STUDIES ON SAPROBIC RHABDITID NEMATODES AND THEIR ASSOCIATED BACTERIA AFFECTING MUSHROOM CULTURE

BY

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A thesis submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy of the University of London and
for the Diploma of the Imperial College of Science, Technology and Medicine

September 1990

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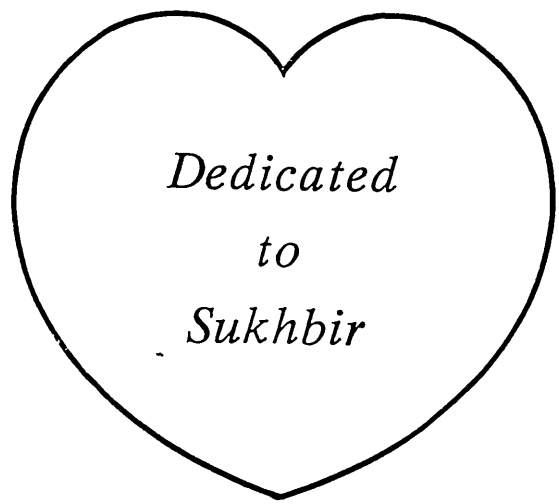
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ABSTRACT

Samples of compost and/or casing received from mushroom growers in the British Isles contained thirteen species (eleven genera) of rhabditoid nematodes. This research has concentrated on the most common of these, *Caenorhabditis elegans*. A simple method, based on the use of agar as a cover-glass support for mounting nematodes was developed and also an optimum procedure for killing, fixing and mounting parthenogenetic adult female *C. elegans* for taxonomic identification devised.

Bacteria associated (both externally and internally) with a strain of *C. elegans* were isolated and identified. In monoxenic cultures, bacterial food source and temperature affected reproductive capacity of parthenogenetic female *C. elegans*. Bacteria affected the migration of *C. elegans* on agar plates; the extent to which this migratory behaviour was changed depended on the species and on the age of the bacterial colony. The role of both the diffusible and/or the volatile substances produced by bacteria in the attraction of nematodes was studied.

The effects of monoxenically mass-produced *C. elegans* on *Agaricus bisporus* (strain U3) were assesed in mushroom growth chambers. *C. elegans* did not multiply in well-prepared, pasteurized, spawned compost, but it reproduced rapidly in casing material. When inoculated in casing, *C. elegans* had far-reaching effects on mushroom crops. It not only delayed the onset of mushroom production and disrupted the growth pattern of crops, but contributed to discolouration of mushrooms, caused some to become physically distorted and, most importantly, resulted in significant yield losses.

The bacteria isolated from *C. elegans* (especially *Enterobacter amnigenus, E. cloacae* and *Serratia liquefaciens*) inhibited the mycelial growth of *A. bisporus* in agar cultures. Bacteria also contributed to yield losses and quality deterioration; however, the presence of *C. elegans* resulted in much greater effects. Morphological distortion of sporophores was only observed when *C. elegans* was present. The nematodes significantly reduced the incidence of the bacterial blotch pathogen *Pseudomonas tolaasii* on sporophores.

ACKNOWLEDGEMENTS

It gives me immense pleasure to express gratitude to my supervisor, Paul Richardson for his valuable guidance, constructive criticism and keen interest in my project. His meticulous editing ability and supportive attitude to my quixotic ideas will be remembered with everlasting memory.

I am grateful to my external supervisor, Dr Denis Wright (Imperial College, Ascot, Berks) for his help and constant encouragement throughout the course of this study. I also thank Dr Chris Payne for his interest in the project and Dr Keith Sunderland for his ever-helpful attitude.

I feel contented to have this opportunity to express my sincere thanks to Dr M. R. Siddiqi and Dr David Hunt (CAB-International Institute of Parasitology, St. Albans) for their help during taxonomic studies of nematodes. It is my privilege to thank Dr Paul Hand for help and discussions during identification of bacteria.

I thank Phil White, Richard Gaze, the ADAS staff and mushroom growers who sent me the samples of infested material that formed the basis of this interesting project. All staff at the Mushroom Unit are thanked for their help during trials and contributions from all my collegues are thankfully acknowledged.

I sincerely thank Miss Cathy Townley and Mike Ledieu for excellent computing facilities and Rodney Edmondson (always willing) and Dr John Fenlon for statistical advice. I am grateful to Andy Smith (ever cordial) for photography, Brenda Barnard for art work (Figures 4.1, 4.2, 7.1 & 7.2), Rhona Floate and Jackie Van de Poll for literature and Sue Bewsey (service with a smile and perfection) for help during typing.

I can not describe the untiring help by Sukhbir without which this task would have been simply impossible. I feel guilty for Parry who sacrificed his Dad for the cause he did not know. I shall be failing in my duties if I do not remember my brother-in-law, Avtar at this moment of joy, who was there whenever I needed him during this ordeal. Above all, the moral support from parents and other family members was necessary. I must thank all my friends

who bore with me during this long-lasting task.

I am grateful to the Committee of Vice-Chancellors and Principals of British Universities (Royal Commission for the Exhibition of 1851) for the offer of a most prestigious scholarship.

(Parwinder Singh Grewal)

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CHAPTER 1

General introduction

The mushroom *Agaricus bisporus* (Lange) Imbach is the single most important protected crop in the UK. There are more than 500 commercial mushroom farms which together produce about £150m worth of crop every year (Anon., 1989). Mushrooms are grown in purpose-built houses with partially to completely controlled environmental conditions (Flegg *et al.*, 1985). The mushroom substrate is prepared from composted wheat straw which is pasteurised before being 'spawned' by the fungal mycelium. The colonised substrate is covered with a mixture of peat and chalk ('casing material') which stimulates sporophore initiation and the mushrooms are produced in distinct flushes.

The dark and humid environment that allows rapid mushroom growth also favours the development of many pests and diseases. The most serious insect pest problems are posed by three groups of Diptera - Phoridae, Cecidomyiidae and Sciaridae. In each case, the fly larvae feed on mycelium. Adult phorids and sciarids also annoy mushroom pickers (White, 1985) and act as vectors of nematodes and diseases (Haglund & Milne, 1973; Fletcher *et al.*, 1989). Bacterial blotch caused by *Pseudomonas tolaasii*, and the 'dry bubble' caused by *Verticillum fungicola* var. *fungicola*, are two common and serious diseases affecting mushroom culture (Fletcher *et al.*, 1989).

However, nematodes are the most important pests of mushrooms due to their microscopic size, rapid multiplication and insidious damage. Apart from direct damage to the mushroom mycelium, nematodes are also known to spread bacterial blotch disease in the mushroom beds (Steiner, 1933). The nematodes that affect mushroom culture are of two types: mycophagous and saprobic (or saprophagous). Mycophagous nematodes e.g. *Aphelenchoides composticola* Franklin and *Ditylenchus myceliophagous* Goodey, feed and destroy mushroom mycelium with the aid of a needle-like organ, the stylet. This group posed a

major threat to mushroom cultivation in the UK during 1950s-1970s (Goodey, 1960). However, with the introduction of modern pasteurization techniques and the construction of purpose-built mushroom growing houses, this problem has been alleviated considerably (Hesling, 1979).

In contrast, during the last decade saprobic nematodes that usually feed on decaying plant material, seem to have acquired pest status in the industry. Many reports concerning saprobic rhabditid nematodes causing mushroom crop losses have appeared from countries with modern mushroom growing facilities including UK, USA, Canada, Australia and New Zealand (Ingratta & Olthof, 1978; McLeod & Nair, 1981; Ross & Burden, 1981; Sanderson *et al.*, 1981; Kaufman *et al.*, 1984).

However, there are conflicting reports of the significance of rhabditid nematodes in the mushroom industry (Hesling, 1966, 1979; Gerrits, 1973; Ross & Burden, 1981; Kaufman *et al.*, 1984) mainly because the stoma (buccal cavity) of such saprobic rhabditid nematodes is structurally incapable of causing any significant physical damage to the mushroom mycelium. Furthermore, the pathogenicity of such nematodes to mushroom is difficult to demonstrate because eelworms need live bacteria as food for maturation and reproduction (Nicholas, 1984) and because *A. bisporus* requires the activity of bacteria for its sporophore induction (Eger, 1972).

It has been suggested in the past that the toxins produced by nematodes and/or bacteria may inhibit the mycelial growth of the mushroom (Cairns & Thomas, 1950; Sarazin, 1951; Kux & Rempe, 1954). Kaufman *et al.* (1983) observed that the extracts from compost infested with *Caenorhabditis elegans* suppressed the mycelial growth of *Agaricus brunnescens*. Furthermore, they observed that an extract from uninfested compost also inhibited mycelial growth but to a lesser degree. They therefore suggested that the role of compost bacteria and other microorganisms may be important. Bloom & Levine (1985) suggested that bacteria associated with saprophagous nematodes may contribute to mushroom yield losses and the bacteria are spread by the activity of nematodes.

However, it is evident from the literature (Chapter 2) that the qualitative analysis of bacteria associated with saprobic rhabditid nematodes and

of the interactions between bacteria, nematodes and the mushroom mycelium have not been made. These interactions may be crucial in acquiring a better understanding of the processes of pathogenesis of saprobic nematodes to mushrooms. Furthermore, information on the species of nematodes involved (especially in the UK) and on their biology is lacking.

The present investigation aimed to determine:

(i) the species of rhabditid nematodes infesting the UK mushroom industry.

(ii) the species of bacteria associated with the commonest nematodes.

(iii) the relationships between bacteria and nematodes in agar cultures.

(iv) the effects of monoxenically mass-produced nematodes on *A. bisporus*.

(v) the effects of bacteria on the mycelial growth of *A. bisporus* in agar cultures

(vi) the relative effects of bacteria and/or nematodes on the yield and quality of mushrooms.

(vii) the effects of nematodes on the spread of the bacterial blotch pathogen *P. tolaasii*.

CHAPTER 2

Review of literature

This chapter provides a brief review of literature on saprobic rhabditid nematodes infesting mushroom culture. The available information is divided into four categories: nematode species, effects on mushroom production, sources of infestation and processes of pathogenesis.

2.1 Nematode species

Appendix 2.1 lists the species of saprobic rhabditoid nematodes associated with mushroom culture all over the world. So far, 69 species (34 genera) of Rhabditoidea and 15 species (10 genera) of Diplogasteroidea have been identified from mushroom compost/casing material during cropping and/or during the preparation of the substrate. Nineteen species belonging to 14 genera have been implicated as detrimental to the mushroom production.

2.2 Effects on mushroom production

Steiner (1931) was the first to appreciate the possible effects of non-stylet bearing free-living nematodes on mushroom production. He suggested that a saprophagous nematode *Cruznema tripartitum* (=*Rhabditis lambdiensis)* acted as a carrier of a bacterial disease in mushrooms. Apart from the role of nematodes as vectors of bacteria, Cairns and Thomas (1950) added another dimension to their effects on mushrooms. They suggested that the combined activity and/or metabolic products of eelworms and bacteria may cause inhibition of the normal development of fruiting bodies of the mushrooms. Their suggestions seemed to stimulate interest in saprophagous nematodes and resulted in conflicting views of their significance in the mushroom industry.

In the next few years many reports appeared on the occurrence of

Rhabditis-like nematodes on many mushroom farms (Thomas & Mitchell, 1951; Cairns, 1952; Lambert & Ayers, 1953; Moreton, 1953; Kux & Rempe, 1954; Ritter, 1957 and Staniland, 1957). Although no experimentation was done, most of these reports associated saprophagous nematodes with a degenerative disease condition of the mushroom wherein a sudden break-down of mycelium resulted in considerable crop losses.

van Haut (1956) and Blake & Conroy (1959) were the first to demonstrate that the saprophagous nematodes (*Rhabditis* spp.) extracted from diseased mushroom beds damaged mycelium cultured on grain ('spawn'). In contrast, Moreton *et al.* (1956) were unable to prove that *Panagrolaimus* sp. was harmful to mushroom mycelium growing on sterile compost.

Cayrol (1962) reported that saprophytic nematodes principally belonging to the genus *Rhabditis* can produce secondary attacks on mushrooms by transporting bacteria, by feeding on compost or by producing secretions harmful to the mushrooms. Juhl (1966) showed that *Cruznema tripartitum (=Pelodera lambdiensis), Acrobeloides* sp. and *Rhabditis* sp. reproduced and destroyed mushroom mycelium growing on malt extract agar. In contrast, Hesling (1966) reported that when added to spawned mushroom compost, microphagous saprobic nematodes, *Coarctadera cylindrica* (*=Pelodera cylindrica*), *Mesorhabditis spiculigera* and *Acrobeloides butschlii* did not affect mycelial growth or compost pH (however, in the same work the existence of a significant relationship between mycelial decline and logarithmic eelworm (*M. spiculigera*) density in compost and in casing is presented, p 483). Furthermore, he observed that *M. spiculigera* added to the casing had no effect on mushroom yield but the mushroom 'flushes' were less pronounced. McLeod (1968) similarly reported that *Panagrolaimus* sp. did not significantly affect mushroom yield, number of sporophores produced or duration of cropping.

Gerrits (1973, 1980) supported the views of Hesling (1966) and McLeod (1968) and considered rhabditid nematodes of secondary importance. In contrast, Ingratta & Olthof (1978) reported a positive correlation between nematode populations (predominantly *Acrobeloides* sp., *Rhabditis* sp. and *Choriorhabditis* sp.) at the time of casing and mushroom yield and suggested that saprophagous nematodes may spread mushroom 'pinning' bacteria (*Pseudomonas* sp.) in the

casing layer and induce uniform fruiting.

From 1974-84, many reports appeared demonstrating the detrimental effects of saprophagous nematodes on mushrooms. Han *et al.* (1974) observed complete disintegration of mycelia growing in compost whereas Bloom and Bookbinder (1978) reported mycelial growth decline and sizable yield losses due to the infestations of *Rhabditis*-like nematodes in many mushroom farms. Klingler and Tschierpe (1980) found a variation in response of two strains of the cultivated mushroom to infestation by *Rhabditis (Choriorhabditis)* sp. They observed a significant negative correlation between the initial population of nematodes and the yield of one of the strains (A 3.2) whereas the other strain (A 8.8) was resistant. On the other hand, McLeod and Nair (1981) observed variations in the effects elicited by nematode isolates; four out of the six isolates of *Rhabditis (Rhabditoides* or *Rhabditella*) sp. reduced mushroom yield when inoculated into compost at spawning and into the casing at casing.

Sanderson *et al.* (1981) showed that when inoculated in casing, *Mesorhabditis* sp. significantly reduced mushroom yields and were the first to demonstrate the combined effects of saprophagous nematodes and bacteria (*Pseudomonas cichorii*) on mushroom quality. They found that 45% of the mushrooms harvested from nematode infested boxes during the first flush showed 'bacterial browning'. Ross and Burden (1981) reported a severe problem associated with rhabditid nematodes (predominantly *Rhabditis (Cephaloboides)* sp.) on a commercial mushroom farm. After extensive experimentation, they concluded that saprophagous nematodes can cause significant yield losses to the mushroom and that their effects varied along a scale which ranged from complete suppression of the mushrooms at one end to complete suppression of the nematodes at the other. Furthermore, the variable susceptibility of mushroom strains to attack by free-living nematodes was once again demonstrated; white strains showed a considerable degree of resistance whereas off-white strains were susceptible.

Chongti (1982) reported that *Pelodera teres* and *M. spiculigera* were particularly pathogenic to the cultivated mushroom. Kaufman *et al.* (1984) observed that the initial compost moisture was a critical factor influencing the potential damage caused by *Caenorhabditis elegans* infestations and in a high

moisture compost (78%) all four cultivated strains (cultivars) of *Agaricus brunnescens* (=*A. bisporus*) were adversely affected.

2.3 Sources of infestation

A large number of nematode species are shown to infest the mushroom substrate ('compost'), during its preparation (Choleva, 1966, 1968; Farkas, 1972, 1977; Farkas & Balazs, 1975). Pasteurization generally eliminates most of these nematodes (Hesling, 1972, 1979). However, compost becomes a source of nematode infestation if: (i) it is not pasteurized (eg. in India and some other neighbouring countries the compost is prepared by the 'long' method and is not pasteurized, Grewal, 1989); (ii) pasteurization is incomplete; or (iii) it is reinfested.

The second and perhaps the most important source, is the casing material which is used to cover mushroom beds to initiate fructification. Casing material is generally not sterilized because of the desirable attributes of sporophore-inducing bacteria (Eger, 1972). Moss-peat which is most widely used as the main ingredient of casing material may harbour large numbers of saprophagous nematodes (Farkas & Koronczy, 1974; Hesling & Gaze, 1975; Khair & McLeod, 1978). Alternate materials used for the preparation of 'casing' such as spent-compost, hotbed soil, sand or field soil have been shown to carry potentially harmful mycophagous and/or saprophagous nematodes (Farkas & Koronczy, 1974; Thapa *et al.*, 1981; Grewal & Grewal, 1988).

Cayrol (1962) reported that nematode infestation may also occur either through contaminated spawn or through the soil upon which the beds are made. According to Moreton and John (1957), eelworms may get into the beds through the woodwork of shelves and trays. Hesling (1962) suggested that eelworms are spread throughout the mushroom house not only by implements, containers, hands and the footwear of workers, but in the first place by flies and mites. Jankowska (in Bukowski, 1967) reported that eelworms may also be introduced into a mushroom house in the water used to sprinkle the beds.

After extensive survey of 106 mushroom farms, Haglund and Milne (1973) concluded that neither farm workers nor wind-blown debris were the

major sources of contamination. Dipterous insects, principally Sphaerocerid flies, were the primary means by which the spawn-running rooms were contaminated with nematodes. Rinker & Bloom (1982) experimentally demonstrated that sciarid flies (*Lycoriella mali*) can phoretically disseminate *Caenorhabditis elegans* and *Cruznema tripartitum* (=*C. lambdiensis*).

2.4 Processes of pathogenesis

Although no experimental evidence is yet available, many theories have been suggested to explain the degenerative disease condition observed in cultivated mushrooms associated with saprophagous nematode infestations. Cairns and Thomas (1950) suggested that the combined effects and metabolic products of nematodes and bacteria may cause inhibition of the normal development of fruiting bodies of the mushrooms. Sarazin (1951) reported that nematode-free water leached from nematode infested mushroom beds caused a suppression in yield when it was applied to uninfested beds. Kux and Rempe (1954) concluded that the sudden breakdown of mycelium in heavily infested beds was probably due to the accumulation of toxins or ferments released by eelworms in the substratum. However, Hesling (1966) and Han *et al.* (1974) could not find any effects of nematode infestations on compost pH.

Kaufman *et al.* (1983) observed that extracts from compost infested with *C. elegans* suppressed mycelial growth of *A. brunnescens*. Furthermore, they observed that an extract from uninfested compost also inhibited mycelial growth but to a lesser degree. They suggested that the role of compost bacteria and other micro-organisms may be important. Cayrol and B'chir (1972) reported that in many cultures, *Dolichorhabditis dolichura* (=*Caenorhabditis dolichura*) and *Rhabditella* sp. are associated with specific bacteria that are necessary for their nutrition and the bacterial multiplication is enhanced by secretions from the nematodes. Bloom & Levine (1985) suggested that bacteria associated with saprophagous nematodes may contribute to mushroom yield losses and that the bacteria are spread by the activity of nematodes.

CHAPTER 3

Isolation and identification of nematodes

3.1 Introduction

Many species of saprophagous rhabditid nematodes are associated with mushroom culture (Appendix 2.1). However, the current incidence of these nematodes in the British mushroom industry is not known. In this part of the project, rhabditid nematodes infesting mushroom farms in the British Isles were isolated and identified. A new method was developed for the quick preparation of nematode mounts; the effects of several killing, fixing and mounting techniques on the taxonomic characters of parthenogenetic adult female *Caenorhabditis elegans* were also examined.

3.2 Materials and methods

3.2.1 Collection of samples

Samples of compost and/or casing material were acquired from various mushroom farms in the British Isles during 1987-88 (Table 3.1).

3.2.2 Extraction of nematodes and their preparation for identification

Nematodes were extracted from infested material using the Baermann funnel technique (Hooper, 1986a). Adult nematodes, were killed and fixed in hot Triethanolamine-formaldehyde (TAF, Courtney *et al.*, 1955) processed to glycerol by the glycerol-ethanol method (Seinhorst, 1959) and mounted in anhydrous glycerol on glass slides using pieces of agar as cover-glass supports.

Nematode larvae were inoculated on to plates of 3% (w/v) nutrient agar

Table 3.1 List of samples of compost and/or casing collected from mushroom farms in the British Isles

Farm locality	Code	Type of material
Eire		
Carbury, Kildare	Ca	casing
Drumree, Meath	Dr	casing
Dublin	Du	casing
England		
Bungay, Suffolk	Bu	casing
Chichester, West Sussex	Ch	casing
Isle of Wight	Iw	casing
Lee Valley, Hertfordshire	Lv	casing
Littlehampton, W. Sussex	Lt-1	casing/compost
Littlehampton, W. Sussex	Lt-2	casing/compost
Preston, Lancashire	Pr-1	casing/compost
Preston, Lancashire	Pr-2	casing/compost
Shackleford, Surrey	Sh-1	casing
Shackleford, Surrey	Sh-2	casing
Taunton, Somerset	Tn	casing
Wales		
Kilgetty, Dyfed	Wa	casing

(Oxoid Ltd) then allowed, together with the associated bacteria, to grow to adults (at 22°C). The larvae were then processed using similar methods of fixation and mounting. Nematodes were identified (Goodey, 1963a; Andrassy, 1983) using measurements from at least 20 specimens.

3.2.3 Development of a new method for mounting nematodes

(i) *Preparation of agar*

2 g of agar (Technical grade No. 3, Oxoid Ltd) and 50 mg of cupric sulphate ($CuSO_4.7H_2O$) was dissolved in I00 ml of distilled water by heating gently for 2 to 3 min. It was then autoclaved at 121°C for 15 min., allowed to cool and whilst still warm poured into Petri-plates.

(ii) *Preparation of nematode mounts*

1. Killed specimens were placed in a drop of mountant (water or fixative) on a glass slide.

2. A small quantity of the agar (prepared as above) was then taken from the Petri-plate with clean forceps and placed on a slide adjacent to the drop of mountant containing nematodes.

3. The agar was divided into three to five similarly-sized portions with forceps and the agar portions were arranged at equal intervals around the outer margin of the drop of mountant.

4. A clean cover glass was carefully placed on the drop of mountant so that it rested evenly on all the pieces of agar.

5. While observing the slide under a low power stereoscope the cover-glass was gently pressed with forceps, to the extent that it almost touched the nematodes.

6. Permanent preparations were then made by sealing the cover-glass

with Thorne's cement ("Glyceel").

Twenty females each of *C. elegans* and *Tylenchus davainei* were processed to glycerol (according to Seinhorst, 1959) and mounted on glass-slides using agar pieces or glass-wool as cover-glass supports. Measurements of body diameter at the anus and vulva were recorded and data were subjected to Analysis of Variance (ANOVA).

3.2.4. Effects of killing, fixing and mounting methods on taxonomic characters of adult female *C. elegans*.

(i) *Selection of nematode specimens*

The Taunton strain of *C. elegans* (Tables 3.1 & 3.2) was cultured on 3% nutrient agar (Oxoid Ltd) in Petri plates along with the associated bacterial flora at 22°C. Only similar pre-egg-laying parthenogenetic adult females (with fully developed eggs in the uterus) from 6-day-old cultures were selected for this study. Males were extremely rare in this isolate.

(ii) *Killing and fixing*

Nematodes were either killed in hot water and fixed in cold fixative or killed and fixed in hot fixatives according to Seinhorst (1966). Hot water-killed, unfixed nematodes served as controls. The following fixatives were used: (a) FA 4:1 (10 ml 40% formaldehyde, 1 ml glacial acetic acid, 89 ml distilled water); (b) FP 4:1 (10 ml 40% formaldehyde, 1 ml propionic acid, 89 ml distilled water); (c) TAF (7 ml 40% formaldehyde, 2 ml triethanolamine, 91 ml distilled water); (d) FG (8 ml 40% formaldehyde, 2 ml glycerol, 90 ml distilled water); (e) FAA (6 ml 40% formaldehyde, 20 ml 95% ethanol, 1 ml glacial acetic acid, 40 ml distilled water).

Water and fixatives were contained in separate glass test tubes and were maintained at 95°C in a water bath. Selected specimens, placed in a very small drop of saline (0.85% w/v sodium chloride) in a glass cavity block, were flooded with 4 ml of hot water or fixative to ensure rapid death and fixation of nematodes. For cold fixation of nematodes, excess water from the cavity blocks

containing hot water-killed nematodes was removed and the specimens were flooded with about 4 ml of fixative (at room temperature). Temporary mounts of nematodes (i.e. in water or fixative) were prepared using pieces of agar (Section 3.2.3) as cover-glass supports and were sealed with glyceel. Hot water-killed, unfixed nematodes were examined for taxonomic details and were measured immediately after killing whereas the fixed specimens were maintained at 22°C for 10 days and then studied.

(iii) *Processing to mounting medium*

Nematodes were killed and fixed in hot TAF and after 10 days in fixative at 22°C were processed to lactophenol or glycerol using one of the following techniques: (a) Rapid lactophenol method (Franklin & Goodey, 1949); (b) Rapid method to glycerol (Baker, 1953); (c) Glycerol-ethanol method (Seinhorst, 1959); (d) Slow method to glycerol (Goodey, 1963b). Permanent mounts were also prepared using agar pieces as cover-glass supports and were sealed with glyceel.

(iv) *Data recording and analysis*

A compound microscope was used for observations of both qualitative and quantitative effects. Drawings and measurements were made on twenty individual nematodes in each treatment using differential interference contrast optics. Observations on twenty different body parameters, most of which are commonly used in rhabditid taxonomy (Andrassy, 1983) were made. Taxonomic ratios including, a, b, c, c', V, m (promesostom length/promesostom width) and t (distance from posterior flexure of gonad to tail end as a percentage of body length) were calculated and the data were subjected to two-way ANOVA. Observations on qualitative features including the appearance of cuticle and hypodermis and the clarity of structures such as the nerve ring, glottoid apparatus, oesophageal collar and excretory duct were made at high magnification using oil immersion objectives.

3.2.5 Calibration of the Baermann funnel technique for the extraction of *C. elegans* from casing soil

(i) *Sample size*

Autoclaved casing-soil was weighed (13, 26 or 39 g corresponding to 10, 20 or 30 cc, respectively) and placed in plastic beakers. Freshly extracted *C. elegans* (at 20,000 nematodes/sample; approx. 1500 adults + 18,500 larvae) in one ml of water were sprinkled on the soil which was then mixed thoroughly with a glass rod. Six replicates were prepared for each treatment. After one hour, the samples were placed in Baermann funnels on a milk filter (Clares Ltd) supported on aluminium wire gauze and were flooded with water. The room-temperature during this experiment varied between 22 and 23.5°C. After 24 hours, 30 ml suspension was collected from each funnel and the nematodes were fixed in 2% (v/v) formaldehyde solution and counted.

Another experiment was conducted with a similar design, level of replication and infestation technique except that the density of nematodes (i.e. the numbers of nematodes/g of soil) was kept constant. The nematodes were added to each sample of casing-soil according to its size (i.e. 16,000, 32,000 and 48,000 *C. elegans* were mixed with 13, 26 and 39 g of soil, respectively). The nematode suspension contained approx. 1000 adult + 15,000 larvae per ml. The nematodes were extracted for 24 h and counted as above.

(ii) *Level of water*

The effects of different water levels in the funnels on the extraction of *C. elegans* were examined. Each casing sample (13 g) was infested with 20,000 *C. elegans* (approx. 1500 adults + 18,500 larvae) as described in the above experiment. The samples were placed in funnels and three different levels of water: flooding, half-flooding (i.e. some of the solids were above the level of water), and just in contact with the bottom of the sample, were maintained. Six replicates were prepared for each treatment. Only one milk filter was used in each funnel. After 24 hours, 30 ml suspension was collected from each funnel, the nematodes were fixed in 2% (v/v) formaldehyde solution and counted.

(iii) *Extraction time*

Each casing sample (13 g) was infested with 16,000 *C. elegans* (approx. 1000 adults + 15,000 larvae) contained in one ml of water. Eleven sets of six samples were prepared and each set was extracted for a different time using Baermann funnels. One milk filter was used in each funnel and the samples were flooded with water. After extraction, 30 ml suspension was collected from each funnel and the nematodes were fixed in 2% formaldehyde solution and counted.

3.3 Results

3.3.1 Identification of nematodes

The nematode genera and species isolated from compost or casing samples collected from various parts of the British Isles are listed in Table 3.2. Of the fifteen sites studied, six were single species infestations; the others containing two or more species.

Thirteen species of rhabditoid nematodes (eleven genera) representing two sub-orders were identified. *Caenorhabditis elegans* (Maupas) Dougherty (Plates 3.1 & 3.2) was most frequently found. The other common nematodes were *Acrobeloides buetschlii* (de Man,) Steiner & Buhrer; *Bursilla labiata* (Volk) Andrassy, *Diplogaster maupasi* (Potts) Goodey and *Hemidiplogaster* sp.

3.3.2 New method for mounting nematodes

Measurements of body diameter at the anus and at the vulva showed that the agar-bit method did not differ significantly from the glass-wool technique (Table 3.3).

Commonly used media including Malt Extract Agar, Nutrient Agar, Yeast Extract Agar, Potato Dextrose Agar (all at recommended concentrations, Tuite, 1969) were tried and were found equally effective for routine preparation of temporary nematode mounts. But there is the danger of microbial contamination if the slides are to be kept for long. Distilled water agar did not

Table 3.2 List of rhabditoid nematodes identified from the samples of compost/casing material collected from mushroom farms in the British Isles.

Nematodes	Locality codes*
Rhabditina	
Acrobeloides apiculatus	Wa
A. buetschlii	Pr-1, Lt-2, Dr
Bursilla labiata	Pr-2, Lt-1
Caenorhabditis elegans	Lv, Tn, Iw, Sh-2, Wa, Pr-1
Cuticularia oxycera	Sh-1
Panagrolaimus rigidus	Pr-2
Panagrolaimus sp.	Pr-2
Rhabditella pseudoelongata	Ca
Rhabditis sp.	Pr-2
Diplogasterina	
Butlerius sp.	Ca
Diplogasteritus nudicapitatus	Ca
Diplogaster maupasi	Lv, Pr-2
Hemidiplogaster sp.	Sh-2, Du

*See Table 3.1

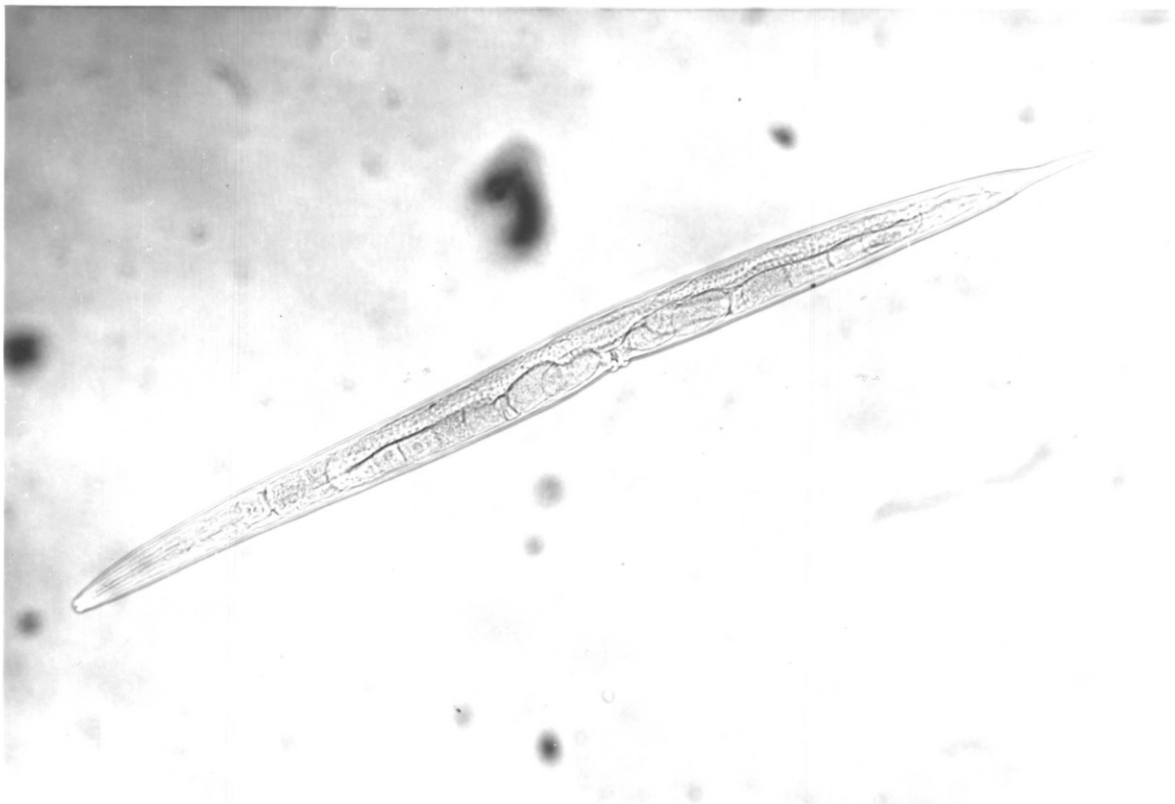


Plate 3.1 Adult female *Caenorhabditis elegans* (x 150)

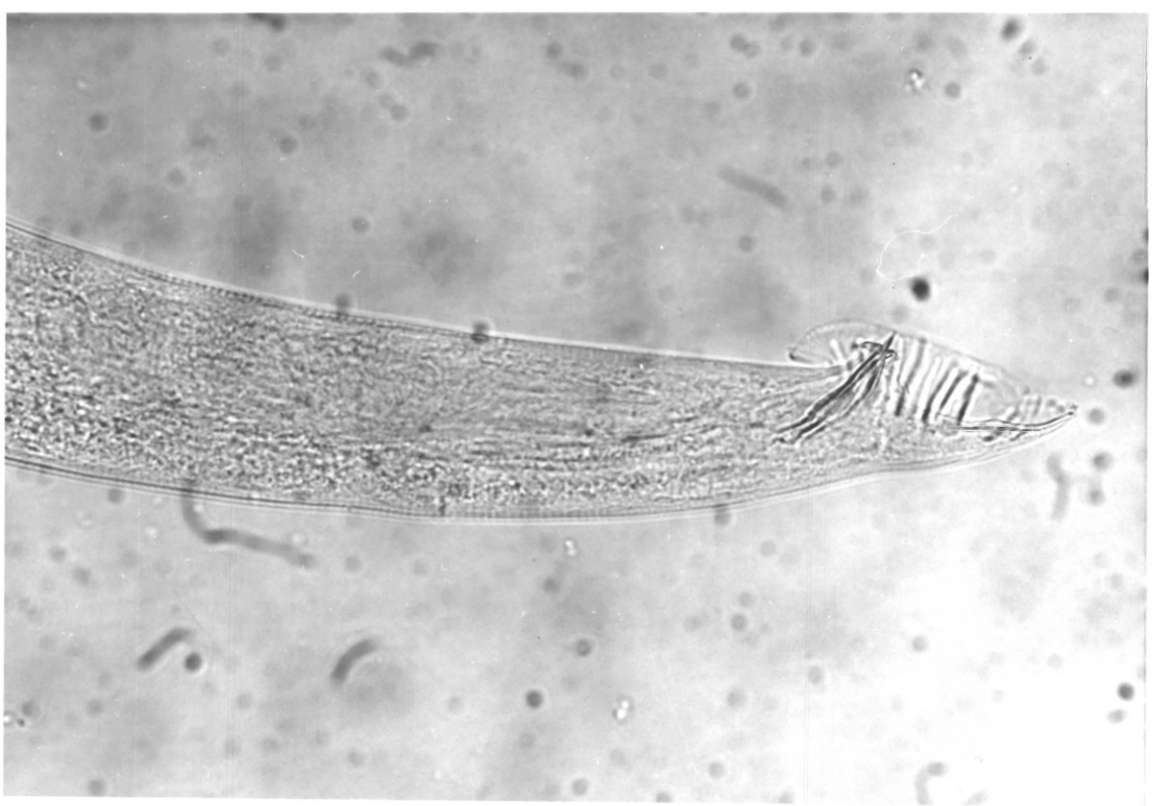


Plate 3.2 Adult male *C. elegans*: tail region (x 500)

Table 3.3 Comparative mean body widths (μm)* at anus and at vulva of 20 adult female *Caenorhabditis elegans* and *Tylenchus davainei* mounted in glycerol using glass-wool or agar as cover-glass supports

		X ± S.E. (range)	
Species		Glass-wool	agar
C. elegans	anus	20.6 ± 3.2 (19 - 28.4)	20.5 ± 2.9 (18.6 - 29)
	vulva	59.8 ± 7.4 (48 - 72)	60.1 ± 6.9 (50 - 73)
T. davainei	anus	16.1 ± 3.4 (12.4 - 20.2)	15.9 ± 3.5 (12.2 - 20.6)
	vulva	24.3 ± 5.5 (20 - 33.4)	24.4 ± 5.6 (19.8 - 34.5)

* Mean width was not significantly different at $P > 0.05$ with glass-wool and agar as cover-glass supports

have the required consistencies as the pieces of agar slipped out of the cover-glass rather than spreading below. Consequently, a number of salts including copper sulphate, sodium ethylmercurithiosalicylate ("Thimerosal") and sodium hypochlorite were tried at a range of concentrations (0.01 - 0.4% w/v) to break the agar gel to the extent required and at the same time be toxic to microbes. In these respects copper sulphate at 0.05% (w/v) concentration proved ideal.

3.3.3 Effects of killing, fixing and mounting methods on taxonomic characters of adult female *C. elegans*

(i) *Killing and fixing*

(a) Quantitative effects. The effects of methods of killing and fixing on certain dimensions of adult female *C. elegans* are summarised in Figs. 3.1, 3.2 & 3.3. Neither of methods of preparation involving the use of fixatives produced nematodes that were similar to those killed in hot water and unfixed. All fixatives caused significant ($P<0.05$) shrinkage in most characters measured and this was particularly marked with regard to: body length, length from anterior end to vulva (Fig. 3.1) and anal body width (Fig. 3.2). Most of the commonly-used taxonomic ratios that were considered were also significantly ($P<0.05$) altered (Fig. 3.3).

Killing and fixing in hot fixative gave better results overall than killing with hot water and fixing in cold fixative (Table 3.4). Out of the 20 parameters evaluated, the former process had significant ($P<0.05$) adverse effects on 13 of them whereas the latter process significantly ($P<0.05$) affected 18 characters. Interactions between the two methods were significant for most characters indicating that a particular fixative had different effects when used hot or cold.

TAF significantly ($P<0.05$) affected the fewest parameters. Eleven parameters were affected when nematodes were killed and fixed in hot TAF and 14 were altered when the specimens were killed in hot water and fixed in cold TAF (Figs. 3.1, 3.2 & 3.3). The greatest changes were found with FP 4:1 and FAA: each fixative significantly ($P<0.05$) affected 18 parameters when specimens were killed and fixed in hot fixative and 19 parameters when hot water-killed nematodes were fixed in cold fixative.

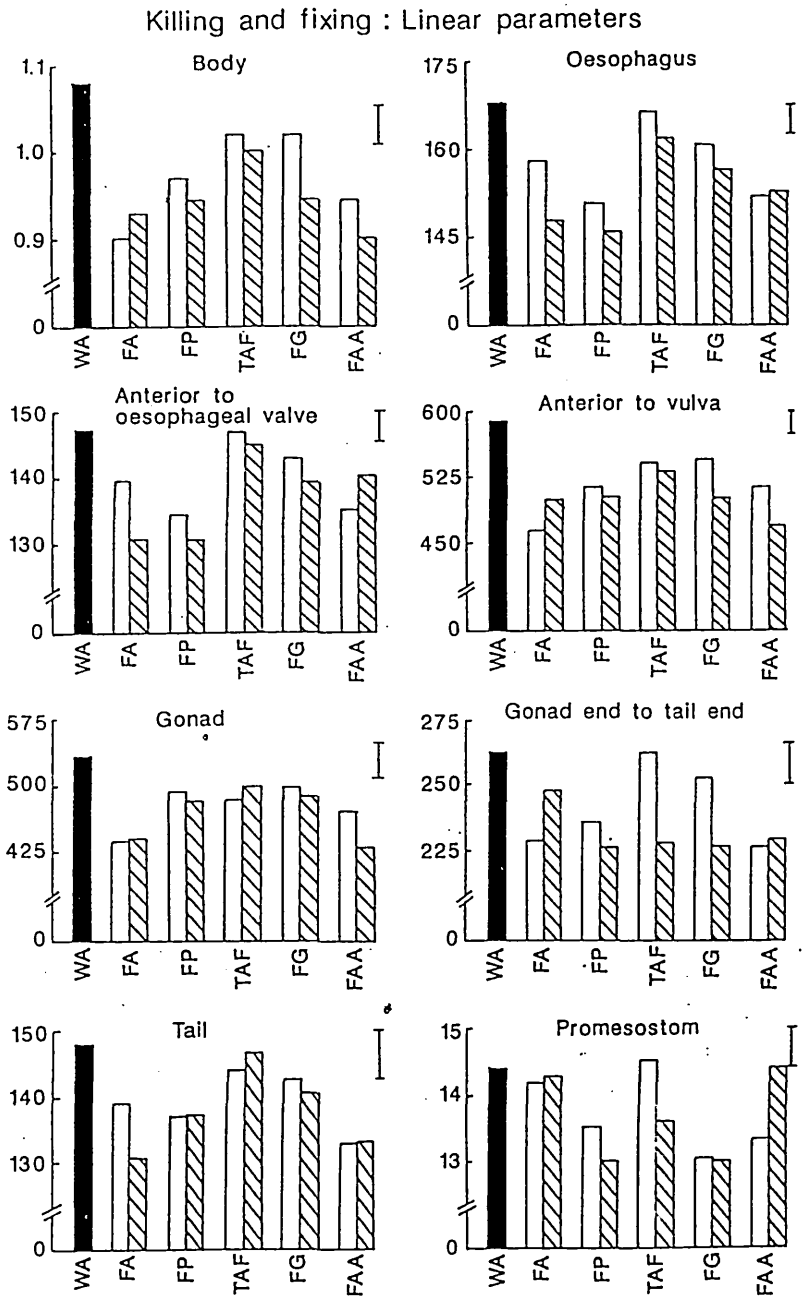


Fig. 3.1 Effects of killing and fixing methods on linear dimensions of parthenogenetic adult female *C. elegans*. For a key to methods refer to Fig. 3.2 and for the fixatives see Section 3.2.4. Bars represent LSD ($P<0.05$). All body measurements are in μm except body length (mm) (n = 20).

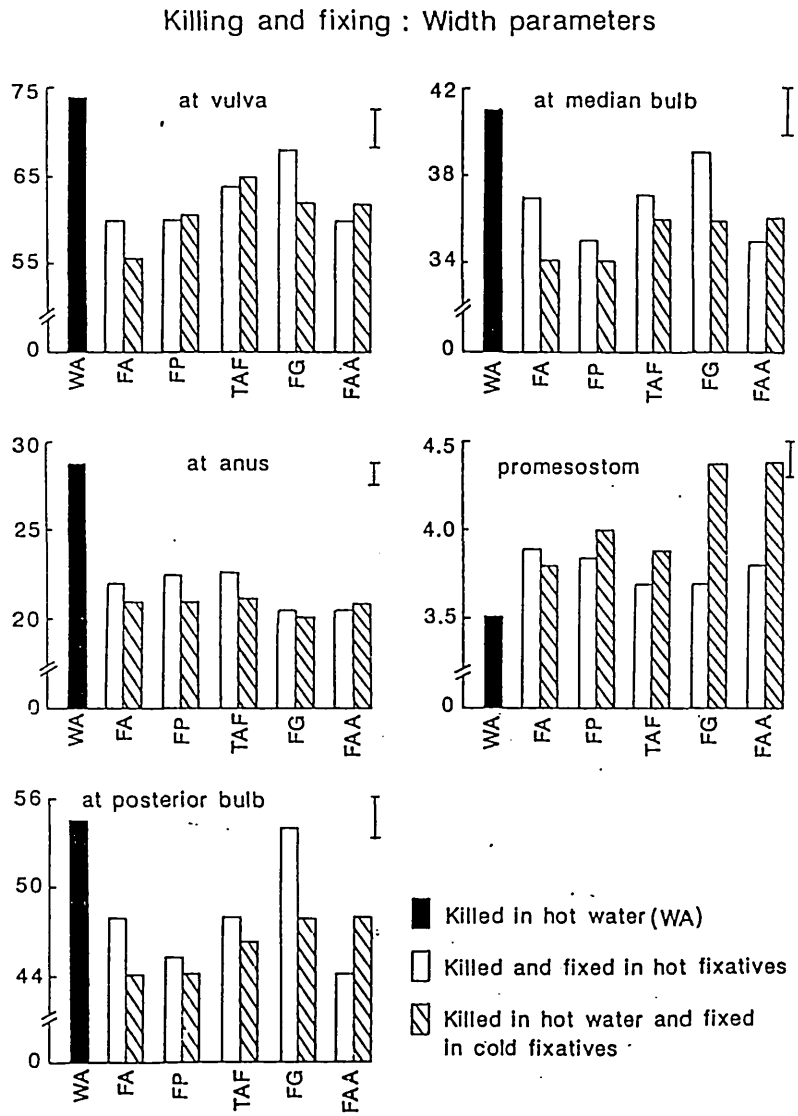


Fig. 3.2 Effects of killing and fixing methods on width parameters of parthenogenetic adult female *C. elegans*. For key to the fixatives see Section 3.2.4. Bars represent LSD ($P<0.05$). All body measurements are in μm (n = 20).

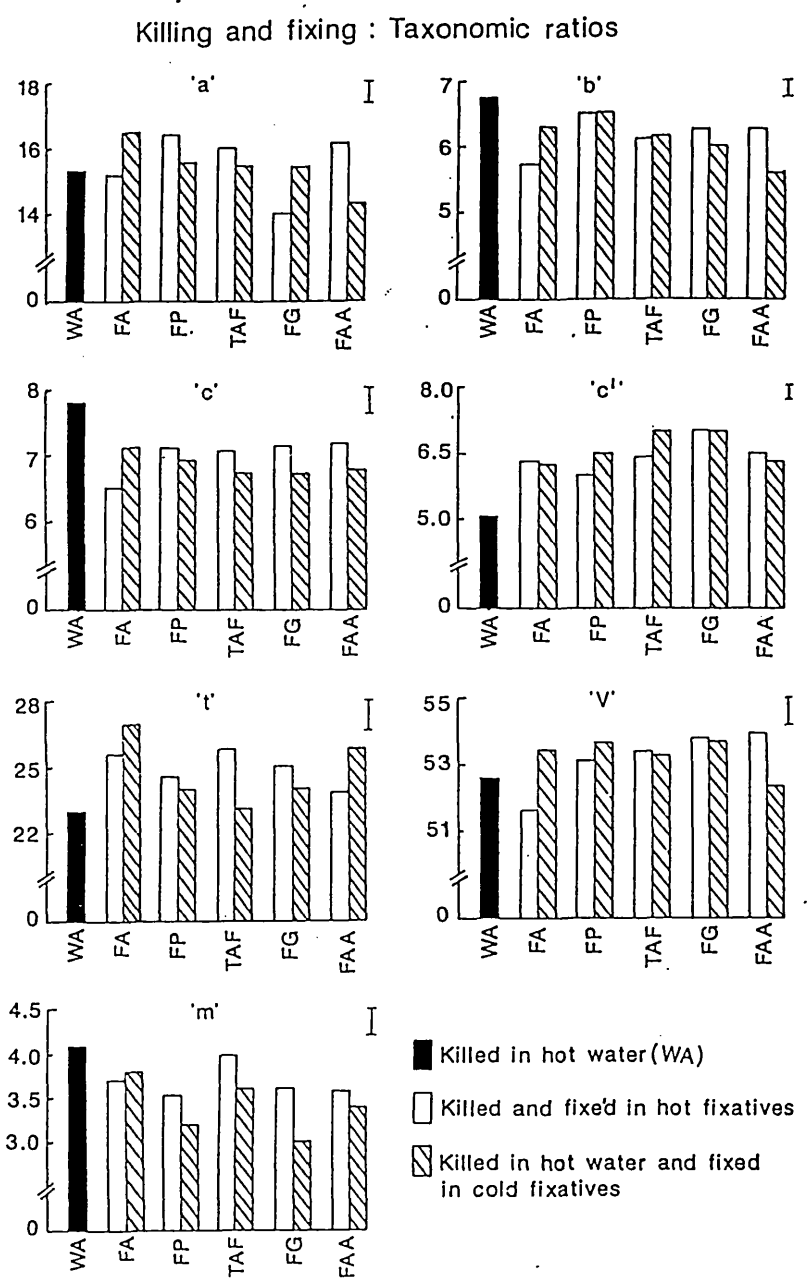


Fig. 3.3 Effects of killing and fixing methods on taxonomic ratios (see Table 3.4 for details) of parthenogenetic adult female *C. elegans*. For a key to the fixatives see Section 3.2.4. Bars represent LSD ($P<0.05$) (n = 20).

Table 3.4 Effects of killing and fixing methods on morphometrics of adult female *C. elegans*: Analysis of Variance

Body measurements	Significance		
	HF	CF	HFxCF
Linear parameters			
Body length	**	***	*
Oesophagous length	**	***	***
Promesostom length	NS	***	***
Length from anterior end to oesophageal valve	*	***	***
Length from anterior end to vulva	**	***	***
Gonad length (anterior to posterior flexure)	NS	***	**
Length from posterior flexure of gonad to tail end	**	**	***
Tail length	NS	***	NS
Width parameters			
At median bulb	**	**	*
At posterior bulb	***	***	***
At vulva	*	***	***
At anus	**	**	NS
Promesostom width	***	NS	*
Taxonomic ratios			
a (= body length/greatest body width)	NS	***	***
b (= body length/oesophagous length)	NS	***	***
c (= body length/tail length)	*	NS	***
c' (= tail length/body width at anus)	**	**	*
V (= length from anterior end to vulva as percentage of body length)	NS	**	***
m (= promesostom length/promesostom width)	***	***	*
t (= length from posterior flexture of gonad to tail end as percentage of body length)	NS	***	***

HF = killed and fixed in hot fixative (95°C); CF = Killed in hot water (95°C) and fixed in cold fixative (22°C); HFxCF = interaction between hot and cold fixatives; NS = not significant ($P>0.05$); * $P<0.05$ ** $P<0.01$ *** $P<0.001$

(b) Qualitative effects. Cold and hot fixation in FA 4:1, FP 4:1, FG or FAA caused considerable swelling of the cuticle, and in extreme cases resulted in the cuticle tearing away from the hypodermis, distortion of the hypodermis and darkening (browning) of the specimens. However, TAF caused clearing of the specimens and improved the appearance of most features. Formalin-glycerol fixative (FG) had the most pronounced effects on cuticle thickness and this was reflected in mean width of the specimens (Fig. 3.2). TAF caused no such qualitative distortion and produced the most acceptable specimens. Structures including the nerve ring, promesostom, glottoid apparatus (metastom), denticles and tri-radiate oesophageal valve were most distinct in TAF-fixed specimens (observed after 10 days in fixative) but the excretory duct and oesophageal collar were more distinct in all other fixatives.

(ii) *Processing to mounting media*

(a) Quantitative effects. When compared with nematodes killed and fixed with the optimal method (i.e. in hot TAF) all subsequent processing methods caused significant shrinkage ($P<0.05$) in most characters measured (Figs. 3.4, 3.5 & 3.6). Characters including the lengths of promesostom, oesophagus, and tail which were not significantly ($P<0.05$) affected by the killing and fixing in hot TAF were affected adversely by all the mounting methods. Processing of nematodes to glycerol by the slow method had significant ($P<0.05$) adverse effects on the fewest parameters - 13 out of 20 studied. The rapid lactophenol method significantly ($P<0.05$) affected the most parameters - 17 out of 20 evaluated.

Table 3.5 lists the dimensions (including mean ± standard error and range) of adult female *C. elegans* when hot TAF-killed and -fixed specimens were processed to mounting medium (lactophenol or glycerol) by four different techniques.

(b) Qualitative effects. Both the rapid lactophenol method and the rapid method to glycerol caused the greatest distortion of specimens and the loosening of cuticle away from the hypodermis. Although maximum distortion occurred in the region of the oesophagus the effects extended to the vulval region. The slow

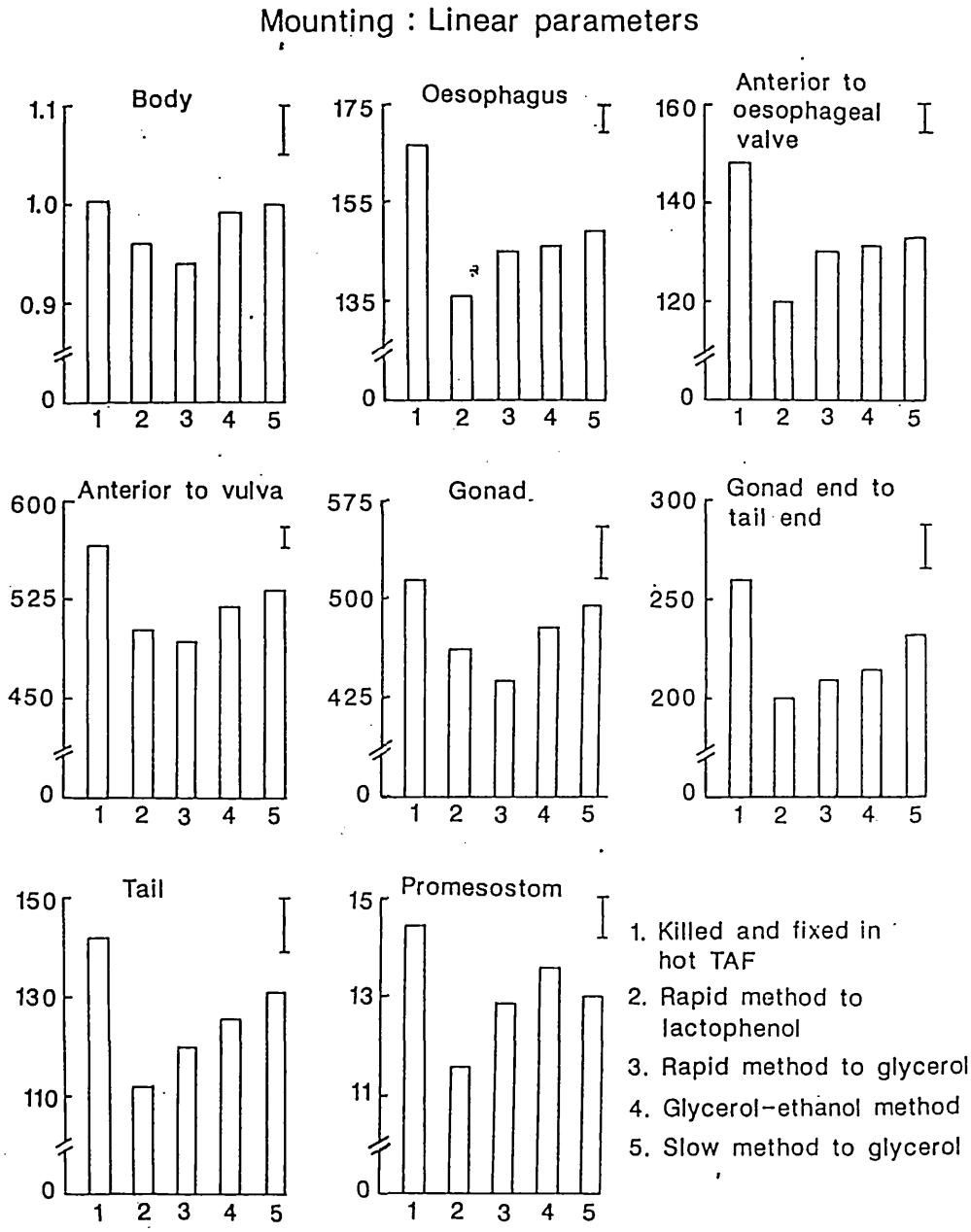


Fig. 3.4 Effects of mounting methods on linear dimensions of parthenogenetic adult female *C. elegans*. Bars represent LSD ($P<0.05$). All body measurements are in μm except body length (mm) (n = 20).

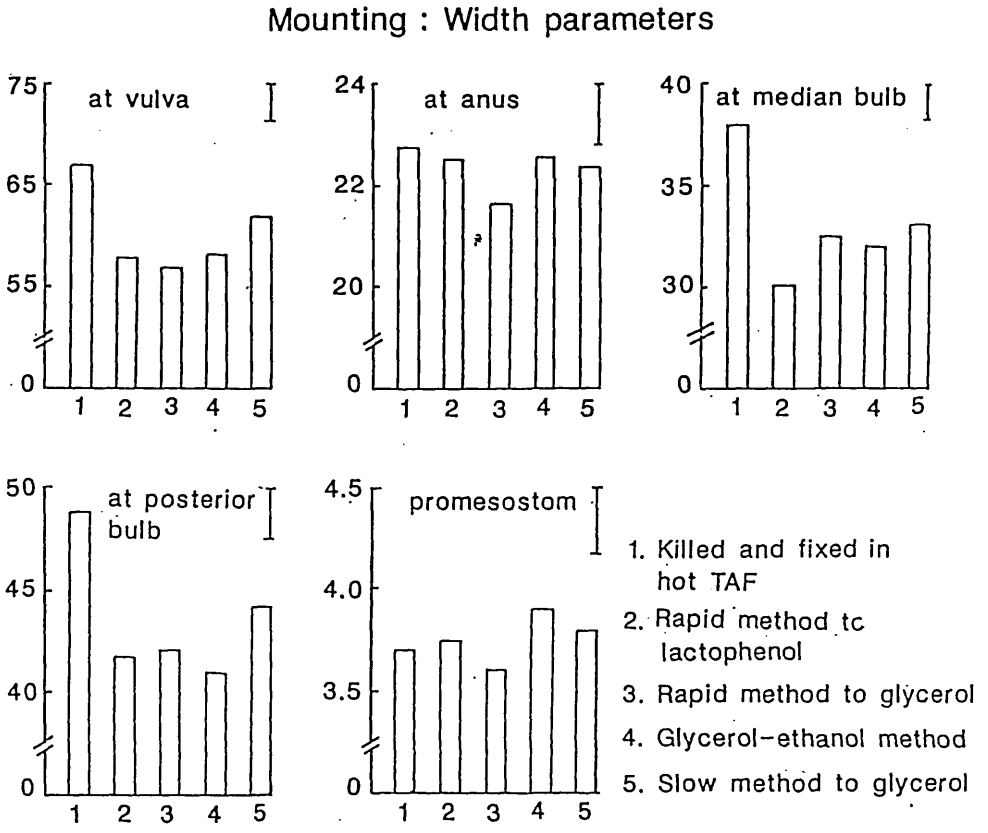


Fig. 3.5 Effects of mounting methods on width parameters of parthenogenetic adult female *C. elegans*. Bars represent LSD ($P<0.05$). All body measurements are in μm (n = 20).

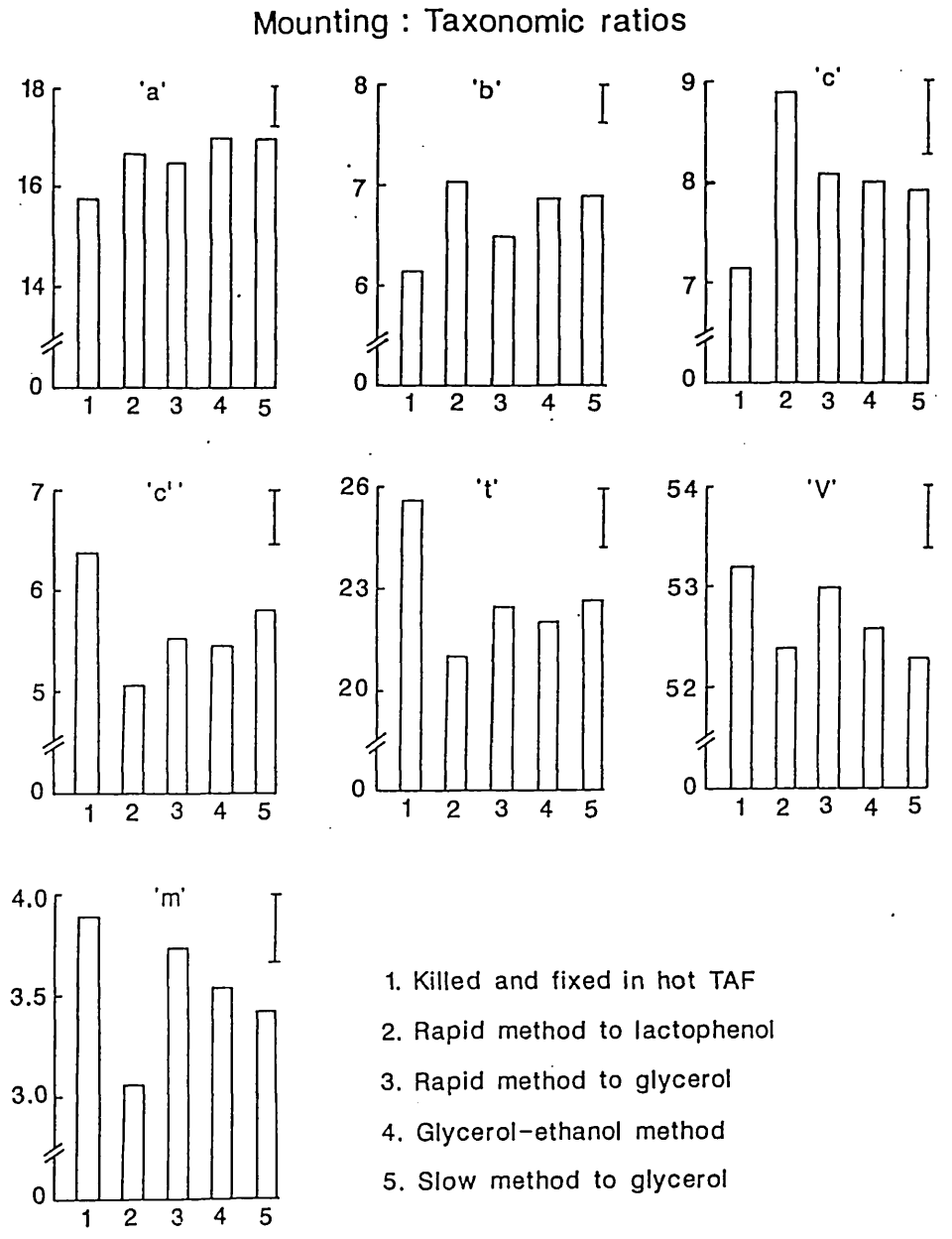


Fig. 3.6 Effects of mounting methods on taxonomic ratios (see Table 3.4) of parthenogenetic adult female *C. elegans*. Bars represent LSD ($P<0.05$) (n = 20).

Table 3.5 Dimensions of parthenogenetic adult female *C. elegans* killed and fixed in hot TAF (95°C) and processed to mounting medium by four different techniques (n = 20)

	Mean ± S.E. (range)			
Parameters	Rapid method to lactophenol	Rapid method to glycerol	Glycerol-ethanol method	Slow method to glycerol
Body length (μm)	957 ± 22 (728-1070)	937.2 ± 18 (734-1015)	984.3 ± 8.7 (900-1037)	1016 ± 12 (878-1097)
Ratio a	16.7 ± 0.39 (13.4-19)	16.5 ± 0.26 (13.8-17.9)	17 ± 0.19 (15.6-18.5)	17 ± 0.29 (14.9-19.6)
Ratio b	7 ± 0.14 (5.7-7.8)	6.5 ± 0.12 (5.6-7.8)	6.7 ± 0.06 (6.2-7.3)	6.8 ± 0.11 (6.1-7.8)
Ratio c	8.8 ± 0.37 (6-12.5)	8.1 ± 0.32 (7-12.5)	8 ± 0.14 (7-8.9)	7.9 ± 0.13 (7-9.2)
Ratio c'	5.7 ± 0.22 (3-6.6)	5.6 ± 0.23 (3-7.2)	5.5 ± 1 (4.4-6.2)	5.8 ± 0.11 (5-6.8)
Ratio V	52.4 ± 0.44 (49.5-57.5)	53 ± 0.74 (44.6-62.8)	52.6 ± 0.36 (47.3-54.9)	52.3 ± 0.39 (49.5-54.6)

method to glycerol did not cause any such cuticular distortions. The glycerol-ethanol method (Seinhorst, 1959) also produced acceptable specimens.

3.3.4 Calibration of the Baermann funnel technique for the extraction of *C. elegans* from casing

(i) *Sample size*

Casing sample size significantly ($P<0.05$) affected extraction efficiency of *C. elegans* by the Baermann funnel method (Fig. 3.7). Most effective extraction of nematodes (about 84%) was attained with the smallest casing samples (13 g): extraction efficiency decreased as sample size increased.

When the same number of nematodes were added to different sized soil samples (i.e. variable density) there was no significant ($P>0.05$) difference in the extraction efficiency of adult or larval stages of *C. elegans*. However, when the nematodes were added according to the sample size (i.e. constant density) the extraction of larval stages was significantly ($P<0.05$) lower than that of the adult stages, especially when larger-sized samples were used (26 or 39 g).

(ii) *Level of water*

The level of water in the funnel, significantly affected extraction efficiency (Fig. 3.8). Flooding the casing samples with water resulted in maximum extraction of nematodes (about 84-86%) whereas half flooding and minimal contact resulted only in 52-60% and 45-46% extraction, respectively. Different life stages of *C. elegans* showed similar response pattern to different levels of water in the funnel.

(iii) *Extraction time*

Fig. 3.9 shows trends in nematode extraction after different durations. More then 50% of nematodes were extracted within three hours of the start of the extraction process and about 78% were extracted in just seven hours. Maximum extraction (about 84%) was reached after 12 hours.

Different life stages of *C. elegans* showed differential response to the exposure period; initially (i.e. after 2 and 3 hours) the extraction of the adult

stages was significantly better ($P<0.05$) than the larval stages but from five hours onwards the extraction of larval stages remained significantly ($P<0.05$) greater than the adults.

3.4 Discussion

From this work, it is evident that mushroom compost is a rich source of nematode fauna and *C. elegans* is the most frequently found. In contrast, there are only few reports of the recovery of *C. elegans* from mushroom beds (Appendix 2.1). This species is ecologically adapted (because of its self fertilization and rapid growth rate) to exploit rich but temporary resources consisting of high concentrations of bacteria (Dusenbery, 1980). Th erefore, soon after the 'outbreak' of its population, *C. elegans* produces 'dauer' larvae that help in its survival during adverse conditions. This absence of adults coupled with the rare occurrence of males probably undermines the presence of *C. elegans* in most taxonomic surveys. However, growing of larval stages to adults on nutrient agar facilitated the identification of this widespread nematode species.

Esser (1988) embedded nematode cysts in agar blocks to examine the vulva area and the present study provides an additional use of agar that overcomes some commonly-encountered problems during preparation of slides of nematodes. The wax-ring method of sealing mounts (De Maeseneer & D'Herde, 1963) is very useful for lactophenol or glycerol based permanent mounts, and can probably be adapted for temporary mounts (in water or fixatives). However, the heat required to melt the wax could be detrimental to specimens especially narcotised ones and it might also affect comparative studies of methods of killing and fixing.

Another advantage of the described agar method is that it removes the need for additional cover-glass supports when the area occupied by the mountant exceeds a quarter of the cover-glass area (Hooper, 1986b) as it often does in the case of aqueous or fixative mountants. Furthermore, oil-immersion objectives may be more easily used, as thick mounts which can result from the wax-ring method (Hooper, 1986b) can be avoided using agar pieces.

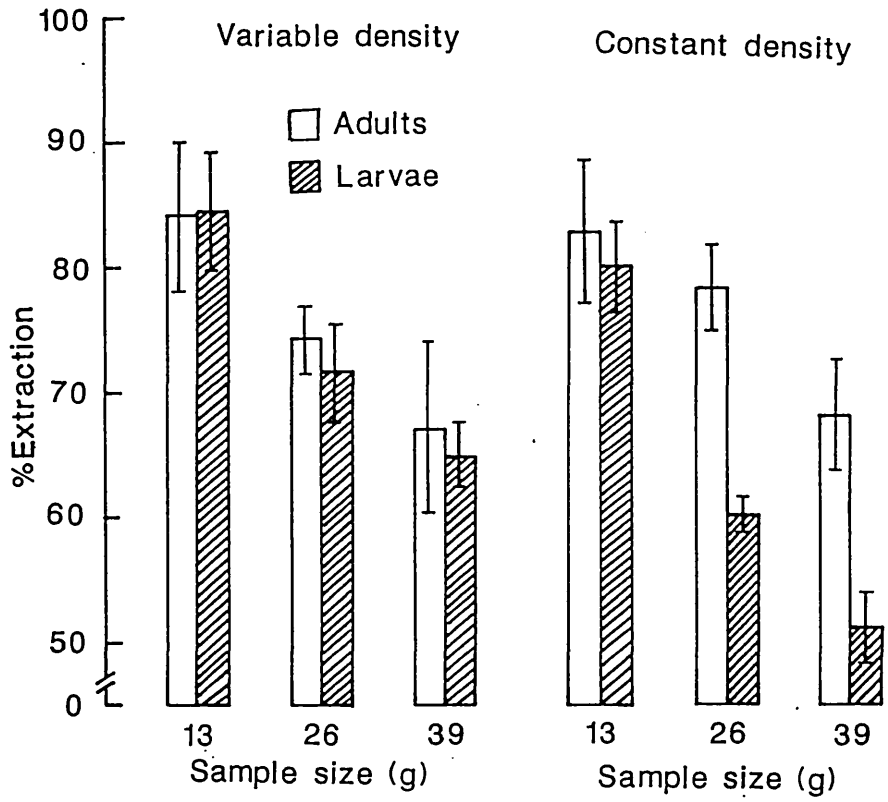


Fig. 3.7 The Baermann funnel technique for the extraction of *C. elegans* from casing material: effects of sample size and nematode density. Data are mean ± S.E. (n = 6).

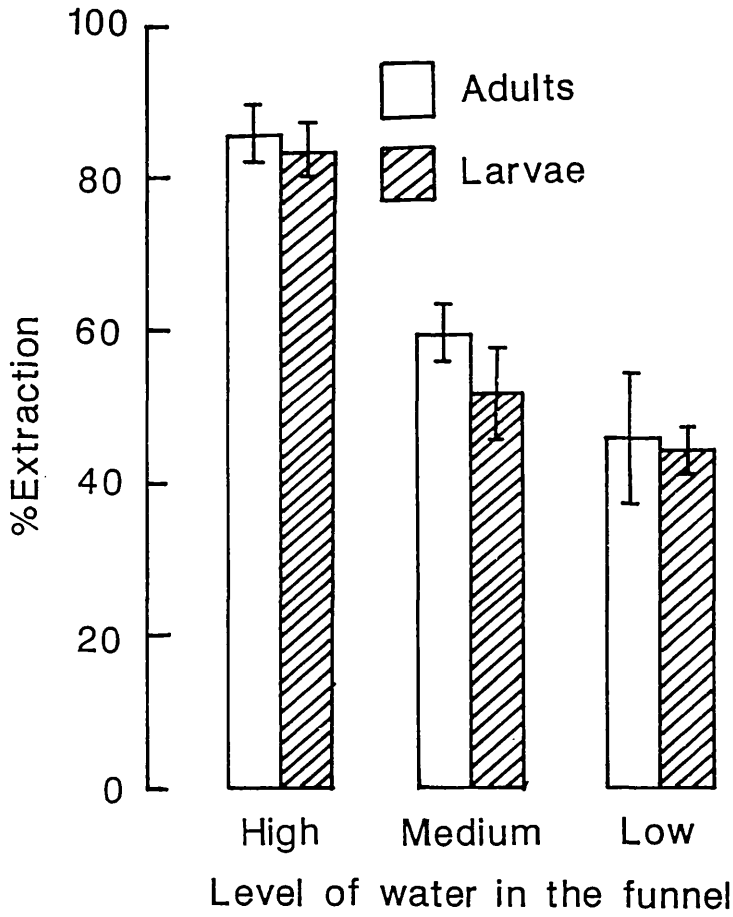


Fig. 3.8 The Baermann funnel technique for the extraction of *C. elegans* from casing material: effects of water level. Data are mean ± S.E. (n = 6).

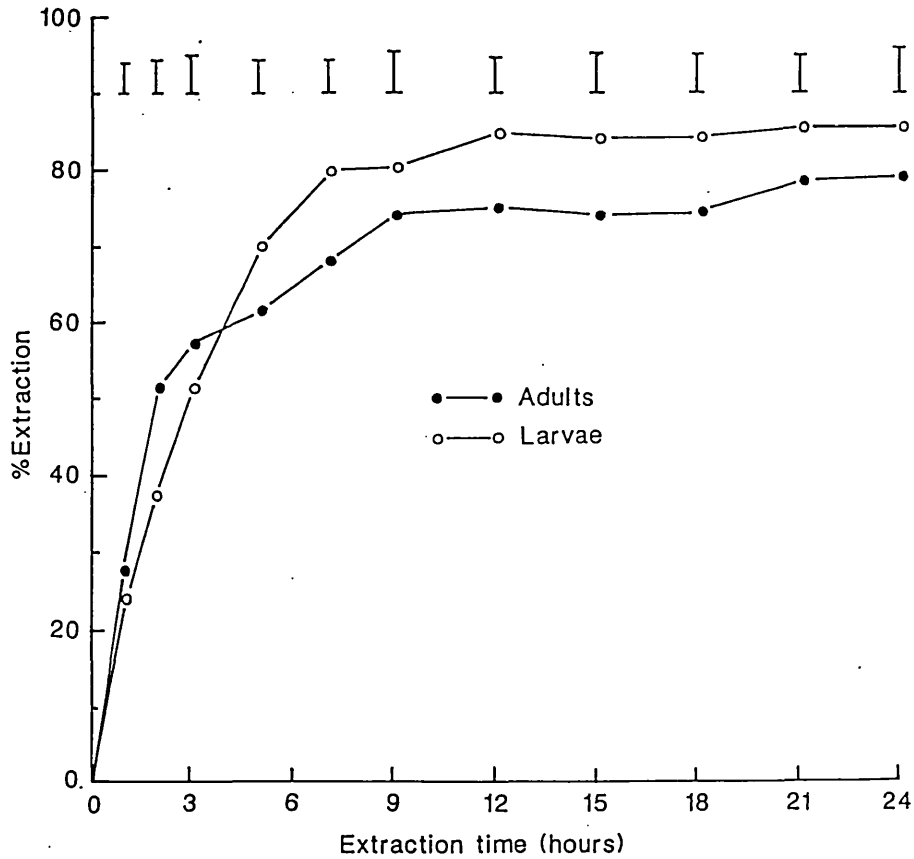


Fig. 3.9 The Baermann funnel technique for the extraction of *C. elegans* from casing material: effects of extraction time. Data are mean ± S.E. (n = 6).

Permanent nematode mounts prepared by the agar method were very successful. Esser (1973) recommended Thorne's cement ("Zut") as a cover-glass support for preparation of permanent nematode mounts. However, agar appears to be more useful because of the problems associated with the viscosity of cement (which is a critical factor). Also cement firmly seals the cover-glass to the slide making it difficult to recover/remount specimens. Agar did not provide any such problems to remounting. Moreover, the latter method is simple, quick and inexpensive.

As expected there was no single combination of methods ranging from killing to mounting without any adverse effects on the specimens. Every stage in the process resulted in some degree of shrinkage and/or distortion of the specimens. Killing and fixing with hot TAF by Seinhorst's method and mounting in glycerol by the slow method produced the best specimens with measurements closest to those that were killed in hot water and not fixed. Lamberti and Sher (1969) recommended the same method to process *Longidorus africanus* for taxonomic study, but they used FAA for killing and fixing the specimens.

Curran and Hominick (1981) obtained the most life-like specimens of *Romanomermis culicivorax* and *Gastromermis* sp. by killing in water at 65°C for three seconds, fixing in hot TAF, stored for one week at room temperature and processing to glycerol by Seinhorst's technique. However, the present study revealed that killing and fixing (in one process) with hot TAF adversely affected fewer parameters when compared with those killed in hot water (by Seinhorst's method) and fixed in cold TAF. This may have been due to two factors: (i) killing with hot water distorted some specimens (as the genital tracts came out through the vulva) probably due to osmotic differences, and (ii) fixing the water-killed specimens in cold TAF caused shrinkage of specimens (due to the action of formaldehyde; Fagerholm, 1979).

Body measurements expressed as ratios are used in nematode taxonomy as they are presumed to reduce biological variability and hence are considered useful for discrimination between taxa (Cobb, 1913). However, in the present work it was observed that body measurements, even when considered as ratios, show significant differences ($P < 0.05$) when specimens of the same species are

processed by different mounting techniques. Differential shrinkage of body tissues during the various processing methods may have been the cause of changing ratios. For instance, killing and fixing with hot TAF (our best method) resulted in specimens with a mean c ratio (mean ± S.E.) of 7.08 ± 0.11 (for water killed-specimens it was 7.77 ± 0.26), while further processing by the rapid lactophenol method produced specimens with a mean c ratio of 8.77 ± 0.37. Furthermore, differential shrinkage sometimes produces ratios that have no comparative value. For example, the ratio between mean body length and mean spicule length for *Hydromermis conopophaga* has been reported to be 44 (Poinar, 1968); 35 (Mulvey & Nickle, 1978) and 64 (Hominick & Welch, 1971).

Length and width of stoma are important characters in rhabditid taxonomy (Andrassy, 1983). This study revealed, however, that processing methods are liable to alter these parameters. For instance, the mean length and breadth of the promesostom ranged from 11.5-14.5 μm and 3.5-4.4 μm, respectively in specimens of *C. elegans* processed through different mounting techniques. Significant differences ($P<0.05$) were observed even when the ratio between length and breadth of promesostom was used as a parameter for comparing various processing techniques.

The validity of the use of ratios in nematode taxonomy has been discussed extensively (Roggen & Asselberg, 1971; Fortuner, 1984 and Roggen *et al.*, 1986). According to Fortuner (1984) a ratio is considered as taxonomically valid when the characters which constitute it are biologically related. This relationship must be verified by the study of the significance of the correlation between the two characters. In addition to its validity, a ratio is considered useful when its variability in a sample is lower than the variability of its constituent characters. It is evident from the present study that the processing of nematodes through various mounting techniques differentially alters the constituent characters of a ratio and thereby affects the ratios significantly.

Mounting techniques may alter the specimens to the extent that processing artifacts may sometimes be interpreted as taxonomic characters. For example, *R. culicivorax* is morphologically similar to *R. iyengari* and has been distinguished primarily by the more acute angle of the papillary tract to the oesophagus and a tendency for a thinner cuticle and egg-shell (Ross & Smith,

1976). However, Curran and Hominick (1981) have concluded that the above characteristics are processing artifacts rather than real differences and considered *R. culicivorax* as a species inquirenda. In the present study, such effects of processing were also evident. Hot formalin-glycerol fixative (FG), for example, increased the cuticle thickness considerably and thus resulted in an apparent increase in mean width at the vulva (Fig. 3.2).

Geographically isolated populations of the same species show considerable morphometric variations. For instance, the present population of *C. elegans* (processed for microscopy by the optimal method) was much smaller (L= 878-1097 μm; a= 14.9-19.6; b= 6.1-7.8; c= 7-9.2) when compared with the originally described (Maupas, 1900) Algerian population (L= 943-1700 μm; a= 20-22; b= 5-8.5; c= 7-10). While van den Berg (1988) observed great variation in body measurements of various populations of two rhabditid nematodes, *Elaphonema messinae* and *E. mirabile* collected from different regions in South Africa. Body measurements of *Helicotylenchus dihystera* are also known to vary with the host (Fortuner & Queneherve, 1980) and those of *Aphelenchoides composticola* with the amount of nutrition and population density (Franklin, 1957). Stephenson (1942) found that the measurements of *Rhabditis terrestris* varied so greatly because of culture conditions that individuals from opposite ends of a specific range could be mistaken for different species. These population variations coupled with the differential effects of mounting techniques further complicate the situation and may lead to false conclusions.

Fortuner and Wong (1984) developed a computer programme (NEMAID) for the identification of species of *Helicotylenchus* in which intraspecific variability of measurements were taken into account. The present study tends to widen the definition of intraspecific variability to include not only naturally-occurring differences caused by culture methods, host type or geographic locality, but also induced variability (i.e. the effects of handling and mounting techniques). It is therefore suggested that measurements, if used for diagnostic purposes, indicate specific differences only if those differences exceed natural and induced (including fixation artefacts) intraspecific variability.

The effects of methods of killing, fixing and mounting on particular groups of nematodes vary greatly. Effects of processing techniques also depend

on the species involved, the combination of methods used, the concentration of the preservatives and the time of preservation (Lamberti & Sher, 1969; Stone, 1971; Boag, 1982; Olowe & Corbett, 1983). The results of this work further support the need for concise and detailed accounts of the methods used by taxonomists when describing new species or real differences between nematode populations.

The present study has shown that the extraction efficiency of the Baermann funnel technique is affected by the size of soil sample. As this technique is based on the principle that nematodes actively leave the flooded soil habitat (Kimpinski & Welch, 1971), their movement may be restricted in the larger soil samples. Furthermore, the lack of aeration in the a deep zone of water-saturated soil may also adversely affect nematode mobility.

In the Baermann funnel technique, water provides the medium for sedimentation of nematodes and is therefore a critical factor for their extraction. Maximum nematode extraction was achieved when the samples were flooded with water. It may be due to the excess water that might have filled most of the macro- and micro pores in the soil and ultimately faciliated the separation of nematodes from the soil colloids.

Because of the variable sedimentation rates of different nematode genera, species and geographic populations, the time required for their extraction differs greatly (Viglierchio & Schmitt, 1983). About 84% extraction of *C. elegans* was achieved in 12 hours with only a slight increase in subsequent 24 hours. The differential response shown by different life stages of *C. elegans* to extraction time can also be explained in terms of their variable sedimentation rates. Furthermore, temperature is known to influence the efficiency of Baermann funnel technique (Adams, 1965; Kerr & Vythilingam, 1966; Flegg, 1967; Robinson & Heald, 1989). In the present study the temperature varied between 22-23.5°C i.e. well within the range (15-25°C) that Adams (1965) reported to be optimum for maximum recovery of nematodes from soil.

CHAPTER 4

Interactions between *C. elegans* and associated bacteria

4.1 Introduction

Saprobic rhabditid nematodes need live bacteria as food resource for maturation and reproduction (Nicholas, 1984). However, qualitative analysis of the bacterial flora associated with mushroom infesting rhabditid nematodes and the specific interactions between nematodes and bacteria have not been made. As these interactions may be crucial in understanding the processes of pathogenesis by eelworms to mushrooms, in this part of the project, bacteria associated with *C. elegans*, the commonest nematode (Section 3.3.1), were isolated and identified. The relationships between nematodes and bacteria were also examined in agar cultures.

4.2 Materials and methods

4.2.1 Isolation and identification of bacteria

Bacteria associated with the Taunton strain of *C. elegans* (both externally and in the gut) were isolated immediately after the nematodes had been extracted from compost. Adult *C. elegans* were separated from the suspension under a low power stereobinocular microscope (25x) and placed in drops (one ml) of water in sterile cavity blocks. The bacterial flora externally associated with the adult nematodes was isolated by inoculating freshly-extracted nematodes onto five plates (about 20 nematodes/plate) of 3% (w/v) nutrient agar (Oxoid Ltd). Bacteria from the gut of the nematodes were isolated by surface-sterilization with 'Thimerosal' (Section 4.2.2) and homogenization in a sterile tissue-homogeniser (Akhurst, 1980). The homogenate was diluted to 1:10,000 in

a saline solution (0.85% w/v sodium chloride) and the suspension plated on 3% (w/v) nutrient agar plates (0.1 ml/plate). After 48h incubation at 25°C, 26 isolates of bacteria (13 from each lot) were selected on the basis of colony morphology for further study. The chosen isolates were purified by 2 or 3 sub-cultures at 25°C for 24h on 3% (w/v) nutrient agar plates, transferred to nutrient agar slopes and stored at 4°C for further testing.

The form of the colonies of each isolate was then examined. Colony appearance, motility, spore formation and morphology (Doetsch, 1981) were noted. Gram reactions (Gregersen, 1978) and tests for both catalase (Lelliott & Stead, 1987) and oxidase reactions (Kovacs, 1956) were made. The oxidative and fermentative ability of each isolate was determined by inoculating two tubes of oxidation-fermentation media (Hugh & Leifson, 1953). The isolates were further characterised using the appropriate Analytical Profile Index (API Products Ltd) and a range of other confirmatory tests including LOPAT (Lelliott *et al.*, 1966), growth at 4 and 41°C and gelatin liquefication (Izard *et al.*, 1981), citrate utilisation and lecithinase production (Parry *et al.*, 1983) were done.

4.2.2 Axenization of nematodes

For all the experiments, gravid females were collected from 7 to 8-day-old agar cultures and placed in saline solution (0.85% w/v sodium chloride) in cavity-blocks for five minutes. The female nematodes were washed three times with 0.1% (w/v) 'Thimerosal' (w/v sodium ethylmercurithiosalicylate, Sigma Ltd) and left in sterile distilled water over-night during which time egg-laying and hatching occurred. Newly-hatched larvae were washed four times each with 'Thimerosal' and with antibiotics (Streptomycin 100 μl/ml and Chloramphenicol 50 μl/ml, Sigma Ltd) together with alternate washes of sterile distilled water every five minutes. Sintered glass funnels (pore size 50 μm, Plate 4.1) were used to wash the nematodes with the sterilants. Sterile larvae were collected in a drop of sterile water and placed on plates of 3% (w/v) nutrient agar; they were incubated at 25°C for 48h and checked for bacterial contaminants. Nematodes from bacteria-free plates were washed with sterile distilled water and used immediately.

Plate 4.1 Sintered glass funnels (pore size 50 μm, Sigma Ltd) used to wash nematodes with sterilants.

4.2.3 Reproduction of *C. elegans* on associated bacteria

Ten species of bacteria, isolated from *C. elegans*, were compared as food substrates supporting nematode reproduction. The bacteria were: *A. calcoaceticus* var. *anitratus*, *A. calcoaceticus* var. *lwoffi*, *Bacillus cereus*, *Bacillus* sp., *Enterobacter amnigenus*, *E. cloacae*, *Pseudomonas aeruginosa*, *P. maltophilia*, *Pseudomonas* sp. and *Serratia liquefaciens*. Each species was streak inoculated and grown on 3% (w/v) nutrient agar in 100 mm square Petri plates (18 mm deep), each with 25 equal compartments (Sterilin, UK), for 24h at 25°C. Sterile, second-stage nematode larvae were individually inoculated (one larva contained in 10 μl of sterile distilled water/compartment) on to the bacterial streaks, using a Pasteur pipette, and were incubated at 20°C. Fifteen compartments were prepared for each bacterium. After four, eight and twelve days, the size of the nematode population (eggs, larvae and adults) in five compartments of each of the ten treatments was determined by washing, with distilled water, the contents of each compartment into a separate beaker. Specimens were fixed in 2% (v/v) formalin solution. Eggs and nematodes were counted and the data subjected to ANOVA.

4.2.4 Effects of temperature and bacterial food source on nematode fecundity and generation time

(i) *Nematode fecundity*

The reproductive capacity of parthenogenetic female *C. elegans* was studied at a range of constant temperatures (5, 10, 15, 20, 22, 25, and 28°C) in monoxenic cultures of the three bacteria (species that supported vigorous reproduction): *A. calcoaceticus* var. *anitratus*, *A. calcoaceticus* var. *lwoffi* and *S. liquefaciens*. Bacteria were streak inoculated and grown at 25°C for 24h on 3% (w/v) nutrient agar in square Petri plates each with 25 equal compartments. Sterile, second-stage larvae were inoculated onto the bacterial streaks in 10 μl sterile distilled water using a sterile Pasteur pipette. One larva was inoculated into each compartment and five replicates of each bacterium at each temperature were prepared. Larvae were allowed to develop to adults and to lay eggs. As soon as the eggs started to hatch, the adults were transferred, until

death, to similarly-prepared fresh bacterial streaks for further egg-laying. Eggs and hatched larvae (from both batches) were washed with distilled water into 100 ml beakers, fixed in 2% (v/v) formalin solution and counted.

Data were square-root transformed, analysed using a factorial design and subjected to ANOVA. Cubic equations were fitted using a third degree polynomial regression model (Draper & Smith, 1966). The cubic equation was:

$$Y = a + bt^1 + ct^2 + dt^3$$

Where Y = no. of eggs laid, t = temperature and a, b, c and d were regression coefficients. Differentiation of cubic equations enabled the optimum temperature for nematode reproduction with each bacterium to be calculated.

(ii) *Nematode generation time*

The duration of a single generation of *C. elegans* was studied in similar conditions of temperature and bacterial inoculum. The experiment was done using square Petri plates with one larva in each compartment. Generation time was recorded from the time of inoculation to the time of hatching of the first egg in each compartment. As soon as the egg-laying started, observations on five nematodes in each treatment were recorded at 30 minute intervals. Data were log transformed, analysed using a factorial design and subjected to ANOVA.

4.2.5 Effects of bacteria on the migration of *C. elegans* larvae

(i) *Bacterial cultures*

The ten species of bacteria, out of 13 species studied, were isolated from the Taunton strain of *C. elegans*; these were nine species mentioned in Table 4.3.1 plus *P. fluorescens* biovar *reactans* (Section 8.3.5). Reference strains of *Bacillus thuringiensis* (HD1), *Escherichia coli* (ATCC9001) and *Pseudomonas tolaasii* (Pt51) obtained from the IHR culture collection were also included. All the bacteria except *P. tolaasii* were maintained on 3% (w/v) nutrient agar plates, stored at 4°C and were sub-cultured at monthly intervals. *P. tolaasii* was maintained on King's B medium (King *et al.*, 1954).

(ii) *Quantification of nematode response*

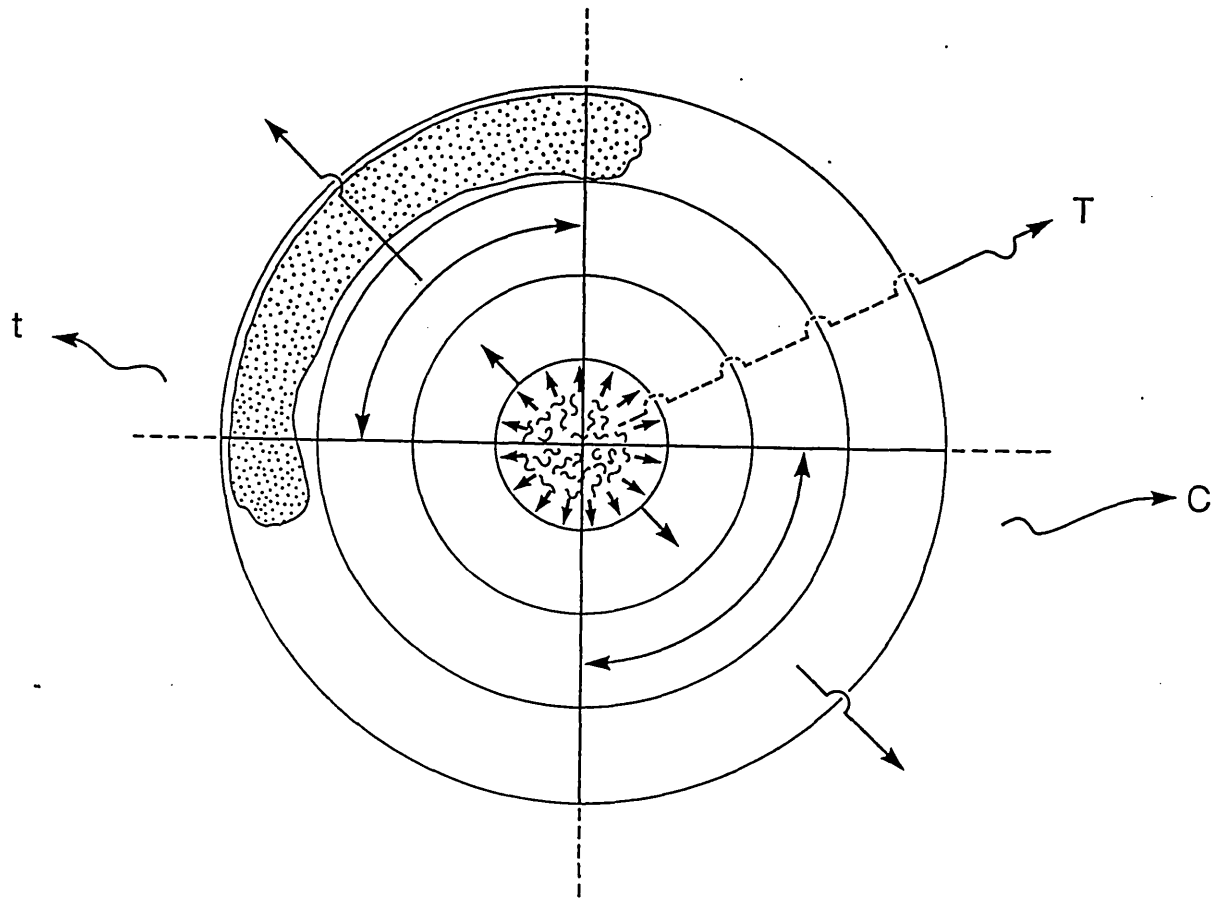
Quadrant plates were prepared according to Andrew and Nicholas (1976) with concentric rings marked at intervals of one cm on the bottom of each Petri plate (8 cm diam.). Nutrient agar was poured in plates and a loop-full of bacteria, from 24h old cultures grown in nutrient broth (Oxoid Ltd) in shaker flasks at 25°C, were streaked along half the periphery (Fig. 4.1). The plates were then incubated at 25°C for 24h. Nematodes (Mean ± standard error, 60 ± 1.3) contained in 30 μl of sterile distilled water were placed in the centre of each plate, the drops were then allowed to evaporate for 5 minutes on a sterile-air laminar flow cabinet. The numbers of nematodes moving out of the central ring towards the bacterial streak (test side) and the bacteria-free side (control side) were recorded periodically. Mean distance travelled by nematodes towards bacteria after each time interval in each plate (replicate) was calculated using the equation given in Fig. 4.1.

(iii) *Relative attractiveness of bacterial species*

All thirteen species of bacteria were randomly divided into four groups; one group contained four species and the others three species each. Three replicate plates were prepared for each species of bacteria and three bacteria-free control plates were also run simultaneously. Room temperature varied between 20-21°C during this experiment. Data on mean distance travelled by nematodes were recorded periodically and were subjected to ANOVA.

(iv) *Response of nematodes to a 'choice' of three attractive bacteria*

Quadrant plates were inoculated with three attractive bacteria including, *A. calcoaceticus* var. *anitratus*, *P. maltophilia* and *S. liquefaciens*. The bacteria were inoculated in three of the four quadrants in all the three possible combinations (Section 4.3.4), with a gap of 0.5 cm between bacterial streaks. The fourth quadrant was kept blank. The plates were incubated at 25°C for 24h. Three replications were kept for each combination and three similarly prepared bacteria-free control plates were also run simultaneously. Nematodes were placed in the centre of each plate and the numbers of nematodes in each arc of every quadrant recorded periodically. Mean distance travelled by the



Attraction index ($\bar{X}$)

$$\bar{X} = [\ 1t_1 + 2t_2 + 3t_3\ -\ (\ 1C_1 + 2C_2 + 3C_3\ )]\cdot T^{-1}$$

Fig. 4.1 A quadrant plate used to quantify nematode response to the test material. T = total number of nematodes; t_1 - t_3 are the respective arcs in the test quadrant (t) and C_1 - C_3 are the respective arcs in the control quadrant (C). Attraction index is defined as the mean distance travelled by nematodes at any time from the central arc towards the periphery.

nematodes towards each bacterial streak and the control quadrant was determined as described earlier. The data were square-root transformed and subjected to ANOVA.

(v) *Effects of age of bacterial streak on nematode attraction*

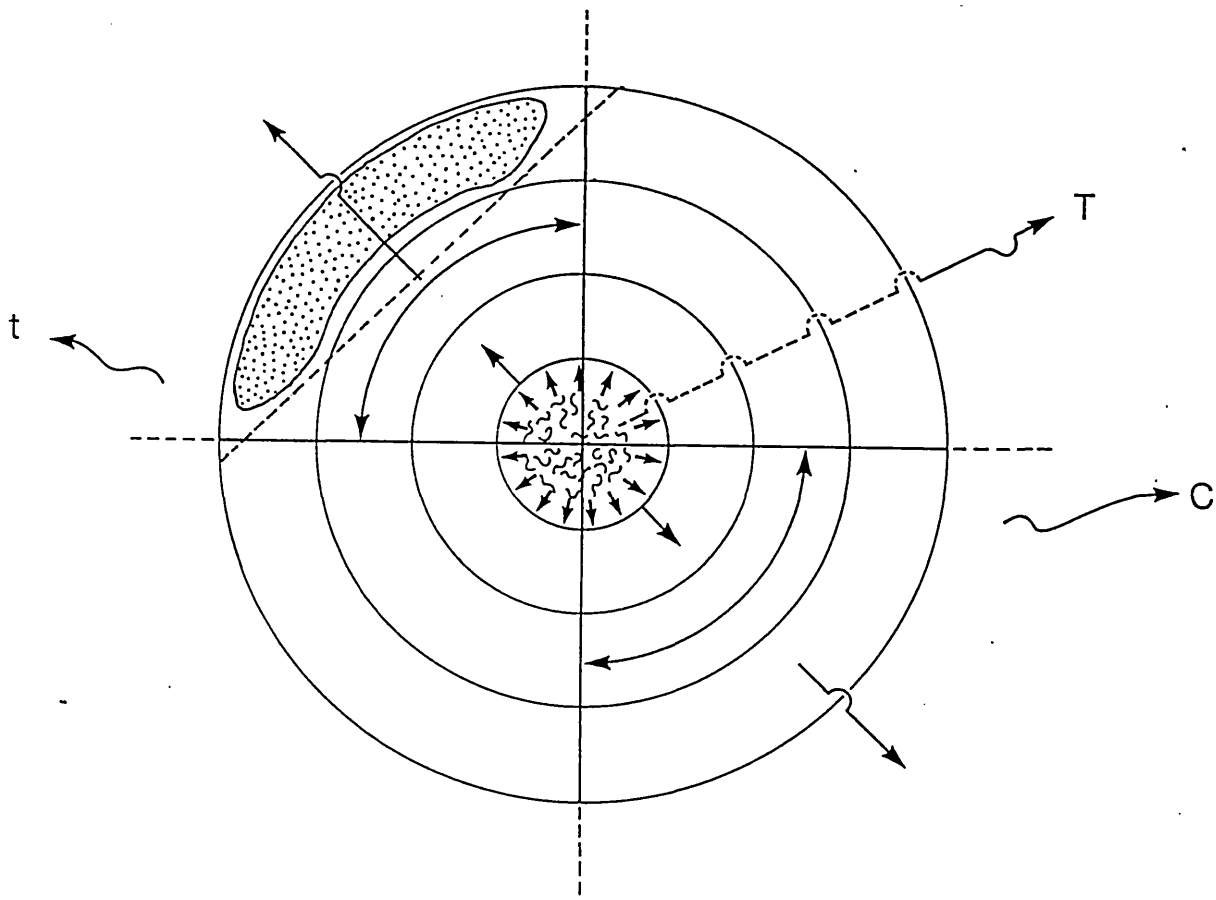
Streaks of four types (24, 48, 96 and 192h-old) of two attractive bacteria, *A. calcoaceticus* var. *anitratus* and *S. liquefaciens* were grown at 25°C. Their effects on nematode attraction were tested. One species of bacteria was studied at a time with three replications for each treatment. Data on the mean distance travelled by nematodes in each plate were recorded periodically and subjected to ANOVA.

(vi) *Attraction of nematodes to dead bacteria*

Four species of bacteria attractive to *C. elegans*, including *A. calcoaceticus* var. *anitratus, E. amnigenus, P. maltophilia,* and *S. liquefaciens* were streak-inoculated around half the periphery of the plates. Three plates were used for each species and the plates were incubated at 25°C for 24h. The bacterial streaks were then killed *in situ* by exposing the plates to drops of chloroform (50 μl each) for 30 minutes in a fume cupboard. To test the effectiveness of chloroform treatment, loop-full of bacteria from each plate was inoculated onto fresh nutrient agar plates and examined for next three days for any growth of the bacteria. The response of *C. elegans* to the dead bacterial lawns was studied (immediately after killing) as described earlier and the data were subjected to a similar analysis.

(vii) *Nature of attractants*

(a) Volatile attractants. Non-vented, partitioned quadrant plates were divided into two unequal portions by fitting aseptic impermeable plastic barriers to the signs of the plate with 'UHU' glue (Beacham, Brentford, UK) before pouring of the agar (Fig. 4.2). The level of the agar was kept a little below the partition wall. Bacteria were streaked in the partitioned area and incubated at 25°C for 24h. Attraction of nematodes rowards the streaks of all 13 species of bacteria was studied similarly.



Attraction index ($\bar{X}$)

$$\bar{X} = [\ 1t_1 + 2t_2 + 3t_3\ - (\ 1C_1 + 2C_2 + 3C_3\ )] \cdot T^{-1}$$

Fig. 4.2 A partitioned quadrant plate used to assess the role of volatiles produced by bacteria in nematode attraction. The dotted line in the test quadrant (t) represents the position of a plastic barrier. For other details see Fig. 4.1.

(b) Diffusible attractants. For detection of diffusible attractants, bacteria were grown on nutrient agar plates in straight lines at an interval of one cm. After 24h incubation at 25°C the inter-streak portions were taken out with a sterile scalpel and placed on half the periphery (test side) of pre-poured nutrient agar quadrant plates. In other half of the periphery (control side), similarly-sized nutrient agar blocks obtained from the bacteria-free plates were placed. Control plates received agar blocks from the bacteria-free plates in both halves of the periphery. Relative migration of nematodes towards both the test and control sides were estimated similarly.

(c) Diffusible-volatile attractants. Agar blocks containing the bacterial diffusates were prepared in similar conditions of incubation. These agar-blocks were then placed on thin pieces of cover-glass in half the periphery of pre-poured nutrient agar plates. The other half of these plates (i.e. the control sides) and also the separate control plates received agar-blocks from bacteria-free plates. Four species of attractive bacteria (exhibiting the ability to produce diffusible attractive substances) were studied simultaneously with three replications for each species. The bacteria tested were: *A. calcoaceticus* var. *anitratus, E. amnigenus, E. cloacae,* and *S. liquefaciens*. Relative migration of nematodes towards the test or control sides was assessed periodically and the data were subjected to ANOVA.

4.2.6 Mass-culture of *C. elegans*

A technique described by Bedding (1981) for the mass-production of insect-parasitic rhabditid nematodes was tested for the production of *C. elegans* for use in large scale trials. Only the modifications made during the present study are described here. Diet was prepared using pig's kidneys (60%), beef-fat (20%) and tap water (20%) and the homogenate was added to crumbed polyether polyurethane sponge (12:1, w/w) and incorporated evenly by stirring and squeezing. About 60 g of the finished homogenate was added to each 500 ml conical flask. Thirty flasks were prepared and autoclaved for one hour at 122°C.

Five species of bacteria: *A. calcoaceticus* var. *anitratus, E. amnigenus, E. cloacae, P. maltophilia* and *S. liquefaciens* were tested as food substrates supporting nematode reproduction. The bacteria were streaked on 3% (w/v) nutrient agar plates (three plates for each bacterium) and incubated at 25°C for 24h. Appoximately 60 axenized second stage *C. elegans* larvae were introduced in 20 μl of sterile distilled water near to the edge of each bacterial streak in each plate and were incubated at 22°C for 48h. When pre-egg laying adults were observed, portions of agar containing bacteria and eight female nematodes were added to each culture flask (six flasks per species) and the flasks were incubated at 22°C (Plate 4.2).

Seven days post-inoculation, nematodes were extracted from the flasks, with the use of milk filters placed in sieves, into water. The nematodes and eggs extracted over 10h were collected, settled, and washed four or five times before being counted.

4.3 Results

4.3.1 Identification of bacteria

Ten species of bacteria were distinguished (Appendix 4.1) from the 26 isolates collected from *C. elegans* immediately after its extraction from compost. Bacteria were identified as *Acinetobacter calcoaceticus* var. *anitratus*, *A. calcoaceticus* var. *lwoffi*, *Bacillus cereus*, *Bacillus* sp., *Enterobacter amnigenus*, *E. cloacae*, *Pseudomonas aeruginosa*, *P. maltophilia*, *Pseudomonas* sp. and *Serratia liquefaciens*. No differences were observed between external and internal bacterial flora from the nematodes.

Plate 4.2 Flasks containing crumbed polyether polyurethane sponge impregnated with beef-fat and pig's kidney diet used for the mass-production of *C. elegans*.

4.3.2 Reproduction of *C. elegans* on associated bacteria

Results of this study are summarised in Fig. 4.3 and Appendix 4.2. Nematode growth was supported by all the bacteria and adult female *C. elegans* were observed 3 days after inoculation. However, reproductive capacity varied greatly. Four days after inoculation the greatest numbers of offspring (mean = 230-358) were evident in cultures containing *A. calcoaceticus* var. *anitratus*, *A. calcoaceticus* var. *lwoffi*, *E. amnigenus* and *S. liquefaciens*; whereas only 14-75 eggs were found in compartments containing *B. cereus, Bacillus* sp., *Pseudomonas* sp. and *P. aeruginosa.*

Five species of bacteria (*A. calcoaceticus* var. *anitratus*, *A. calcoaceticus* var. *lwoffi*, *E. cloacae, P. maltophilia* and *S. liquefaciens*) supported nematode growth and reproduction for several generations. Over a period of 12 days monoxenic cultures of *A. calcoaceticus* var. *anitratus*, and *A. calcoaceticus* var. *lwoffi* resulted in the highest yields.

E. amnigenus and *P. aeruginosa* supported nematode growth and reproduction for 2-3 generations but multiplication then either declined or stopped. *C. elegans* populations increased slightly but steadily with *B. cereus* and *Pseudomonas* sp. With *Bacillus* sp., inoculated larvae grew to adults and started to lay eggs but most of them either did not hatch or the larvae died soon after hatching.

4.3.3 Effects of temperature and bacterial food source on nematode fecundity and generation time

(i) *Nematode fecundity*

When the mean yields of nematodes from the three bacteria were compared at a range of temperatures (Table 4.1), it was found that female *C. elegans* laid maximum numbers of eggs at 15°C and minimum numbers at 10°C. However, when mean yields for all the temperatures were considered, nematode fecundity was greatest when *A. calcoaceticus* var. *anitratus* was present. Interactions between temperatures and bacteria were significant ($P<0.05$), suggesting that the effects of temperature on reproduction of *C. elegans* differ

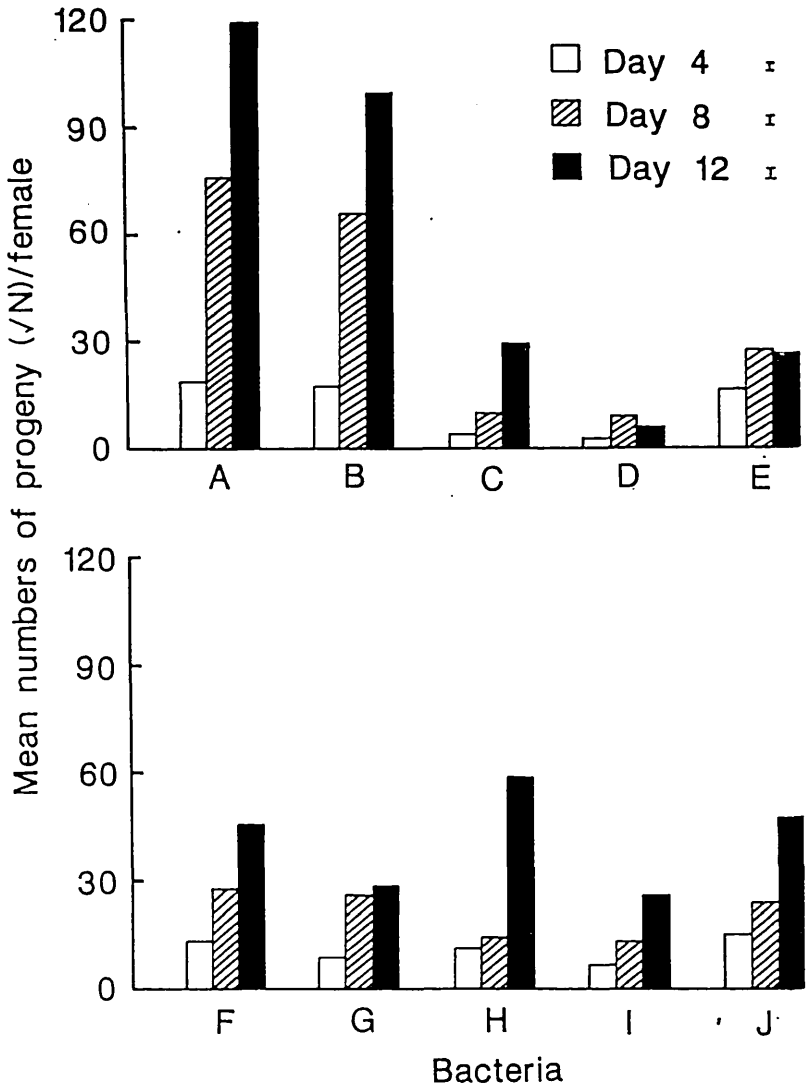


Fig. 4.3 Reproduction of *C. elegans* at 20°C: effects of associated bacteria. A = *A. calcoaceticus* var. *anitratus*; B = *A. calcoaceticus* var. *lwoffi*; C = *Bacillus cereus*; D = *Bacillus* sp.; E = *E. amnigenus*; F= *E. cloacae*; G = *P. aeruginosa*; H = *P. maltophilia*; I = *Pseudomonas* sp. and J = *S. liquefaciens*. Bars represent LSD ($P<0.05$) for comparing bacteria (36 df).

Table 4.1 Effects of temperature and bacterial food source on fecundity of parthenogenetic female *C. elegans*. Data are square-root transformations of the mean total numbers of eggs laid.

	Bacteria*			
°C	Aa	Al	Sl	Grand mean
5	0	0	0	0
10	7.29	5.28	6.70	6.42
15	14.05	13.08	13.71	13.61
20	14.44	11.52	12.39	12.78
22	10.96	10.74	9.55	10.42
25	9.99	9.95	9.74	9.89
28	0	0	0	0
Grand mean	11.34	10.11	10.42	

* Aa = *A. calcoaceticus* var. *anitratus*, Al = *A. calcoaceticus* var. *lwoffi*, Sl = *S. liquefaciens*.

LSD ($P<0.05$) at 60 df:

(a) for comparing grand means = 0.21

(b) for comparing grand means with the same level of temperature = 0.48

in monoxenic cultures of the three bacteria.

Females laid maximum numbers of eggs at 15°C when cultured with *A. calcoaceticus* var. *lwoffi* and *S. liquefaciens*, and at 20°C when cultured with *A. calcoaceticus* var. *anitratus* (Table 4.1). *C. elegans* did not reproduce at 5 or at 28°C with any of the three bacteria and the larvae died 3-4 days after inoculation.

Cubic regression equations for the estimation of optimum temperature for *C. elegans* fecundity (i.e. the temperature at which maximum eggs were laid) with each bacterium are given in Fig. 4.4. The estimated temperature optima were: 16.5, 16.1 and 15.5°C for *A. calcoaceticus* var. *anitratus, A. calcoaceticus* var. *lwoffi* and *S. liquefaciens,* respectively.

(ii) *Nematode generation time*

The effects of temperature on the duration of one generation of *C. elegans* in monoxenic cultures of three bacteria are summarised in Table 4.2. Mean generation time was significantly decreased ($P<0.05$) by increased temperature. *C. elegans* completed one mean generation in 54.2h at 25°C, 59.6h at 22°C, 73.6h at 20°C, 106.2h at 15°C and 233.3h at 10°C. The three species of bacteria did not differ significantly ($P>0.05$) in their effects on generation time.

4.3.4 Effects of bacteria on the migration of *C. elegans* larvae

(i) *Relative attractiveness of different bacteria*

The effects of various bacteria on the behaviour of *C. elegans* are summarised in Fig. 4.5. All the species of bacteria studied altered the normal random migration of the test nematode to a more directed and precise orientation towards bacterial colonies. The degree of alteration in the pattern of nematode migration varied with the bacterial species. For instance, *A. calcoaceticus* var. *anitratus, E. amnigenus, E. cloacae, P. maltophilia* and *S. liquefaciens* elicited significantly vigorous ($P<0.05$) and rapid response of the nematodes when compared to any other bacteria.

Bacillus species including *B. cereus, B. thuringiensis* and *Bacillus* sp. showed the least effects on the nematode movement and attracted fewest

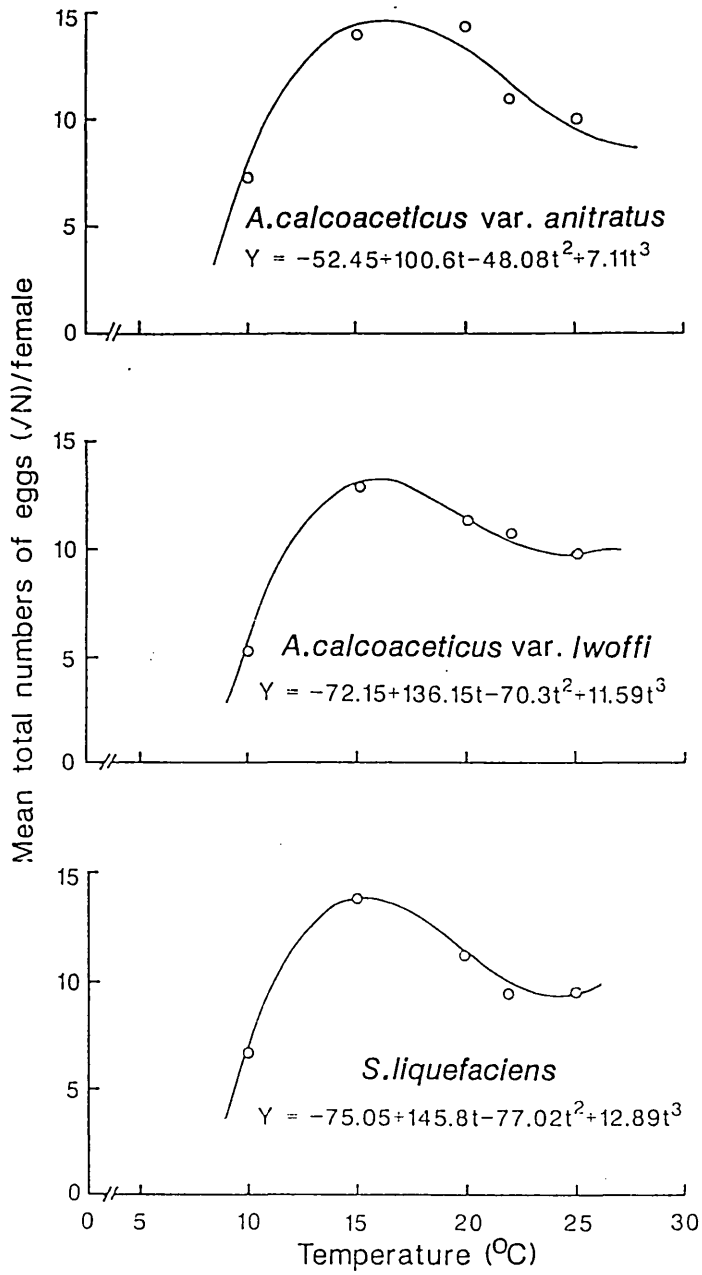


Fig. 4.4. Fitted cubic equations and regression lines showing the effects of temperature and bacterial food source on fecundity of *C. elegans*.

Table 4.2 Effects of temperature and bacterial food source on mean generation time (hours) of *C. elegans*. Data are log transformations.

	Bacteria*			
°C	Aa	Al	Sl	Grand mean
5	0	0	0	0
10	2.346	2.372	2.357	2.364
15	2.026	2.034	2.019	2.027
20	1.867	1.876	1.859	1.867
22	1.779	1.772	1.765	1.772
25	1.725	1.738	1.725	1.730
28	0	0	0	0
Grand mean	1.952	1.958	1.94	

* Aa = *A. calcoaceticus* var. *anitratus*, Al = *A. calcoaceticus* var. *lwoffi*, Sl = *S. liquefaciens*.

LSD ($P<0.05$) at 60 df:

(a) for comparing grand means = 0.014

(b) for comparing grand means with the same level of temperature = 0.032

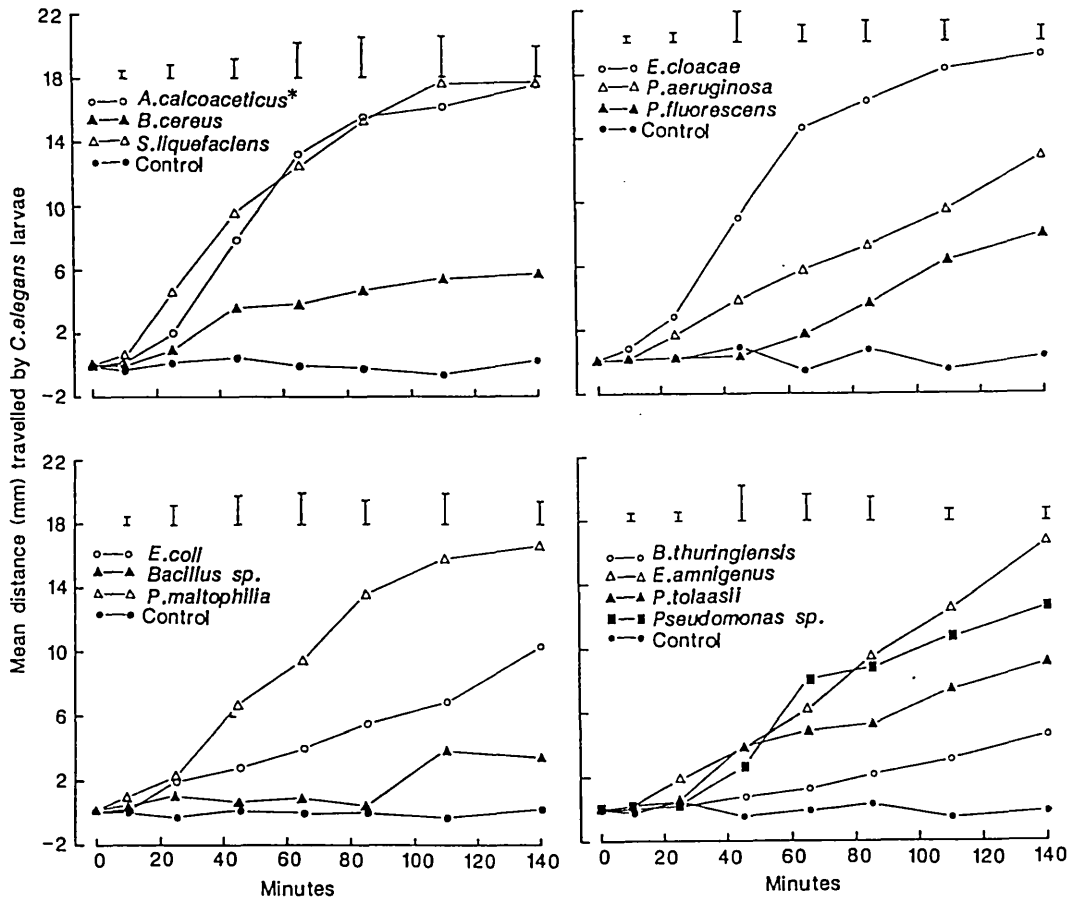


Fig. 4.5 Effects of intact bacterial streaks of different species on the migration of *C. elegans*. Data are mean distance (mm) travelled by third stage larvae towards or away from the bacterial streak. Bars represent LSD ($P<0.05$). * *A. calcoaceticus* var. *anitratus*

nematodes. Other bacteria such as *E. coli, P. aeruginosa* and *P. fluorescens* biovar *reactans, P. tolaasii* and *Pseudomonas* sp. produced intermediate effects.

(ii) *Response of nematodes to a 'choice' of three attractive bacteria*

Behaviour of the nematodes was studied when subjected to three species of bacteria known to be attractive to *C. elegans* (inoculated in all the three possible combinations) simultaneously and the results are presented in Fig. 4.6. Calculation of mean attraction by each species of bacteria in all three combinations revealed that *A. calcoaceticus* var. *anitratus* was significantly ($P<0.05$) more attractive when compared with *P. maltophilia* or *S. liquefaciens*. However, ANOVA elucidated that the combinations also had a significant effect on nematode behaviour. For instance, in combinations I and II, *A. calcoaceticus* var. *anitratus* attracted significantly ($P<0.05$) more nematodes than any other bacteria but in combination III, *S. liquefaciens* was most attractive.

(iii) *Effects of age of bacterial streak on nematode attraction*

Differential response was observed when the nematodes were exposed to different-aged streaks (24, 48, 96, 192h old) of two attractive bacteria (Fig. 4.7). The 24h old streaks of *A. calcoaceticus* var. *anitratus* were most attractive whereas 48h-old streaks of *S. liquefaciens* elicited significantly vigorous response. Furthermore, the streaks of *A. calcoaceticus* var. *anitratus* remained attractive even after 192h incubation whereas the same aged streaks of *S. liquefaciens* were not attractive.

(iv) *Attraction by dead bacteria*

Nematodes were attracted towards dead streaks of all the bacteria tested (Fig. 4.8). Three bacteria (*A. calcoaceticus* var. *anitratus, E. cloacae* and *S. liquefaciens)* were significantly ($P<0.05$) more attractive than *P. maltophilia.*

(v) *Nature of attractants*

(a) Volatile attractants. Fig. 4.9 shows the effects of bacteria on the orientation of *C. elegans* on partitioned quadrant plates when the bacterial streak was separated from the rest of the plate (using an impermeable barrier) to avoid

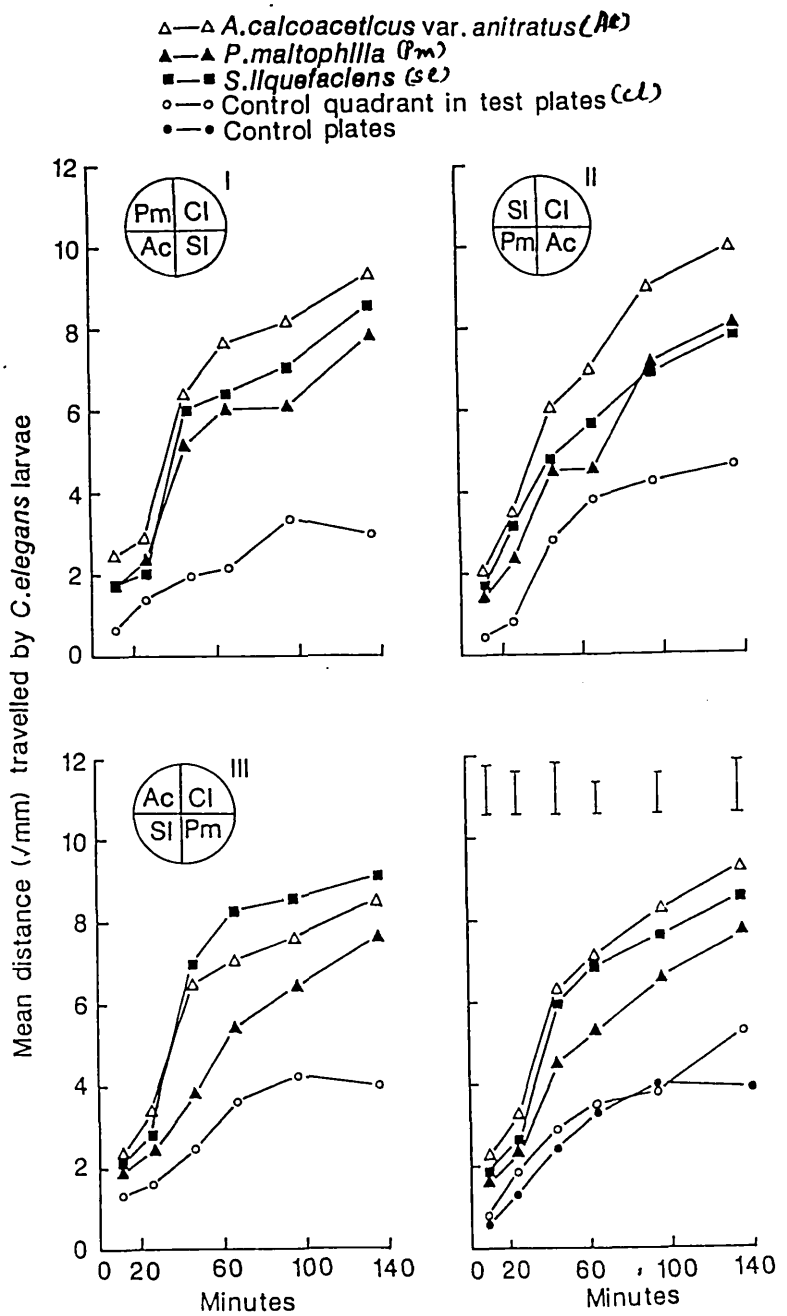


Fig. 4.6 Response of *C. elegans* to a 'choice' of three attractive bacteria. Bacteria were streaked in all the three possible combinations (I, II & III) on each plate. Data are square-root transformations of the mean distance (mm) travelled by third stage larvae. Bars represent LSD ($P<0.05$).

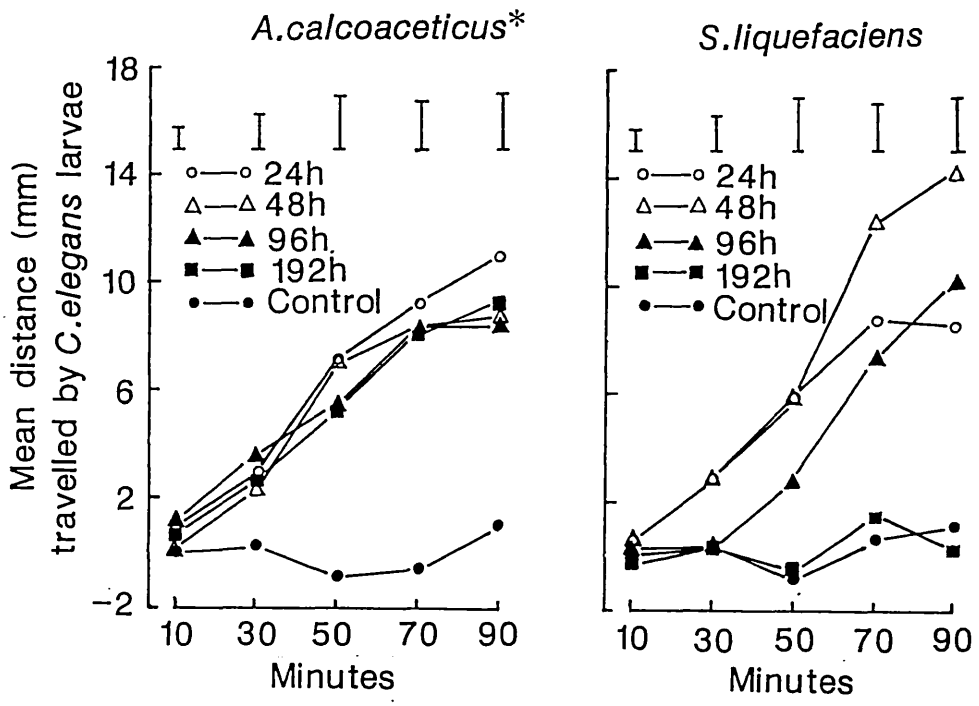


Fig. 4.7. Effects of age of bacterial streaks on the migratory behaviour of *C. elegans*. Data are mean distance (mm) travelled by third stage larvae towards or away from the bacterial streaks.

* *A. calcoaceticus* var. *anitratus*

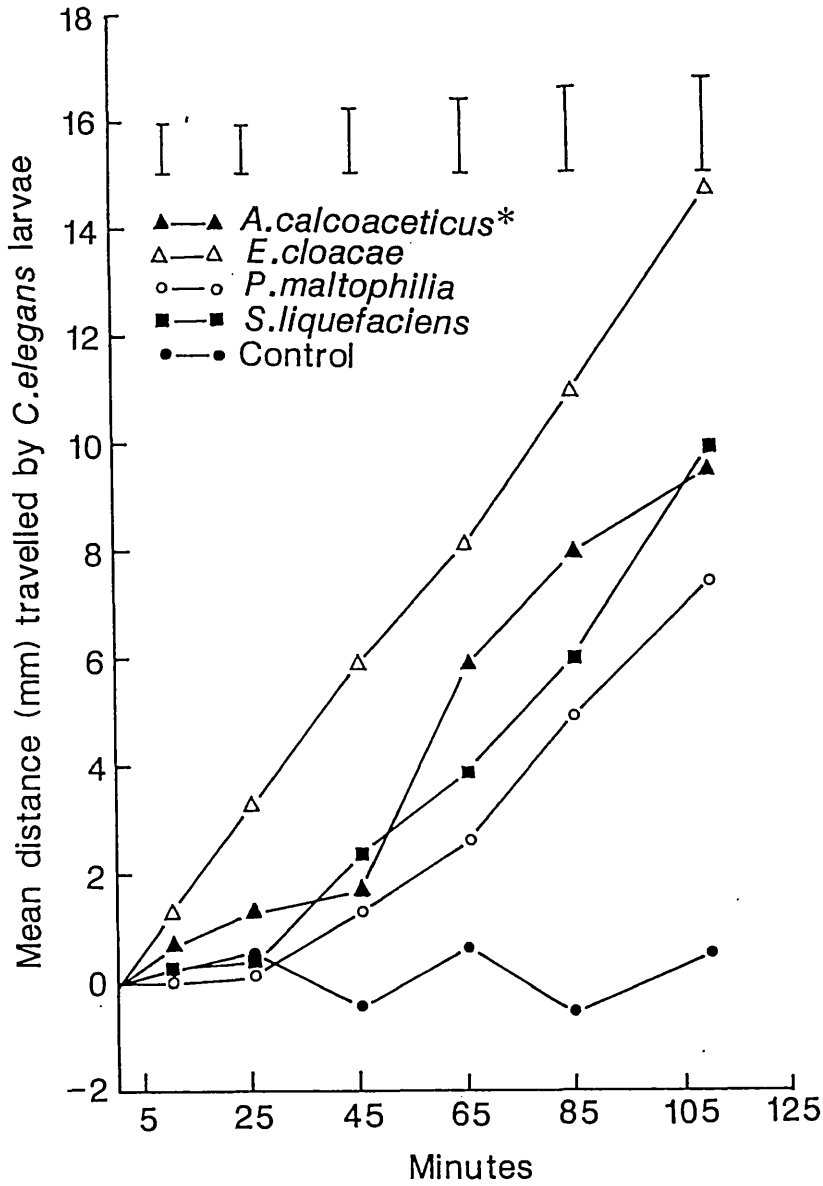


Fig. 4.8 Effects of dead bacteria (killed *in situ*) on the migratory behaviour of *C. elegans*. Data are mean distance travelled by the third stage larvae toward and away from the bacterial streak. Bars represent LSD ($P<0.05$). * *A. calcoaceticus* var. *anitratus*

any diffusion through the agar. All the species of bacteria except, *Bacillus* sp. and *P. fluorescens* biovar *reactans* produced volatile attractants which significantly ($P<0.05$) affected nematode migration. *B. thuringiensis* seemed to be more attractive to the nematodes on partitioned plates than on plates where diffusion through the agar was unimpeded (See Fig. 4.5).

(b) Diffusible attractants. Agar-blocks containing the bacterial diffusates elicited differential responses from nematodes (Fig. 4.10). Diffusates of *A. calcoaceticus* var. *anitratus, E. amnigenus, E. cloacae, P. aeruginosa, P. fluorescens* biovar *reactans, P. maltophilia, P. tolaasii, Pseudomonas* sp. and *S. liquefaciens* were significantly ($P<0.05$) attractive to nematodes whereas those of *B. cereus, B. thuringiensis, Bacillus* sp. and *E. coli* did not elicit any significant ($P>0.05$) response.

(c) Diffusible-volatile attractants. Fig. 4.11 shows that the agar blocks containing bacterial diffusates emitted volatile substances that attracted nematodes. The agar blocks containing diffusates of the bacteria, *E. amnigenus, E. cloacae* and *S. liquefaciens* attracted significantly ($P<0.05$) more nematodes than *A. calcoaceticus* var. *anitratus*.

4.3.5 Mass-culture of *C. elegans*

Yields obtained from the flask cultures are listed in Table 4.3. There was strong evidence that bacteria influence yields: mean yields ranging from 7.2 x 10^6 - 25.7 x 10^6 per flask were obtained from 8 adult female nematodes in one week. *C. elegans* produced the greatest number of progeny with *A. calcoaceticus* var. *anitratus* and the least with *S. liquefaciens.*

The frequency of various life stages of *C. elegans* seven days post-inoculation are shown in Fig. 4.12. Structure of *C. elegans* populations varied with the species of bacteria and seven days after inoculation the mean frequency of second stage juveniles (J2) ranged from 40.8-52% and that of J3 larvae from 31.8-45.5%.

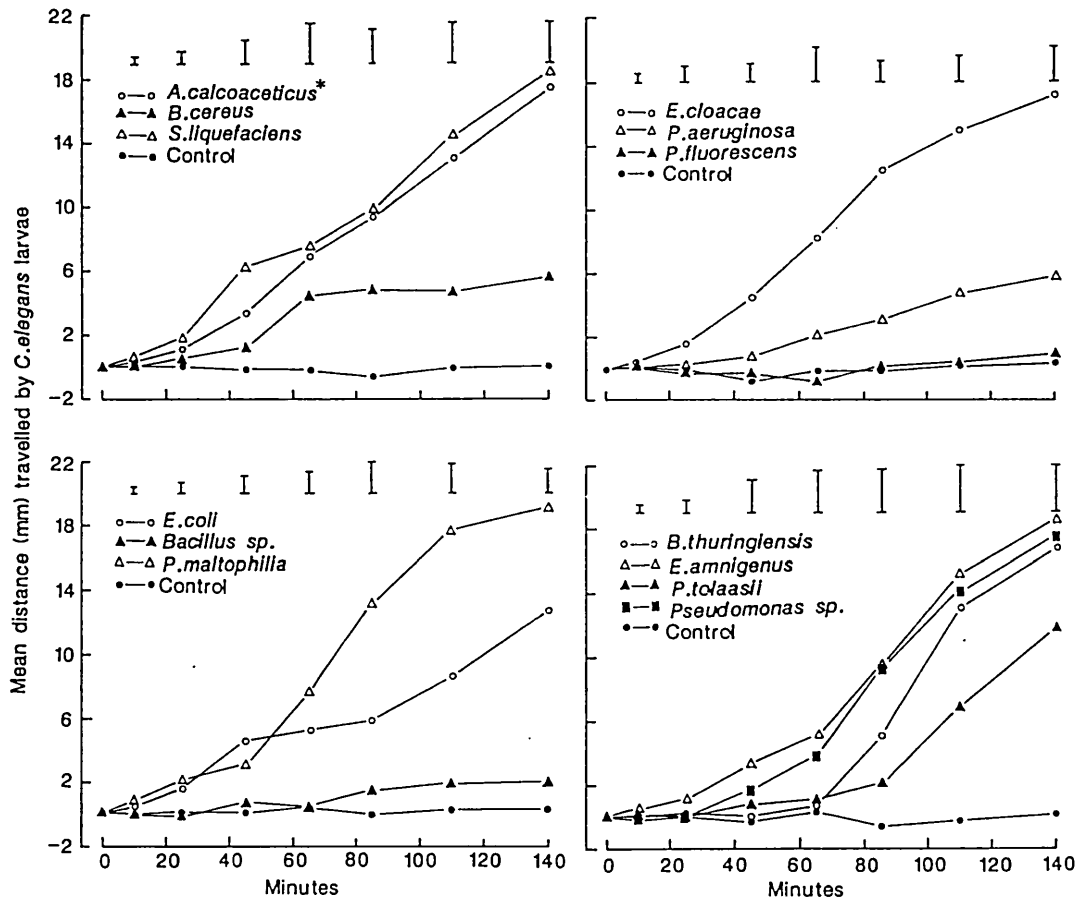


Fig. 4.9 Effects of different bacteria on the migratory behaviour of *C. elegans* when diffusion through the agar was impeded. Data are mean distances (mm) travelled by third stage larvae towards or away from the bacterial streak. Bars represent LSD ($P<0.05$). * *A. calcoaceticus* var. *anitratus*

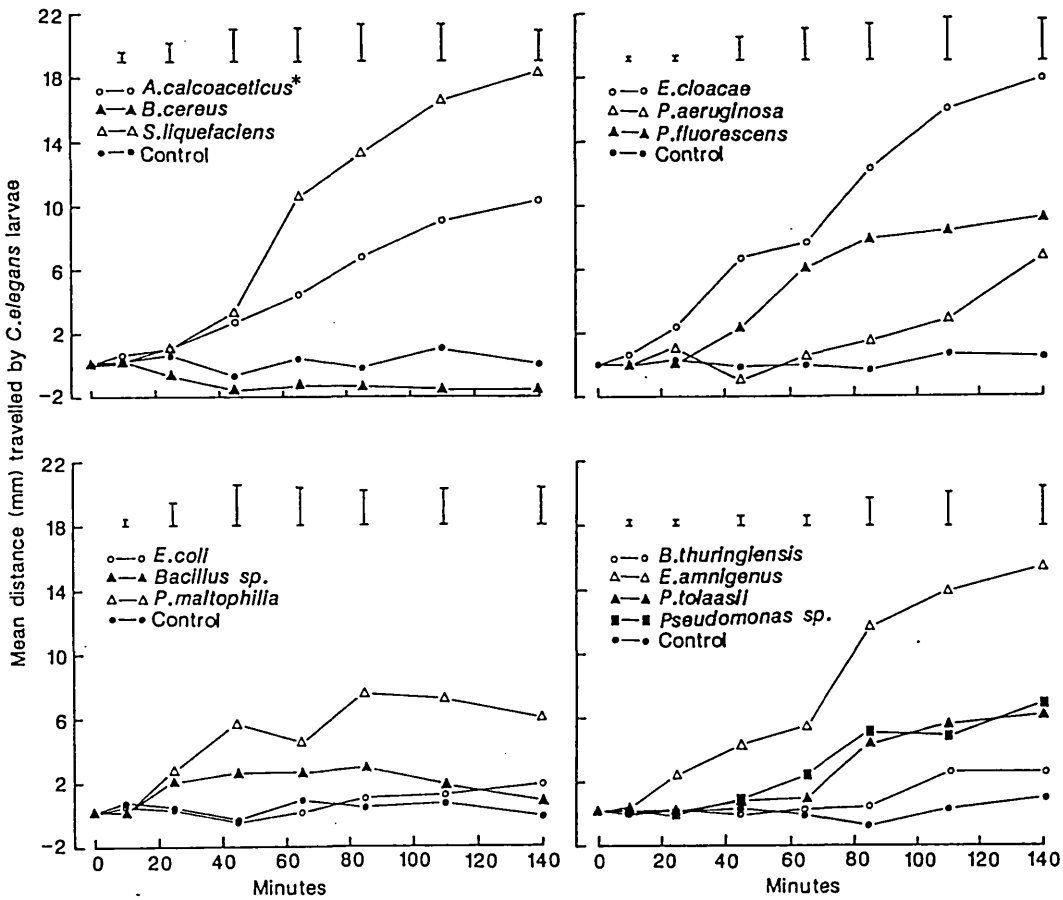


Fig. 4.10 Effects of diffusates (contained in agar blocks) of different species of bacteria on the migratory behaviour of *C. elegans*. Data are mean distances (mm) travelled by third stage larvae towards and away from the test blocks. Bars represent LSD ($P<0.05$).

* *A. calcoaceticus* var. *anitratus*

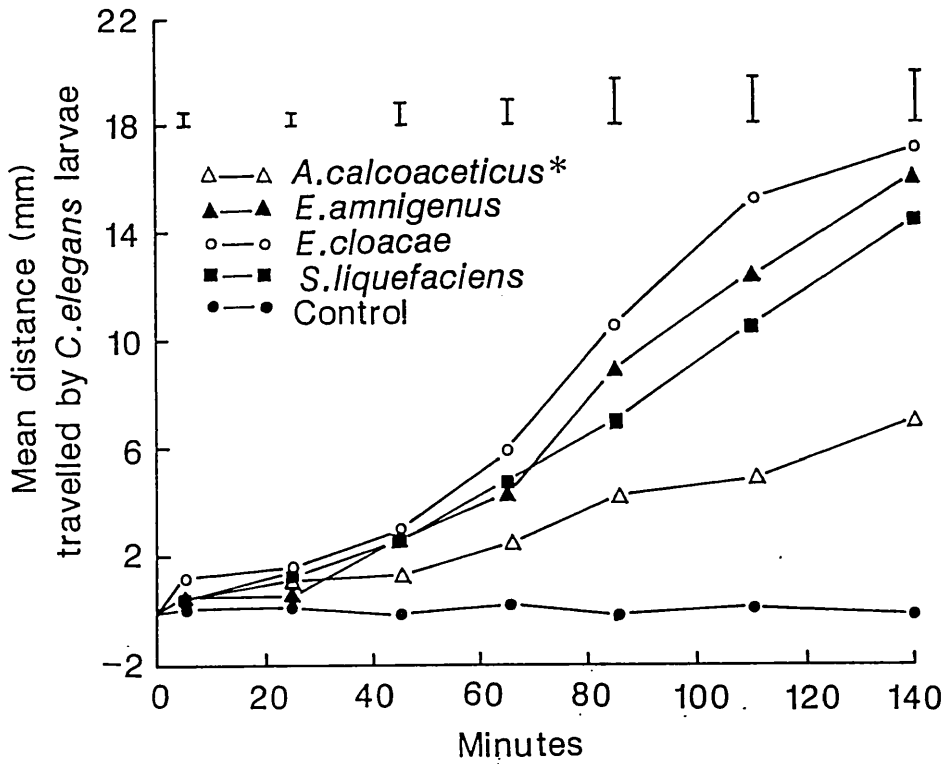


Fig. 4.11 Effects of diffusates (contained in agar blocks) of different species of bacteria on the migratory behaviour of *C. elegans* when further diffusion through the agar was impeded. Data are mean distances travelled by third stage larvae towards and away from the test blocks. Bars represent LSD ($P<0.05$). * *A. calcoaceticus* var. *anitratus*

Table 4.3 Mass production of *C. elegans*: mean numbers of nematodes (eggs, larvae and adults)/flask seven days after inoculation at 22°C.

Bacteria	Mean population ($x10^6$)/flask
A. calcoaceticus var. *anitratus*	25.69
E. amnigenus	15.05
E. cloacae	13.97
P. maltophilia	7.28
S. liquefaciens	7.20
LSD ($P<0.05$)	1.34

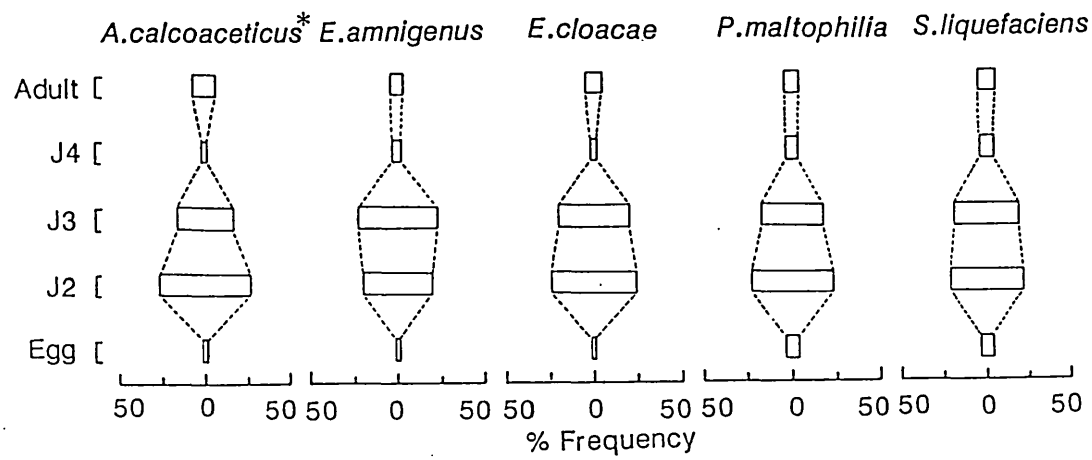


Fig. 4.12 Structure of *C. elegans* populations (% frequency of various life stages) seven days after inoculation on artificial sponge impregnated with beef-fat and pig's kidney diet and different bacteria.

* *A. calcoaceticus* var. *anitratus*

4.4 Discussion

Differential responses of various rhabditid nematodes to bacterial species have been observed in the past (Sohlenius, 1968; Tietjen *et al.*, 1970; Andrew & Nicholas, 1976; Anderson & Coleman 1981). The present study showed that all ten species of bacteria tested supported the growth and development of *C. elegans* and suggested that the nematode is not a selective bacteria feeder. However, when the nematodes continued feeding monoxenically, much variation in their reproductive capacity was observed. For instance, *A. calcoaceticus* var. *anitratus* and *A. calcoaceticus* var. *lwoffi* sustained vigorous reproduction of the nematode for several generations, *E. amnigenus* and *P. aeruginosa* supported reproduction only for 2-3 generations and *C. elegans* could not reproduce at all on *Bacillus* sp.

In comparative studies, Andrew and Nicholas (1976) found that *Escherichia coli* and *P. fluorescens* supported excellent growth and reproduction of *C. elegans* for several generations and resulted in very large adults. *P. aeruginosa* supported growth and reproduction but produced smaller populations than *P. fluorescens*. Reproduction was less vigorous on *Bacillus subtilis*. *B. mycoides* supported growth, but not reproduction, of the nematode. The present results confirm these findings.

The inhibition/retardation of *C. elegans* reproduction after the first few generations by bacteria such as *E. amnigenus* and *P. aeruginosa* could be due to accumulation of bacterial by-products toxic to the nematodes. Bacteria are known to produce nematicidal metabolic products (Johnson, 1957; Bergmann & van Duuren, 1959). Complete inhibition of *C. elegans* reproduction by *Bacillus* sp. suggests the involvement of a toxin. Ignoffo and Dropkin (1977) reported that a thermostable toxin from *Bacillus thuringiensis* (beta exotoxin) was active against *Aphelenchoides avenae, Meloidogyne incognita* and *Panagrellus redivivus*. Bottjer *et al.* (1985) found that *B. thuringiensis* subsp. *kurstaki* and *B. thuringiensis* subsp. *israelensis* were toxic to eggs of the nematode, *Trichostrongylus culubriformis*.

The response of *C. elegans* (in monoxenic cultures of *E. coli*) to temperature has been studied by Byerly *et al.* (1976) who reported that egg-laying was significantly affected by temperature. The present study revealed that

the response of *C. elegans* to temperature is affected by the bacterial food source and that temperature optima for maximum egg-production vary with the species of bacteria used as food. This finding has a direct relevance to mushroom culture as (i) the bacterial flora in casing material changes during cropping (Eger, 1972; Doores *et al.*, 1987) and (ii) temperatures vary during different phases of mushroom growth (Flegg & Wood, 1985). So the development of *C. elegans* populations in mushroom compost/casing material is not only governed by temperature, or total bacterial biomass, but also by the quality of food (i.e. the relative frequency of bacterial species).

Anderson and Coleman (1982) found that *Caenorhabditis* sp. isolated from a short grass prairie in Colorado had a niche breadth of 20-30°C in monoxenic cultures with *Pseudomonas cepacia*. In contrast, in the present study, the Taunton isolate of *C. elegans* could not reproduce at 28°C. Sudhaus (1980) found that species collected from the tropics always had higher lethal temperature tolerances than sibling species from temperate regions. The present investigation also corroborates the findings of Lyons *et al.* (1975) who reported that *C. elegans* did not tolerate temperatures below 10°C.

This study has shown that the size of *C. elegans* populations can be determined by the nature of the bacterial flora. There are two broad sources of bacteria in mushroom culture which affect rhabditid nematodes: (i) the resident bacterial flora in compost and casing material (Hayes *et al.*, 1969; Eger, 1972); (ii) 'foreign' bacteria which are probably introduced in or on the body of nematodes by flies especially fungus gnats (Sciaridae) from rotting vegetable matter. As sources of casing material (especially of peat) and of fly infestations differ, the bacterial flora at each farm will be different and so will have differential effects on nematode populations. This may determine the effects of saprobes on mushroom yield and, in part, explain why there are conflicting reports on whether or not rhabditid nematodes are pests in the mushroom industry.

Interactions between bacteria/nematodes and mushroom mycelium are complex. *A. bisporus* is known to produce volatile metabolites with antibiotic effects (Tschierpe & Sinden, 1965). Some sporophore-inducing bacteria can metabolize the volatile products of *A. bisporus* (Hayes *et al.*, 1969; Eger, 1972).

Therefore, the bacteriostatic environment created by the mushroom imparts selectivity on bacterial populations in the casing material. Barron (1988) reported that *A. bisporus* and some other Basidiomycetes can parasitise living colonies of bacteria and utilise them as a nutrient source. These interactions determine the quantity and quality of bacterial populations in compost or casing and ultimately the size of the populations of bacteria-feeding rhabditid nematodes.

Furthermore, saprobic rhabditid nematodes are known to encourage bacterial populations by (i) providing nutrition in the form of their excretory products and/or dead nematode tissues (Novogrudsky, 1948; Ingham *et al.*, 1985), and (ii) by spreading bacteria in the substrate and consequently providing them with fresh and new food resources (Griffiths, 1986; Poinar & Hansen, 1986).

Kaufman *et al.* (1983) showed that extracts obtained from nematode-infested compost inhibited the mycelial growth of *Agaricus brunnescens*. The present study shows that if the bacterial flora is favourable to rhabditid nematodes, nematode multiplication will be enhanced with a consequent increase in the level of production of inhibitory products which result in yield losses. However, the question of whether or not the nematodes, bacteria or both, are responsible for the inhibitory substance remains unanswered.

The present study has shown that the presence of bacteria affected the pattern of *C. elegans* migration on agar plates and the degree of alteration depended on the species of bacteria. Similar observations have been reported in the past on *C. elegans* (Andrew & Nicholas, 1976) and on *Neoaplectana carpocapsae* (Pye & Burman, 1981). Andrew and Nicholas (1976) reported that *Escherichia coli, Pseudomonas fluorescens* and *P. aeruginosa* were the most attractive bacteria for *C. elegans. Bacillus mycoides* and *B. subtilis* were comparatively less attractive and *B. megatherium* was a repellent. The present results expanded this list and had shown that some of the bacteria e.g. *A. calcoaceticus* var. *anitratus, E. amnigenus, E. cloacae, P. maltophilia* and *S. liquefaciens* were even more attractive than *E. coli* and *P. fluorescens* biovar *reactans*. The bacteria including, *E. coli, P. aeruginosa, P. fluorescens* biovar *reactans, P. tolaasii* and *Pseudomonas* sp. represented an intermediate group and

that *B. cereus, B. thuringiensis* and *Bacillus* sp. were the least attractive. No bacteria repelled *C. elegans. B. thuringiensis* and *Bacillus* sp. showed no significant effects on nematode migration up to 85 minutes after the start of the experiment and thereafter the response was significant, probably because of the absence of any other food source on plates the nematodes tended to stay in the bacterial streaks.

In nature, nematodes are probably exposed to a range of bacterial flora growing in close proximity to each other rather than individual species at a time. Under such circumstances the attractants/repellents are simultaneously produced and released into the micro-environment. A more natural situation was simulated by exposing the nematodes to three attractive bacteria simultaneously. Although the differences in attractiveness of the three bacteria were small the nematodes invariably detected the most attractive bacteria.

The ability of bacteria to attract nematodes is probably related to their growth conditions: 24h-old colonies of *A. calcoaceticus* var. *anitratus* being more attractive than older colonies. In *S. liquefaciens* the 48h-old colonies were more attractive than 24h-old ones which may be due to a higher optimum growth temperature for this bacterium (28-35°C, Grimont & Grimont, 1984). This indicates that the attractants are produced in abundance only when the bacteria are in active growth phase. The differential response of *C. elegans* to the older cultures (i.e. 192h-old) of *A. calcoaceticus* var. *anitratus* and *S. liquefaciens* suggested that the two bacteria produce different types of attractants.

Andrew and Nicholas (1976) observed that smears of autoclaved bacteria were not attractive. Therefore, the attractiveness of bacterial lawns that were killed *in-situ* by fumes of choloroform may be due to prior establishment of gradients of attractants in the agar. This view was supported when the agar blocks containing the bacterial diffusates attracted nematodes.

There is evidence of chemical attraction of nematodes to bacteria although the identity of the chemicals involved is uncertain. Ward (1973) reported that *C. elegans* was attracted to cyclic AMP, which is released by bacteria; and to other unidentified substances in the media where bacteria have grown. Andrew and Nicholas (1976) reported that the attractive bacteria produced an alkaline environment in their vicinity. Therefore, it is also possible

that the attraction is based simply on the pH gradient as *C. elegans* is attracted to high pH (Ward, 1973).

Another interesting aspect of this study was the observation that the agar-blocks containing bacterial diffusates emitted volatile attractive substance(s). On the partitioned-quadrant plates, eleven species of bacteria (out of the 13 studied) produced volatiles that affected nematode migration. Furthermore, the pattern of *C. elegans* response to the volatiles was almost comparable to that observed when unimpeded diffusion through the agar occurred. These results suggest that the volatile substances may be the main component(s) of the attractants produced by bacteria. In nature, the volatile attractants may be more important than the diffusible substances in affecting the nematode migration significantly because the diffusion through substrate is generally impeded.

Because it can occur in high enough concentrations in nature, carbon dioxide can also be an attractant. Klingler (1965) reported that plant parasitic nematodes are attracted to the roots of plants which produce CO_2. Gaugler *et al.* (1980) demonstrated that infective stage juveniles of *N. carpocapsae* migrated up the CO_2 gradient. Furthermore, the response of nematodes to CO_2 depends upon its concentration in the medium (Balan & Gerber, 1972) and also of one of its hydrated forms on the type of other ions present (Dusenbery, 1983).

Conflicting reports exist about the possibility of ammonia as a nematode attractant. Katznelson and Henderson (1963) found that some soil nematodes though not *Caenorhabditis briggsae* were attracted to ammonium ions whereas Ward (1973) demonstrated that *C. elegans* was neither attracted nor repelled by ammonium ions. Andrew and Nicholas (1976) found that the more attractive bacteria released ammonium ions into peptone-water solution in which they were grown and also produced an alkaline gradient in agar cultures. Schmidt and All (1979) demonstrated that the dauer larvae of *N. carpocapsae* are attracted towards various constituents of insect faeces including ammonia. These authors observed that the nematodes migrated onto the tops of ammonia treated filter paper squares whereas with the other attractants such as allantoin, arginine and adenine they aggregated in a dense mass directly beneath the square. This probably explains the effects of volatile nature of ammonia on nematode

behaviour.

Axenic liquid cultures of *C. elegans* are reported to produce large populations only when shallow; depth crucially affects gaseous exchange and influences accumulation of ammonia in the medium (Hansen & Cryan, 1966; Buecher & Hansen, 1971; Vanfleteren, 1976; Skimming *et al.*, 1984). Using such a method, Vanfleteren (1976) obtained 10^9 *C. elegans*, or 40 g wet weight in six weeks from 50 nematodes using spinner flasks in a partially defined medium (3% soypeptone, 3% yeast extract, 1% dextrose, 1% casein hydrolysate and 500 μg/ml haemoglobin).

Yields of up to 25.7 x 10^6 *C. elegans* in monoxenic cultures of *A. calcoaceticus* var. *anitratus* per 60 g diet (i.e. over 500,000 nematodes/g of diet) from eight nematodes in seven days were achieved using the present technique. Sponge thinly coated with medium provided a large three dimensional surface area and allowed adequate interstitial space for migration and ventilation of nematodes. The sponge technique might prove useful when large numbers of nematodes are required quickly. Having developed the sponge technique, Bedding (1981) obtained 29-55 million *N. carpocapsae* from monoxenic culture with a bacterial symbiont, *Xenorhabdus nematophilus* in 2-3 weeks at 20-28°C. Although even greater yields of *C. elegans* (Skimming *et al.*, 1984) have been achieved with the use of fermentors, the sponge technique is less cumbersome, cost-effective and quicker.

This study showed that the yields of *C. elegans* varied considerably with species of bacteria. This confirmed the previous results of plate cultures (Section 4.2.3) and may be due to differential suitability of various bacteria as a food substrates for the nematodes and/or due to accumulation of toxic by-products (Nicholas, 1984, Poinar & Hansen, 1986).

Assessment of the structure of *C. elegans* populations yielded interesting information. The greater proportion of dauer larvae (J3) in the population may be useful for handling and long-term storage of nematodes as the 'dauer' is a non-feeding and developmentally arrested stage which has the ability to tolerate environmental stress (Golden & Riddle, 1984; Riddle, 1988). Based on these observations it was decided to extract the nematodes from flasks eight days after inoculation (not seven days). This considerably improved the proportion of J3

larvae in *C. elegans* populations during subsequent mass-production. However, there is considerable scope for improvement of this technique for mass-production of *C. elegans* and other rhabditid nematodes.

CHAPTER 5

Effects of *C. elegans* on *A. bisporus*

5.1 Introduction

While the effects of some saprobic rhabditid nematodes on the mushroom *A. bisporus* have been studied previously (Chapter 2.2), these reports have resulted in conflicting views of their significance in the mushroom industry. In all these earlier studies, the nematode inoculum was obtained from naturally-infested compost and/or casing material collected from mushroom farms. Thus, the nematode inoculum almost certainly contained an unknown flora of micro-organisms which may have been partly responsible for the conflicting results obtained by these workers.

In this study, the effects of monoxenically (with *A. calcoaceticus* var. *anitratus*) mass-produced *C. elegans* (Section 4.2.6), the commonest species found, on the yield and quality of U3 strain of *A. bisporus* were evaluated.

5.2 Materials and methods

5.2.1 Preparation of the mushroom substrate

The mushroom substrate, prepared from composted wheat straw (Randle, 1974), was pasteurised and inoculated with mushroom grain spawn (*A. bisporus* strain Darmycel U3) at a rate equivalent to 0.5% (w/w) of the fresh-weight of the substrate.

For Experiment 1, spawned compost was placed in milk crates (7.5 kg compost/crate), each lined with a polythene sheet. The compost was incubated for 14 days at 25°C to allow colonization by the fungal mycelium ('spawn-run').

In all the other experiments, spawned compost was filled into

commercial growing trays (90 x 60 x 15 cm), incubated under similar spawn-run conditions and then, 14 days later, transferred into milk crates. The crates were arranged on an aluminium framework in a purpose-built mushroom growth chamber, in a Latin square design in two rows and three layers (Plate 5.1).

In order to minimise natural infestations by saprobes, thoroughly pasteurised compost was used in all the experiments. To avoid inter-plot spread of nematodes by flies, frequent knock-down aerial sprays of a synthetic pyrethroid, 'Pynosect 30' were made during cropping.

Colonized substrate was cased with a mixture of peat and chalk at a rate of 3.75 kg casing/crate. Diflubenzuron (Dimilin 25 WP) was incorporated into the casing material at 30 ppm to control potential sciarid fly infestations. After casing, the temperature of the chamber was maintained at 22°C for seven days, then lowered to 16-18°C for a further seven weeks during which time mushrooms were produced. The environment (temperature, humidity and aeration) in the growth chamber was maintained by a computerised system (Plate 5.2) and the other cultural practices were followed according to Flegg *et al.* (1985).

5.2.2 Compost treatments

There were six replicates of four compost treatments; three nematode inocula (10^4, 10^5 and 10^6 nematodes/crate) and a control treatment (no nematodes). For each replicate, 7.5 kg of spawned compost was weighed and spread on to a polythene sheet. A nematode inoculum (adults:larvae = 1:19) in 250 ml of water was sprinkled on to the compost and mixed thoroughly. Controls received an equal amount of water.

5.2.3 Casing treatments

Two more experiments were conducted with a similar layout, level of replication and infestation technique except that the nematodes, for each replicate, were applied to 3.75 kg of casing. In the first experiment, the inocula were 10^4, 10^5 and 10^6 nematodes/crate and in the second they were 10^6, 10^7 and 2×10^7 nematodes/crate.

Plate 5.1 The milk-crates used for mushroom growth trials and their arrangement in the chamber.

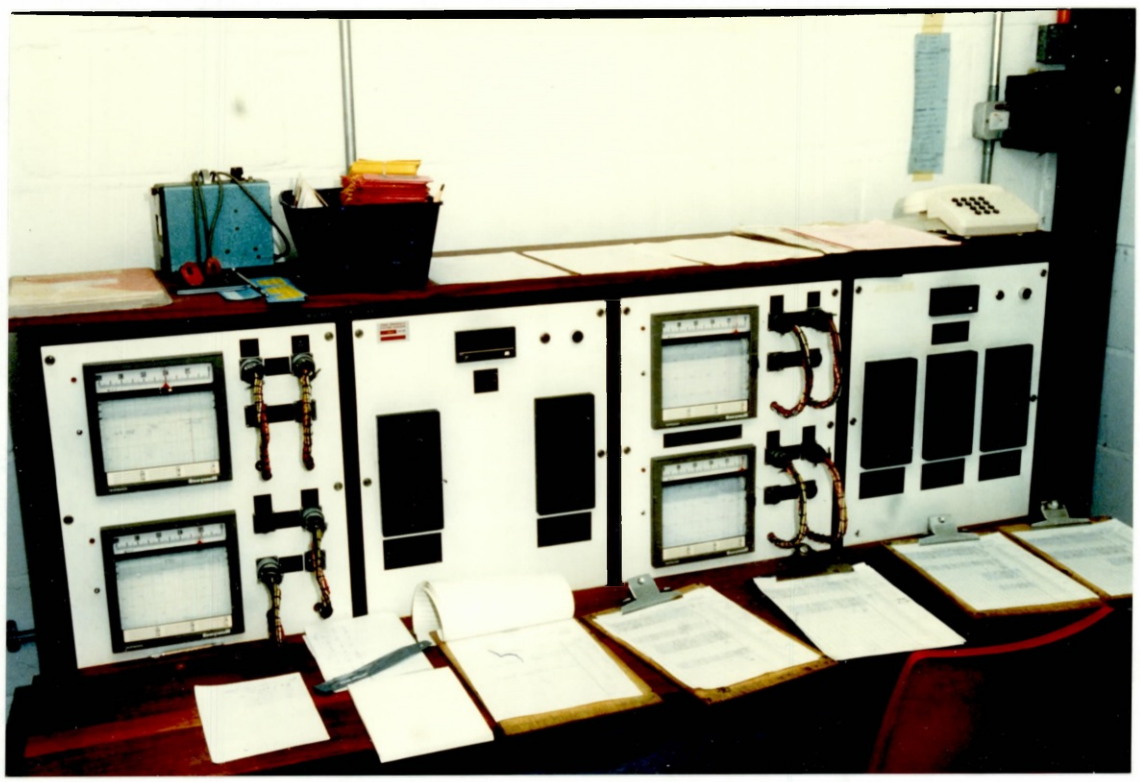


Plate 5.2 The central environment control facility for computerised maintenance of temperature, humidity and aeration in the mushroom growth chambers.

5.2.4 Data recording and analysis

(i) *Compost temperature*

In Experiment 1, probes were used to record air and compost temperatures during spawn-run and cropping. Means of three readings were recorded daily. In all other experiments, air and compost temperatures were recorded only during cropping (see Appendices 5.2 & 5.3).

(ii) *Casing pH*

In Experiment 2, casing samples (10 g each) were taken at weekly intervals from each crate. After thoroughly mixing, 2 g of casing was drawn from each sample, suspended in 100 ml of distilled water for one hour and its pH determined with a pH meter.

(iii) *Nematode populations in compost or casing*

Populations of *C. elegans* in compost or casing were estimated at weekly intervals after inoculation. Samples of either compost (20 g) or casing (25 g) were taken from each crate and nematodes were extracted using the Baermann funnel technique (See Section 3.3.4). They were fixed in a 2% (v/v) formaldehyde solution and counted after appropriate dilutions were made. Data were square-root transformed and subjected to ANOVA.

(iv) *Nematodes on mushroom sporophores*

In the third experiment only, numbers of *C. elegans* on mushroom sporophores were estimated once during each flush, the onset of which was estimated from the yield data. Picked mushrooms (approx. 200 g) from each crate were selected at random and soaked in one litre of tap water. They were agitated every 30 minutes and removed after two hours. Nematodes in the suspension were allowed to settle and excess water siphoned off. The nematodes were fixed in a 2% (w/v) formaldehyde solution and counted. Data analysis was as above.

(v) *Mushroom yield*

Mushrooms were harvested on alternate days at the point of market acceptability, i.e. stages 3-5 (Hammond & Nichols, 1976). Their stipes were trimmed off and numbers and weights recorded using the computerised programme (Plate 5.3). Total numbers and weight of mushrooms were subjected to ANOVA. The relationship between the yield and the initial nematode population was calculated using regression analysis.

Plotting of cumulative yields from the nematode treatments (Y-axis) against the controls (X-axis) allowed to evaluate the effects of *C. elegans* on flushing patterns of *A. bisporus*. Slopes of the resulting curves were subjected to regression analysis (Edmondson, 1989).

(vi) *Distortion of mushroom sporophores.*

In the third experiment, where the highest nematode inocula were applied, data on the numbers and weights of healthy and distorted mushrooms were recorded separately at each pick and were similarly analysed.

5.3 Results

5.3.1. Compost treatments

(i) *Compost temperature*

Trends in the air and compost temperatures during spawn-run and cropping are shown in Fig. 5.1 (also see Appendix 5.1). Compost temperatures rose rapidly after spawning and remained higher than air temperatures during most of the cropping period.

(ii) *Nematode populations in compost and casing*

Populations of *C. elegans* in compost initially declined (Fig. 5.2) but in the casing started to increase 28 days after inoculation (two wk after casing). Populations reached their peaks by 56 or 63 days after inoculation depending on treatment type and then declined.

Plate 5.3 Data recording assembly: a programmed computer, a balance and a printer.

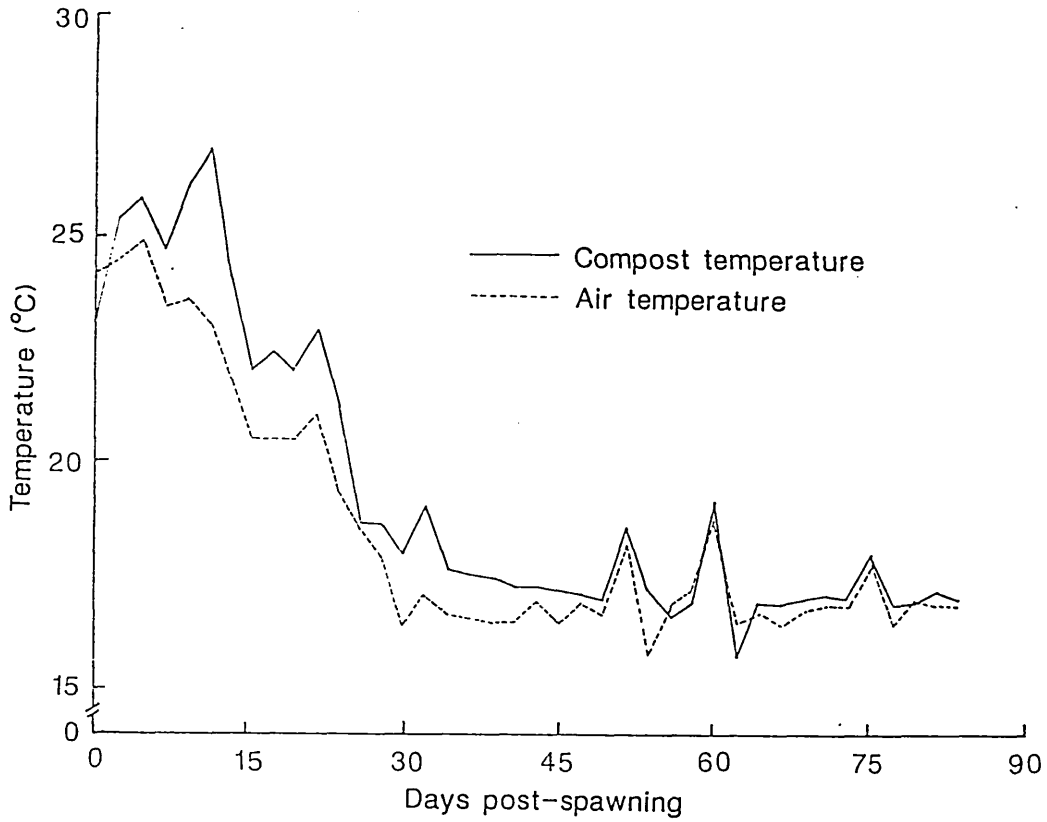


Fig. 5.1 Average air and compost temperatures in mushroom growth chambers during spawn-run and cropping (n = 3).

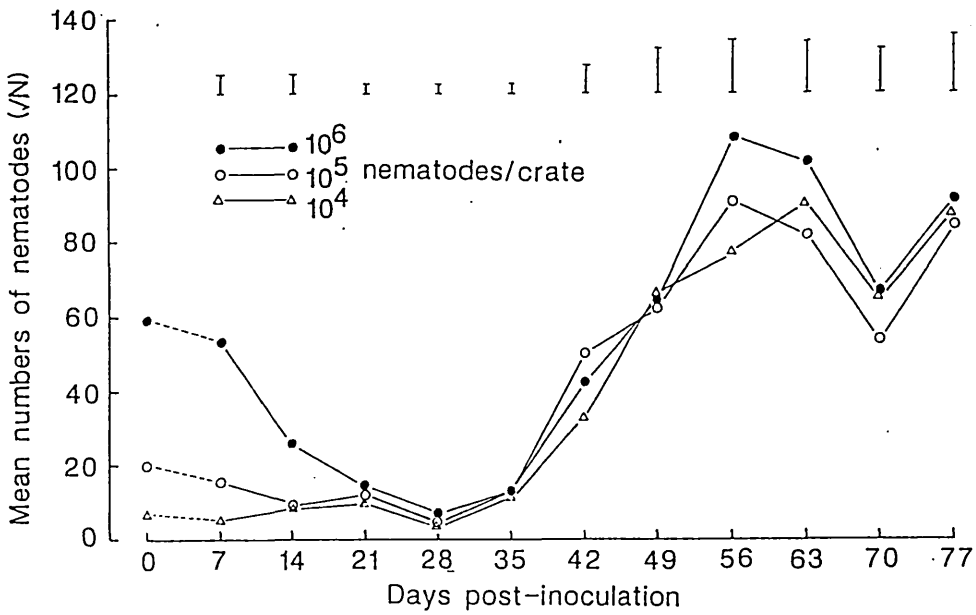


Fig. 5.2 Mean number of *C. elegans* extracted from 20g of compost (0, 7 and 14 days post-inoculation) or 25g of casing (21-70 days post-inoculation): nematodes added to compost during spawning (casing applied at day 14). Bars represent LSD ($P<0.05$) at 10 df.

(iii) *Mushroom yield*

C. elegans inoculated in compost during spawning did not result in significant effects ($P>0.05$) on the total mushroom yields (Table 5.1).

5.3.2 Casing treatments-lower inocula

(i) *Casing pH*

The level of nematode inoculum appeared to have little subsequent effect on the pH of casing (Table 5.2). After 1, 5, 6 and 8 weeks post-inoculation, significant differences ($P<0.05$) between nematode treatments and controls were observed, but the results could not be related to nematode dosage rate.

(ii) *Nematode populations in casing*

When assessed seven days after inoculation, an increase in the *C. elegans* population was observed at the highest dose (10^6 nematodes/crate) (Fig. 5.3). This declined over the next 14 days and then fluctuated around an equilibrium from 28 days after inoculation. In the other two treatments (10^4 and 10^5 nematodes/crate), populations only started to increase 14 days after inoculation, reaching their respective peaks 35 and 42 days after inoculation and then stabilising around a similar equilibrium.

(iii) *Mushroom yield*

Nematodes introduced in the casing showed some effects on the total mushroom yield (Table 5.1). All the three inocula (10^4, 10^5, and 10^6 nematodes/crate) significantly reduced ($P<0.05$) the total numbers of mushrooms. *C. elegans* applied at 10^6 nematodes/crate also caused a significant reduction ($P<0.05$) in total weight of mushrooms picked.

5.3.3 Casing treatments-higher inocula

(i) *Nematode populations in casing*

Trends in *C. elegans* populations in casing are shown in Fig. 5.4. All

Table 5.1 Effects of *C. elegans* on *A. bisporus*: mean total weight and numbers of mushrooms/crate

Nematodes ($x10^4$)/crate	Nematodes added to compost		Nematodes added to casing	
	Mean weight (kg)	Mean numbers	Mean weight (kg)	Mean numbers
100	2.10	181.2	2.013	179.8
10	2.06	193.0	2.130	185.2
1	2.29	208.5	2.251	186.5
0	2.16	198.7	2.226	208.7
SED (15 df)	0.95	10.8	0.063	11.3

Table 5.2 Changes in the pH of mushroom casing material with or without *C. elegans*.

Nematodes ($x10^4$)/crate	Weeks post-casing							
	1	2	3	4	5	6	7	8
100	7.43	7.51	7.18	7.37	7.23	7.23	7.08	7.20
10	7.43	7.53	7.09	7.45	7.14	7.32	7.09	7.23
1	7.42	7.60	7.18	7.46	7.24	7.19	7.09	7.24
0	7.52	7.54	7.17	7.49	7.47	7.33	7.15	7.36
SED (15 df)	0.03	0.06	0.05	0.08	0.06	0.07	0.06	0.06

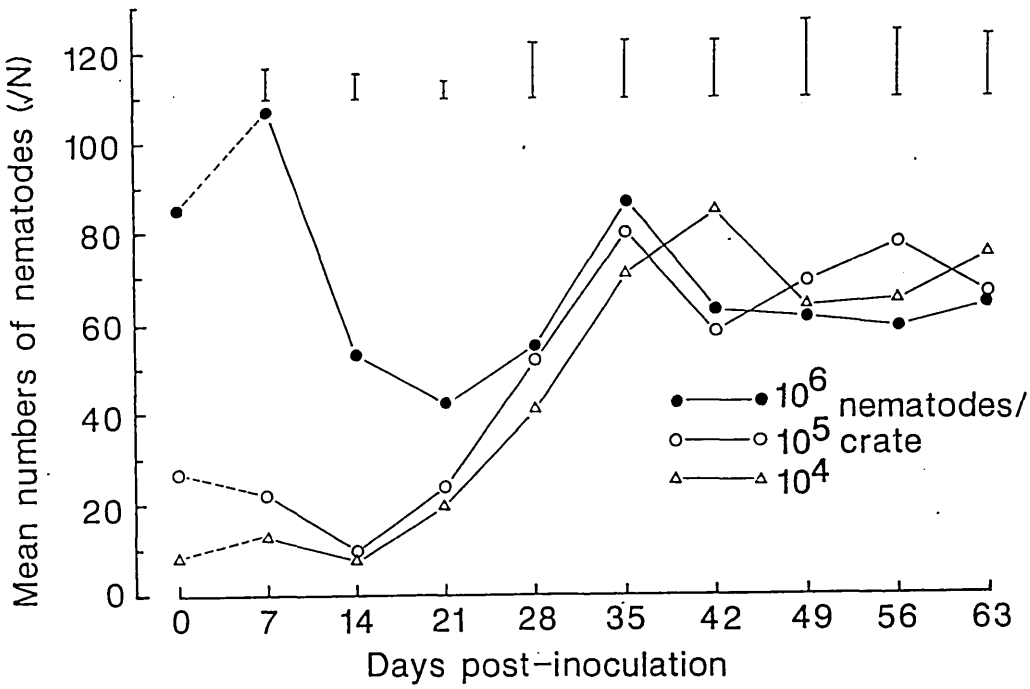


Fig. 5.3. Mean number of *C. elegans* extracted from 25g of casing: nematodes added to casing (lower inocula). Bars represent LSD ($P<0.05$) at 10 df.

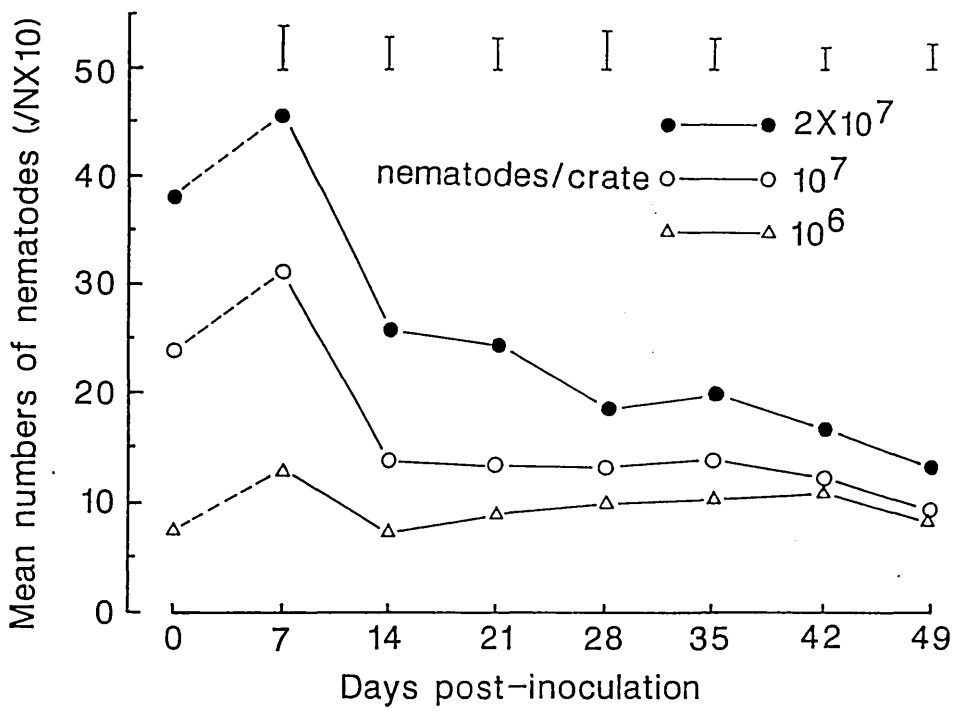


Fig. 5.4. Mean number of *C. elegans* extracted from 25g of casing: nematodes added to casing (higher inocula). Bars represent LSD ($P<0.05$) at 10 df.

three inocula resulted in an initial rise in nematode populations which then declined slowly throughout the cropping period. One to two wk after inoculation, massive out-breaks of nematode populations were observed which resulted in the production of classical 'winking' columns of nematodes on the casing layer (Plate 5.4).

(ii) *Nematodes on sporophores*

Washed mushrooms revealed the presence of *C. elegans* whose numbers varied according to treatment level or mushroom flush (Fig. 5.5). Swarming of nematodes was observed on the sporophores and populations in excess of 17,000 nematodes/sporophore were recorded at the highest inoculum level.

(iii) *Mushroom yield*

Yields of *A. bisporus* obtained from the third experiment are listed in Table 5.3. All three rates of inocula resulted in significant decreases ($P<0.05$) in both the weight and number of mushrooms picked. Mean losses of 11, 20 and 26 % in total mushroom weight were observed at inocula of 10^6, 10^7 and 2×10^7 nematodes/crate, respectively.

Regression analysis of the data showed that there was a significant linear relationship ($P<0.05$) between the total weights and numbers of mushrooms and initial nematode inoculation level (Table 5.4).

(iv) *Flushing patterns*

Plotting cumulative mean yields from the nematode treatments against controls, revealed that the flushing patterns of *A. bisporus* were significantly affected ($P<0.05$) by *C. elegans* (Fig. 5.6). Regression analysis indicated that the slope of the curves for the three nematode inocula were significantly ($P<0.05$) different from an expected straight line curve. In the untreated controls, the mushrooms appeared in distinct flushes. However, all three nematode treatments caused a suppression of this natural phenomenon and resulte in the production of fewer mushrooms.

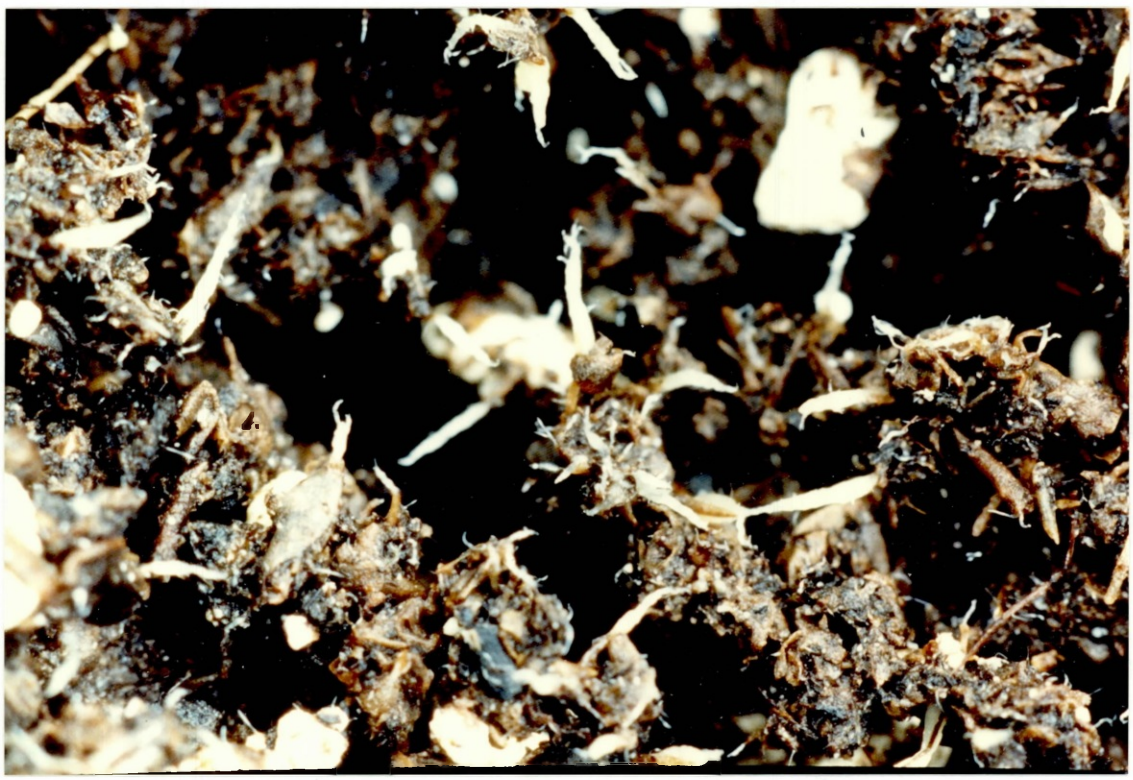


Plate 5.4 'Winking' or 'climbing' columns of nematodes on casing layer indicative of high populations of *C. elegans* (x 5).

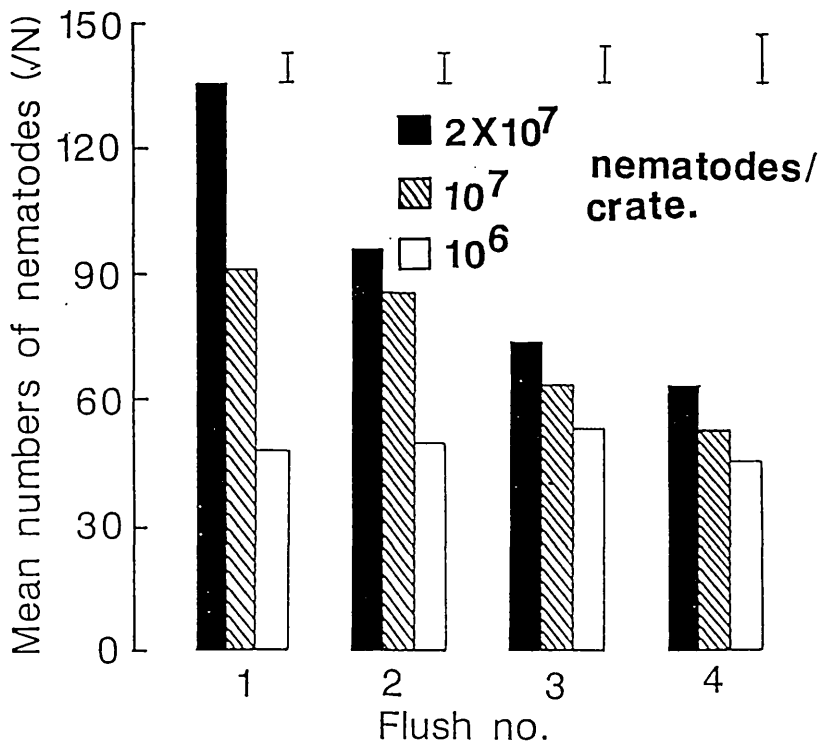


Fig. 5.5 Mean number of *C. elegans* obtained from washes of 200g of mushrooms: nematodes added to casing (higher inocula). Bars represent LSD ($P<0.05$) at 10 df.

Table 5.3 Effects of *C. elegans* on *A. bisporus*: nematodes added to casing (higher inocula)

Nematodes ($x10^6$)/crate	Mean total weight (kg) of mushrooms/crate	Mean total numbers of mushrooms/crate
20	2.254	198.7
10	2.438	221.2
1	2.710	237.8
0	3.042	266.2
SED (15 df)	0.079	15.6

Table 5.4 Regression equations and correlation coefficients (r) between initial *C. elegans* population (X); and weight and number of total (see Table 5.3) and distorted (see Table 5.5) mushrooms (Y)

Parameter	Regression equation (10 df)
Total wt (g) of mushrooms	Y = 2714.3 - 23.9X (r = -0.98)
Total number of mushrooms	Y = 240.6 - 2.1X (r = -0.99)
Total wt (g) of distorted mushrooms	Y = 88.7 + 4.5X (r = 0.99)
Total number of distorted mushrooms	Y = 8.3 + 0.7X (r = 0.99)

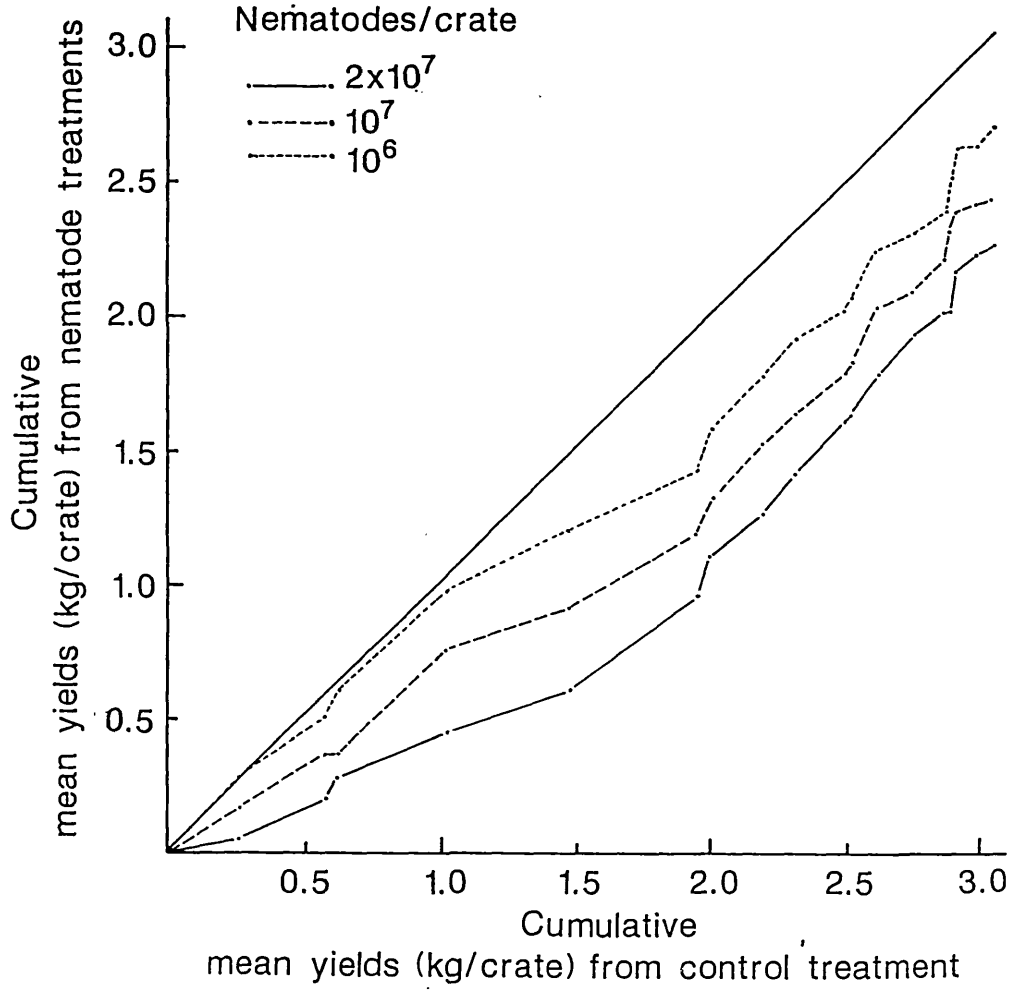


Fig. 5.6 Effects of *C. elegans* on flushing patterns of *A. bisporus*. Line drawn through the origin represents the (hypothetical) expected relationship.

(v) *Distortion of sporophores*

Infesting the casing with *C. elegans* resulted in characteristics symptoms on the mushroom sporophores (Plate 5.5). A proportion of the mushrooms were morphologically distorted, notched and mis-shapen (Table 5.5). Gills of the distorted mushrooms turned violet or brown and, on closer examination, revealed the presence of actively-reproducing populations of *C. elegans* (Plate 5.6). There was a significant linear relationship ($P<0.05$) between the numbers and weights of distorted mushrooms and initial nematode population (Table 5.4).

5.4 Discussion

The present study showed that when inoculated into a properly prepared and pasteurised compost the populations of *C. elegans* fell during the spawn-running period and had no significant effects on mushroom production. This confirms the findings of Hesling (1966) who reported that rhabditid nematode species *Coarctadera cylindrica* (=*Pelodera cylindrica*), *Mesorhabditis spiculigera* and *Acrobeloides buetschlii* were unable to compete with a healthy growth of mushroom mycelium.

There could be three reasons for the above findings: (i) quick-growing mycelium absorbs the free moisture of compost (Flegg & Wood, 1985) which consequently limits nematode activity; (ii) *A. bisporus* creates a bacteriostatic environment which reduces the availability of bacteria as a food resource for saprobes and thus limits nematode reproduction; and (iii) temperature optima for the 'spawn-run' and for nematode reproduction differ.

Evidence for the bacteriostatic nature of mycelial growth comes from Tschierpe and Sinden (1965) who reported that *A. bisporus* produces volatile products with antibiotic activity. Furthermore, Barron (1988) found that *A. bisporus*, and some other lignicolous fungi, can feed on living colonies of bacteria and utilise them as a nutrient source. An optimum 'spawn-run' is achieved with an air temperature of 25°C (Flegg & Wood, 1985) whereas the optimum temperature for *C. elegans* (Taunton strain) fecundity is between 15-17°C (Section 4.3.3). In addition, extra heat produced by actively-growing mycelium

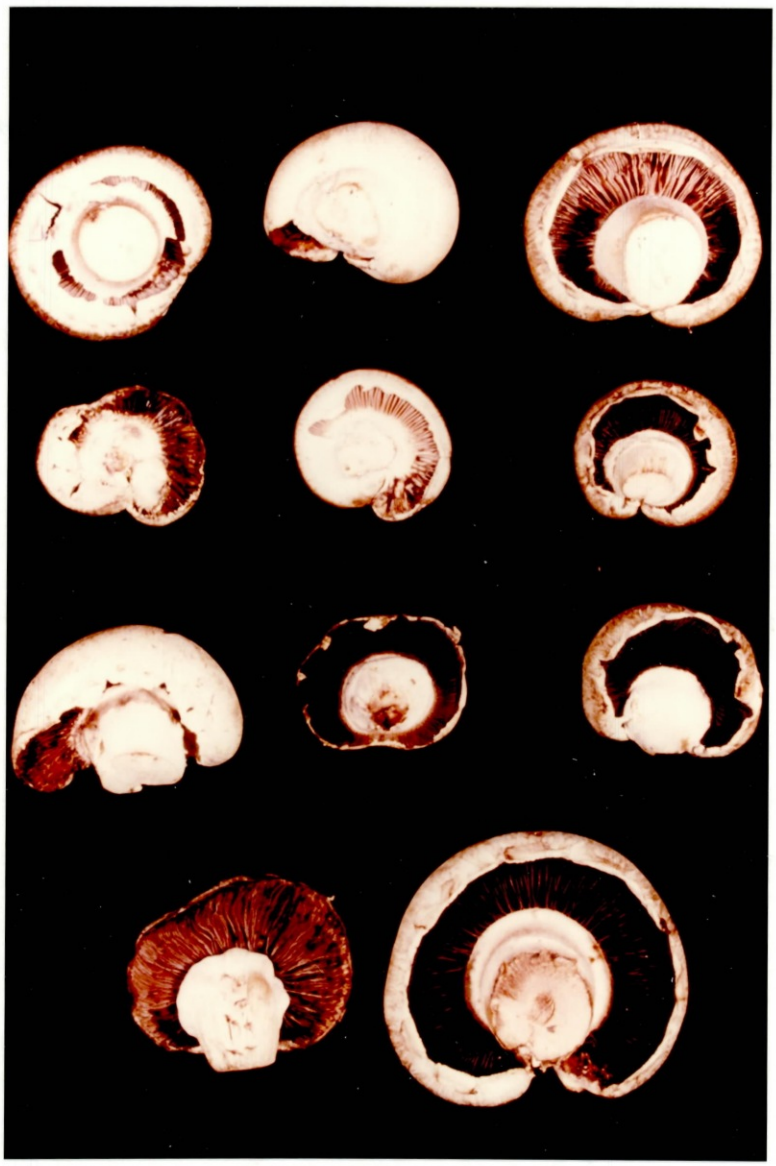


Plate 5.5 Mushroom distortion due to *C. elegans* infestation.

Table 5.5 Incidence of sporophores distorted by *C. elegans*: nematodes added to casing (higher inocula). *Data are mean total weight and number of distorted mushrooms/crate (% distorted in brackets)

Nematodes ($x10^6$)/crate	Weight of distorted mushrooms (g)*	Number of distorted mushrooms*
20	179.3 (10.8)	21.5 (8.0)
10	131.7 (6.7)	14.7 (5.4)
1	94.3 (3.8)	9.0 (3.5)
0	0	0
SED (10 df)	7.8	1.9

Plate 5.6 Enlargement of gill region from a distorted sporophore to show colonisation by *C. elegans*.

further raises the compost temperature by 2-3°C; 28°C is lethal to this strain of *C. elegans*.

In contrast, other reports have shown that rhabditid nematodes, along with their associated organisms, damage mushroom mycelium (van Haut, 1956; Blake & Conroy, 1959; Hesling, 1966; Ross & Burden, 1981; Kaufman *et al.*, 1984) which may have been due to competition between organisms that occupy the same niche (i.e. compost). Ross and Burden (1981), after extensive experimentation, concluded that the effects of saprobic rhabditid nematodes may vary along a scale which ranges from complete suppression of the mushroom at one end, to the complete suppression of the nematodes at the other. Normally, every effort is made to favour the mushroom, especially with regard to the creation of a selective compost. The nematodes, therefore, rarely acquire favourable conditions for their multiplication. Furthermore, rhabditids cannot degrade organic matter without their bacterial associates. The existence and activity of bacterial populations is, in turn, greatly affected by the selective micro-environment created by the mushroom.

In contrast, casing material provides a highly suitable environment for nematode activity because of its water-holding capacity, low nutritive status (which allows only sparse mycelial colonization) and inherent natural bacterial flora (casing material is generally not sterilized because of the desirable attributes of sporophore-inducing bacteria, Hayes *et al.*, 1969; Eger, 1972). The present trials show that the competition also occurs in the casing layer. Thus, if initial numbers of *C. elegans* are low, the nematode's multiplication rate is initially restricted and no significant effects on weight of mushrooms are found. However, if initial inocula are high, the nematodes rapidly colonize the casing layer, produce large populations which can be seen as 'winking' columns on top of the casing and consequently result in significant reductions in mushroom yield. Similar out-breaks and swarming of nematode populations are commonly experienced in the mushroom industry (Hesling, 1979).

The stoma of saprobic rhabditid nematodes is not structurally capable of causing any direct physical damage to mushroom mycelium. The data relating to nematode populations in the casing layer (Fig. 5.4) support the hypothesis that it is the accumulation of nematode by-products which chemically inhibit mycelium

and/or sporophore growth that is the main cause of yield loss. An initial peak in the *C. elegans* population clearly was necessary if any significant effects on mushroom yield were to develop. When initial inocula of *C. elegans* were equal to, or higher than about 267 nematodes/g of casing material (i.e. 10^6 nematodes/crate) the resulting population explosion was sufficiently rapid to cause competition with *A. bisporus*. However, nematode populations started to decline two weeks after inoculation, indicating that they should perhaps best be described as 'opportunistic substrate modifiers'. This view is substantiated by Kaufman *et al.* (1983) who showed that extracts of a nematode-infested compost inhibited the growth of mushroom mycelium. The present study, however, could not relate this substrate modification to a simple pH shift. Recording the pH of casing-soil during Experiment 3 when highest inocula were used may have yielded different results.

Normally, mushrooms appear in distinct flushes at intervals of about 7-14 days (Flegg & Wood, 1985). This study showed that *C. elegans* infestations significantly affected this type of flushing pattern and that the mushrooms appeared more regularly and in fewer numbers throughout the cropping period. This confirms the earlier observations of Hesling (1966, 1979).

Another interesting aspect of this study was the recognition of mushroom sporophore distortion. A characteristic malformation of the sporophores was observed only in nematode-infested treatments. The numbers and weights of distorted mushrooms showed a significant linear relationship and a high positive correlation with initial *C. elegans* populations proving that the damage was the direct result of nematode infestation.

Apart from yield loss and quality deterioration due to malformation of mushrooms, the physical presence of nematodes on even healthy sporophores was another factor. This could have three implications for the grower: (i) the nematodes may spread bacteria on to sporophores and could, therefore, play an important role in post-harvest deterioration of mushrooms (Doores *et al.*, 1987); (ii) the nematodes may spread the bacterial blotch pathogen, *P. tolaasii* onto sporophores (Steiner, 1933); and (iii) transportation of infested mushrooms may spread the nematodes to new environments.

These experiments also showed that *C. elegans* reduced the numbers of

mushrooms produced, indicating that the nematodes and/or their by-products disturbed the sporophore initiation process. This may be due to nematodes feeding on sporophore-inducing bacteria or to inhibition of the induction of sporophores and/or of their growth by nematode by-products.

It is known that *A. bisporus* regulates populations of bacteria in the vicinity of the hyphae by producing volatile antibiotic products and/or by feeding on bacterial colonies. This may explain the variation in response of different mushroom strains to nematode damage reported in the literature (Klingler & Tschierpe, 1980; Ross & Burden, 1981). Nematode resistant strains may have a greater capacity to limit bacterial populations and thus have a direct effect on nematode multiplication.

Due to their ability to spread sporophore-inducing bacteria, and thus to even-out the process of sporophore-induction, low numbers of saprobic rhabditid nematodes in the casing layer are probably beneficial. Cayrol *et al.* (1981) demonstrated that *Cephalobus emarginatus* and *Dolichorhabditis dolichura (=Cephalobus dolichura)* were able to disperse *Pseudomonas putida* through the casing and thus induce fruiting. The increase in mushroom yields due to an infestation with rhabditid nematodes observed by Ingratta and Olthof (1978) may corroborate this. The results reported here (Table 5.1; 10^4 nematodes/crate added in compost) also tend to lend support to this theory although they were not significant. More extensive experimentation with even lower initial inocula and perhaps with different rhabditid species may be necessary before conclusive evidence is acquired.

Although the term 'saprobic' describes the habit of feeding on dead organic matter, Nicholas (1984) emphasised the importance of bacteria in the diet of rhabditid nematodes. Therefore, studies of the bacterial flora associated with rhabditids in mushroom culture and of their potential interactions with the nematodes are of primary significance in gaining a better understanding of the processes of pathogenesis.

It is concluded that *C. elegans* has the potential to cause significant losses in mushroom yield when inoculated, during casing, at about 267 nematodes/g of casing material. It also reduces mushroom quality by causing sporophore distortion and/or by its physical presence on sporophores. Similar or

even greater numbers of rhabditid nematodes/g of compost or casing material have been reported from many mushroom farms all over the world (Ingratta & Olthof, 1978; Hesling, 1979; McLeod & Nair, 1981; Sanderson *et al.*, 1981; Kaufman *et al.*, 1984) and it is, therefore, a matter of speculation as to the extent of annual crop losses incurred by commercial growers.

CHAPTER 6

Effects of bacteria associated with *C. elegans* on mycelial growth of *A. bisporus*

6.1 Introduction

The results described in Section 5 illustrated that *C. elegans* has the potential to cause significant losses in yield and quality of mushrooms. However, the processes of pathogenesis are poorly understood mainly because the stoma (buccal cavity) of saprobic rhabditid nematodes is structurally incapable of causing any significant physical damage to the mushroom mycelium.

Kaufman *et al.* (1983) have demonstrated that the extracts obtained from *C. elegans*-infested compost inhibited the mycelial growth of *A. brunnescens*. Furthermore, they observed that an extract from uninfested compost also inhibited mycelial growth but to a lesser degree and suggested that the role of compost bacteria and other micro-organisms may be important. In this chapter, the effects of bacteria, isolated from *C. elegans* (Section 4.3.1), on the mycelial growth of *A. bisporus* in agar cultures are described.

6.2 Materials and methods

6.2.1 Mushroom cultures

Three strains of the mushroom *A. bisporus*, smooth-white (C43), off-white (C54) and a hybrid strain (Horst U3) were obtained from the IHR culture collection and subcultured on malt extract agar (MEA, Oxoid Ltd) in 90 mm diam. Petri plates at 25°C before use.

6.2.2 Assay for the effects of bacteria on mycelial growth of *A. bisporus*

The response of *A. bisporus* strain C43 to nine species of bacteria was studied on malt extract agar medium in Petri plates. One mycelial disc (5 mm diam.) of the test fungus was placed in the centre of each plate. At the same time, a loopful of a test bacterium from a previously inoculated (for 24h at 25°C) nutrient broth (Oxoid Ltd) flask was streaked in one quadrant near the edge of the plate (Plate 6.1). Four replicate plates were prepared for each bacterium and the plates were incubated at 25°C. Mycelial growth (colony radius) towards and away from the bacterial colony was recorded 21 days after inoculation. Per cent inhibition in radial growth (over control) was calculated in each treatment and the data were subjected to ANOVA.

6.2.3 Effects of bacteria on different strains of *A. bisporus*

Based on the above assay, the effects of three species of bacteria, which produced more than 50% inhibition, were also studied on the mycelial growth of three commercial strains (C43, C54 & U3) of *A. bisporus* on malt extract agar and compost malt medium (CMM, Rainey, 1989). Data on colony diameter measurements perpendicular to the bacterial streak were recorded 21 days after inoculation, per cent inhibition was calculated and subjected to ANOVA.

6.2.4 Assay for toxin production

The three chosen species of bacteria were grown in 250 ml flasks containing 100 ml nutrient broth in a shaker at 200 rpm for 24h at 28°C. After the incubation period, each bacterial culture was centrifuged at 5500 g for 20 minutes (MSE Mistral 3000) and the supernatant divided into two halves. One half was filtered through a micro-pore filter (pore size 0.2 μm) and the other was autoclaved at 121°C for 15 minutes. The response of *A. bisporus* strain C43 to the cell-free extracts was studied by the 'diffusion drop' and 'well' methods.

In the 'diffusion drop' method, drops (30 μl) of the bacterial extracts (filtered or autoclaved) were placed (only once) at 15 mm distance from culture

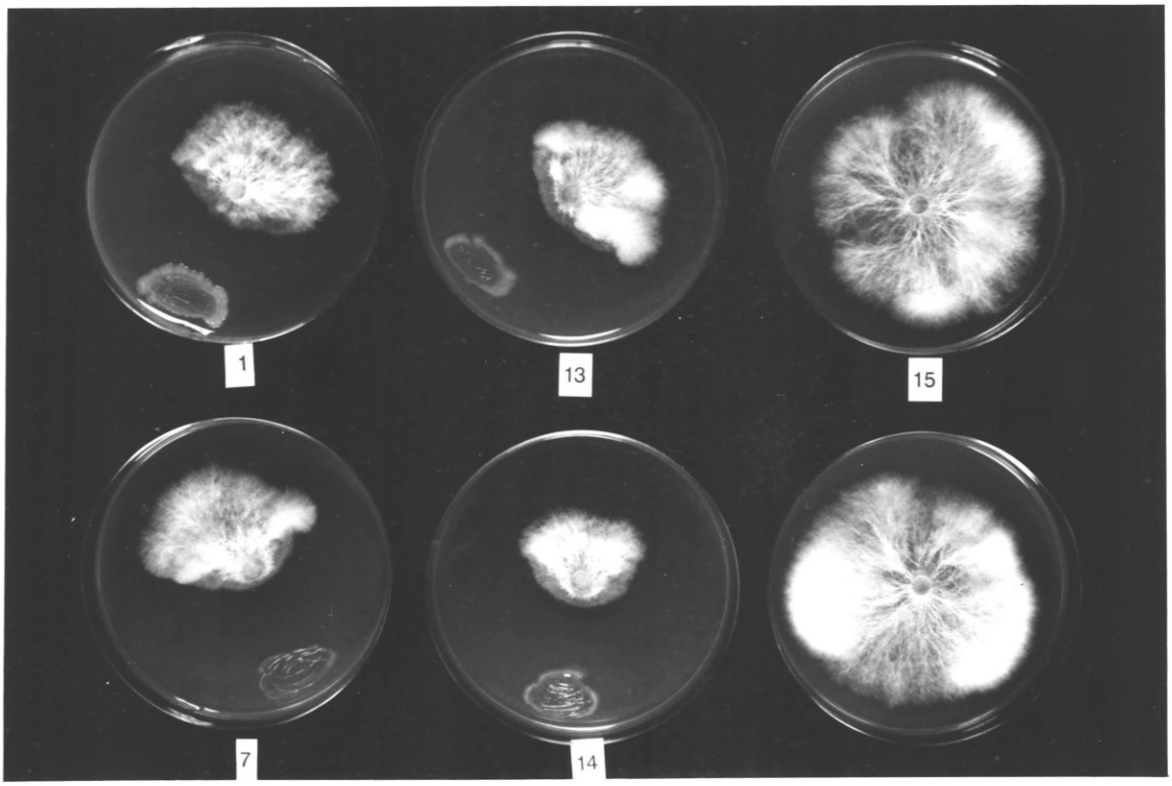


Plate 6.1 Inhibition of mycelial growth of *A. bisporus* by the bacteria associated with *C. elegans*. 1 = *S. liquefaciens*, 7 = *E. cloacae*, 13 & 14 are two isolates of *E. amnigenus* (only isolate 14 was used in all subsequent experiments), 15 & unlabelled are controls.

discs (5 mm diam.) of the test fungus on MEA plates. In the 'well' method, wells of 5 mm diam. were cut with a sterile cork borer at a distance of 15 mm from culture discs of the fungus placed in the centre of MEA plates. The bacterial extracts (40 μl) were applied to each well after every two days. In all experiments, filtered/autoclaved nutrient broth was used to prepare control plates. Plates were inoculated with culture discs of the test fungus and incubated at 25°C. Colony diameter was measured 21 days after inoculation, per cent inhibition calculated and the data were subjected to ANOVA.

The effects of concentrated cell-free extracts of the three bacteria on mycelial growth of C43 strain were also studied. The bacteria were grown in 250 ml flasks of nutrient broth, as described above. Fifteen flasks were used for each bacterium. Cultures (approx. 1.5 l for each bacterium) were centrifuged at 5500 g for 20 minutes. The supernatant was concentrated to 15 ml using ultra-filtration membranes (Amicon, PM 10). The concentrated extracts were divided into two halves: one half was passed through a micropore filter and the other was autoclaved. The effects of the concentrated extracts on mycelial growth of C43 strain were studied by the 'diffusion-drop' and the 'well' method. In all these experiments the data on the fungal colony diameter were recorded 21 days after inoculation, per cent inhibition calculated and data were subjected to ANOVA.

In a further series of experiments, the three inhibitory bacteria were grown for 24, 48, 96 and 192h at 28°C and 200 rpm in conical flasks, each containing 100 ml of nutrient broth. After each incubation period, the culture media were centrifuged at 6000 rpm for 20 minutes and the supernatant divided into two halves. One half was filter-sterilised and the other was autoclaved as before. The effects of the extracts were studied by both methods described above. The data on fungal colony diameter were recorded 21 days after inoculation, per cent inhibition was calculated and subjected to ANOVA.

6.2.5 Chitinase production test

The three species of bacteria were tested for the production of the cell wall-degrading enzyme chitinase. The bacteria were streaked on chitin agar (0.2% w/v chitin, Sigma Ltd, and 1.5% w/v agar) plates; incubated at 25°C for

seven days and observed for the production of a clearing zone around the bacterial colonies.

6.2.6 Assay for volatile inhibitors

Non-vented, Petri plates were divided into two unequal portions by fitting impermeable plastic barriers with 'UHU' glue (Beecham, Brentford, UK) before pouring the MEA medium. Culture discs of the test fungus were inoculated onto the centre of the plates and bacteria were streaked onto the agar at the far side of the plastic barriers. Four plates were prepared for each bacterium and incubated at 25°C. The plates were observed periodically (without opening the lid) for any effects on mycelial growth and the data on colony diameter were recorded at seven-day intervals after inoculation. Per cent inhibition in mycelial growth was calculated and the data were subjected to ANOVA.

6.2.7 Effects of D-glucose on the production of volatile inhibitors

As the incorporation of some sugars in the growth medium are shown to eliminate the production of antifungal volatiles by some bacteria, partitioned plates were prepared with MEA or MEA + D-glucose. Three concentrations of D-glucose were used: 1, 2 and 3% (w/v) and two bacteria were tested. Bacteria and the test fungus were inoculated as above and three replicate plates were prepared for each treatment. Plates were incubated at 25°C and mycelial growth was recorded 21 days after inoculation. The data were analysed using split-plot design and subjected to ANOVA.

6.3 Results

6.3.1 Mycelial growth inibition by bacteria

All nine species of bacteria inhibited the mycelial growth of *A. bisporus* strain C43 (Table 6.1 & Appendix 6.1). When observed 21 days post-inoculation, mycelial growth towards bacteria was strongly inhibited (62-90.8% mean inhibition was caused by four species of bacteria, *Bacillus* sp., *E. amnigenus, E. cloacae* and *S. liquefaciens*). Mycelial growth of the test fungus away from the bacterial streak was less affected. Calculation of mean per cent inhibition in total mycelial growth after 21 days of inoculation (i.e. diameter of the fungal colony perpendicular to the bacterial lawn) showed that three bacteria: *E. amnigenus, E. cloacae* and *S. liquefaciens* caused more than 50% inihibition (Plate 6.1).

6.3.2 Effects of bacteria on different strains of *A. bisporus*

All three mushroom strains showed a similar response pattern to the presence of bacteria and this response was not significantly ($P>0.05$) altered by the nutrient media used (Fig. 6.1 & Appendix 6.2).

6.3.3 Toxin production

The cell-free extracts obtained from 24h old cultures of bacteria did not show any significant ($P>0.05$) effects on the mycelial growth of *A. bisporus* (data not shown). Only the concentrated extracts from *S. liquefaciens* using the 'well' method produced significant effects on colony diameter of the test fungus (Fig. 6.2). Autoclaving reduced the inhibitory effects of concentrated extracts.

Cell-free extracts obtained from bacterial cultures of different ages did not show any signficant ($P>0.05$) effects on the mycelial growth of *A. bisporus* when tested by 'diffusion drop' method (data not shown), whereas the 'well' method resulted in significant ($P<0.05$) inihibitory effects (Fig. 6.3).

The 'well' method showed that filtrates from 96h and 192h old cultures of all three bacteria caused significant ($P<0.05$) inhibitory effects on mycelial

Table 6.1. Effects of bacteria on mycelial growth of *A. bisporus* (strain C43) on malt extract agar medium. Data are mean % inhibition in radial growth to or away from the bacterial streak 21 days after inoculation.

	% inhibition		
Bacteria	To	Away	Mean
A. calcoaceticus var. *anitratus*	28.9	19.0	23.9
A. calcoaceticus var. *lwoffi*	27.7	20.5	24.1
B. cereus	49.5	14.0	31.5
Bacillus sp.	62.1	27.8	44.7
E. amnigenus	82.1	28.0	54.7
E. cloacae	90.8	31.0	60.5
P. maltophilia	38.5	11.8	24.9
Pseudomonas sp.	49.2	17.5	33.2
S. liquefaciens	78.7	24.0	51.0
SED (24 df)	4.2	3.8	3.4

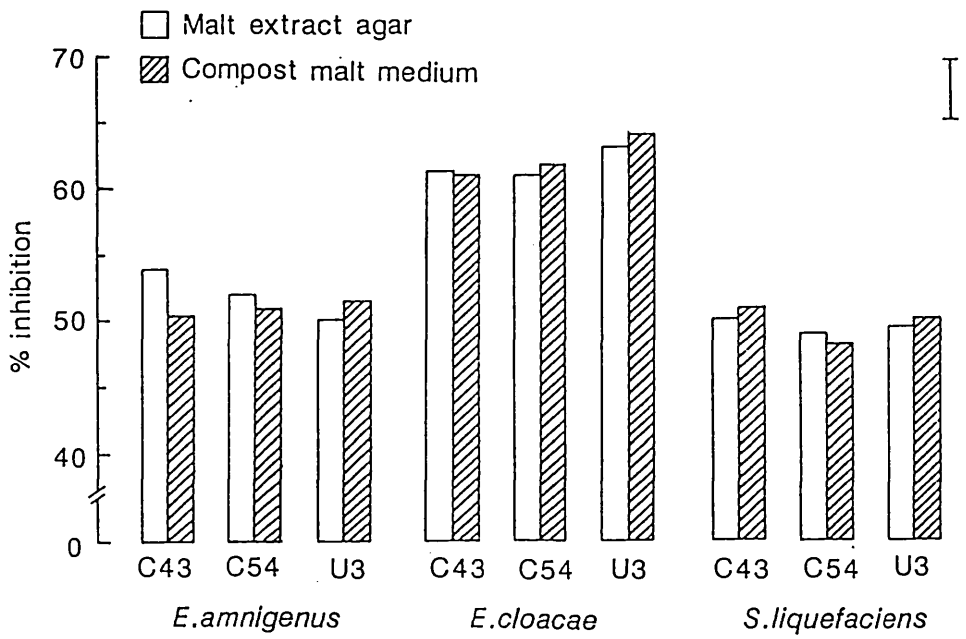


Fig. 6.1 Mean % inhibition in mycelial growth of the three strains of *A. bisporus* (C43, C54, & U3) 21 days after inoculation by the three inhibitory bacteria associated with *C. elegans*. Bars represents LSD ($P<0.05$).

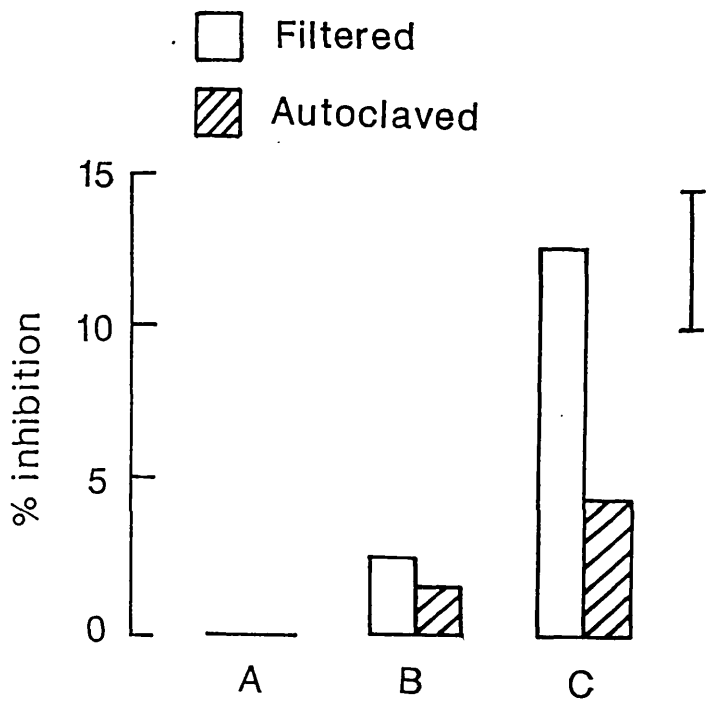


Fig. 6.2. Mean % inhibition in mycelial growth of *A. bisporus* 21 days after inoculation with concentrated filtrates from cultures of bacteria associated with *C. elegans*. A = *E. amnigenus*; B = *E. cloacae*; C = *S. liquefaciens*. Bar represents LSD ($P<0.05$).

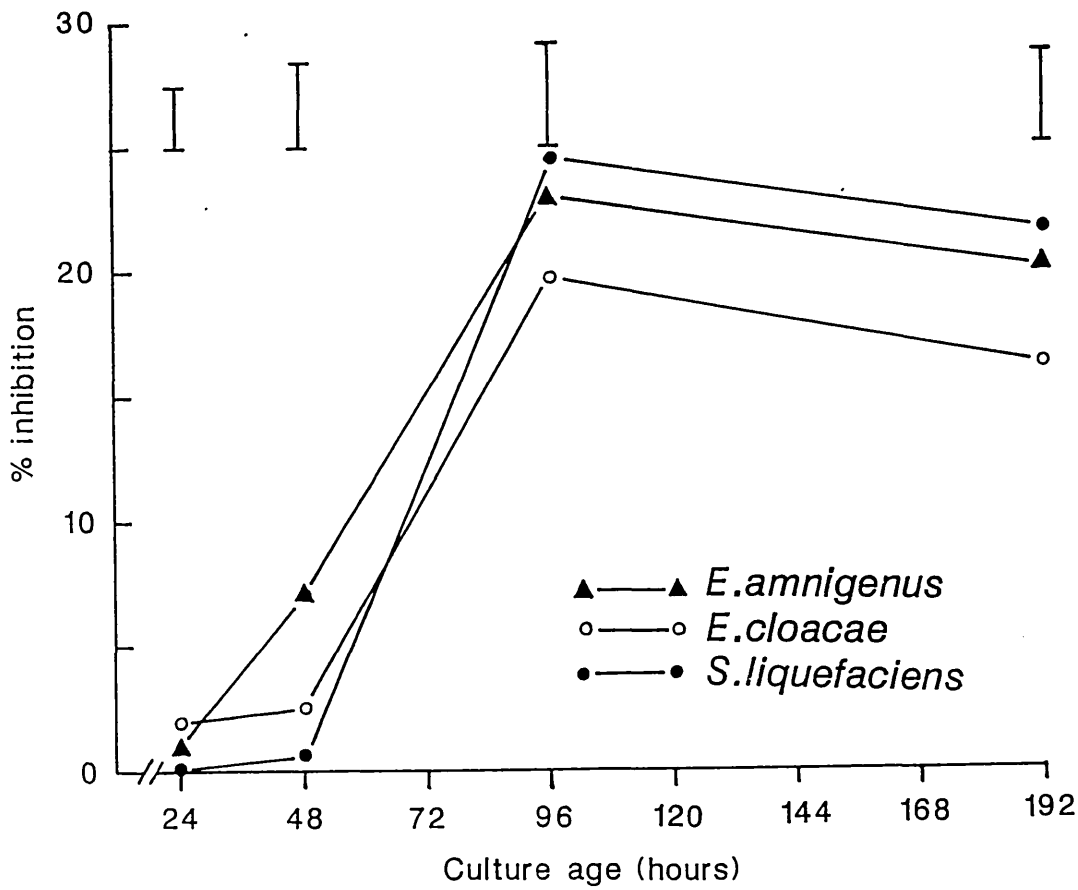


Fig. 6.3. Mean % inhibition in mycelial growth of *A. bisporus* 21 days after inoculation by filtrates from different aged cultures of bacteria associated with *C. elegans*. Bars represent LSD ($P<0.05$).

growth and that the three bacteria did not differ significantly ($P>0.05$). The filter-sterilised extracts were more inhibitory than the autoclaved ones.

6.3.4 Chitinase production

Of the three bacteria tested for the production of chitinase on chitin agar, only *S. liquefaciens* produced the enzyme (Plate 6.2).

6.3.5 Production of volatile inhibitors

Partitioned plates revealed that *E. amnigenus* and *E. cloacae* produced volatile fungal inhibitors (Plate 6.3). Fourteen days after inoculation the two bacteria resulted in significant inhibition ($P<0.05$) in colony diameter of the test fungus and 21 days after inoculation the two bacteria resulted in 45-48% inhibition (Fig. 6.4). *S. liquefaciens* did not cause any significant effects ($P>0.05$) on mycelial growth on partitioned plates.

6.3.6 Effects of D-glucose on the production of volatile inhibitors

Incorporation of D-glucose in the nutrient medium did not result in any significant effects ($P>0.05$) on the production of volatile inhibitors by bacteria (Table 6.2). Even at 3% (w/v) glucose concentration, the bacteria caused significant ($P<0.05$) reduction in colony diameter of the test fungus when assessed 21 days after inoculation (Plate 6.4). The higher glucose concentrations tested (2 and 3%) significantly reduced ($P<0.05$) the mycelial growth of *A. bisporus*.

Plate 6.2. Production of chitinase by *S. liquefaciens* on chitin agar: the presence of a halo around the bacterial colonies indicates chitin degradation.

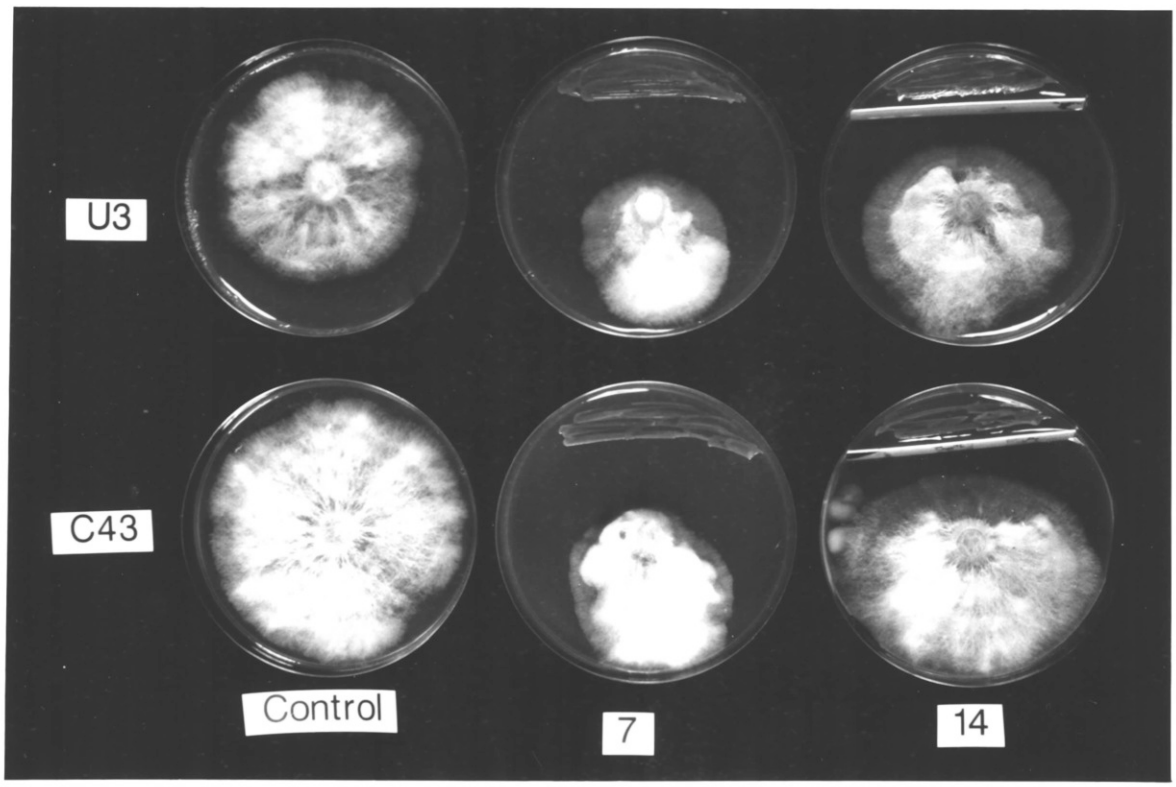


Plate 6.3. Production of volatile inhibitors by *E. amnigenus* (14) and *E. cloacae* (7). C43 & U3 are the two strains of *A. bisporus*.

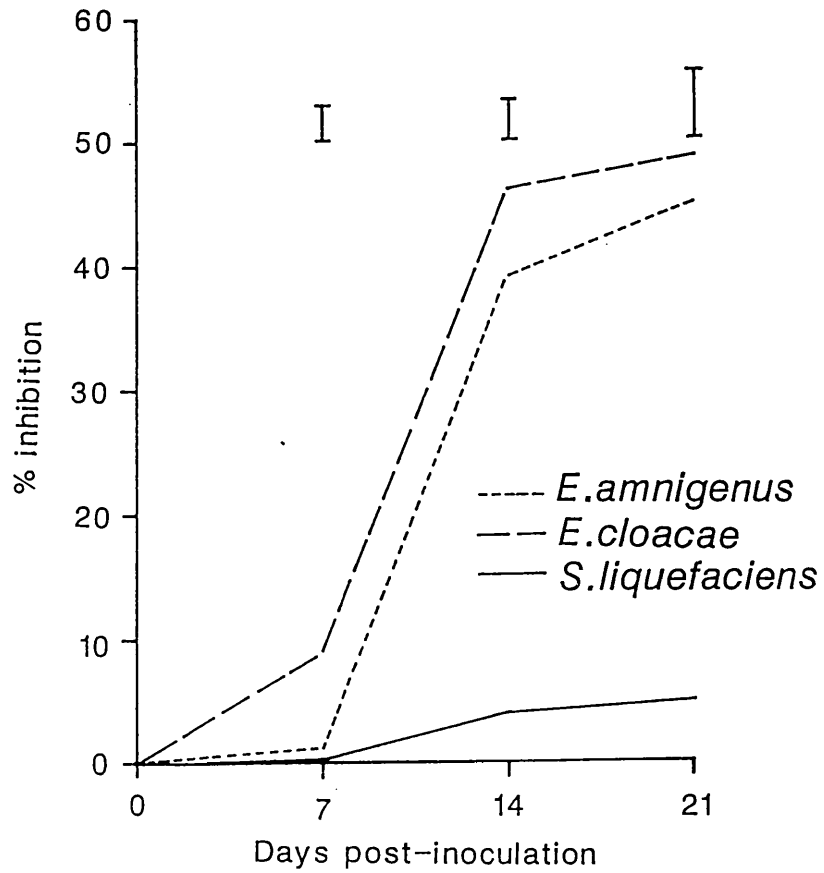


Fig. 6.4. Mean % inhibition in mycelial growth of *A. bisporus* by volatile substances produced by the bacteria associated with *C. elegans*. Bars represent LSD ($P<0.05$).

Table 6.2 Effects of D-glucose on the production of volatile inhibitors by bacteria. Data are mean colony diam. (mm) of *A. bisporus* (strain C43) 21 days after inoculation.

Glucose conc. (% w/v)	*E. amnigenus*	*E. cloacae*	Control	Mean
0	36.3	30.3	81.3	49.3
1	38.0	30.7	77.0	48.6
2	33.3	29.3	64.3	42.3
3	24.0	19.7	38.7	27.4
				SED (22 df) = 1.29
Mean	32.9	27.5	65.3	
				SED (22 df) = 1.12

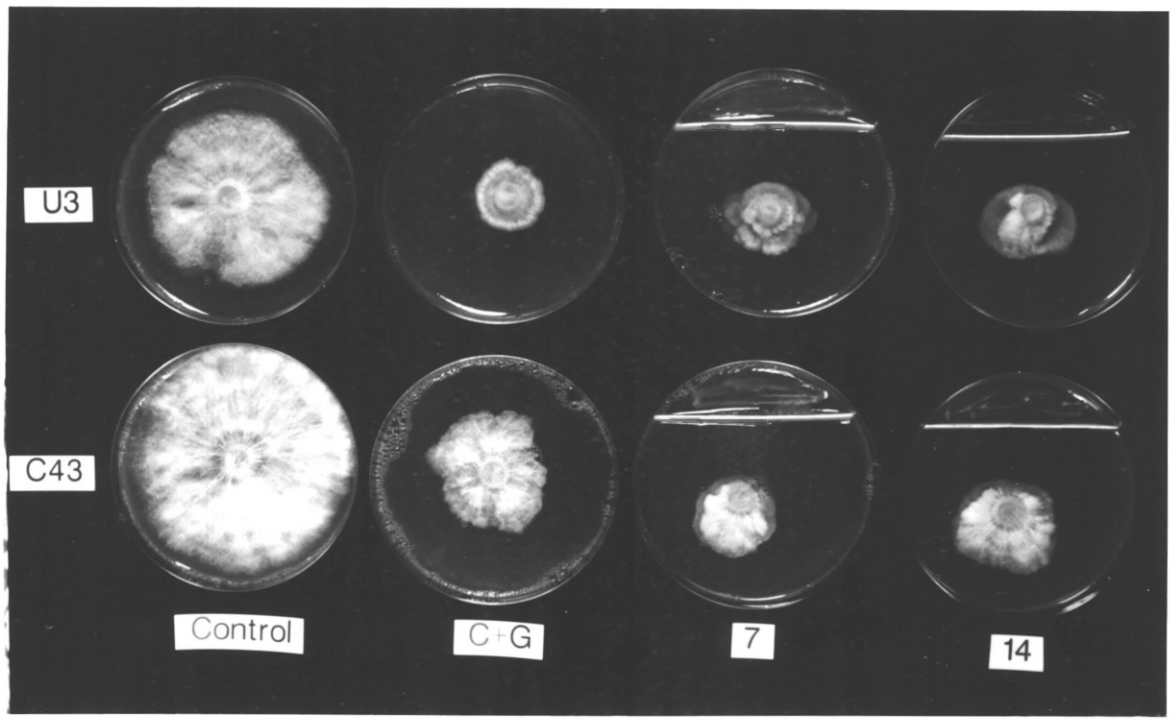


Plate 6.4 Effects of D-glucose incorporated into MEA medium on the production of volatile inhibitors by *E. amnigenus* (14) and *E. cloacae* (7). C+G shows the growth of *A. bisporus* in the presence of D-glucose. C43 and U3 are the two strains *A. bisporus*.

6.4 Discussion

All the bacteria isolated from *C. elegans* inhibited the mycelial growth of *A. bisporus* in the range of 23.9-60.5%. Three bacteria; *E. amnigenus, E. cloacae* and *S. liquefaciens* produced strong inhibition zones in dual cultures and caused more than 50% inhibition in mycelial growth of the test fungus. The three strains of the mushroom tested during this study showed a similar response pattern to the presence of these bacteria on two different media.

Two assay methods: 'diffusion drop' and 'well' method, used to detect the presence of growth inhibitors in cell-free extracts resulted in differential inhibition in mycelial growth of *A. bisporus*. This was probably a dose effect: the test mycelium was exposed to the greater amount of extract by the 'well' method than the 'diffusion drop' technique. The reduction in inhibition observed on autoclaving the extracts suggest that the inhibitory substance(s) is/are volatile or heat-labile.

The three bacteria investigated were found to produce inhibitor(s) after different periods of shake flask culture. However, the observed delay of 96h for *S. liquefaciens* and 44h for *E. amnigenus* and *E. cloacae* suggested that inhibitor production occurred after bacterial multiplication ceased under the culture conditions employed.

Species of *Enterobacter* (e.g. *E. cloacae*) and *Serratia* (e.g. *S. marcescens*) are known to inhibit mycelial growth of a wide spectrum of fungi and have been suggested as potential biocontrol agents of economic soil-borne plant pathogens. (Howell *et al.*, 1988; Ordentlich *et al.*, 1988). The mechanisms of fungal growth inhibition by such bacteria have been investigated recently. Ordentlich *et al.* (1988) showed that chitinase is the main constituent of fungal growth inhibitors produced by *S. marcescens*. *S. liquefaciens* also produced a chitinolytic enzyme when grown on chitin agar. However, chitinase is an inducible enzyme and is secreted by the bacteria in response to the presence of chitin in their environment (Oranusi & Trinci, 1985; Ordentlich *et al.*, 1988). During the present study, no physical contact between bacteria and fungal hyphae occurred. Furthermore, the presence of inhibitory activity in the concentrated filtrates obtained from 24h old cultures and also in the unconcentrated filtrates from 48,

96 and 192h old cultures of *S. liquefaciens* grown in nutrient broth (i.e. in the absence of chitin or fungal cell walls) suggest the production of some other kind of inhibitor by this bacterium.

The present results suggest that *E. amnigenus* and *E. cloacae* do not produce any chitinolytic enzymes in the presence of chitin. The use of partitioned plates revealed the production of volatile inhibitors by these bacteria but not by *S. liquefaciens*. Howell *et al.* (1988) found that *E. cloacae* produced a volatile fungal inhibitor, ammonia. They suggested that under conditions of low concentrations of readily metabolised sugars, *E. cloacae* deaminates amino acids to obtain a carbon source and thus ammonia is produced as a byproduct. The presence of antifungal activity in the filtrates of aged cultures (96 or 192h old) of *E. amnigenus* and *E. cloacae* tends to support this hypothesis.

Howell *et al.* (1988) reported that the production of ammonia by *E. cloacae* was eliminated by the addition of D-glucose and other sugars (at 40 mM) in the medium. However, during the present investigation the elimination/reduction of the antifungal activity of *E. amnigenus* and *E. cloacae* was not achieved even when 3% D-glucose (166 mM) was incorporated into the medium. This may be due to two reasons: (i) *A. bisporus* being a slow growing fungus produces a colony of a reasonable size in a minimum of 7-10 days during which period the bacteria probably utilise the additional sugars in the medium and start to deaminate the amino acids and this result in fungal growth inhibition; or (ii) ammonia is not involved in the interaction between these bacteria and *A. bisporus*.

The present study has shown that bacteria associated with *C. elegans* inhibit mycelial growth of *A. bisporus*. Due to their ability to carry bacteria superficially and in the gut (Cairns & Thomas, 1950; Cayrol *et al.*, 1981, 1987; Griffiths, 1986), rhabditid nematodes may limit the growth of mushroom mycelium by spreading inhibitory bacteria in the compost environment.

CHAPTER 7

Effects of bacteria and/or *C. elegans* on mushroom production

7.1 Introduction

It was demonstrated in Chapter 6 that some of the bacteria isolated from *C. elegans* significantly inhibited the mycelial growth of *A. bisporus* in agar cultures. In this part of the project, an effort was made to differentiate the effects of *C. elegans* and of its associated bacteria on flushing patterns, quality and yield of *A. bisporus*.

7.2 Materials and methods

7.2.1 Nematode culture

The nematode *C. elegans* was mass-produced monoxenically with *A. calcoaceticus* var. *anitratus* as described in Section 4.2.6.

7.2.2 Bacterial cultures

Three species of bacteria isolated from the Taunton strain of *C. elegans* (Section 4.3.1) were selected for this study; one of the species, *A. calcoaceticus* var. *anitratus* supported most vigorous reproduction of the nematode (Section 4.3.2) and the other two species, *Enterobacter cloacae* and *Serratia liquefaciens* significantly inhibited the mycelial growth of *A. bisporus* (Section 6.3.1). For inoculations, cell suspensions of bacteria were prepared in nutrient broth. The bacteria were inoculated in 250 ml flasks containing 100 ml of the medium and the flasks were incubated in a shaker incubator (125 rpm) at 25°C for 24h. The optical density of the bacterial suspension was determined using a spectro-

photometer and the numbers of cells/ml were estimated using pre-calibrated curves for each bacterium.

7.2.3 Preparation of the mushroom substrate

The preparation of the mushroom substrate and the other cultural practices followed are described in Section 5.2.1.

7.2.4 Treatments and experimental design

There were six replicates of each of the following eight treatments; three bacteria (*A. calcoaceticus* var. *anitratus, E. cloacae* and *S. liquefaciens*), three bacteria + *C. elegans, C. elegans* and a control treatment. Casing for each crate was weighed separately (3.75 kg) and spread onto a polythene sheet. *C. elegans* (at 2 X 10^7 nematodes/crate, adults:larvae = 1:1) in 200 ml of water + 100 ml of water or bacterial suspension containing 10^8 cells/ml were sprinkled onto the casing which was then mixed thoroughly. Controls received an equal amount of water. Immediately after the casing was applied, all 48 crates were arranged in a Latin square design in four rows, and three layers, on an aluminium framework in a purpose-built mushroom growth chamber.

Thoroughly pasteurised compost was used in order to minimise contamination by natural infestation of saprobes. To prevent inter-plot spread of nematodes by flies frequent knock-down aerial sprays of pyrethroid 'Pynosect 30' were carried out during cropping.

7.2.5 Data recording and analysis

(i) *Mushroom flushing patterns*

Flushes were distinguished by means of weighted moving averages as described by Wyatt (1977). Total yield for each flush was computed and the data were subjected to ANOVA. In the nematode treatments, where flushes could not be demarcated, the flushing pattern was analysed by plotting the cumulative yields from the nematode + bacteria treatments (Y-axis) against the nematode

treatment (X-axis). The slopes of the resulting curves were subjected to regression analysis (Edmondson, 1989).

(ii) *Timing of flushes*

Effects of treatments on the timing of flushes were determined according to White (1986). The mean date of each flush in each treatment was computed using the following equation:

$$\text{Mean date, } d = (EYx.dx).(EYx)^{-1}$$

where Yx is the yield of each day (dx) of the flush, dates being measured from a common first day of picking. The data were subjected to ANOVA.

(iii) *Mushroom size*

Mean weight/mushroom was calculated during each flush in each replicate of the eight treatments. Flush intervals were determined on the basis of yield data in controls for all the treatments. The data were subjected to ANOVA.

(iv) *Sporophore colour*

Degree of whiteness of the top and of the four sides of the mushroom cap was assessed by the Hunter Colourmeter (Hunter Associates Laboratory, Inc., Virginia, USA, Plate 7.1) in the L, a, b mode (colour parameters). Once during each flush (flushes determined from the yield data in the untreated plots), the spectral colour of ten randomly selected harvested sporophores from each replicate of each treatment was determined and the average recorded. The data were subjected to ANOVA.

(v) *Mushroom distortion*

The numbers and weights of healthy and distorted mushrooms were recorded separately at each pick and subjected to ANOVA.

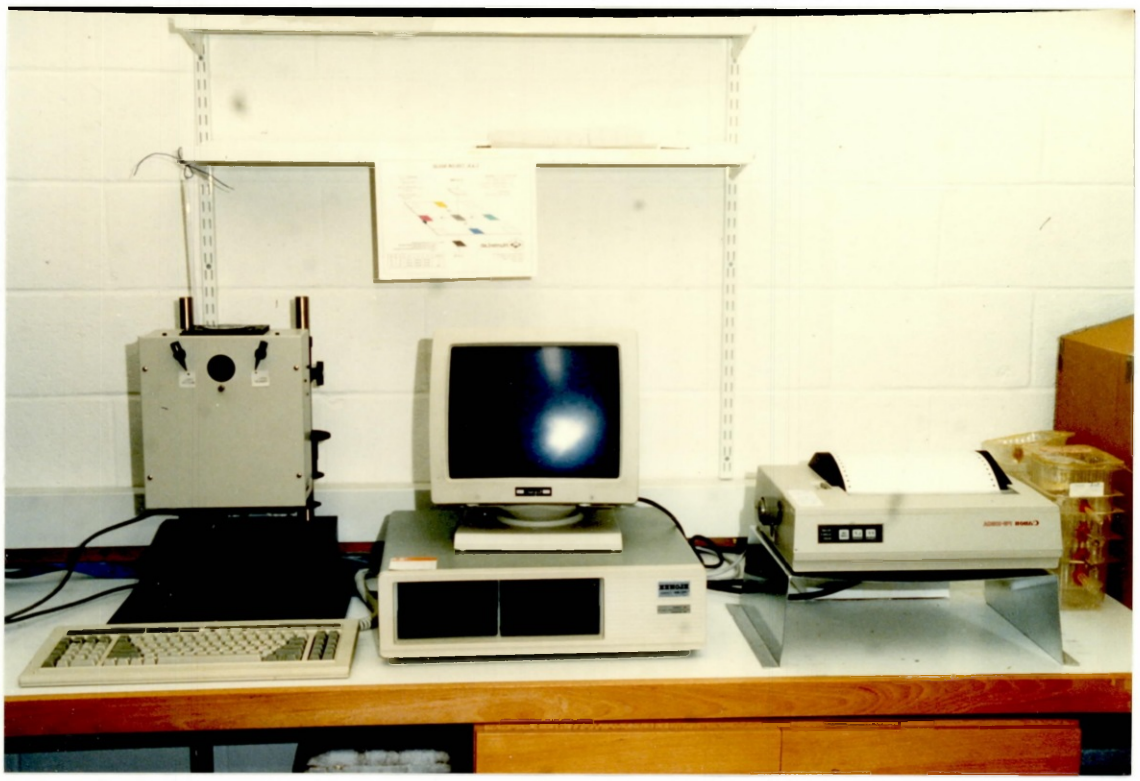


Plate 7.1 Assembly for recording mushroom colour: Hunter colour meter, a programmed computer and a printer.

(vi) *Nematodes on sporophores*

Once during each flush, numbers of *C. elegans* on mushroom sporophores were estimated as described in Section 5.2.4.

(vii) *Nematode population in casing layer*

Populations of *C. elegans* in casing were estimated at weekly intervals after inoculation as described in Section 5.2.4

(viii) *Mushroom yield*

Mushrooms were harvested on alternate days at the point of market acceptability, i.e. stages 3-5 (Section, 5.2.4). Their stipes were trimmed off and numbers and weights recorded. Total yields of both the numbers and weights of mushrooms were subjected to ANOVA.

7.3 Results

7.3.1 Mushroom flushing patterns

Mushrooms appeared in distinct flushes in the control and bacteria alone treatments (Figs. 7.1 & 7.2). In *C. elegans* treatments (i.e. with or without bacteria) the mushrooms appeared almost regularly and flushes were not distinguished. Both the weights (Fig. 7.1) and numbers (Fig. 7.2) of mushrooms showed a similar pattern.

(i) *Bacteria vs control treatments*

(a) Mushroom yield. Three bacteria showed differential effects on total mushroom yields during each flush (Table 7.1). When compared to controls, significantly lower ($P<0.05$) yields were recorded in *A. calcoaceticus* var. *anitratus* treatments during the first and the fifth flush. *S. liquefaciens* resulted in significantly ($P<0.05$) lower yields during the fifth flush only.

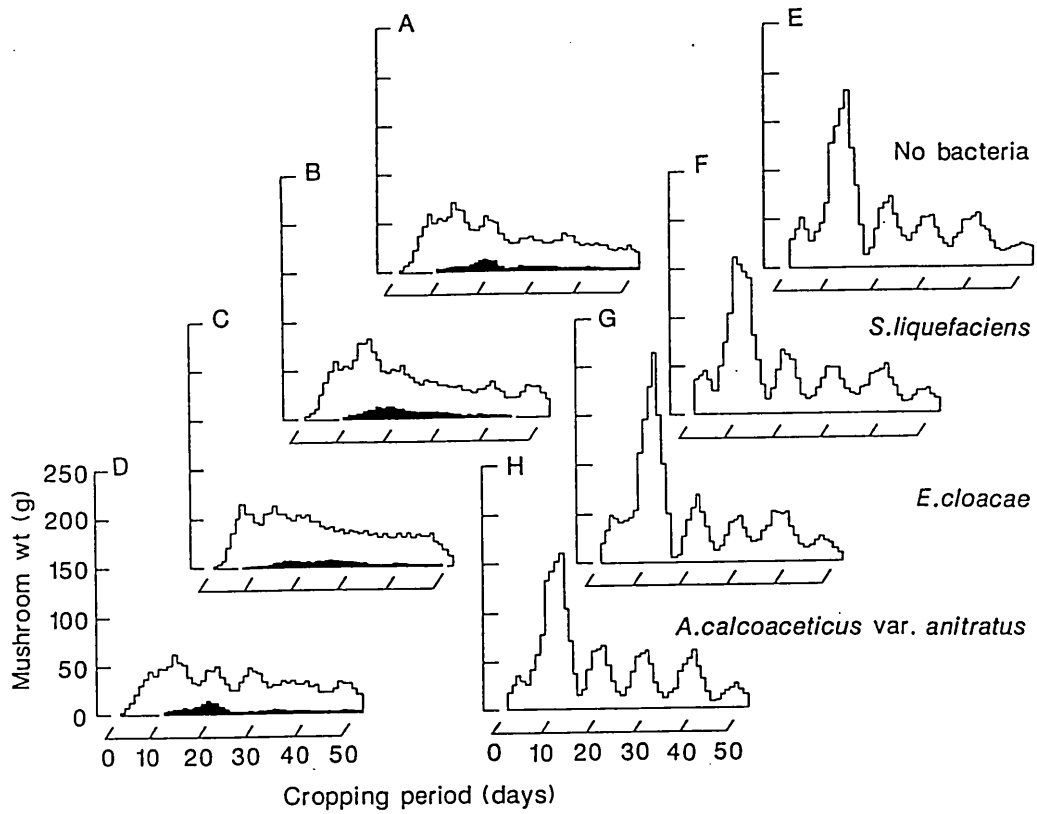


Fig. 7.1 Effects of bacteria and/or *C. elegans* on flushing patterns of *A. bisporus*. Daily mushroom yields (mean wt) smoothed by weighted moving averages. Treatments A-D contain nematodes (B-D also contain bacteria), treatment E is an untreated control and F-H contain only bacteria. Solid areas represent the proportion of distorted mushrooms.

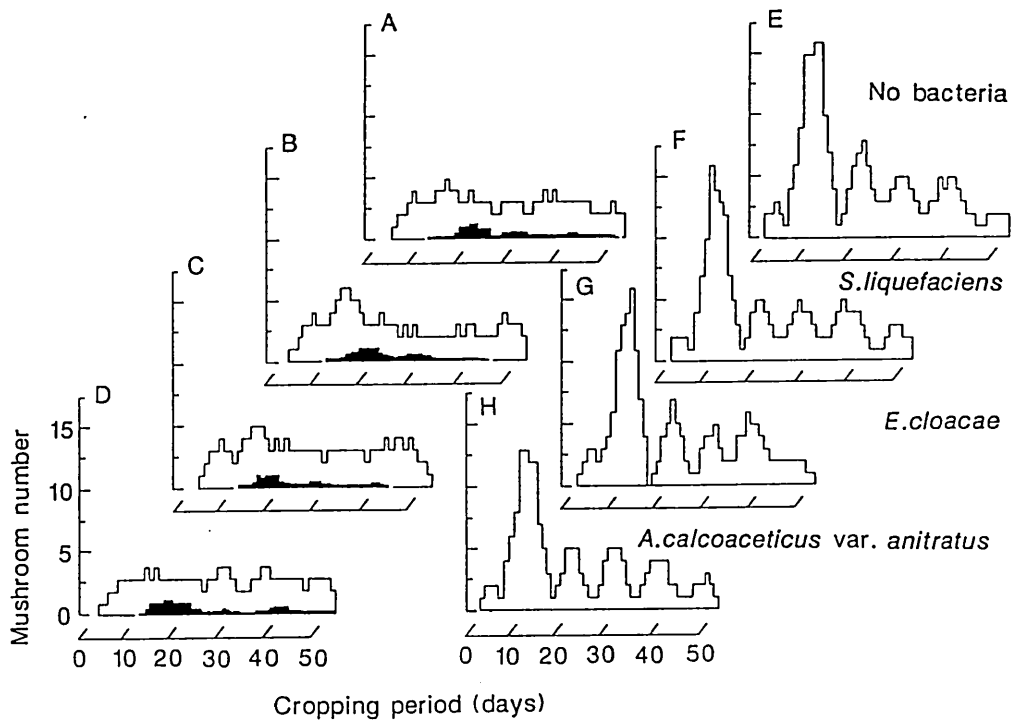


Fig. 7.2 Effects of bacteria and/or *C. elegans* on the flushing patterns of *A. bisporus*. Daily mushroom yields (mean no.) smoothed by weighted moving averages. Treatments A-D contain nematodes (B-D also contain bacteria), treatment E is an untreated control and F-H contain only bacteria. Solid areas represent the proportion of distorted mushrooms.

Table 7.1 Effects of bacteria associated with *C. elegans* on flushing patterns of *A. bisporus*: mean total weight (g) of mushrooms/crate during flushes (1-6)

Treatments	Flushes					
	1	2	3	4	5	6
A. calcoaceticus[$]	172.2	953.5	338.0	337.3	337.0	115.7
E. cloacae	235.2	1065.2	314.7	267.8	393.5	134.7
S. liquefaciens	226.2	995.0	326.5	316.2	354.7	151.3
Untreated	219.8	1020.5	376.7	373.0	403.3	147.0
SED (15 df)	22.0	59.5	19.9	29.1	20.8	25.7

[$] *A. calcoaceticus* var. *anitratus*

(b) Mushroom numbers. Table 7.2 summarises the effects of bacteria on the production of numbers of mushrooms during each flush. When compared with controls, *A. calcoaceticus* var. *anitratus* significantly reduced ($P<0.05$) the numbers of mushrooms that appeared during the first flush. In contrast, significantly ($P<0.05$) greater numbers of mushrooms were picked from crates inoculated with *E. cloacae*. During the third flush, *A. calcoaceticus* var. *anitratus* and *S. liquefaciens* resulted in a significant reduction ($P<0.05$) in mushroom numbers.

(c) Timing of flushes. When compared to controls, *A. calcoaceticus* var. *anitratus* did not show any significant effects ($P<0.05$) on the timing of flushes (Table 7.3). *E. cloacae* treatments resulted in a significant ($P<0.05$) early appearance of the first flush and a delayed sixth flush. *S. liquefaciens* resulted in a significant delay ($P<0.05$) in the appearance of the second flush.

(ii) *Nematode vs nematode + bacteria treatments*

(a) Mushroom yield. A plot of cumulative yields from *C. elegans* + bacteria treatments against *C. elegans* treatment is given in Fig. 7.3. Regression analysis indicated that the slopes of curves for the three bacteria + *C. elegans* treatments were significantly different ($P<0.05$) from an expected straight line response, suggesting that the effects of *C. elegans* on mushroom flushing pattern are influenced by its bacterial associates.

(b) Mushroom numbers. A similar plot for cumulative numbers of mushrooms showed no significant differences ($P>0.05$) between *C. elegans*, and *C. elegans* + bacteria treatments (data not shown).

(c) Timing of flushes. All the *C. elegans* treatments delayed the appearance of the first flush (Plate 7.2) and subsequent flushes were not demarcated. The mushrooms appeared continuously and in fewer numbers throughout the cropping period.

Table 7.2 Effects of bacteria associated with *C. elegans* on flushing patterns of *A. bisporus*: mean total numbers of mushrooms/crate during flushes (1-6)

Treatments	Flushes					
	1	2	3	4	5	6
A. calcoaceticus[$]	9.8	84.3	26.3	30.2	29.7	13.2
E. cloacae	13.8	87.7	30.7	25.2	41.5	10.8
S. liquefaciens	11.8	93.3	26.2	30.5	34.3	16.0
Untreated	11.7	99.8	37.5	34.0	38.7	15.3
SED (15 df)	0.9	6.6	4.4	4.6	3.5	1.6

[$] *A. calcoaceticus* var. *anitratus*

Table 7.3 Effects of bacteria associated with *C. elegans* on timing of mushroom flushes. Figures represent differences in mean day of each flush over control: + = early and - = delay.

Treatments*	Flushes					
	1	2	3	4	5	6
A. calcoaceticus[$]	+0.3	-0.3	-0.3	+0.2	+0.1	-0.3
E. cloacae	+0.7*	+0.1	+0.3	-0.5	-0.5	-1.1*
S. liquefaciens	-0.4	-0.8*	-0.7	0	-0.3	-0.5

* Significant ($P<0.05$); [$] *A. calcoaceticus* var. *anitratus*

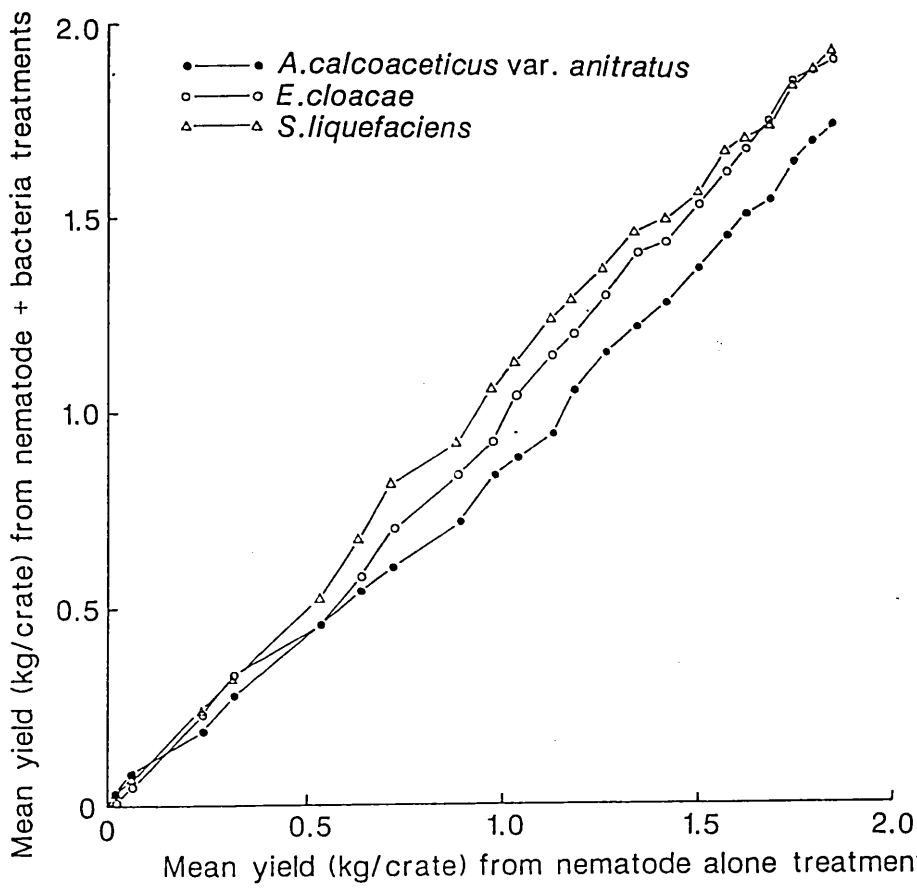


Fig. 7.3 Effects of *C. elegans* with or without the presence of associated bacteria on flushing patterns of *A. bisporus*: regression analysis.

Plate 7.2. Effects of bacteria and/or *C. elegans* on timing of mushroom flushes. Photo taken 15 days after casing. 1 = *C. elegans*, 2 = *A. calcoaceticus* var. *anitrarus* + *C. elegans*, 3 = *A. calcoaceticus* var. *anitratus*, 4 = Untreated.

7.3.2 Mushroom size

Table 7.4 lists the mean sporophore size (defined as the mean weight/mushroom) in the eight treatments. During the first flush, all the crates containing *C. elegans*, produced significantly smaller ($P<0.05$) mushrooms when compared with the controls or any other treatment. In contrast, during flushes 2 and 3 all the nematode treatments resulted in significantly ($P<0.05$) larger mushrooms. However, during fourth flush, only *E. cloacae* + *C. elegans* and *S. liquefaciens* + *C. elegans* treatments produced significantly ($P<0.05$) larger mushrooms.

Among bacteria treatments, *E. cloacae* resulted in significantly ($P<0.05$) larger mushrooms during the second flush and *A. calcoaceticus* var. *anitratus* had a similar effect during the third flush.

7.3.3 Sporophore colour

Marked effects of bacteria on the relative whiteness of mushrooms were observed and the effects varied with mushroom flush (Fig. 7.4). *S. liquefaciens* caused a significant reduction ($P<0.05$) in sporophore whiteness (except during the third flush) and the effects substantially increased in the later flushes. However, no such trends were demonstrated with *A. calcoaceticus* var. *anitratus* and *E. cloacae*.

The magnitude of quality deterioration (i.e. the reduction in the whiteness of sporophores) was much greater in the presence of *C. elegans* (Fig. 7.5). Except for the *C. elegans* + *A. calcoaceticus* var. *anitratus* treatment during the second and third flush and the *C. elegans* treatment during the third flush all the other treatments resulted in a significant reduction ($P<0.05$) in sporophore whiteness. The effects were particularly pronounced during the fifth and the sixth flush.

7.3.4 Nematodes on sporophores

Washing of sporophores revealed considerable numbers of *C. elegans*

Table 7.4 Effects of bacteria and/or *C. elegans* on size of mushrooms: mean sporophore weight (g)/mushroom

Treatments*	Flushes					
	1	2	3	4	5	6
A.c.	17.7	12.1	13.2	11.6	11.8	9.4
E.c.	17.3	12.6	10.0	11.3	9.9	11.3
S.l.	19.9	10.8	12.4	10.3	12.4	9.2
A.c.+ C.e.	13.4	15.0	15.7	11.9	9.1	9.4
E.c.+ C.e.	9.5	15.0	14.7	14.8	10.4	9.3
S.l.+ C.e.	12.1	14.0	14.4	14.3	10.7	9.2
C.e.	12.8	16.2	13.8	12.9	9.6	9.1
Untreated	18.8	9.7	10.0	11.5	10.9	9.6
SED (35 df)	2.3	1.3	1.4	1.2	0.9	0.8

* A.c. = *A. calcoaceticus* var. *anitratus*, E.c. = *E. cloacae*, S.l. = *S. liquefaciens*, C.e. = *C. elegans*

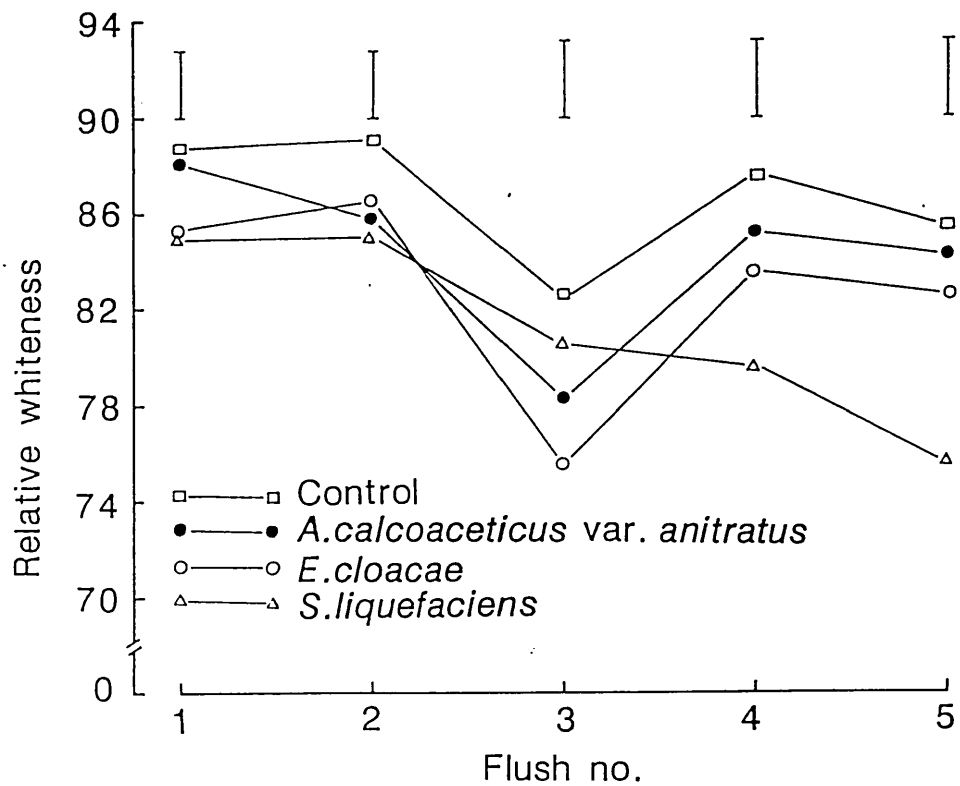


Fig. 7.4 Effects of bacteria associated with *C. elegans* on relative whiteness of mushrooms. Bars represent LSD ($P<0.05$).

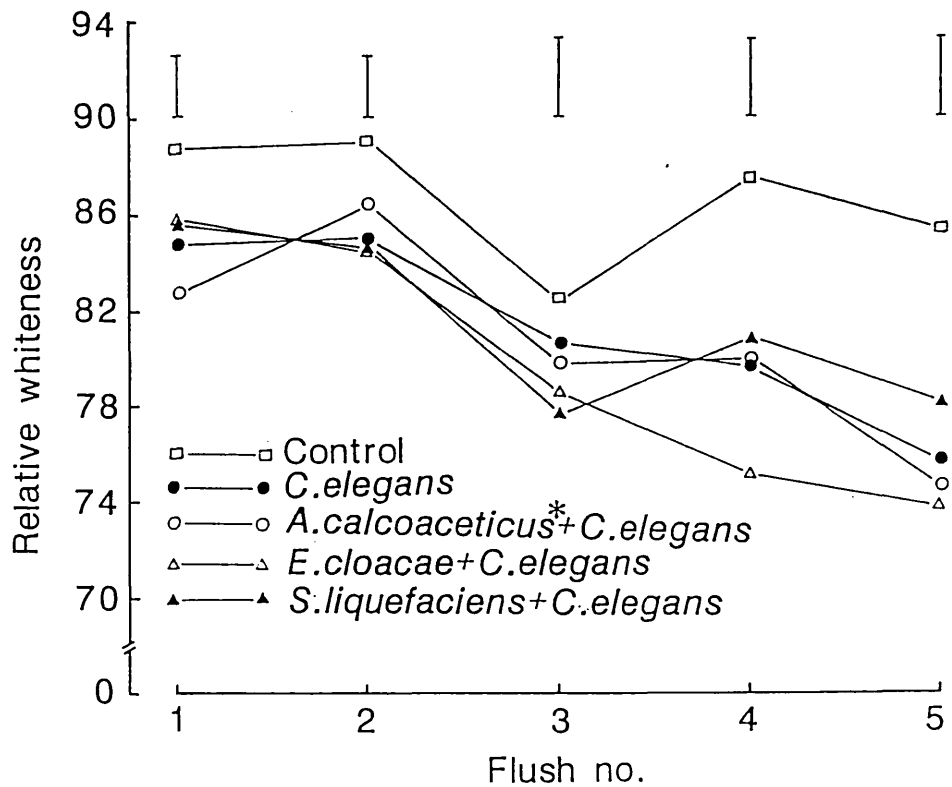


Fig. 7.5 Effects of *C. elegans* and/or associated bacteria on relative whiteness of mushrooms. Bars represent LSD ($P<0.05$).

* *A. calcoaceticus* var. *anitratus*

(Fig. 7.6); their numbers varied according to the treatment type and the mushroom flush. When compared with the *C. elegans* treatment, the sporophores from *C. elegans* + *A. calcoaceticus* var. *anitratus* and the *C. elegans* + *E. cloacae* treatments harboured significantly greater ($P<0.05$) numbers of nematodes during the first flush. The differences in the treatments were less marked in the later flushes. Swarming of nematodes was observed on sporophores and up to 14,000 nematodes/sporophore were observed.

7.3.5 Nematode population in casing layer

The trends in *C. elegans* populations in the casing layer during cropping are shown in Fig. 7.7. An initial rapid increase in nematode populations was observed in all treatments. Nematode 'outbreak' resulted in 'climbing' columns of nematodes on the surface of the casing and were followed by population declines throughout the cropping period. One week after inoculation, populations of *C. elegans* were significantly larger in the presence of both *A. calcoaceticus* var. *anitratus* and *E. cloacae* than they were in any other treatment.

7.3.6 Mushroom distortion

C. elegans infestations resulted in characteristic distortion of mushroom sporophores. A proportion of the mushrooms were morphologically distorted, notched and mis-shapen (Table 7.5). Such symptoms were not observed in treatments where bacteria were applied alone.

7.3.7 Mushroom yield

The yields of *A. bisporus* obtained from this experiment are listed in Table 7.6. When compared with controls, all the treatments, except *S. liquefaciens*, significantly reduced ($P<0.05$) the total yields (wt) of mushrooms. When compared with bacteria only treatments, the magnitude of yield loss was significantly ($P<0.05$) greater in nematode treatments (i.e. either nematodes alone or in combination with bacteria). The combination of *C. elegans* and

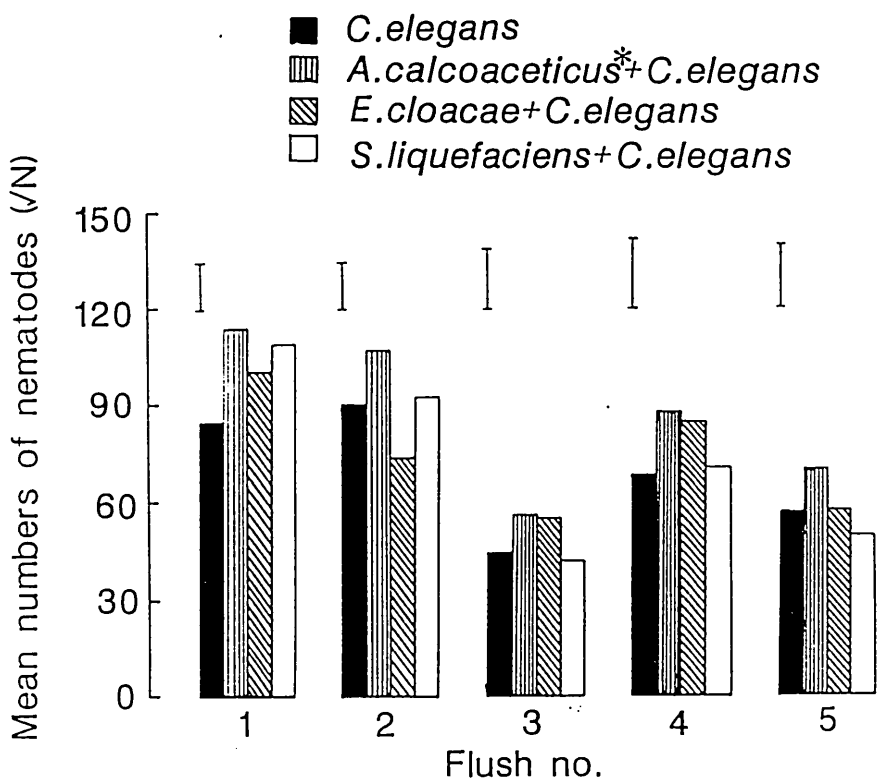


Fig. 7.6 Nematodes on sporophores. Data are mean numbers of *C. elegans*/200g sporophores during each flush. Bars represent LSD ($P<0.05$).

* *A. calcoaceticus* var. *anitratus*.

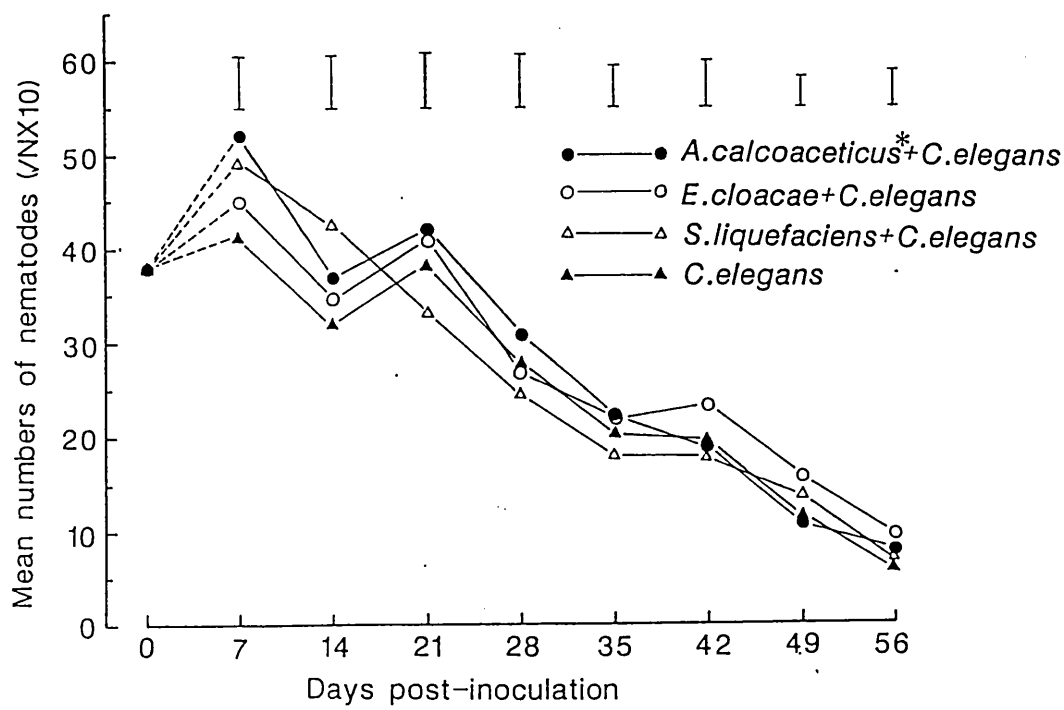


Fig. 7.7 Mean numbers of *C. elegans* per 25g of casing material in different treatments. Bars represent LSD ($P<0.05$). * *A. calcoaceticus* var. *anitratus*

Table 7.5 Effects of *C. elegans* and/or associated bacteria on mushroom sporophores: mean total weight and number of distorted mushrooms/crate

Treatments	Mushroom wt (g)*	Mushroom numbers*
A. calcoaceticus$	0	0
E. cloacae	0	0
S. liquefaciens	0	0
A. calcoaceticus$ + *C. elegans*	181.8 (10.5)	14.7 (10)
E. cloacae + *C. elegans*	140.7 (7.4)	13.0 (8.6)
S. liquefaciens + *C. elegans*	187.7 (9.8)	14.0 (9.3)
C. elegans	176.2 (9.6)	14.0 (9.6)
Untreated	0	0
SED (35 df)	21.8	1.7

* Figures in brackets = % distorted; $ *A. calcoaceticus* var. *anitratus*

Table 7.6. Effects of *C. elegans* and/or associated bacteria on yield (mean total weight and numbers of mushrooms/crate) of *A. bisporus*

Treatments	Mushroom wt (kg)	Mushroom numbers
A. calcoaceticus[$]	2.265	191.7
E. cloacae	2.411	209.3
S. liquefaciens	2.370	211.5
A. calcoaceticus[$] + *C. elegans*	1.744	146.5
E. cloacae + *C. elegans*	1.902	151.3
S. liquefaciens + *C. elegans*	1.921	150.3
C. elegans	1.838	145.8
Untreated	2.540	238.8
SED (35 df)	0.085	12.5

[$] *A. calcoaceticus* var. *anitratus*

A. calcoaceticus var. *anitratus* resulted in the maximus yield loss.

When compared with controls, all the treatments caused significant ($P<0.05$) reduction in the total numbers of mushrooms produced. The presence of nematodes resulted in these treatments, producing significantly fewer mushrooms ($P<0.05$) than the treatments which received only bacterial suspensions.

7.4 Discussion

In commercial practice, mushrooms appear in distinct flushes at intervals of about 7-14 days (Flegg & Wood, 1985). This study has shown that infestations of *C. elegans* can significantly affect this type of flushing pattern and that when this is the case mushrooms appear more regularly, and in fewer numbers, throughout the cropping period. This corroborates the results reported in Section 5.3.3 and also confirms the observations of Hesling (1966). In the nematode treated crates, the appearance of the first flush was delayed by three to four days and the effects of *C. elegans* on the mushroom flushing patterns were significantly altered by the species of bacteria present. This may be due to the direct differential effects of bacteria on the mushroom and/or indirectly through their effects on *C. elegans* reproduction.

The effects of bacteria on flushing patterns were comparatively less marked; they varied according to the type of bacteria. For instance, *E. cloacae* showed stimulatory effects on the mushroom and resulted in significantly more mushrooms being produced during the first flush and also in the early appearance of the first flush. The other two bacteria showed inhibitory/suppressive effects. *A. calcoaceticus* var. *anitratus* showed no significant effects on the timing of flushes but significantly reduced the total weight of mushrooms during the first and the fifth flush and the total numbers of mushrooms during the first and the third flush. *S. liquefaciens* resulted in significantly lower yields during the fifth flush and in fewer mushrooms during the third flush. These results suggest that the bacteria probably did not disperse effectively in the substrate in the absence of nematodes.

Nematodes and their associated bacteria affected the size of mushrooms. *C. elegans* infestations (either alone or in combination with bacteria) resulted in significantly larger mushrooms especially during the second and the third flush. However, the effects of bacteria were less obvious; *E. cloacae* resulted in significantly larger mushrooms during the second flush; *A. calcoaceticus* var. *anitratus* had this effect during the third flush.

Another interesting aspect of this study was the observation that nematodes and/or their associated bacteria can affect the quality (i.e. relative whiteness) of mushrooms. *S. liquefaciens* produced the most pronounced effect on the whiteness of mushrooms; the effect became greater towards the end of the crop. *C. elegans* applied alone or in combination with bacteria (especially *E. cloacae* and *S. liquefaciens*) resulted in a significant reduction in whiteness of mushrooms throughout the cropping period. The mushrooms turned brown and the extent of browning was much greater during the fifth or sixth flush.

Sanderson *et al.* (1981) studied the effects of *Mesorhabditis* sp. on mushroom quality and reported that 45% of all the mushrooms from the nematode infested boxes during the first flush were stained; symptoms that were typical of faulty watering. Furthermore, they isolated a bacterium, *Pseudomonas cichorii* from the stained mushrooms and confirmed its pathogenicity by subsequent reinoculation on fresh mushrooms. Doores *et al.* (1987) and Beelman *et al.* (1989) also reported that the quantity and quality of bacterial populations on sporophores affected the relative whiteness of mushrooms. However, if this phenomenon is only due to the bacteria, and the nematodes merely act as vectors, the uniformity of mushroom browning in combined treatments of nematodes and bacteria is explained. But the magnitude of difference between control and nematode treatments towards the end of the crop when the nematode numbers in casing and on sporophores actually declined suggested that the browning of mushrooms may be the end result of the accumulation of toxic by-products produced by the combination of nematodes and bacteria.

C. elegans infestations result in characteristic distortion of mushroom sporophores (Section 5.3.3). The present study revealed that such symptoms are not produced by bacteria alone. Furthermore, the inoculated bacteria seem to have no synergistic relationship with *C. elegans* in the production of such

distortion of sporophores because the numbers of distorted mushrooms did not differ significantly in the presence/absence of bacteria or with the type of bacteria. Therefore, this type of malformation may be either due to physical damage to mushroom primordia 'pinheads' at their early stages of development by the nematodes (the view supported by Dr D. J. F. Brown, personal communication) and/or to localised accumulation of toxic by-products of the nematodes.

The present study has shown that the bacteria associated with *C. elegans* contribute to yield loss and quality deterioration. The bacteria, *A. calcoaceticus* var. *anitratus* and *S. liquefaciens* cause significant reductions in the total weight of mushrooms of 10.8 and 6.7%, respectively. However, in the presence of *C. elegans* yield losses were much greater; being 24.4 and 31.3% with/or with bacteria, respectively.

The differential effects of *C. elegans* in the presence of different bacteria on the yield of mushrooms may be due to the variable suitability of bacteria as food substrates for the nematode. This view was supported by differences in nematode multiplication in casing treated with different bacteria. Furthermore, *in vitro* studies had shown that *C. elegans* produced larger populations in monoxenic cultures with *A. calcoaceticus* var. *anitratus* than on *E. cloacae* or *S. liquefaciens* (Section 4.3.2).

Nematodes and/or their associated bacteria appear to affect the mushroom sporophore-inducing process. When compared with controls, all the treatments significantly reduced the numbers of mushrooms produced. This can also explain why mushrooms were invariably bigger in the treated plots; *A. bisporus* is able to compensate for the lost 'pinheads' by producing larger mushrooms (Wyatt, 1977).

In Section 6.3.1, it was demonstrated that *E. cloacae* and *S. liquefaciens* caused >50% inhibition in mycelial growth of *A. bisporus* in agar cultures whereas *A. calcoaceticus* var. *anitratus* showed only minor effects. However, this study showed that *A. calcoaceticus* var. *anitratus* and *S. liquefaciens*, and not *E. cloacae*, caused significant reductions in mushroom yields. This further supports the view that the effects of casing treatments are not on the mycelial growth of the mushroom but are on the sporophore-inducing process. The ability of the

bacteria associated with *C. elegans* to inhibit mycelial growth of *A. bisporus* may be more important during the colonisation of compost ('spawn-run') by the mushroom when temperatures are especially suitable for bacterial growth (Section 5.3.1).

CHAPTER 8

Effects of *C. elegans* on the spread of bacterial blotch

8.1 Introduction

Steiner (1931) reported that *Cruznema tripartitum* (=*Rhabditis lamdiensis*) was possibly an important vector of a bacterium *Pseudomonas tolaasii* Paine that caused pits on the mushroom sporophores (Section 2.2). However, since then, this phenomenon has never been confirmed. In this study, the potential role of mass-produced *C. elegans* (Section 4.2.6) as vectors of bacterial blotch pathogen *P. tolaasii* in mushroom beds was examined.

8.2 Materials and methods

8.2.1 Bacterium culture

The reference strain of *Pseudomonas tolaasii* Paine (Pt 51) obtained from the IHR culture collection was maintained on King's B medium (King *et al.*, 1954) and was sub-cultured, at 25°C, at monthly intervals. Suspensions of the bacteria were prepared in King's B broth. Bacteria were inoculated into 250 ml conical flasks containing 100 ml of medium. Flasks were incubated at 25°C for 24h in a shaker-incubator (200 rpm). Optical density of the bacterial suspension was determined at 600 nm using a spectrophotometer and the numbers of cells/ml were estimated using a pre-calibrated standard curve.

8.2.2 Preparation of the mushroom substrate

The preparation of the mushroom substrate and other cultural practices followed are given in Section 5.2.1.

8.2.3 Treatments and experimental design

There were six replicates of four treatments; three *C. elegans* inocula (i.e. 10^5, 10^6 and 10^7 nematodes/crate) and a control treatment. The nematodes (adults:larvae = 1:19) in 100 ml of water were thoroughly mixed into the casing material of each crate. Immediately after the casing was applied, all 24 crates were arranged in a Latin square design in two rows and three layers supported on aluminium frame-work in a purpose-built mushroom growth chamber.

Fourteen days after casing and just before the appearance of the first flush, 100 ml of cell suspension of *P. tolaasii* containing 10^8 cells/ml was applied to the surface of each crate.

8.2.4 Data recording and analysis

(i) *Intensity of bacterial blotch.*

On each day that mushrooms were picked, the intensity of blotch symptoms on sporophores was assessed using a 0-4 scale (Plate 8.1). All mushrooms were assigned to different classes on the basis of per cent area diseased (visual observation). The scale was: 0 = no disease; 1 = 1-25%; 2 = 26-50%; 3 = 51-75%; and 4 = 76-100% surface area diseased, respectively. The per cent disease intensity was computed as:

$$PDI = 100(\text{sum of numerical ratings})(4N)^{-1}$$

where N is the number of observations and 4 is the maximum rating. The data were subjected to ANOVA.

(ii) *Nematodes on mushroom sporophores.*

Once during each flush, numbers of *C. elegans* on approx. 200g of mushroom sporophores from each replicate of each treatment were estimated as described in Section 5.2.4.

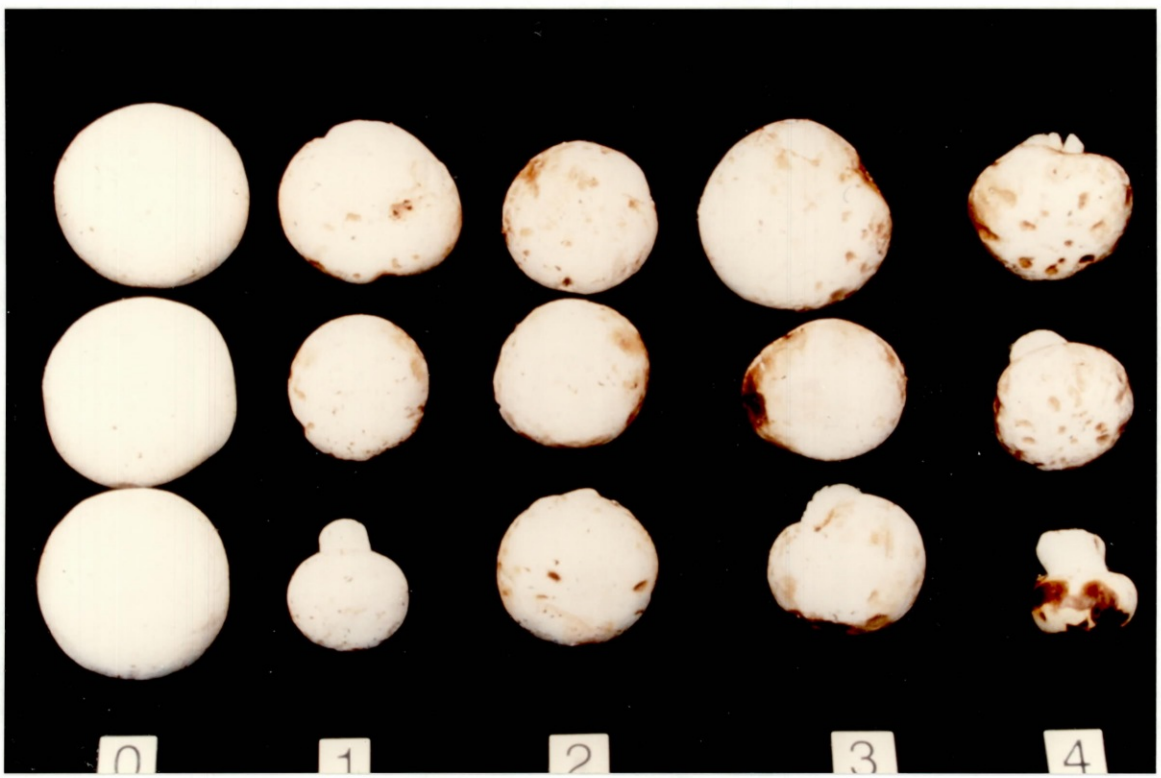


Plate 8.1 Intensity of bacterial blotch on sporophores. Scale represents: 0 = no disease, 1 = 1-25%, 2 = 26-50%, 3 = 51-75%, and 4 = 76-100% suface area diseased, respectively.

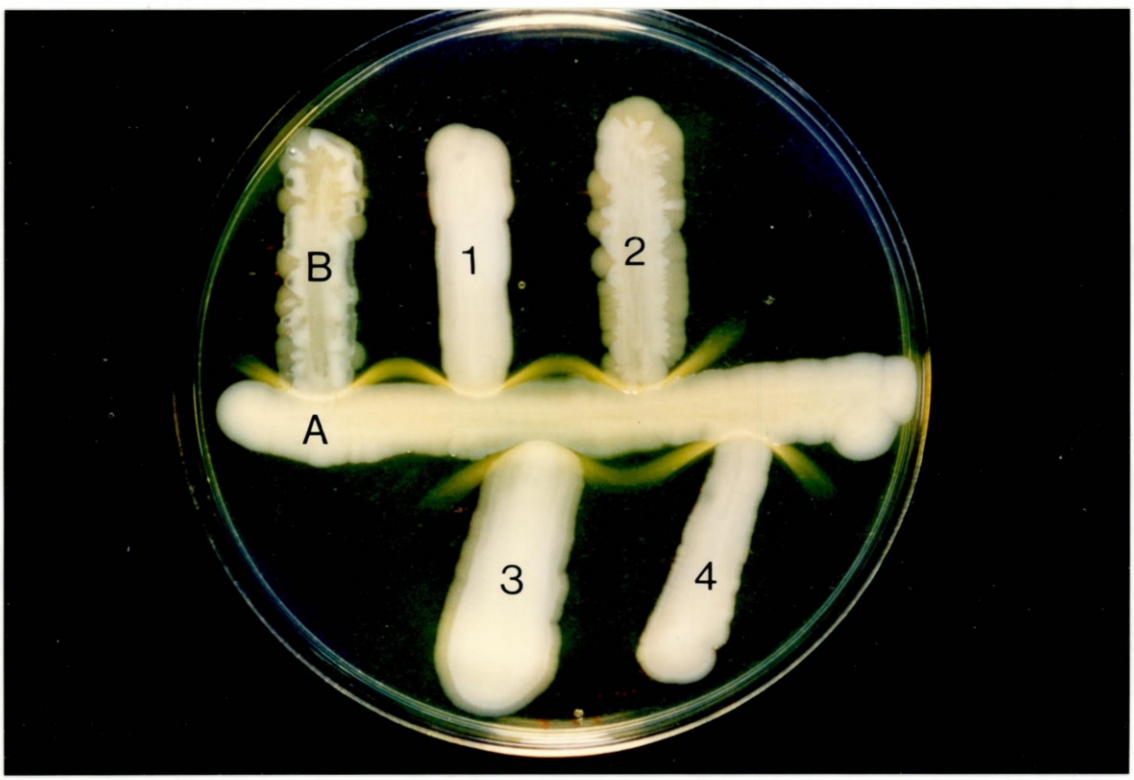


Plate 8.2 The white line test used to identify antagonistic strains of *P. fluorescens*. A = *P. tolaasii* (Pt51); B = *Pseudomonas 'reactans'* NCPPB 3149T2; 1-4 = test cultures.

(iii) *Nematode multiplication in casing layer.*

Populations of *C. elegans* in casing were estimated at weekly intervals after inoculation as described in Section 5.2.4.

(iv) *Mushroom yield.*

Mushrooms were picked on alternate days at the point of market acceptability, that is stages 3-5 (Section 5.2.4). Their stipes were trimmed off and numbers and weights recorded. Total numbers and weights of mushrooms were subjected to ANOVA.

8.2.5 Isolation and identification of fluorescent pseudomonads from *C. elegans* during cropping

Once during each flush, fluorescent species of *Pseudomonas* associated with *C. elegans* were isolated. Nematodes were washed from sporophores with sterile distilled water, surface sterilized (Section 4.2.2), crushed in a sterile tissue homogenizer and the suspension plated on a *Pseudomonas*-selective isolation agar (Difco, USA). The colonies that grew over 48 hours at 25°C were purified by repeated sub-culture and identified using the Analytical Profile Index (API Products Ltd) and a range of other confirmatory tests (Lelliott *et al.*, 1966; Zarkower *et al.*, 1984).

8.2.6 White line test

The identity of the pathogen and of the antagonists was confirmed using the method described by Wong and Preece (1979). The reference strain *Pseudomonas 'reactans'* NCPPB 3149T2 was streaked on King's B medium in a Petri dish and the test bacteria were streaked at 90° to it, leaving a small gap between. A white line appeared in the agar between the two cultures only when the test culture was *P. tolaasii*. In order to identify the antagonists, *P. tolaasii* (Strain Pt 51) was used in a similar manner to the reference strain (Plate 8.2).

8.2.7 Reproduction of *C. elegans* on *P. tolaasii* and *Pseudomonas fluorescens* biovar *reactans*

P. tolaasii (Pt 51) and *P. fluorescens* biovar *reactans* (isolated during this study, see Section 8.3.5) were compared as food substrates supporting nematode reproduction. The bacteria were streak-inoculated and grown on 3% (w/v) nutrient agar (Oxoid Ltd) in 100 mm square Petri plates (18mm deep and each with 25 compartments) for 24 hours at 25°C. Freshly-hatched, surface-sterilized second-stage *C. elegans* larvae were individually inoculated (one larva/block) in 10 μl of sterile distilled water using a Pasteur pipette and were incubated at 20°C. Fifteen blocks were inoculated for each bacterium. After four, eight and twelve days, the size of the nematode population (eggs, larvae and adults) in five compartments containing either of the two bacteria was determined. The contents of each block were washed, with distilled water, into separate beakers and the specimens fixed in 2% (v/v) formaldehyde solution. Eggs and nematodes were counted; the data were square-root transformed and subjected to ANOVA.

8.3 Results

8.3.1 Intensity of bacterial blotch on sporophores

Application of *C. elegans* to casing significantly reduced ($P<0.05$) mushroom infection by *P. tolaasii* from one week after inoculation (Fig. 8.1 & Appendix 8.2). However, no significant differences ($P>0.05$) in the effects caused by the three rates of nematode inocula were found.

8.3.2 Nematodes on sporophores

Washing of mushrooms revealed the presence of considerable numbers of *C. elegans* (Fig. 8.2); their numbers varied according to treatment type and mushroom flush. Swarming of nematodes was observed on sporophores; up to 20,000 nematodes/sporophore were found on mushrooms picked from plots

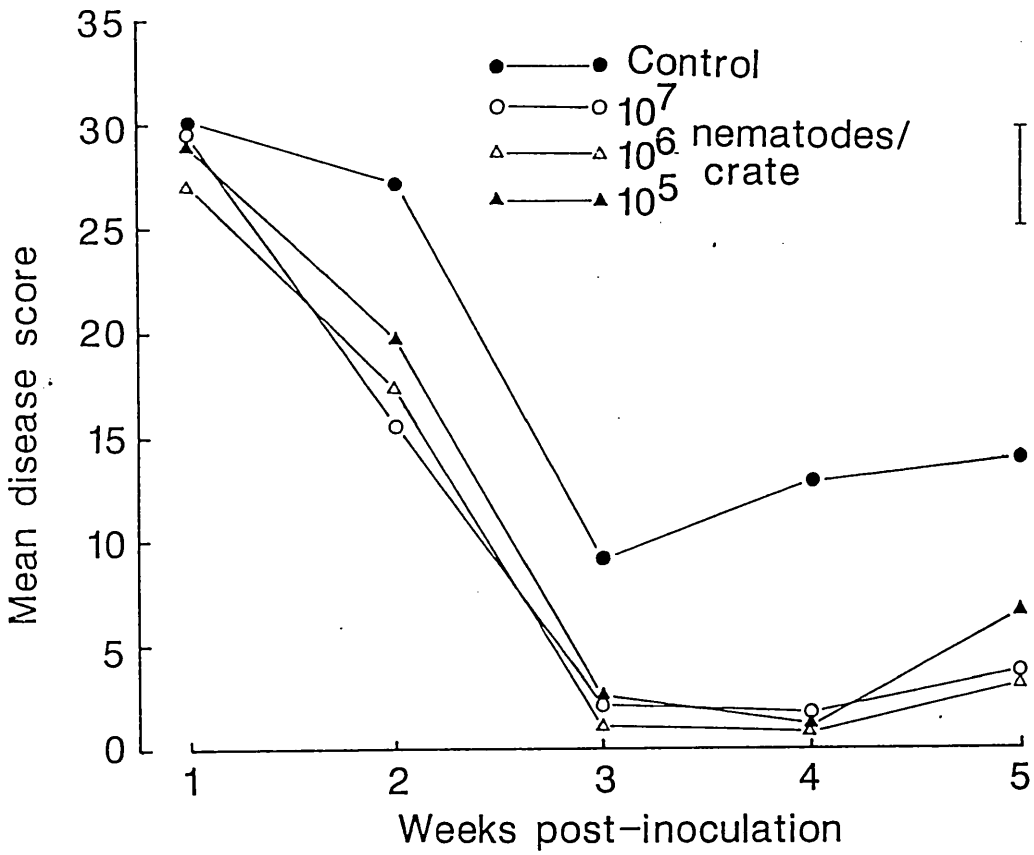


Fig. 8.1 Effects of initial inoculum levels of *C. elegans* on the intensity of bacterial blotch. Bar represents LSD ($P<0.05$) at 15 df.

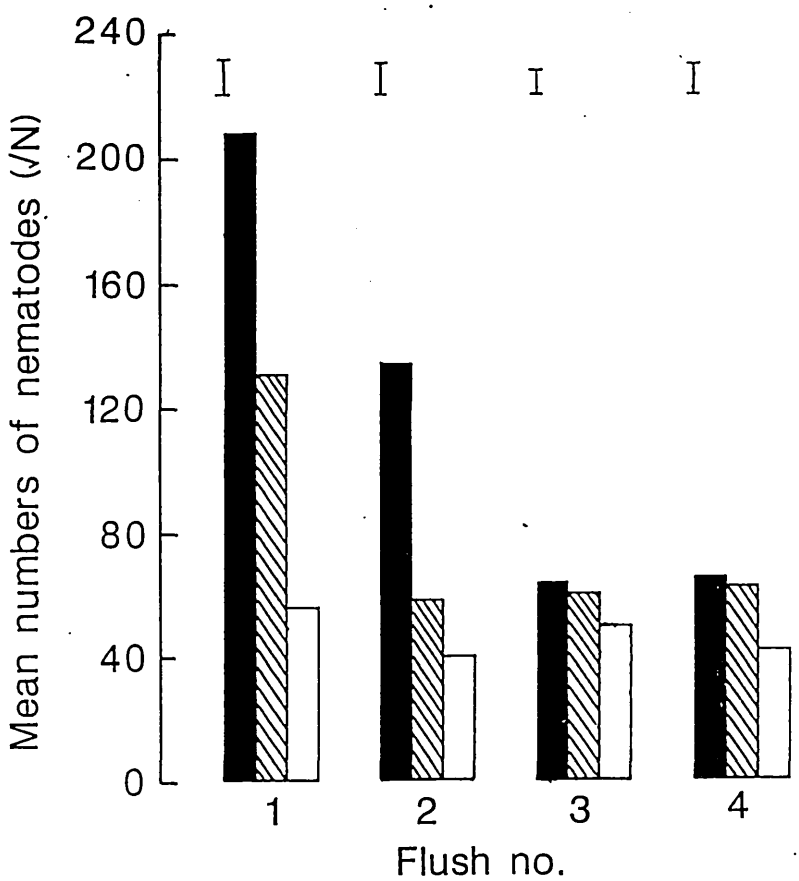


Fig. 8.2 *C. elegans* on sporophores. Data are square-root transformations of the mean numbers of *C. elegans* washed from 200g of sporophores. Bars represent LSD ($P<0.05$) at 10 df.

inoculated at the highest nematode dose.

8.3.3 Nematode population in casing layer

The trends in *C. elegans* populations in casing during cropping are shown in Fig. 8.3. An initial rapid increase in nematode populations was observed at the two highest inocula. This outbreak of nematode populations resulted in characteristic 'climbing' columns of nematodes on the surface of the casing. Fourteen days after inoculation, nematode populations in these two treatments declined and then stabilised. When compared with other treatments, only the highest inoculum resulted in significantly larger ($P<0.05$) populations of *C. elegans* in the casing throughout the cropping period.

8.3.4 Mushroom yield

Yields of *A. bisporus* obtained from this trial are listed in Table 8.1. *C. elegans* applied at or above 10^6 nematodes/7.5 kg of compost significantly reduced ($P<0.05$) both the total numbers and weights of mushrooms picked.

8.3.5 Association of *C. elegans* with fluorescent pseudomonads during cropping

Isolations of the fluorescent pseudomonads from the gut of *C. elegans*, made during cropping, revealed two species; *Pseudomonas fluorescens* biovar *reactans* and *P. tolaasii*. The incidence of *P. fluorescens* biovar *reactans* increased towards the end of the crop, it constituted 27.5%, 65.3%, 83.7% and 93.6% of the colonies isolated during flushes 1-4 respectively.

8.3.6 Reproduction of *C. elegans* on *P. tolaasii* and *P. fluorescens* biovar *reactans*

Growth and reproduction of *C. elegans* was supported by both bacteria. However, the nematode produced significantly larger ($P<0.05$) populations on *P. fluorescens* biovar. *reactans* than on *P. tolaasii* (Table 8.2).

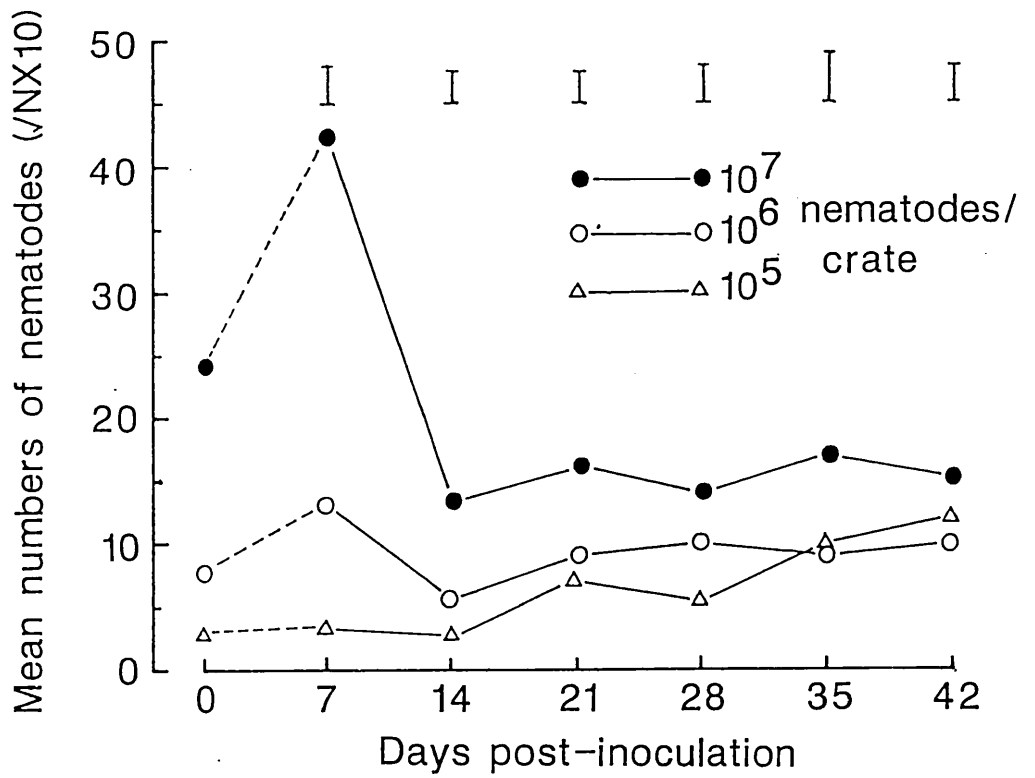


Fig. 8.3. Populations of *C. elegans* in the casing-layer. Data are square-root transformations of the mean numbers of *C. elegans* extracted from 25g casing material. Bars represent LSD ($P<0.05$) at 10 df.

Table 8.1 Effects of *C. elegans* on the yield (kg/crate) of *A. bisporus* in the presence of *Pseudomonas tolaasii*

Nematodes ($x10^5$)/crate	Mean total weight (kg)	Mean total numbers
100	2.343	212.8
10	2.524	221.0
1	2.733	248.3
0	2.754	255.8
SED (15 df)	0.102	16.2

Table 8.2 Reproduction of *C. elegans* on *Pseudomonas tolaasii* and *Pseudomonas fluorescens* biovar *reactans*. Data are square-root transformations of the mean total numbers of progeny (eggs, larvae and adults) produced per inoculated larva.

	Days post-inoculation		
	4	8	12
P. tolaasii	12.5	14.6	54.6
P. fluorescens biovar *reactans*	15.1	120.5	242.2
SED (4 df)	1.1	2.4	9.7

8.4 Discussion

Steiner (1931) reported that *C. tripartitum* (=*R. lambdiensis*) was a possible vector of *P. tolaasii* in mushroom beds. This suggestion has never been confirmed. The present study was conducted to test whether or not *C. elegans* could affect the incidence of blotch in mushroom beds. The results show that *C. elegans* significantly ($P<0.05$) reduced the intensity of blotch on sporophores.

The frequent isolation of *P. fluorescens* biovar *reactans* from the gut of *C. elegans* was interesting because some strains of *P. fluorescens* biovar *reactans* are antagonistic to *P. tolaasii* and are used as biological control agents (Nair & Fahy, 1972; Fermor & Lynch, 1988). The white line tests conducted during this study showed that all the isolates of *P. fluorescens* biovar *reactans* recovered from the nematodes were antagonistic to *P. tolaasii.*

From these observations, it was evident that *C. elegans* probably feeds on *P. fluorescens* biovar *reactans* rather than on *P. tolaasii.* This was confirmed when the reproductive capacity of *C. elegans* on the two species was compared; *C. elegans* produced much larger populations in monoxenic cultures of *P. fluorescens* biovar *reactans* than it did in similar cultures of *P. tolaasii.* Differential responses of *C. elegans* to various bacteria are already well-documented (Andrew & Nicholas, 1976 ; Poinar & Hansen, 1986). Bearing in mind the common occurrence of *P. fluorescens* biovar *reactans* in various raw materials used for mushroom culture (Fermor & Lynch, 1988) and especially in the casing (Samson *et al.*, 1987) one would expect the association of *C. elegans* with *P. fluorescens* biovar *reactans* rather than with *P. tolaasii* which is much less frequently found (Samson *et al.*, 1987).

The present study therefore lends support to the theory that, in the mushroom environment, *C. elegans* tends to feed on the antagonistic strains of *P. fluorescens* biovar. *reactans* (even when *P. tolaasii* was artificially inoculated) and that in this process of feeding it probably also spreads the antagonist. The presence of *C. elegans* on sporophores in large numbers further supports the view that nematodes spread the antagonist to potential sites of pathogen activity.

The efficient spread of bacteria by rhabditid nematodes has already been reported. For instance, Cayrol *et al.* (1981) found that *Cephalobus emarginatus*

and *Dolichorhabditis dolichura* (=*Cephalobus dolichura*) disperse sporophore-inducing bacteria (*Pseudomonas putida)* through casing and thereby increase mushroom production. Furthermore, Cayrol *et al.* (1987) reported that *Cephalobus parvus* can spread *Rhizobium japonicum* and increase the numbers of nodules on soybeans.

C. elegans significantly reduces the intensity of blotch even when quite low inocula are used (i.e. 10^5 nematodes/7.5 kg compost, Fig. 8.1). Such levels of *C. elegans* did not cause any significant loss in mushroom yield. The losses in yield caused by higher nematode inocula in the present study corroborates the results described in Section 5 that *C. elegans* introduced at about 267 nematodes/g of casing material caused a significant reduction in the yield of *A. bisporus.*

CHAPTER 9

General discussion

As standard commercial practice in the UK, the mushroom substrate is prepared from the composted wheat straw (Flegg *et al.*, 1985). The process of composting is carried out in two phases - Phase I and II. During Phase I, the application of manures and/or activators (nitrogen rich compounds) to wheat straw initiates microbial activity. Because of the presence of numerous bacteria rhabditid nematodes also multiply rapidly (Choleva, 1966, 1968; Farkas, 1972; Turner, 1988).

Phase II of the composting process which is also called 'peak-heating' is carried out for two main purposes one of which is to eliminate ammonia from the substrate and to develop a specific type of microflora. This 'selectivity' of the final medium enhances the subsequent growth of the mushroom mycelium. This process is carried out at 50-55°C for 4-8 days. The other aim of phase II is to pasteurize the compost to eliminate mushroom pests and disease organisms - for this a peak temperature of 60°C for 3-6 hours must be attained. Nematodes generally do not survive pasteurization (Hesling, 1979; Turner, 1988) and the presence of nematodes, if any, in compost at the end of phase II indicates that the pasteurization process was ineffective.

Pasteurized compost is cooled to 25°C, 'spawned' with the mushroom spawn and incubated at 25°C to allow colonization by fungal mycelium ('spawn-run') to occur. The present study showed that when inoculated into a properly prepared and pasteurized compost populations of *C. elegans* fell during the spawn-running period and that they had no significant effects on mushroom production (Section 5.3.1). This could be due to the fact that under good growing conditions a vigorous mycelial growth through the compost limits nematode activity and reproduction, since both moisture and bacteria are removed from the compost (Section 5.4).

In contrast, other reports had shown that rhabditid nematodes along with

their associated organisms damage mushroom mycelium (van Haut, 1956; Blake & Conroy, 1959; Hesling, 1966; Ross & Burden, 1981; Kaufman *et al.*, 1983, 1984). After extensive experimentation, Ross & Burden (1981) concluded that the effects of saprophagous rhabditid nematodes may vary along a scale which ranges from complete suppression of the mushrooms at one end to complete suppression of the nematodes at the other.

Normally every effort is made to favour the mushroom, especially with regard to the creation of a selective compost. Nematodes, therefore, rarely acquire favourable conditions for their multiplication. Furthermore, the bacteriostatic nature of *A. bisporus* (Tschierpe & Sinden, 1965; Barron, 1988) reduces the availability of bacteria as a food resource for saprobes and thus limits nematode maturation and reproduction. However, when the conditions for mycelial growth are not optimum (e.g. excessive moisture, Kaufman *et al.*, 1984) the process of substrate colonization by the fungus slows down, allowing rapid growth of bacteria and nematodes which subsequently limit mushroom growth. Ross & Burden (1981) reported that rhabditid nematodes could develop and multiply in unspawned, pasteurized compost and suggested that the nematodes compete for nutrition with the mushroom mycelium. However, the nematode inoculum used in that study was not sterile.

After a 2 wk 'spawn-run', the colonized substrate is covered with peat and chalk mix ('casing material') in order to initiate fruiting. The casing material is generally not pasteurized because of the beneficial activity of the resident bacterial flora for mushroom sporophore induction (Egar, 1972) and therefore is a potential source of nematode infestation (Farkas & Koronczy, 1974; Hesling & Gaze, 1975; Khair & McLeod, 1978). In addition, the casing material provides a highly suitable environment for nematode activity because of its water-holding characteristics, low nutritive status (that allows only sparse mycelial colonization) and inherent natural bacterial flora.

After the mushroom beds are cased, bacterial multiplication in the casing material starts and so does colonization by mycelium. The temperature during this phase is maintained around 22-24°C to allow rapid mycelial growth. The ventilation is increased to lower the ambient CO_2 levels and to stimulate the initiation of fruiting and normal morphogenesis of mushrooms (Flegg *et al.*,

1985). Mushroom beds are frequently watered and the relative humidity in cropping houses is maintained around 90-95%. These conditions also favour the development of nematodes. Therefore, this 2 wk period after casing is critical for both mushroom growth and nematode multiplication.

The results of the present study suggest that the competition between *C. elegans* and mushroom mycelium also occurs in casing. Thus, if initial numbers of *C. elegans* are low, the nematode's multiplication rate is initially restricted and no significant effects on total weight of mushrooms are found. However, if the initial inocula are high and the subsequent population explosion results in the development of 'climbing' columns of nematodes on the surface of casing within one to two weeks after inoculation, then the mushroom growth and sporophore production is significantly affected (Section 5.3).

When inoculated in casing, *C. elegans* showed considerable effects on mushroom yield and quality. The total weight and numbers of mushrooms picked were significantly reduced when 10^6 nematodes or more per crate were introduced into the casing material during casing. Up to 26% loss in total mushroom yield was recorded at the highest inoculum level (2×10^7 nematodes/crate). Apart from yield losses, infestation of *C. elegans* at casing resulted in considerable distortion of mushroom sporophores. At the highest inoculum level (2×10^7 nematodes/crate) up to 8% of the total number and 11% of the total weight of mushrooms were distorted. This malformation of mushrooms directly adds to the unmarketable yield.

The physical presence of nematodes in large numbers even on healthy sporophores is another factor to be considered in commercial cultivation of the mushrooms. Nematodes are known to enhance bacterial multiplication by their phoretic transport (Griffiths, 1986) and by providing some essential growth factors (Ingham *et al.*, 1985). This extra bacterial load may therefore lead to greater post-harvest deterioration of mushrooms (Doores *et al.*, 1987).

Due to their ability to spread sporophore-inducing bacteria and thus, to even-out the process of sporophore-induction, low numbers of saprophagous rhabditid nematodes in the casing layer are probably beneficial. Cayrol *et al.* (1981) demonstrated that, when artificially infested, *C. emarginatus* and *D. dolichura* were able to disperse *Pseudomonas putida* through the casing and

thus induce mushroom fruiting.

The role of *C. elegans* in restricting of the spread of the bacterial blotch pathogen, *P. tolaasii*, is another interesting aspect of the present investigation. The ability of nematodes to significantly reduce the intensity of blotch on sporophores at an initial inoculum level that has no significant effect on mushroom yield is important. The probable association of nematodes with antagonistic strains of *P. fluorescens* biovar *reactans* may thus contribute to the natural 'biological' control of blotch in the mushroom industry.

The pathogenicity of saprophagous nematodes to mushroom is difficult to demonstrate satisfactorily mainly because they lack a stylet and therefore are unable to cause any significant physical damage to mushroom mycelium. Bacteria constitute a main component of the diet of saprophagous nematodes in nature (Nicholas, 1984; Wood, 1988). Furthermore, the bacteria present in the casing material provides the necessary 'stimulus' for the induction of mushroom sporophores (Eger, 1972) and the production of fruit bodies by *A. bisporus* in sterile cultures has yet not been reported.

Bacteria serve as a food substrate for saprophagous nematodes in nature and are necessary for nematode maturation and reproduction (Hesling, 1979; Nicholas, 1984). In monoxenic cultures the reproduction of *C. elegans* varied greatly on different species of bacteria. Bacteria also affected the migratory behaviour of *C. elegans* and the degree of alteration in nematode behaviour depended upon the species of bacteria. Nematodes are shown to enhance bacterial multiplication by their phoretic transport thereby providing them with new food avenues (Griffiths, 1986) and also by providing essential growth factors (Ingham *et al.*, 1985).

It has been suggested that saprophagous nematodes and their associated micro-organisms inhibit mycelial growth of mushrooms (Blake & Conroy, 1959; Hesling, 1966). Kaufman *et al.* (1983) showed that the extracts obtained from *C. elegans*-infested compost inhibited the mycelial growth of *A. brunnescens*. However, they observed that an extract from uninfested compost also inhibited mycelial growth but to a lesser degree and suggested that the role of compost bacteria and other micro-organisms may be important. Bloom & Levine (1985) also suggested that the bacteria may contribute to the losses of mushroom yield

associated with saprophagous nematodes. The inhibition of mycelial growth of *A. bisporus* by the bacteria (*E. amnigenus, E. cloacae* and *S. liquefaciens*, Section 6.3.1) isolated from *C. elegans* in agar cultures support the above reports and suggest that the role of inhibitory bacteria may be important in limiting the mycelial growth of *A. bisporus* especially during the 'spawn-run' phase.

Phoretic association of saprophagous nematodes with flies especially the fungus gnats (Sciaridae) is another important factor that contributes to primary infestation and also to the secondary spread of nematodes in the mushroom houses (Haglund & Milne, 1973; Rinker & Bloom, 1982). Nematodes also bring in 'foreign' bacteria and can probably disturb the natural balance between the resident bacterial flora and the mushroom mycelium. Since the sources of fly infestation on mushroom farms differ, the nematode species and their associated bacteria will be different and may have different effects on mushroom cropping.

Bacteria associated with *C. elegans* contribute to yield losses and quality deterioration (browning) and affect flushing patterns of *A. bisporus*. When mixed in casing, two of the three species of bacteria ; *A. calcoaceticus* var. *anitratus* and *S. liquefaciens* caused significant reduction in the total weight of mushrooms picked. All three species of bacteria significantly reduced the total numbers of mushrooms produced. Furthermore, all the bacteria caused browning of mushrooms and *S. liquefaciens* produced the most pronounced effects on whiteness of mushrooms. Bacteria also showed some effects on normal flushing patterns of *A. bisporus*.

However, the magnitude of yield and quality loss was much greater in the presence of nematodes. *C. elegans* greatly suppressed the natural phenomenon of distinct mushroom flushes in such a way that the mushrooms were produced continuously but in smaller numbers. The effects of *C. elegans* on mushroom flushing patterns were affected by the species of bacteria present. *C. elegans* infestations result in maximum yield loss (33%) when introduced with *A. calcoaceticus* var. *anitratus* and this suggests a kind of synergistic relationship. Both in agar culture and *in-vivo C. elegans* produced its largest populations in the presence of *A. calcoaceticus* var. *anitratus*.

Distortion of mushrooms, however, certainly require the presence of *C. elegans*. None of the bacteria, when added alone, produced such distortion of

mushrooms. But, how such a malformation of mushrooms is produced is not known. It seems likely that the distortion results either from some kind of physical injury by nematodes to mushroom primordia at their early stages of development or is due to the localized accumulation of toxic by-products from the nematodes.

Because of its self fertilization and rapid growth rate, *C. elegans* is ecologically adapted to exploit the rich but temporary resources furnished by high concentrations of bacteria (Dusenbery, 1980). The nematode is capable of producing large populations immediately after its inoculation into casing material (an environment rich in bacteria) and can subsequently compete with mushroom growth. This in depth study of the bionomics and effects on mushrooms of a single nematode species throws open an interesting new area for research. Other species such as *Acrobeloides* spp, *Bursilla labiata* and *Diplogaster maupasi* appear at the present time to be less common than *C. elegans* in the mushroom industry. Similar intensive studies of these rhabditids are needed now that the full impact of *C. elegans* is more fully understood.

Chapter 10

Summary and conclusion

The cultivated mushroom, *Agaricus bisporus* is the most valuable protected crop grown in the UK. In commercial culture throughout the world, mushrooms production is affected by two types nematodes: mycophagous and saprophagous. Although economic losses caused by mycophagous nematodes are well documented the impact of saprophagous nematodes, that usually feed on decaying plant material, is not fully understood in the industry.

Samples received from mushroom growers in the British Isles contained thirteen species (eleven genera) of rhabditoid nematodes. This research has concentrated on the most common of these, *Caenorhabditis elegans*. A simple and new method, based on the use of agar, for mounting nematodes was developed. Several commonly-used killing, fixing and mounting techniques were compared and the optimum procedure for the preparation of parthenogenetic adult female *C. elegans* for taxonomic identification devised. Although all the fixatives caused significant shrinkage and/or distortion, killing and fixing in hot TAF (95°C) produced the least-affected specimens. Subsequent processing of hot TAF-killed and -fixed specimens to lactophenol or glycerol caused further significant shrinkage and/or distortion. Processing of nematodes to glycerol by the slow method affected the fewest parameters and produced the most acceptable specimens. The Baermann funnel technique was calibrated for the extraction of *C. elegans* from casing soil and the effects of sample size, nematode density, water level, and duration of extraction on extraction efficiency were evaluated.

The bacteria associated (both externally and internally) with the Taunton strain of *C. elegans* were isolated and identified. Reproduction of *C. elegans* in monoxenic cultures of all ten species of bacteria in agar cultures was studied at 20°C. Five species of bacteria *Acinetobacter calcoaceticus* var. *anitratus, A. calcoaceticus* var. *lwoffi, Enterobacter cloacae, Pseudomonas*

maltophilia and *Serratia liquefaciens* sustained the growth and reproduction of *C. elegans* for several generations. *Bacillus cereus* and *Pseudomonas* sp. supported growth and reproduction of the nematode, but resulted in smaller populations. *E. amnigenus* and *P. aeruginosa* could support nematode growth and reproduction for the first 2-3 generations; *Bacillus* sp. could support growth but not reproduction. The reproductive capacity of parthenogenetic female *C. elegans* varied with temperature and with bacterial food source. Cubic equations were fitted to the data on nematode fecundity. Temperature optima for reproduction were estimated. Temperature significantly affected nematode generation but bacterial species had little effect.

The possible influence of bacteria on the migratory behaviour of third stage *C. elegans* larvae was studied in agar plates. All thirteen species of bacteria affected the pattern of nematode migration; the extent to which this migratory behaviour was changed depended on the species of bacteria. Some of the bacteria including *A. calcoaceticus* var. *anitratus*, *E. amnigenus*, *E. cloacae*, *P. maltophilia* and *S. liquefaciens* were more attractive than either *E. coli* or *Pseudomonas fluorescens* biovar *reactans*. *B. cereus*, *B. thuringiensis* and *Bacillus* sp. affected nematode migration least. Younger bacterial streaks (24-48h old) were more attractive than the older ones (96-192h old). Bacteria killed *in-situ* by chloroform fumes remained attractive. Simple agar-plate assay methods were developed to investigate the role of diffusible and/or volatile substances produced by bacteria in the attraction of *C. elegans*. The results suggested that volatile substances are probably the main components of the attractants produced by bacteria and that the volatiles are atleast partially able to diffuse through agar.

Bedding's nematode mass-culture technique was evaluated and modified for the mass-production of *C. elegans*. Five species of bacteria were evaluated as food substrates and found to strongly influence nematode yields: mean yields ranging from 7.2×10^6 - 25.7×10^6 nematodes (eggs, larvae and adults) per 500 ml flask were obtained from 8 pre-adult female nematodes in one week at 22°C. *C. elegans* produced the greatest number of progeny when cultured with *A. calcoaceticus* var. *anitratus* and the lowest number with *S. liquefaciens*. The structure of *C. elegans* populations varied with the species of bacteria used as

food; seven days after inoculation the mean frequency of second stage juveniles (J2) ranged from 40.8-52% and that of J3 larvae from 31.8-45.5%.

The effects of monoxenically mass-produced *C. elegans* (with *A. calcoaceticus* var. *anitratus*) on the cropping of *A. bisporus* (strain U3) were studied in mushroom growth chambers. *C. elegans* did not multiply in well-prepared, pasteurized, spawned compost, but it reproduced rapidly in casing material. An initial inoculum of 10^6 nematodes/crate of compost (7.5kg) applied to the casing, caused a significant reduction in mushroom yield. Losses in total mushroom yields of 11, 20 and 26% were caused by inocula of 10^6, 10^7, and 2×10^7 nematodes/crate, respectively. Yields were negatively correlated with nematode inoculation level and regression equations were derived. The nematode treatments caused fewer mushrooms to be produced; the usual distinctive flushing patterns were absent.

C. elegans caused considerable deterioration in mushroom quality and characteristic distortion of sporophores. The latter were mis-shapen, notched and had brown or violet coloured gills. Up to 3.8, 6.7 and 10.8% of the total weight, and 3.5, 5.4 and 8% of the total numbers of mushrooms were distorted at the three highest nematode inocula tested. Weights and numbers of distorted mushrooms were positively correlated with the initial nematode population. *C. elegans* commonly colonised sporophores.

The effects of bacteria isolated from *C. elegans* on the mycelial growth of *A. bisporus* (strain C43) were studied on malt extract agar medium. Three species of bacteria: *E. amnigenus, E. cloacae* and *S. liquefaciens* caused more than 50% inhibition in mycelial growth. The three inhibitory bacteria caused similar growth inhibition in three strains of *A. bisporus* (C43, C54 and U3) on both malt extract and compost malt media. *S. liquefaciens* produced the cell wall-degrading enzyme chitinase on chitin agar and *E. amnigenus* and *E. cloacae* produced volatile fungal inhibitors.

The effects of three bacteria (either with or without *C. elegans*), *A. calcoaceticus* var. *anitratus* (the isolate that supported the most vigorous reproduction of *C. elegans*), *E. cloacae* and *S. liquefaciens* (both of which inhibited mycelial growth of *A. bisporus*) on the yield, quality and flushing patterns of *A. bisporus* were studied. When inoculated alone (at casing), all three

species of bacteria significantly reduced the total numbers of mushrooms produced. Two of the three bacteria: *A. calcoaceticus* var. *anitratus* and *S. liquefaciens* also caused significant reductions in the total weight of mushrooms of 10.8 and 6.7% respectively. However, in the presence of *C. elegans*, yield losses were much greater (24.4 and 31.3% with or without bacteria, respectively).

C. elegans almost completely suppressed the natural phenomenon of distinct mushroom flushes whereas the effects of bacteria were much less pronounced. When compared with untreated or bacteria alone treatments, *C. elegans*-treated crates produced fewer, but larger, mushrooms especially during flushes 2, 3 and 4. Bacteria contributed to the browning of mushrooms (i.e reductions in the relative whiteness of sporophores) and the most significant effect was produced by *S. liquefaciens*. However, the presence of nematodes resulted in a more uniform browning of mushrooms. Typical morphological distortion was only observed in the *C. elegans* treatments.

The effects of *C. elegans* on the spread of the bacterial blotch pathogen, *Pseudomonas tolaasii*, were studied in mushroom growth chambers. *C. elegans* significantly reduced the intensity of blotch on sporophores. Repeated isolations of the bacterial flora from the gut of *C. elegans* that had recovered from mushroom sporophores during cropping, revealed the presence of *Pseudomonas fluorescens* biovar *reactans*. All the isolates of *P. fluorescens* biovar *reactans* isolated from nematodes were antagonists of *P. tolaasii*.

C. elegans produced much larger populations in monoxenic cultures with *P. fluorescens* biovar *reactans* than with *P. tolaasii*. It is suggested that as *C. elegans* selects *P. fluorescens* biovars *reactans* rather than *P. tolaasii* as a food substrate it probably spreads the antagonist in the mushroom crop and may contribute to the control of bacterial blotch.

Chapter 11

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Appendices

Appendix 2.1 List of saprobic nematodes infesting cultivated mushrooms.

Nematode species	*Reference*
Rhabditoidea	
Ablechroiulus gongyloides (Reiter,1928) Andrassy,1966 = *Rhabditis gongyloides* Reiter,1928	Reiter (1928)
Acrobeles ciliatus (Linstow,1877) Thorne,1925	Choleva (1966); Farkas (1972); Kermarrec (1973)
Acrobeloides apiculatus (Thorne,1925) Thorne,1937 = *Acrobeles apiculatus* Thorne,1925	Kaufman *et al.* (1983)
Acrobeloides buetschlii (de Man,1884) Steiner & Buhrer,1933 = *Cephalobus buetschlii* de Man,1884 = *C. (Acrobeles) buetschlii* de Man,1884 (de Man,1921)	Hesling (1966); Farkas (1972); Kaufman *et al.* (1983)
Acrobeloides enoplus Steiner,1938	Farkas (1972)
Acrobeloides nanus (de Man,1880) Anderson,1968 = *Cephalobus nanus* de Man,1880	Choleva (1966); Farkas (1972)
Acrobeloides sp.*	Juhl (1966)*; Gerrits (1973); Ingratta & Olthof (1978)

*species implicated as pathogenic to mushrooms

Aspidonema ruchmi (Sachs,1949) Goodey, 1963 = *Bunonema ruchmi* Sachs,1949	Sachs (1949)
Aspidonema stammeri (Sachs,1949) Andrassy,1958 = *Bunonema (Aspidonema) stammeri* Sachs,1949	Sachs (1949)
Bunonema multipapillatum Stefanski,1914	Farkas (1972)
Bunonema richtersi Jagerskiold,1905	Choleva (1966); Kermarrec (1973)
Bursilla monhystera (Butschli,1873) Andrassy,1976 = *Rhabditis (Mesorhabditis) monhystera* Butschli,1873 Osche,1952	Choleva (1968); Farkas (1972)
Caenorhabditis elegans (Maupas,1899) Dougherty,1953* = *Rhabditis elegans* Maupas,1899	Choleva (1966); Farkas (1972); Cayrol (1978); Kaufman *et al.* (1983)*
Cephaloboides musicola (Rahm,1928) Masssey,1974 = *Rhabditis (Cephaloboides) musicola* Rahm,1928	Kermarrec (1973)
Cephaloboides pseudoxycera (Goodey,1929) Andrassy,1983 = *Rhabditis (Choriorhabditis) pseudoxycera* Goodey,1929 (Osche,1952)	Choleva (1966)
Cephaloboides sp.*	Ross & Burden (1981)*
Cephalobus emarginatus de Man,1880	Cayrol (1981)
Cephalobus parvus Thorne,1937	Cayrol (1978)
Cephalobus persegnis Bastian,1865	Choleva (1966); Farkas (1972); Chongti (1982)

Cervidellus devimucronatus Sumenkova,1964	Sumenkova (1964)
Chiloplacus demani (Thorne,1925) Thorne,1937 = *Acrobeles demani* Thorne,1925 = *Cephalobus bisexualis* de Man,1928	Farkas (1972)
Chiloplacus symmetricus (Thorne,1925) Thorne,1937 = *Acrobeles (Acrobeloides) symmetricus* Thorne,1925 = *Cephalobus symmetricus* (Thorne,1925) Steiner,1935	Farkas (1972)
Coarctadera coarctata (Leuckart,1891) Andrassy,1976 = *Rhabditis (Pelodera) coarctata* Leuckart,1891 (Sudhaus,1976)	Farkas (1972)
Coarctadera cylindrica (Cobb,1898) Andrassy,1983* = *Rhabditis (Pelodera) cylindrica* Cobb,1898 (Sudhaus,1976) = *Pelodera (Cylindridera) cylindrica* (Cobb,1898) Dougherty,1953	van Haut (1956)*; Hesling (1966); Choleva (1966,68); Farkas (1972); Kermarrec (1973)
Coarctadera icosiensis (Maupas,1916) Andrassy,1983 = *Rhabditis (Pelodera) icosiensis* Maupas,1916 (Sudhaus,1976) = *Pelodera (Cylindridera) icosiensis* (Maupas,1916) Dougherty,1955	Farkas (1972)
Coarctadera serrata (Korner in Osche,1952) Andrassy,1983 = *Rhabditis (Pelodera) serrata* Korner in Osche,1952 (Sudhaus,1976)	Farkas (1972)

Cruznema tripartitum (Linstow,1906) Zullini,1982* = *Rhabditis tripartita* Linstow,1906 = *Rhabditis lambdiensis* Maupas,1919 = *Pelodera (Cruznema) lambdiensis* (Maupas,1919) Dougherty,1953 = *Rhabditis monohysteroides* Skwarra,1921 = *Pelodera (Cruznema) monohysteroides* (Skwarra,1921) Dougherty,1955 = *Rhabditis (Mesorhabditis) cryptocercoides* (Wollenweber,1921) Dougherty,1955 = *Cruznema cruznema* Artigas,1927 = *Epimenides extricatus* Gutierrez,1949 = *Pelodera (Cruznema) melisi* Marinari,1957 (Meyl,1961)	Steiner (1933)*; Haseman & Ezell (1934); Choleva (1966); Juhl (1966); Farkas (1972); Kermarrec (1973); Cayrol & Quiles (1983); Kaufman *et al.* (1983)
Curviditis curvicaudata (Schneider,1866) Andrassy,1983* = *Leptodera curvicaudata* Schneider,1866 = *Rhabditis (Choriorhabditis) curvicaudata* (Schneider,1866) Linstow,1878 (Osche,1952) = *Cephaloboides curvicaudata* (Schneider,1866) Zullini,1982	van Haut (1956)*
Cuticularia oxycera (de Man,1895) Andrassy,1983* = Rhabditis brevispina (Claus,1862) Butschli,1873	van Haut (1956)*; Kaufman *et al.* (1983)
Diploscapter coronata (Cobb,1893) Cobb,1913	Choleva (1966); Farkas (1972); Kermarrec (1973)

Dolichorhabditis dolichura (Schneider,1866) Andrassy,1983* = *Leptodera dolichura* Schneider,1866 = *Rhabditis (Caenorhabditis) dolichura* (Schneider,1866) Butschli,1876 (Osche,1952) = *Rhabditis (Pellioditis) dolichura* (Schneider,1866) Butschli,1873 (Sudhaus,1976)	Cayrol & B'chir (1972)*; Farkas (1972); Kermarrec (1973); Cayrol *et al.* (1981); Chongti (1982)
Eucephalobus elongatus (de Man,1880) Thorne,1937 = *Cephalobus (Neocephalobus) elongatus* (de Man,1880) Steiner,1936	Choleva (1966)
Eucephalobus longicaudatus (Butschli,1873) Andrassy,1958 = *Cephalobus longicaudatus* (Butschli,1873) Steiner,1936	Farkas (1972)
Eucephalobus oxyuroides (de Man,1876) Steiner,1936 = *Cephalobus oxyuroides* de Man,1876	Choleva (1968); Farkas (1972)
Eucephalobus steineri (Andrassy,1952) Goodey,1963 = *Tricephalobus steineri* (Andrassy,1952) Ruhm,1956	Farkas (1972)
Evaginorhabditis agaricus Sultan, Kaul & Chhabra, 1985	Sultan *et al.* (1985)
Mesorhabditis inarimensis (Meyl,1953) Dougherty,1955 = *Rhabditis inarimensis* Meyl,1953	Farkas (1972)
Mesorhabditis oschei (Korner in Osche,1952) Dougherty,1955	Farkas (1972)

Mesorhabditis spiculigera (Steiner,1976) Dougherty,1953* = *Rhabditis spiculigera* Steiner,1936 = *Pseudorhabditis acuminata* Kreis,1929 = *Trilabiatus acuminatus* (Kreis,1929) Goodey,1963	Hesling (1966)*; Farkas (1972); Kermarrec (1973); Chongti (1982)
Mesorhabditis ultima (Korner in Osche,1952) Dougherty,1955 = *Rhabditis (Mesorhabditis) ultima* Korner in Osche,1952	Farkas (1972)
Mesorhabditis sp.*	Gerrits (1973); Sanderson *et al.* (1981)*
Panagrolaimus fuchsi Ruhm,1956	Farkas (1972)
Panagrolaimus longicaudatus (Sumenkova,1965)	Sumenkova (1965); Farkas (1972)
Panagrolaimus mycophilus (Steiner,1934) Steiner,1935	Cayrol & Quiles (1983)
Panagrolaimus rigidus (Schneider,1866) Thorne,1937	Choleva (1966,68); Farkas (1972); Kermarrec (1973); Chongti (1982); Kaufman *et al.* (1983)
Panagrolaimus subelongatus (Cobb,1914) Thorne,1937 = *Cephabolus subelongatus* Cobb,1914 = *Panagrolaimus superbus* Fuchs,1930	Choleva (1968); Farkas (1972)
Panagrolaimus sp.	Moreton *et al.* (1956); Bukowski (1967); McLeod (1968)

Pellioditis pellio (Schneider,1866) Timm,1960 = *Pelodera pellio* Schneider,1866 = *Rhabditis (Choriorhabditis) pellio* (Schneider,1866) Butschli,1873 (Osche,1952)	Kaufman *et al.* (1983)
Pelodera strongyloides (Schneider,1860) Schneider,1866 = *Rhabditis strongyloides* (Schneider,1860) Linstow,1878	Choleva (1966); Farkas (1972); Kaufman *et al.* (1983)
Pelodera teres Schneider,1866* = *Rhabditis (Pelodera) teres* (Schneider,1866) Butschli,1873 (Sudhaus,1976)	van Haut (1956)*; Choleva (1968); Farkas (1972); Kermarrec (1973); Chongti (1982); Cayrol & Quiles (1983)
Plectus rhizophilus de Man,1880	Farkas (1872)
Rhabdibicauda jagdishi Sultan, Chhabra & Kaul,1985	Sultan *et al.* (1985)
Rhabditella pseudoelongata (Micoletzky,1913) Andrassy,1983* = *Rhabditis (Rhabditella) pseudoelongata* Micoletzky,1913 (Sudhaus,1976) = *Rhabditis (Rhabditella) axei* (Cobbold,1884) Dougherty,1955 = *Rhabditis elongata* (Schneider,1866) Butschli,1876	van Haut (1956)*; Choleva (1966,68); Farkas (1972); Kermarrec (1973)
Rhabditella sp.*	Cayrol & B'chir (1972); McLeod & Nair (1981)*
Rhabditis gracilicauda de Man,1876 = *Rhabditis (Choriorhabditis) gracilicauda* de Man,1876 (Osche,1952)	Farkas (1972)

Rhabditis intermedia de Man,1880 = *Rhabditis (Choriorhabditis) intermedia* de Man,1880 (Osche,1952)	Farkas (1972)
Rhabditis longicaudata Bastian,1865 = *Rhabditis (Choriorhabditis) longicaudata* Bastian,1865 (Osche,1952)	Choleva (1966,68); Farkas (1972); Kaufman *et al.* (1983)
Rhabditis producta (Schneider,1866) Linstow,1878* = *Leptodera producta* Schneider,1866 = *Rhabditis (Choriorhabditis) producta* (Schneider,1866) Linstow,1878 (Osche,1952)	van Haut (1956)*
Rhabditis terricola Dujardin,1845*	Blake & Conroy (1959)*; Farkas (1972); Kaufman *et al.* (1983)
Rhabditis sp.*	Cairns & Thomas (1950)*; Moreton (1953); Blake & Conroy (1959)*; Cayrol (1962); Juhl (1966)*; Ingratta & Olthof (1978); Han *et al.* (1974)*; Thapa *et al.* (1981); Grewal & Grewal (1988)
Rhabditis (Choriorhabditis) sp.*	Ingratta & Olthof (1978); Klingler & Tschierpe (1980)*
Rhabditoides longispina (Reiter,1928) Dougherty,1953 = *Rhabditis (Rhabditoides) longispina* Reiter,1928 (Sudhaus,1976)	Andrassy (1983)

Rhabditoides sp.*	McLeod & Nair (1981)*
Rhitis inermis (Schneider,1866) Andrassy,1983 = *Rhabditis (Rhabditoides) inermis* (Schneider,1866) Linstow,1878 (Sudhaus,1976)	Farkas (1972)
Rhomborhabditis stammeri (Volk,1950) Andrassy,1983 = *Rhabditis (Pelodera) stammeri* Volk,1950 (Sudhaus,1976)	Farkas (1972)
Serronema dentatus (Peasler,1957) Goodey,1963 = *Bunonema (Serronema) dentatus* Peasler,1957	Peasler (1957); Choleva (1966)
Teratorhabditis marianne Farkas,1973	Farkas (1973)
Trilabiatus franzi (Ruhm,1956) Goodey,1963	Farkas (1972)
Trilabiatus lignicolus (Korner,1954) Goodey,1963	Farkas (1972)

Diplogasteroidea

Butlerius butleri Goodey,1929	Farkas (1972)
Butlerius filicaudatus Adam,1930	Kermarrec (1973)
Butlerius spirifer (Skwarra,1921) Zullini & Loof,1980 = *Diplogaster (Paroigolaimella) spirifer* Skwarra,1921 (Weingartner,1955)	Choleva (1966,68)
Diplogaster sp.*	Blake & Conroy (1959)*
Demaniella cibourgensis Steiner,1914	Kermarrec (1973)
Diplogasteritus consobrinus (de Man,1920) Paramonov,1952	Farkas (1972)
Diplogasteritus nudicapitatus (Steiner,1914) Paramonov,1925	Choleva (1966,68)

Diplogastrellus gracilis (Butschli,1876) Paramonov,1952	Choleva (1966); Farkas (1972)
Diplogastrellus monhysteroides Butschli,1876 (Weingartner,1955)	Choleva (1968)
Mesodiplogaster lheritieri (Maupas,1919), Goodey,1963 = *Diplogaster (Pristionchus) lheritieri* Maupas,1919 (Volk,1950)	Kermarrec (1973)
Metadiplogaster inaequidens (Peasler,1946) Meyl,1960	Kermarrec (1973)
Monochoides leptospiculum Weingartner,1955	Farkas (1972)
Monochoides striatus (Butschli,1876) Goodey,1963 = *Eudiplogaster striatus* (Butschli,1876) Paramonov,1952	Choleva (1966,68); Farkas (1972)
Paroigolaimella micrura (Weingartner in Meyl,1956) Andrassy,1958	Choleva (1968)
Tylopharynx foetidus (Butschli,1874) Sachs,1950 = *Aphelenchus (Paraphelenchus) foetidus* Butschli, 1874 (Micoletzky, 1922)	Chongti(1982)

Appendix 4.1 Identification of bacteria isolated from *C. elegans*: biochemical profile. * 1 = *Acinetobacter calcoaceticus* var. *anitratus*; 2 = *A. calcoaceticus* var. *lwoffi*; 3 = *Bacillus cereus*; 4 = *Bacillus* sp.; 5 = *Enterobacter amnigenus*; 6 = *E. cloacae*; 7 = *Pseudomonas aeruginosa*; 8 = *P. maltophilia*; 9 = *Pseudomonas* sp.; 10 = *Serratia liquefaciens.*

	Bacteria*									
Tests	1	2	3	4	5	6	7	8	9	10
Gram reaction	-	-	+	+	-	-	-	-	-	-
Motility	-	-	+	+	+	+	+	-	-	+
Spore formation	-	-	+	+	-	-	-	-	-	-
Rods/Cocci (R/C)	R	R	R	R	R	R	R	R	R	R
Catalase test	+	+	+	+	+	+	+	+	+	+
Oxidation	+	+	+	+	+	+	+	+	+	+
Fermentation of glucose	-	-	-	+	+	+	+	-	-	+
Oxidase test on filter paper	-	-	-	-	-	-	-	-	-	-
Nitrate reductase	-	+	+	+	+	+	-	+	-	+
Nitrite reductase	-	-	-	-	-	-	-	-	-	-
Indole production (tryptophane)	-	-	-	-	-	-	-	-	-	-
Acidification (glucose)	-	-	-	-	n	n	-	-	-	-
Arginine dihydrolase	+	-	+	-	+	+	+	-	-	-
Urease	-	-	-	-	-	-	-	-	-	-
Hydrolysis (B-glycosidase)	-	-	+	+	n	n	+	+	+	n
Hydrolysis (proteases), geletinase	-	-	+	+	-	(+)	+	+	+	-
B-galactosidase (PNPG)	-	-	-	+	n	n	n	+	+	n
Cytochrome oxidase	-	-	-	-	n	n	n	-	-	n
Ortho-nitrophenyl-galactosidase (ONPG)	n	n	n	n	+	+	-	n	n	+
Lysine decarboxylase	n	n	n	n	-	-	-	n	n	+
Ornithine decarboxylase	n	n	n	n	+	+	-	n	n	+
H_2S production	n	n	n	n	-	-	-	n	n	-

contd...

Tryptophane deaminase	n	n	n	n	-	-	-	n	n	-
Acetoin production (Sodium pyruvate, VP)	n	n	n	n	+	-	-	n	n	+
Fluorescence test	-	-	-	-	-	-	-	-	-	-
Growth in 7% NaCl	n	n	+	+	n	n	n	n	n	n
Lecithinase production (Egg Yolk)	-	-	+	+	n	n	-	n	n	+
Assimilation of sugars:										
1. Glucose	+	-	+	+	+	+	+	+	+	+
2. Arabinose	-	-	-	-	+	+	-	-	-	+
3. Mannose	-	-	-	-	n	n	-	+	+	n
4. Mannitol	-	-	-	-	+	+	-	-	+	+
5. N-acetyl glucosamine	-	-	+	+	n	n	+	+	+	n
6. Maltose	-	-	-	+	n	n	-	+	+	n
7. Gluconate	+	-	+	+	n	n	+	-	+	n
8. Caprate	+	+	-	-	n	n	+	-	-	n
9. Adipate	-	-	-	-	n	n	+	-	-	n
10. Malate	+	+	+	+	n	n	+	+	+	n
11. Citrate	+	+	+	-	+	+	+	+	+	+
12. Phenyl-acetate	+	-	-	-	n	n	-	-	+	n
13. Inositol	n	n	n	n	-	-	-	n	n	+
14. Sorbitol	n	n	n	n	-	+	-	n	n	+
15. Rhamnose	n	n	n	n	+	+	-	n	n	-
16. Sucrose	n	n	n	n	-	-	-	n	n	+
17. Melibiose	n	n	n	n	+	+	-	n	n	+
18. Amygdalin	n	n	n	n	+	+	-	n	n	+

+ = positive test; - = negative test; (+) = test positive after 7 days;
n = not determined

Appendix 4.2 Reproduction of *C. elegans* on bacteria at 20°C. Data are mean number of nematodes (eggs, larvae and adults) at days after inoculation. Figures in brackets are square-root transformations.

	Days post-inoculation		
Bacteria	4	8	12
Acinetobacter calcoaceticus var. *anitratus*	358.0 (18.9)	5716.2 (75.6)	14364.6 (119.9)
A. calcoaceticus var. *lwoffi*	334.2 (18.3)	4322.0 (65.7)	10140.4 (100.7)
Bacillus cereus	15.8 (4.0)	113.2 (10.6)	860.0 (29.3)
B. thuringiensis (HD1)	13.0 (3.6)	260.4 (16.1)	394.6 (19.9)
Bacillus sp.	14.4 (3.8)	83.6 (9.1)	34.4 (5.9)
Enterobacter amnigenus	282.4 (16.8)	726.0 (26.9)	723.0 (26.9)
E. cloacae	173.2 (13.2)	770.4 (27.8)	2064.6 (45.4)
Escherichia coli	354.6 (18.8)	9539.6 (97.7)	50548.8 (224.8)
Pseudomonas aeruginosa	75.3 (8.7)	675.0 (26.0)	791.2 (28.1)
P. fluorescens biovar *reactans*	226.2 (15.0)	14524.2 (120.5)	59040.0 (243.0)
P. maltophilia	138.0 (11.8)	210.2 (14.5)	4215.2 (64.9)
P. tolaasii (Pt51)	160.0 (12.7)	214.0 (14.6)	2990.2 (54.7)
Pseudomonas sp.	40.4 (6.4)	174.0 (13.2)	2477.0 (49.8)
Serratia liquefaciens	230.2 (15.2)	556.4 (23.6)	2303.4 (48.0)
LSD ($P<0.05$)	(2.3)	(2.8)	(3.1)

Appendix 5.1 Effects of *C. elegans* on *A. bisporus* (compost treatments): mean air and compost temperatures in the mushroom growth chamber (n = 3)

	Temperature (°C)			Temperature (°C)	
Days post-casing	Air	compost	Days post-casing	Air	compost
1	20.6	22.1	34	16.6	16.9
2	20.4	22.4	35	18.3	18.6
3	20.6	22.5	36	17.1	18.3
4	20.6	22.0	37	15.8	17.2
5	20.6	22.1	38	16.7	16.9
6	22.4	23.9	39	16.9	16.8
7	21.1	23.0	40	17.2	17.2
8	20.4	21.8	41	17.2	17.2
9	19.4	21.2	42	17.6	17.9
10	18.4	19.8	43	18.7	19.1
11	18.5	18.8	44	17.1	18.0
12	18.2	18.8	45	16.5	15.7
13	17.9	18.6	46	16.7	17.0
14	16.8	17.9	47	16.7	17.0
15	16.5	18.0	48	17.0	16.9
16	16.6	18.7	49	16.5	16.9
17	17.1	19.1	50	16.9	16.8
18	16.7	17.8	51	16.8	17.0
19	16.6	17.7	52	17.1	17.2
20	16.6	17.5	53	16.9	17.1
21	16.6	17.7	54	16.9	17.1
22	16.5	17.5	55	16.9	17.0
23	16.6	17.4	56	17.5	17.4
24	16.5	17.1	57	17.8	18.0
25	16.4	17.3	58	18.4	19.0
26	17.0	17.4	59	16.5	16.9

contd...

27	16.7	17.3	60	17.0	17.0
28	16.5	17.2	61	17.0	17.2
29	16.6	17.0	62	17.1	17.1
30	17.0	17.1	63	16.9	17.2
31	16.7	17.5	64	16.8	16.8
32	16.7	16.8	65	16.9	17.1
33	16.7	17.1			

Appendix 5.2 Effects of *C. elegans* on *A. bisporus* (casing treatments - lower inocula): mean air and compost temperatures in the mushroom growth chamber (n = 3)

	Temperature (°C)			Temperature (°C)	
Days post-casing	Air	Compost	Days post-casing	Air	Compost
1	20.4	23.2	34	16.7	17.0
2	20.3	22.8	35	17.2	18.0
3	20.6	22.2	36	16.2	17.8
4	20.4	22.4	37	17.0	17.6
5	20.6	22.1	38	16.8	17.2
6	21.8	22.9	39	16.6	17.3
7	22.0	23.6	40	17.0	17.8
8	20.4	22.0	41	16.8	17.4
9	20.0	21.6	42	17.2	17.6
10	18.4	19.7	43	17.4	17.9
11	18.2	18.9	44	16.6	17.8
12	17.8	18.7	45	16.8	17.3
13	17.2	17.8	46	16.7	17.2
14	16.8	17.6	47	17.0	17.6
15	16.9	18.0	48	16.8	17.5
16	17.2	19.2	49	16.9	17.6
17	16.8	18.0	50	17.2	18.0
18	16.9	17.6	51	16.8	17.4
19	16.6	17.5	52	17.0	17.8
20	16.7	17.6	53	16.8	17.4
21	16.5	17.9	54	16.5	17.3
22	16.4	17.4	55	16.7	17.2
23	16.5	17.8	56	17.2	17.9
24	16.4	17.9	57	16.8	17.5
25	16.2	18.0	58	16.7	17.2

contd...

26	16.8	17.6	59	16.9	17.4
27	16.7	17.8	60	16.8	17.8
28	16.5	17.9	61	16.9	17.4
29	16.7	17.2	62	17.0	17.6
30	17.0	17.4	63	17.0	17.9
31	16.8	17.3	64	17.1	18.0
32	16.5	17.2	65	16.8	17.5
33	17.7	17.0			

Appendix 5.3 Effects of *C. elegans* on *A. bisporus* (casing treatments - higher inocula): mean air and compost temperatures in the mushroom growth chamber (n = 3)

	Temperature (°C)			Temperature (°C)	
Days post-casing	Air	compost	Days post-casing	Air	compost
1	20.6	24.4	28	17.0	17.3
2	21.6	22.5	29	16.9	17.3
3	20.4	21.9	30	16.3	17.1
4	20.4	22.1	31	16.9	17.1
5	20.3	21.7	32	16.8	17.2
6	20.9	22.5	33	16.7	17.1
7	20.8	22.0	34	16.7	17.1
8	20.3	21.5	35	16.6	17.1
9	20.2	21.4	36	15.6	17.1
10	20.4	21.3	37	16.5	16.9
11	17.3	17.1	38	16.3	17.0
12	17.0	17.6	39	16.6	16.8
13	16.6	17.4	40	16.5	17.0
14	16.7	17.4	41	16.6	17.1
15	16.9	17.6	42	17.0	17.2
16	16.8	18.0	43	16.7	17.2
17	16.5	18.1	44	16.3	16.7
18	16.4	17.6	45	17.9	18.3
19	16.5	17.6	46	16.3	16.9
20	16.8	17.6	47	16.6	16.9
21	16.1	17.4	48	16.6	16.8
22	16.3	17.5	49	16.5	16.9
23	17.2	18.9	50	16.4	17.0
24	17.7	18.1	51	16.6	16.8
25	17.2	17.9	52	16.7	17.0
26	17.7	17.9	53	16.4	16.9
27	16.7	17.2	54	16.5	17.2

Appendix 6.1 Effects of bacteria associated with *C. elegans* on the mycelial growth of *A. bisporus* (strain C43). Data are mean colony radius (mm) to or away from the bacterial streak at days post-inoculation (n = 4)

	Days post-inoculation							
	7		11		15		21	
	To	Away	To	Away	To	Away	To	Away
A. calcoaceticus var. *anitratus*	8.0	7.6	14.7	15.3	19.8	22.8	27.7	32.4
A. calcoaceticus var. *lwoffi*	7.7	7.9	13.3	13.0	20.4	19.0	28.2	31.8
B. cereus	9.0	10.0	13.3	14.7	15.8	23.0	19.7	34.4
Bacillus sp.	8.0	8.0	10.2	13.0	12.3	18.4	14.8	28.9
E. amnigenus	5.3	7.7	5.8	14.7	6.7	22.0	7.0	28.8
E. cloacae	3.6	7.5	3.6	15.2	3.6	20.3	3.6	27.6
P. maltophilia	9.0	8.8	13.7	14.0	17.6	22.4	24.0	35.3
Pseudomonas sp.	7.6	8.0	10.8	13.0	14.7	21.0	19.8	33.0
S. liquefaciens	6.3	8.3	8.2	16.7	8.3	23.3	8.3	30.4
Control	9.9	10.0	21.2	19.4	32.4	30.0	39.0	40.0
SED (27 df)	1.4	1.8	1.1	1.5	1.4	1.9	2.0	2.2

Appendix 6.2 Effects of bacteria associated with *C. elegans* on three strains of *A. bisporus* on two different media. Data are mean colony diam. (mm) 21 days after inoculation

		Bacteria			
Media	Strains	*E. amnigenus*	*E. cloacae*	*S. liquefaciens*	Control
MEA	C43	35.7	29.7	38.3	77.0
	C54	38.0	30.7	40.3	78.7
	U3	36.7	27.3	37.0	73.3
CMM	C43	39.3	30.7	39.0	79.3
	C54	39.7	31.0	41.7	80.7
	U3	37.0	27.3	38.0	76.3

Analysis of variance

Tables of means:

	Parameters!				SED 46df	*P*
1.	MEA	CMM				
	45.2	46.7			0.53	**
2.	C43	C54	U3			
	46.1	47.1	44.1		0.65	***
3.	Bac.	Cont.				
	35.4	77.6			0.61	***
4.	Ea	Ec	Sl			
	37.7	29.4	39.1		0.75	***
5.		C43	C54	U3		
	MEA	45.2	46.9	43.6	0.91	NS

contd...

	CMM	47.1	48.3	44.7		

6.		Bac.	Cont.			
	MEA	34.9	76.3	0.86	NS	
	CMM	36.0	78.8			

7.		Bac.	Cont.			
	C43	35.4	78.2	1.05	NS	
	C54	36.9	79.7			
	U3	33.9	74.8			

8.		C43		C54		U3			
		Bac.	Cont.	Bac.	Cont.	Bac.	Cont.		
	MEA	34.6	77.0	36.3	78.7	33.7	73.3	1.49	NS
	CMM	36.3	79.3	37.4	80.7	34.1	76.3		

9.		Ea	Ec	Sl	Cont.		
	MEA	36.8	29.2	38.6	76.3	1.05	NS
	CMM	38.7	29.7	39.6	78.8		

10		Ea	Ec	Sl	Cont.		
	C43	37.5	30.2	38.7	78.2	1.29	NS
	C54	38.8	30.8	41.0	79.7		
	U3	36.8	27.3	37.5	74.8		

! MEA = Malt extract agar; CMM = Compost malt medium; Bac. = Bacteria; Cont. = Control; Ea = *E. amnigenus*; Ec = *E. cloacae*; Sl = *S. liquefaciens*; *P* = significance level; $^{**}P<0.01$; $^{***}P<0.001$; NS = Not significant ($P>0.05$).

Appendix 7.1 Effects of *C. elegans* and/or associated bacteria on *A. bisporus*: mean air and compost temperatures in the mushroom growth chamber (n = 3)

	Temperature (°C)			Temperature (°C)	
Days post-casing	Air	Compost	Days post-casing	Air	Compost
1	20.4	22.6	33	17.5	17.3
2	20.6	22.4	34	17.5	17.3
3	20.3	21.9	35	17.3	17.2
4	19.2	21.3	36	17.3	17.1
5	18.8	20.5	37	17.0	17.2
6	19.6	20.3	38	17.2	17.1
7	19.7	20.0	39	17.8	17.2
8	19.7	20.2	40	17.7	17.1
9	19.5	20.0	41	17.0	17.0
10	19.6	20.0	42	17.2	17.6
11	20.3	20.2	43	17.1	17.1
12	19.6	20.2	44	17.0	17.5
13	20.1	20.6	45	17.2	17.1
14	18.2	18.5	46	17.2	16.9
15	17.4	17.1	47	17.2	17.0
16	16.7	17.4	48	17.4	17.0
17	17.1	17.3	49	17.5	16.9
18	16.7	17.6	50	17.4	16.9
19	16.7	17.3	51	17.2	17.0
20	17.2	17.9	52	17.1	17.0
21	17.1	17.7	53	17.4	16.9
22	16.3	17.3	54	17.3	17.0
23	16.2	16.9	55	17.3	17.1
24	16.5	17.1	56	16.9	17.1
25	16.7	17.4	57	17.2	17.0

contd...

26	17.1	18.2	58	17.2	17.0
27	17.3	17.4	59	18.1	17.1
28	17.0	17.3	60	18.3	16.9
29	17.8	17.3	61	17.5	16.9
30	17.3	17.2	62	17.4	16.8
31	17.1	17.3	63	17.3	16.9
32	17.0	17.2			

Appendix 8.1 Effects of *C. elegans* on the spread of *P. tolaasii*: mean air and compost temperatures in the mushroom growth chamber (n = 3)

	Temperature (°C)			Temperature (°C)	
Days post-casing	Air	Compost	Days post-casing	Air	Compost
1	20.8	22.5	28	16.5	17.2
2	20.7	22.5	29	16.7	17.1
3	20.9	23.2	30	16.6	17.0
4	20.7	22.7	31	16.7	17.0
5	21.0	22.5	32	16.5	17.0
6	20.9	22.1	33	15.5	17.0
7	20.8	22.3	34	16.6	16.8
8	20.7	21.7	35	16.5	16.8
9	20.7	21.8	36	16.5	16.5
10	20.8	21.6	37	16.6	16.6
11	20.9	21.6	38	16.6	16.6
12	20.5	21.4	39	16.3	16.2
13	20.9	21.6	40	15.7	16.3
14	18.3	19.0	41	16.6	16.1
15	17.8	18.7	42	15.1	16.3
16	17.5	17.7	43	16.2	16.1
17	16.7	17.0	44	16.2	16.3
18	16.4	17.1	45	16.4	16.2
19	16.3	17.1	46	16.2	16.4
20	16.5	17.1	47	16.3	16.6
21	16.6	17.1	48	16.8	16.2
22	16.7	17.2	49	16.1	16.3
23	16.6	17.2	50	16.5	16.3
24	16.5	17.4	51	16.3	16.3
25	16.5	17.0	52	16.2	16.4
26	16.5	18.1	53	16.7	16.2
27	16.5	17.3	54	16.4	16.4

Appendix 8.2 Effects of *C. elegans* on the spread of *P. tolaasii*: mean % disease scores on sporophores on each day of mushroom pick

	Nematode inoculum/crate			Control
Day no.	10^5	10^6	10^7	
1	62.11	58.61	65.72	79.57
2	16.37	13.09	15.29	10.99
3	19.95	18.28	11.42	10.49
4	19.11	18.47	27.46	19.64
5	14.47	41.17	36.68	25.92
6	6.91	4.80	7.97	23.29
7	7.54	5.93	2.13	31.44
8	6.67	0.00	0.00	13.58
9	-0.04	3.16	5.76	8.98
10	-1.82	0.00	0.00	3.63
11	-0.53	-1.11	0.52	14.91
12	1.39	1.11	3.35	13.71
13	1.27	0.48	1.61	9.31
14	13.93	7.47	5.25	13.69
15	2.72	1.61	-0.35	11.25
16	4.03	0.40	5.76	16.66

SED (15 df) = 6.557