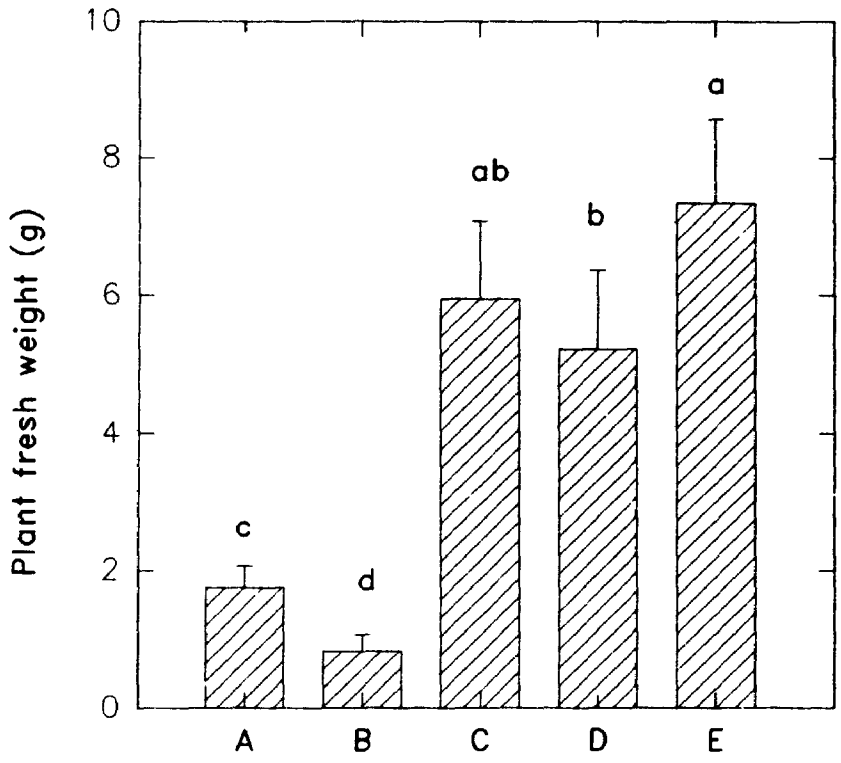


Figure 5.10
The mean fresh weight of eggplants (± 95% C.L., N=13) which were grown for 6 weeks in soil amended with: A No amendment, B microsclerotia, C 1% Bloodmeal, D microsclerotia + 1% Bloodmeal, E microsclerotia + 1% sterilized Bloodmeal. Bars with the same letter were not significantly different at $p<0.001$ by the Student–Newman–Keuls test.

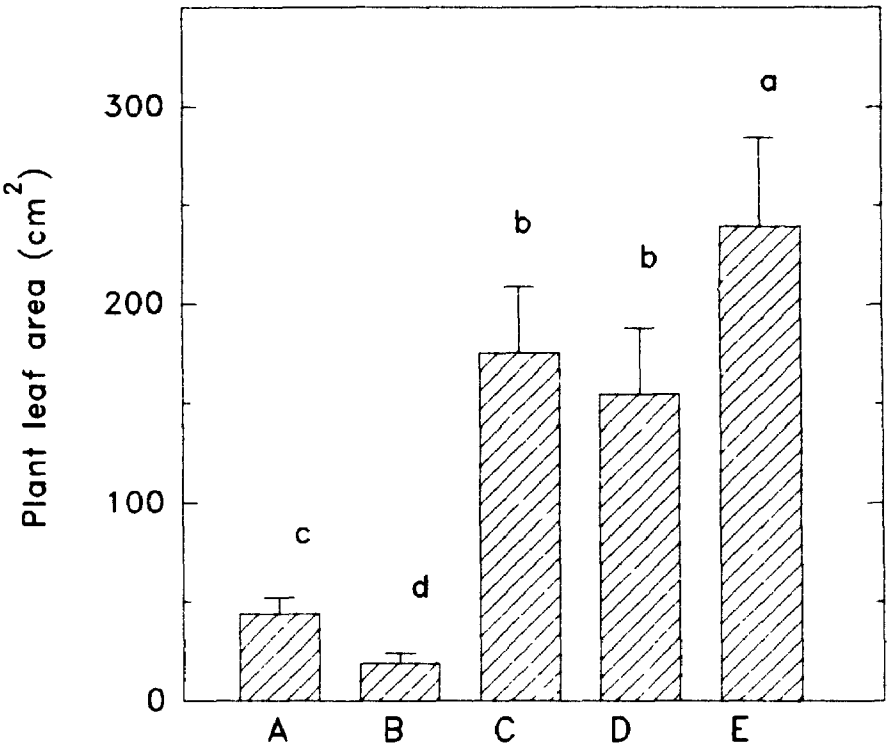


Figure 5.11
The mean leaf area (cm^2) of eggplants (± 95% C.L., N=13) which were grown for 6 weeks in soil amended with: A No amendment, B microsclerotia, C 1% Bloodmeal, D microsclerotia + 1% Bloodmeal, E microsclerotia + 1% sterilized Bloodmeal. Bars with the same letter were not significantly different at $p<0.001$ by the Student–Newman–Keuls test.

Our results indicated that when chitosan or FBK were added to soil at a rate of 1% w/w, they were effective in reducing MS survival to 40% and 43%, respectively. Wilhelm (1951) had reported that no tomato plants were infected with *V. dahliae* when he amended *Verticillium* infested soil with 1% fish meal. Chitin has been shown to be effective against *Rhizoctonia solani* (Sneh & Henis, 1972), *Fusarium, Aspergillus* (Schippers & Palm, 1973) and to produce a volatile fungistatic compound when added to soil (Hora & Baker, 1972).

In our experiments, amending *Verticillium*-infested soil with 1% BM reduced the infection of eggplants from 90% to 20%. There was also a growth-promotion effect of BM on the eggplants since the fresh weight and leaf area of eggplants grown in 1% BM amended soil was significantly higher than plants grown without BM. The eggplants grown in soil alone had developed signs of nutrient deficiency by the end of the experiment, in contrast to the plants receiving BM, which were dark green and healthy (see Plate 5.1). One consequence of BM amendment of soil was that the eggplants were not able to be transplanted into the BM amended soil until at least 10 days after the BM was added or phytotoxic effects occurred. The phytotoxic component, however, also eradicated any of the emerging weeds which germinated in the soils. When the pH changes in the soil were monitored, it was seen that the pH in BM amended soil rose sharply from 5.5 to 8.2 during that time and the pH did not return to original levels until 25 days. A rise and fall of pH has been commonly reported after amendment of soil with low C:N ratios, and it has been related to the processes of ammonification (as pH rises) and nitrification (as pH falls) in the soil (Tsao and Oster, 1981). The equilibrium between ammonia and ammonium in an aqeous medium is described by the equation $NH_3 + H_2O \leftrightarrow NH_4^+ + OH^-$ and at high pH, the equilibrium is shifted in favour of the production of ammonia. Ammonia has been shown to inhibit *Fusarium* and *Aspergillus* (Smiley, Cook & Papendick, 1970; Schippers & Palm, 1973), *Phytophthora* (Tsao & Oster, 1981), *Phymatotrichum omnivorum* (Rush & Lyda, 1982) and other soilborne fungal pathogens

(Pavlica et al., 1978). Ammonia has also been implicated as the mode of action of the biocontrol agent *Enterobacter cloacae* against *Pythium* and *Rhizoctonia* (Howell, Beier & Stipanovic, 1988). Ammonia gas is toxic in low concentrations (1-100 μg/g air) and has been reported to give 100% kill of sclerotia of *P. omnivorum* after 20 sec of exposure (Rush & Lyda, 1982). Several lines of evidence indicated that the lethal effect of BM towards MS was the direct effect of ammonia and not pH. First, the MS were still killed even when they were not in physical contact with the soil. Second, we had observed that BM amended soil contained in Magenta boxes had a very strong ammonia-like smell and the evolution of ammonia in BM amended soil has now been confirmed by subsequent experiments in our laboratory. This is in contrast to the results of Henis & Chet (1967) who reported that it was the pH effect and not the direct effect of ammonia which inhibited the sclerotia of *S. rolfsii* however, they did not test the survival of sclerotia exposed to ammonia as a volatile. Subsequent reports have disputed the idea that it is pH and not ammonia which is directly responsible for the reduction in survival of fungi (Punja & Grogan, 1982; Rush & Lyda, 1982). The exact mechanism by which ammonia is damaging to fungi is unknown, although it has been suggested that ammonia may have a direct effect on survival by disrupting the fungal membrane system or inhibiting the respiration of fungal cells (Rush & Lyda, 1982).

The results of our experiments involving the amendment of soil with various inorganic forms of nitrogen showed that burial of MS for 5 days in 1% urea and 0.5% ammonium acetate completely inhibited the germination of MS but that ammonium sulphate had no effect. These results were similar to the results of others (Henis & Chet, 1968; Henis & Katan, 1975; Tsao & Oster, 1981; Punja & Grogan, 1982). In a greenhouse study, the severity of Verticillium wilt of cotton was inversely related to the concentration of urea applied to the soil (Ranney, 1962). Henis and Chet (1968) reported that the addition of 0.2% w/w of urea or ammonium acetate significantly reduced the survival of sclerotia of *S. rolfsii* compared to unamended soil and that urea (which reduced survival to 0%) was

significantly more effective than ammonium acetate (which reduced survival to 45%). In laboratory studies on agar plates, ammonium sulphate had no effect on the germination of sclerotia of *S. rolfsii* but ammonium acetate reduced germination to zero (Punja & Grogan, 1982). We also found that urea and ammonium acetate were effective in reducing the survival of buried MS, however we did not find a significant reduction in MS survival when the rate of ammonium acetate was 0.25%. While our results showed that ammonium sulphate (at 1% w/w) was not effective in reducing MS survival, Wilhelm (1951) reported that only 8% of tomato plants grown in 0.5% ammonium sulphate amended soil were infected with *V. dahliae* compared to 81% in the unamended soil. The amendment of media with ammonium sulphate was also reported to inhibit the production of conidia in culture by *V. dahliae* (Duncan & Himelick, 1986). Henis and Katan (1975) point out that the results of experiments on the effects of inorganic N fertilizers are contradictory, possibly due to differences in the source of N used, the N requirements of the various pathogens, the physical and biological components of the soil used, the hosts used and other factors.

Chapter Six

General Discussion

The past several decades have brought many changes to agricultural research. Public, and hence political attention has become increasingly directed towards environmental issues and this is reflected in the changing emphasis of agriculturally related scientific research programs (Katan, 1993). As Keister (1991) noted, the empirical approach to plant growth which served well when fertilizers, water and land were abundant and inexpensive is no longer sufficient in an age when soil productivity and crop yields have to be maintained along with environmental quality. In addition to the environmental issues, other factors such as the development of pesticide resistance, explosions of non-target pests and the need for improvement in crop protection also raise concerns (Katan, 1993). Biological control methods are being advocated as cost effective and environmentally aware solutions for the problems presented by soilborne disease. While biocontrol agents do not provide the same rapid and effective control afforded by some agrochemicals, they have a longer residual activity because they do not cause the imbalance in the soil environment characteristic of chemical fumigation (Linderman et al., 1983). The soil environment is a complex ecosystem, with 90% of the biomass in root-free soil being composed of microorganisms (Lethbridge & Lynch, 1987). Development of biocontrol systems requires the manipulation of both indigenous and introduced soil microorganisms (Keister, 1991) and yet our knowledge of the basic biology of many soil inhabiting fungi is still limited.

In trying to identify ways to control *V. dahliae* in the field, there are some useful concepts which can be borrowed from the area of weed ecology. If the fungus is behaving ecologically like a weed, then its propagules (MS) are analogous to seeds. Like many weeds, *V. dahliae* reproduces prolifically, adopting a strategy of producing a large number of very small offspring to create a "seed bank" in the soil. In some plant species, seeds of differing size or morphology are produced (sometimes by the same plant) and may exhibit

distinct patterns of dormancy, germination or vigour (Cavers & Harper, 1966). Seeds may require very specific temperature regimes (stratification) or physical damage (scarification) in order to germinate. Understandably, it would have been next to impossible to study such aspects of seed biology if the seeds could not be isolated from the parent plant material or the soil. Snyder (1970) stated that it would be difficult to overemphasize the importance of techniques in studies on soilborne fungi, since new techniques have made it possible to become more specific in the study of the ecology of these fungi. For *V. dahliae*, the development of a procedure for the production of MS, their isolation from hyphae and conidia and their long term survival in storage was critical to allow the experiments described above to be conducted. The reduction in the variability of results and the additional data which were obtained by, 1) using MS of similar size in experiments, and 2) plating of individual MS after experimental manipulation, were very important modifications to the approach which has generally been taken to study the survival of MS of *V. dahliae* until this time. This procedure allowed both lethal effects (reflected by a failure of the MS to germinate and produce a colony of *V. dahliae*) and sublethal effects (reflected by either a reduction in the diameter of colonies produced or the association of colonies produced by the MS with other fungal species after plating) of experimental treatments to be quantitatively measured. In addition, the plating of individual MS permitted the recovery of specific mycoparasitic fungi from MS buried in soil. Bacteria may also play a significant role in the survival of MS (Baard, VanWyk & Pauer, 1981) and the procedures described here can be readily applied to the selection of such organisms. The procedure of plating individual MS could potentially reduce the immense amount of work involved in screening the many soil fungi and bacteria which are produced on plates made by dilution plating or direct plating of soil for obligate mycoparasites of *V. dahliae* (Adams & Fravel, 1993).

As discussed in the Introduction, soilborne plant pathogens such as *V. dahliae* can be particularly difficult to control because of their persistence in the field in the form of

resting structures like microsclerotia (MS). A one inch piece of diseased plant tissue may contain 20,000 to 50,000 viable MS, and in fields cropped repeatedly with potatoes, it has been estimated that there could be 50 million MS in the soil occupied by the roots of a single potato plant (Menzies, 1970). It is clear that any strategy for managing a monocyclic disease-causing agent like *Verticillium* must involve the development of procedures which reduce the populations of their resting structures. This can most likely be achieved through the targeting of specific adaptations for their survival or through the exploitation of a vulnerability they may possess. The relationship which is purported to exist between the presence of melanin in MS and their ability to survive and perpetuate disease provides a logical point of attack. If the propagules could be deprived of the protection afforded by melanin, their ability to survive under natural conditions should be decreased. The means to inhibit the production of melanin is available in the form of the chemical protectant, tricyclazole (TCZ). TCZ is registered as a pesticide under the trade name "Beam®" for the protection of rice against the blast pathogen *Pyricularia oryzae.* Unlike most pesticides, which act by poisoning the pathogen, TCZ inhibits the processes involved in penetration of the host plant (Howard & Ferrari, 1989). TCZ had also been tested on *V. dahliae*, but only as a tool to elucidate the pathway for melanin biosynthesis (Bell et al., 1976; Bell, Stipanovic & Puhalla, 1976; Wheeler et al., 1978). The use of TCZ as a means of modulating ecological fitness, however, has not been considered. TCZ has a number of desirable features for agricultural pest control. It is not a fungicide, since it does not affect the growth of the fungus at the concentrations required to inhibit melanin (Tokousbalides & Sisler, 1978) and it acts to specifically inhibit the reductase enzymes which are possibly unique to the pentaketide melanin biosynthetic pathway of certain fungal pathogens (Bell & Wheeler. 1986). TCZ is effective at extremely low levels (0.1 or 1 mg/L) and is systemic so it can be applied to either leaves or roots (Froyd, et al., 1976). It could be readily incorporated into potato production, as the plants are top-killed before harvest of the tubers and TCZ could be applied at that time in order to de-melanize the

MS before they become incorporated into the soil. A biocontrol agent of *Sclerotinia minor* is applied to diseased lettuce refuse in a similar manner, so that the sclerotia become infected with the mycoparasite before the plant material is plowed into the soil (Adams & Fravel, 1993).

The results presented in Chapter Four showed that TCZ blocked melanin biosynthesis in laboratory produced MS (Section 4.3.2) and that treatment with TCZ at 1 or 10 mg/L was effective in reducing the survival of MS which were buried in various types of soil (Section 4.3.7). There was evidence to suggest that mycoparasitic activity was involved in reducing their survival. Non-melanized MS, particularly those buried in the Lundy soil, were consistently found to be colonized by other microorganisms. This was particularly interesting because the Lundy soil came from a field which had a history of being disease free in a growing area where 80% of the plants sampled were routinely infected with *V. dahliae*. In contrast, when TCZ treated MS were buried in sand, where the microbial activity might reasonably be expected to be low, the MS were only infrequently colonized by other fungi and their survival was always high (above 84%). Over several experiments, the number of different fungi colonizing the MS was relatively small since the same few organisms were repeatedly associated with the MS recovered from soil. This indicates that, within the limited number of soil types tested, there might be certain specificity of these organisms for *V. dahliae*. There is also some direct evidence that MS of *V. dahliae* are colonized by microorganisms in soil. Ultrastructural studies of normally melanized MS buried in soil for up to 90 days revealed that the MS were colonized by fungi (prevalent at low soil pH) and bacteria (prevalent at high soil pH) and there was evidence of the deterioration of melanin (Baard, Van Wyk & Pauer, 1981). In the results presented here, only fungi were recovered from the MS, since they were plated onto SPT media, which contained antibiotics. One of the fungi found colonizing MS buried in soil amended with bloodmeal, the red pigment-producing *Penicillium*, was subsequently shown to have antagonistic activity towards *V. dahliae* (Section 4.3.9). The potential for control of

soilborne pathogns by mycoparasites has been well recognized for larger sclerotia. Hoes and Huang (1975) found that about half the sclerotia of *Sclerotinia sclerotiorum* isolated from field soil were nonviable and contaminated with the mycoparasites *Coniothyrium minitans* and *Trichoderma viride*. In three years of field trials, *C. minitans* was subsequently demonstrated to reduce Sclerotinia wilt in sunflowers and increase yield (Huang, 1980). *Sclerotinia minor*, the causal agent of lettuce drop, has been controlled in the field by *Sporodesmium sclerotivorum*, which is an obligate mycoparasite of sclerotia (Adams & Fravel, 1993). In addition to the mycoparasites of sclerotia mentioned above, Whipps (1991) also lists *Teratosperma oligocladum* and *Verticillium biguttatum* as obligate mycoparasites of sclerotia of *Sclerotium cepivorum* and *Rhizoctonia solani*, respectively and *Gliocladium* spp. and *Trichoderma* spp. as facultative mycoparasites with wide host ranges. *Gliocladium virens* has been commercialized under the name GlioGard™ and registered for use in the United States to prevent damping off of seedlings caused by species of *Pythium* and *Rhizoctonia* (Lumsden, Lewis & Locke, 1993). It has been suggested that parasitism by a biocontrol agent might be most effective when the strategy of control is centered around the reductio· of the initial inoculum in nonrhizosphere soil, as is the case for pathogens such as *Sclerotinia* and *Verticillium* (Fravel & Keinath, 1991).

The holistic view of plant disease includes the exploitation of various features of the pathogen, crop and biocontrol agent so that the system as a whole can be directed towards higher agricultural productivity (Adams & Fravel, 1993). With this in mind, the combination of targeting specific adaptations for survival of MS of *V. dahliae*, such as the blockage of melanin formation, with the manipulation of the soil environment in a manner which would enhance the activities of biocontrol agents and decrease the survival of MS would possibly be the best way to reduce disease. The survival of MS in which melanin had been inhibited by treatment with TCZ was shown to be reduced when they were buried in soil which had been intentionally inoculated with *Trichoderma, Talaromyces* or

the *Penicillium* which was isolated from MS buried in soil amended with either bloodmeal or fish, bone and kelp meal (Section 4.3.9). After 5 weeks burial, these MS were found to be colonized by the fungal antagonists.

Alteration of the soil microenvironment by the addition of organic amendments to the soil was also investigated as a way to reduce the survival of TCZ treated MS. Initial experiments, however, showed that the presence of melanin had no effect on the survival of MS buried in soil containing certain amendments. Bloodmeal (BM), for example was demonstrated to possess a startling ability to reduce the survival of all MS, whether they were melanized or not. The results indicated that a fungitoxic volatile (probably ammonia) was the factor directly responsible for the death of the MS. This was deduced from a number of observations, including the death of MS which were suspended in the headspace above BM amended soil (Section 5.3.2), the rise and fall of soil pH (Section 5.3.6), the failure of sterilized soil and sterilized BM to reduce MS survival (Section 5.3.3) and the reduction of the killing effect when the BM was incubated in the soil for 4 months prior to the burial of the MS (Section 4.3.8). The burial of MS for 5 days in soil amended with 0.5% urea or ammonium acetate also reduced their survival to zero. Urea, which is degraded by soil microorganisms into ammonia and carbon dioxide, has been shown to reduce the survival of several soilborne fungi at concentrations of 0.25% to 1% w/w and to produce ammonia at a level proportional to the amount of urea applied (Chun & Lockwood, 1985). Addition of large amounts of nitrogen-rich organic matter would be expected to significantly influence the microbial population and indeed, it was found that MS buried in organically amended soil became colonized by fungi that were not apparent in soil receiving DW alone (Plate 4.7). Subsequent experiments in our laboratory have revealed that other bioresiduals, such as fish meal, chicken feather meal and bone and meat meal, elicited the same effects. Use of such materials as soil amendments for the control of soilborne diseases is appealing. They are composed primarily of carbon and nitrogen and are non-toxic until acted upon and then they are converted into fertilizers. Several fish-

based and blood or meat-based amendments have been reported to produce yields comparable to chemical fertilizers in field trials with crops including beans, peas, carrots, corn, broccoli, cabbage and others (Blatt, 1991). Most of these organic products are not water soluble so they do not leach away from the site of application and do not cause environmental damage to water supplies. Finally, many of these amendments are waste byproducts of the seafood, livestock and poultry industries so there is no need for special production methods to create them. Some of the disadvantages of their use include the very high cost and the high level of material required. A rate of 1% seems to be the threshold for many of these amendments to exhibit disease inhibition (Wilhelm, 1955) and this can translate into 5-10 tons of dry matter per acre (Papavizas, 1975).

Despite the knowledge gained during the process of completing Ph.D. research, there seem to be more experiments to be done and questions to be answered at the end than there were at the start. The next logical step in this work, in my opinion, would require going back and repeating the field trials involving the application of TCZ to potato plants which initiated this project four years ago. At that time, the survival of MS could not be accurately determined. With the procedures outlined in this thesis, it should be possible to bury TCZ treated plant material or laboratory produced MS in nylon mesh packets over winter, recover them in the spring and determine their survival. While the effect of adding BM to soil on the level of infection of eggplants was tested in the growth room, there was insufficient time to test the fish, bone and kelp meal (FBK) and chitosan amendments, which showed potential when they were tested in the laboratory (Section 4.3.8). It might be interesting to carry the bioassays into the field for testing, either in microplots or into larger field plots. One of the potential difficulties with the application of BM in the field is the large volume of material required to apply it at the level which was effective under controlled conditions. Also, the results obtained when organic amendments are added to soil (especially under field conditions) are often variable (Papavizas, 1975) and are dependent upon many factors including the soil type. In the laboratory tests described

here, only the sandy loam soil was tested and the efficacy of the treatment in soil types with differing proportions of sand, silt and clay is unknown.

In conclusion, microbial ecology is an interdisciplinary science with the goal of unravelling the properties and activities of microorganisms in their natural environment (Stolp, 1988). Due to the complexity of the relationships within the microbial community and between microorganisms, plants, animals and their environment, it will require the cooperation of scientists with expertise in biochemistry, physiology and ecology, along with the application of the tools of molecular biology to achieve this goal. This project was centered around an autecological approach to answering some questions in microbial ecology and has succeeded in providing some new insights into the survival of microsclerotia of *V. dahliae* in soil. Hopefully, in the future, the tools which are developed and the knowledge which is gained through such autecological studies can be used to design and implement synecological studies which will lead us to a better understanding of microbial ecosystems.

Appendix 1

Recipe for Czapek-Dox (CDX) Agar Growth Medium

35 g Difco Bacto Czapek-Dox broth

15 g agar

10 mL Vitamins solution

10 mL Minerals solution

Combine these ingredients with 1000 mL distilled water (DW) and stir well. Autoclave 20 mins and dispense into Petri plates. Cool until solidified and store in the dark, inverted at 4 C until needed.

Vitamins Solution: add the following to 1 L sterile distilled water (SDW):

0.02 g each of yeast extract, thiamine HCl, adenosine, and P-aminobenzoic acid

0.01 g biotin

0.001 g folic acid

0.005 g each of pyridoxal HCl, nicotinic acid, and riboflavin

Bring the solution to pH 8.5 with NaOH and store at 4 C in the dark.

Minerals Solution: add the following to 1 L sterile distilled water (SDW):

0.308 g $MnSO_4.H_2O$

0.1 g $FeSo_4.7\ H_2O$

0.055 g $CaCl_2$

0.088 g $ZnSO_4.7H_2O$

0.004 g $CuSO_4.5\ H_2O$

0.0047 g $CoSO_4\ 7\ H_2O$

0.018 g $(NH_4)_6Mo_7O_{24}.4\ H_2O$

0.3 mL versenol

1 mL 1 N HCl

Store the solution in the dark at 4 C until needed.

Appendix 2

Recipe for Modified, Semi-solid CDX (MCDX) Growth Medium

35 g Difco Bacto Czapek-Dox broth

0.5 g dextrose

2.5 g pectin

2.5 g agar

10 ml Vitamins solution *

10 ml Minerals solution *

1000 mL Distilled Water (DW)

Bring half of the volume of DW to a boil and add the pectin slowly, stirring vigorously until dissolved. Add the remaining half of the DW and the other ingredients. Autoclave 20 mins and cool.

* Recipes for vitamin and mineral solutions given in Appendix 1.

Appendix 3

Recipe for Soil-Pectate-Tergitol (SPT) Medium *

1.5 g KH_2PO_4	2 g polygalacturonic acid
4 g K_2HPO_4	15 g agar
25 ml soil extract	1000 mL Distilled Water (DW)

Mix the all the ingredients except the agar with 750 mL of the DW and stir well. Adjust the pH to 7.0 with 10 N KOH. Add the agar and remaining 250 mL DW. Stir well and autoclave 20 mins. Before pouring into Petri dishes, add 1 ml of antibiotic solution per litre of medium and stir well. One litre of media should produce approximately 60 Petri plates which can be stored inverted, in a plastic sleeve, in the dark at 4 C until needed.

Soil Extract: Autoclave 1 Kg garden soil in 1 L of tap water for 30 mins. Decant and filter the suspension through several layers of cheesecloth. Extract can be dispensed into 25 mL aliquots and frozen until needed.

Antibiotic Solution: To 0.5 ml Tergitol NPX and 1 ml 95% ethanol add 50 mg each of chloramphenicol, chlortetracycline and streptomycin sulphate. Stir well and store in freezer until needed.

* Modified from Kantzes, J.E. (1980)

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