A STUDY OF *CHALARA ELEGANS* IN MUCK SOILS AND DEVELOPMENT OF BLACK ROOT ROT ON CARROTS IN THE FRASER VALLEY OF BRITISH COLUMBIA

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A STUDY OF CHALARA ELEGANS IN MUCK SOILS AND DEVELOPMENT OF BLACK ROOT ROT ON CARROTS IN THE FRASER VALLEY OF BRITISH COLUMBIA.

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Abstract

Black root rot, caused by the dematiaceous hyphomycete *Chalara elegans* (Nag Raj and Kendrick) [syn. *Thielaviopsis basicola* (Berk. and Br.) Ferr], is an important postharvest disease on fresh market carrots in the Fraser Valley of British Columbia. Black root rot may cause around 10% annual losses to the fresh market carrot industry, which was valued at about $2 million in 1990. To obtain a better understanding of the pathogen and factors influencing disease development, studies were conducted to enumerate populations of *Chalara elegans*, and to determine the distribution and spatial pattern of inoculum in commercial carrot and other vegetable production fields in the Fraser Valley. In addition, the factors influencing survival of *C. elegans* were studied, and the mode of infection and disease development on carrot was studied using light and scanning microscopy.

For quantitative studies, a semiselective medium (TBM-RBA) was developed which contained a range of fungicides and antibiotics. This medium was effective in detecting inoculum levels as low as 20 colony forming units (CFU)/cm^3 of soil. The field sampling studies indicated that *C. elegans* was present in about 80% of the fields that were selected for sampling. The inoculum distribution in one field showed an aggregated spatial pattern within the field. The range of inoculum densities among the fields sampled was <10 to 560 CFU/cm^3. Studies on factors influencing survival of the phialospores (endoconidia) indicated that they could persist in organic soil for more than 20 weeks. Soil planted to onions or flooded and kept at a high temperature (about 25 C) significantly reduced the population of *C. elegans* over time. Survival was not affected by a carrot crop or by flooding at 4 C. The reduction in survival was attributed to an increase of soil microorganisms which were antagonistic to *C. elegans*.

Studies on the mode of infection of carrot roots showed that wounds were required for pathogen growth and it's establishment, and that the periderm of carrot roots was seldom

penetrated directly. Scanning electron microscopy showed rapid development of the pathogen (within 48 hr) and fungal sporulation by 96 hr. The fungus grew both inter- and intra- celluarly.

Dedication

To my parents, who always encouraged me to pursue higher studies.

Acknowledgments

I would like to thank my senior supervisor, Dr. Zamir K. Punja, for his encouragement and support, my supervisory committee for reviewing my thesis and making helpful suggestions, and my lab colleagues Dee Ann Benard, Eric Urquart and others for providing a helpful and friendly work environment and assistance.

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I am also very grateful to the B. C Coast Vegetable Co-operative Association, which provided financial support for my research work, through financial contributions from the Agri-Food Regional Development Subsidiary Agreement Project (ARDSA #11048), from the Natural Science and Engineering Research Council of Canada (NSERC), and from the B.C. Carrot growers research council. I thank Mr. Rick Gilmour and Ms. Mary-Margaret Gaye for providing invaluable information during this project and for their assistance, and to various growers for their cooperation during the field sampling studies.

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CHAPTER 1

INTRODUCTION:

BLACK ROOT ROT ON CARROTS - THE DISEASE:

Black root rot, caused by the dematiaceous hyphomycete *Chalara elegans* (Nag Raj and Kendrick, 1975) [syn. *Thielaviopsis basicola* (Berk. and Br.) Ferr.], is a major postharvest disease on fresh market carrots grown in the Fraser Valley of British Columbia (Fig 1). The pathogen is a soil-borne fungus which has a wide host range, inclusive of cultivated and noncultivated plants (Yarwood, 1981), and it is endemic to organic (muck) soils in the Fraser Valley . In North America, black root rot has been reported to occur on a wide range of plants, including tobacco (Stover, 1950), cotton (King and Presley, 1942), bean (Papavizas and Davey, 1961), pea (Lloyd and Lockwood, 1961), poinsettia (Keller and Shanks, 1955) and holly (Lambe and Willis, 1976). On these plants, the hypocotyls and roots become severely infected under favorable temperature and moisture regimes. Under field conditions, *C. elegans* does not appear to cause any visible disease symptoms on carrot seedlings or on mature carrot roots (Z.Punja, unpublished observations). Apparently, black root rot is also not a major postharvest problem on carrots grown in other parts of the world.

C. elegans survives over a long period of time in soil by producing resistant chlamydospores (Tsao and Bricker, 1966). In culture, two types of spores, which are described as endoconidia (phialospores) and chlamydospores (aleuriospores) are formed (Nag Raj and Kendrick, 1975). Chlamydospores are thick walled, dark brown in color and are produced in chains of 4-6 spores. Phialospores are thin walled, hyaline and are also produced in chains.

Figure 1. Black root rot of carrots, caused by *Chalara elegans*. Typical blackening symptoms of this disease are due to the production of chlamydospores on the root surface.

Wounds on the epidermis of carrots caused by harvesting, washing and especially grading practices, become the infection sites for fungal inoculum (Punja et al., 1992). Both chlamydospores and phialospores are capable of infecting wounded carrots (Punja, 1990). The infections occur only in the upper 4-8 cell layers of the carrot root (see Chapter 5). Within 2-3 days, the carrots develop grey lesions, which expand and blacken due to the spore production on the epidermis. These spores become secondary inoculum and infect other carrots within the polyethylene bags.

The blackening symptoms begin to show 5-7 days after the carrots are packed and sent to the wholesalers and retailers if stored at optimal temperatures (25-27 C) and high moisture (98-100% RH) in the polyethylene bags. In 1989, it was estimated that up to 40% of the carrots were returned to the B.C. Coast Vegetable Co-operative Association during the month of August, when infections were high (Rick Gilmour, Assistant Manager, B.C. Coast Vegetable Co-operative Association, personal communication). This was attributed in part to improper handling practices at the retail end, and resulted in a substantial loss of revenue to the growers.

CARROT HARVESTING AND GRADING:

In the Fraser Valley, fresh market carrots are produced by individual growers on fields ranging from one to 10 ha in size. Harvesting usually takes place during the morning, and the carrots are sent to the B.C Coast Vegetable Co-operative Association in Richmond, B.C., where they are rinsed with water and placed in a large holding bin (Fig 2). The carrots are then washed by roller brushes (scrubber), hand-sorted and sent on conveyor belts through a large hydrocooler, where the carrot surface temperature is brought to 4-6 C for 1-2 min.

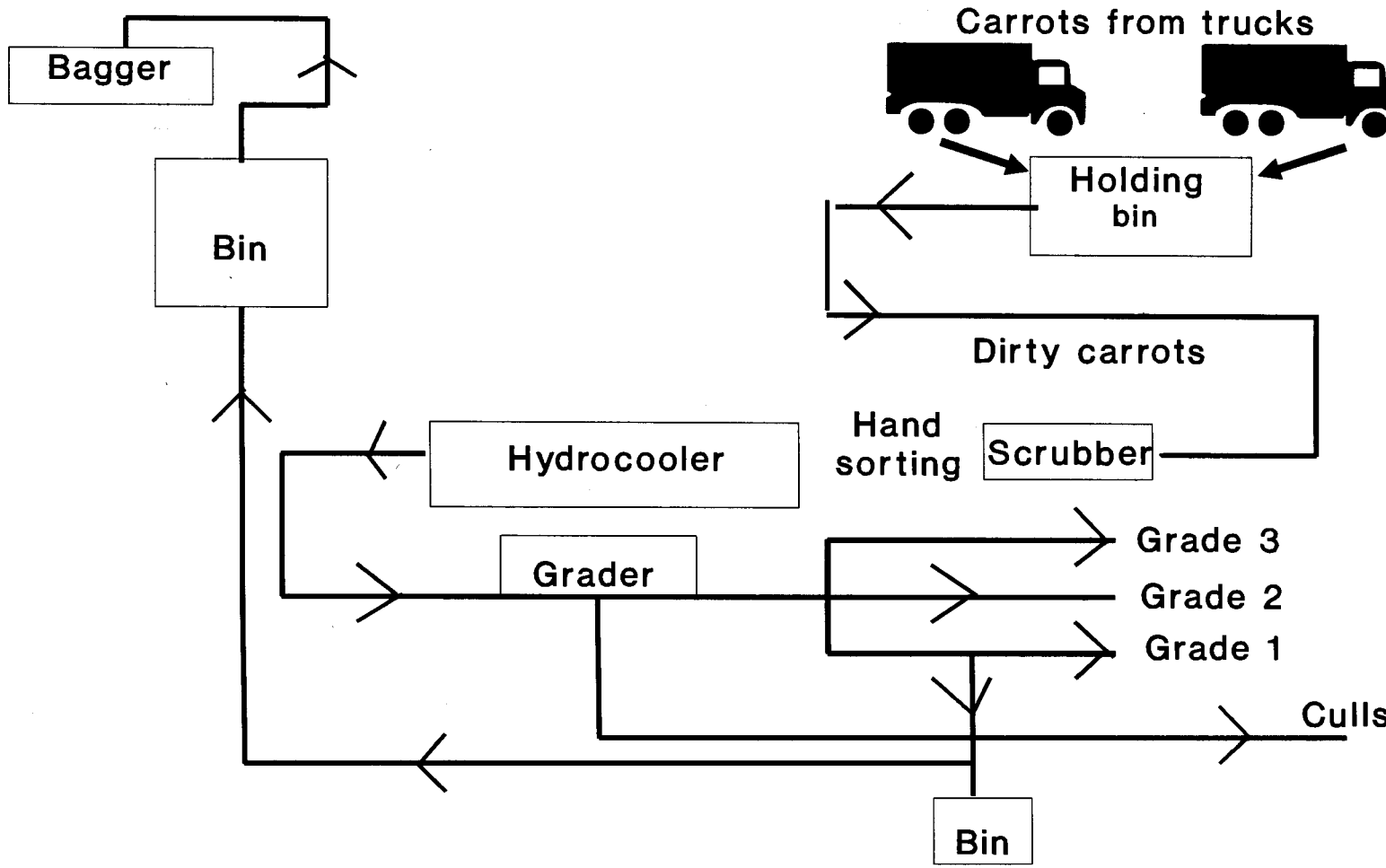


Figure 2. Schematic representation of the processing of carrots at the B.C. Coast Vegetable Co-operative Association in Richmond, B.C., (1991). Arrows indicate direction of movement.

Also, chlorine is maintained at a rate of 80 μg/ml in the hydrocooler. Throughout this process, muck soil can be found in the wash bins and also adhering to the conveyer belts. After hydrocooling, the carrots are conveyed to the grader, where they are sorted according to their diameter. Finally, the carrots are collected and packed into 0.9-1.35 kg polyethylene bags and shipped to wholesalers and retail stores. Bulk carrots in 22.5 kg bags are also produced.

ECONOMIC LOSSES DUE TO BLACK ROOT ROT:

In the lower Fraser Valley, about 350 acres are planted to carrots each year . The commercial carrot production is around 6000 T/year. In 1990, retail sales in the carrot industry were valued at around $2 million. In 1979 and 1982, losses due to black root rot in B.C were as high as $1.2 million. Therefore, a project (D.A.T.E Project No. 135) was initiated in 1984 by the B.C. Ministry of Agriculture and Fisheries to develop possible prevention methods to reduce this disease. It was found that washing carrots with chlorinated water (80 μg/ml concentration of chlorine) and cooling them to 1 C would decrease disease incidence (L. MacDonald and D.J. Ormrod, 1985, B.C. Ministry of Agriculture and Fisheries, Cloverdale, B.C., unpublished).

However, in 1988, losses due to black root rot were estimated to be about $200,000. Studies conducted in 1989-1990 (M.M. Gaye, A.R.D.S.A Project No. 11048,) showed that even after washing with chlorinated water and hydrocooling, 89% of the carrots sampled from 339 loads developed black root rot (M.M. Gaye, 1990, Project Manager, B.C. Coast Vegetable Co-operative Assoc., unpublished). The local carrot industry faces severe market competition, as carrots are imported from the U.S.A. During the growing season (June-November), B.C. carrots achieve

a 65% market share, while California and Washington carrots comprise the rest of the market. When B.C carrots are unavailable, usually from December to June, California imports comprise close to 100% of the B.C market. Black root rot can cause about an average 10% loss to the local fresh market carrot industry. Therefore, between 1988-1990, the estimated revenue loss due to black root rot was $570/acre/year (Rick Gilmour, personal communication). California grown carrots, the major U.S competition, appear to be free from this disease. Therefore, the prevention of black root rot is necessary in order to maintain a representative market share for locally grown carrots.

CONTROL MEASURES FOR BLACK ROOT ROT:

Studies conducted by S. MacDonald, L. MacDonald and D.J. Ormrod (D.A.T.E Project 135, 1984; 1985) showed that washing carrots and hydrocooling and chlorinating immediately after harvest gave the best level of control under laboratory conditions. While these measures reduced disease incidence in those studies, black root rot continues to be a problem. Since muck soil can be found in the hydrocooler, the chlorine can be tied up by the organic matter and carrot plant debris, and the chlorine concentration is never maintained at 80 μg/ml. Also, due to the amount of inoculum brought into the B.C. Vegetable Co-operative during processing, it is difficult to avoid infection of wound sites on the carrots and subsequent disease development. It is clear that more information is needed in several areas in order to obtain a better understanding of disease development and infection by *C. elegans*. These include: the distribution of infested fields, the factors influencing pathogen survival, and disease development on the carrot root.

OBJECTIVES:

Since little information is currently available on black root rot development on fresh market carrots, the objectives of this research project were as follows:

1. To develop a semiselective medium to isolate *Chalara elegans* from muck soils for quantitative studies.
2. To study the distribution and inoculum density of *Chalara elegans* in commercial carrot fields and other vegetable production fields in the lower Fraser Valley of B.C.
3. To study the duration of survival of *Chalara elegans* and the factors influencing survival of phialospores in soil.
4. To determine the mode of infection of carrot roots using scanning and light microscopy.
5. To monitor disease development on carrots at the B.C. Coast Vegetable Co-operative Association.

CHAPTER 2

DEVELOPMENT OF A SEMISELECTIVE MEDIUM FOR ISOLATION AND QUANTIFICATION OF *C.ELEGANS*:

INTRODUCTION:

Chalara elegans can be readily isolated or detected in soil using a modified carrot-disc procedure (Yarwood, 1981). In this assay, freshly cut carrot discs from surface-sterilized roots are pressed onto soil samples collected from the field. After the excess soil is scraped off the discs, they are incubated on moistened filter paper in Petri dishes at room temperature (25 C). If *C. elegans* is present, the typical grey colonies develop within 3-5 days. Although this is a sensitive method to detect the pathogen, it is not a quantitative assay. Another modification of the method of Yarwood's carrot-disc procedure known as "most probable number method" (Tsao and Canetta, 1964) can provide quantitative population data. However, this method requires a large number of replications and, therefore, is time consuming. In addition, it is very difficult to enumerate propagule numbers as discrete colonies using this method (Maduewesi et al., 1976; Specht and Griffin, 1985).

Dilution plating would be the easiest method to quantify propagules of *C.elegans* in natural soil (unsterilized field soil), if a reliable selective medium were available. Papavizas (1964) developed the first medium, VDYA-PCNB, for enumeration of *C. elegans* from natural soil. However, this medium does not give satisfactory results for all soil types (Maduwesi et al., 1976). Two other media, TBM-C (Maduwesi et al., 1976) and TB-CEN (Specht and Griffin, 1985) have been developed for estimating populations of *C. elegans* from natural soils.

However, the mineral soils used in these studies are different from the organic soils found in the Fraser Valley of British Columbia. When all three media were compared in preliminary studies, none gave satisfactory results (S. Chittaranjan, unpublished observations). TBM-C isolated a few *C. elegans* colonies from natural soil, whereas VDYA-PCNB and TB-CEN2 (modified TB-CEN) had no colony forming units (CFU). Therefore, TBM-C was chosen for further modification and subsequent experimental use in this research.

MATERIALS AND METHODS:

MEDIA PREPARATION:

VDYA-PCNB, TBM-C, and modified TB-CEN (TB-CEN2) were prepared for comparison of selectivity as follows:

a) VDYA-PCNB: V-8 juice, 200 ml; CaCO3, 1 g; agar, 20 g; glucose and yeast extract, 2 g of each; oxgall, 1 g; nystatin, 30 mg; streptomycin sulfate, 100 mg; chlorotetracycline HCl, 2 mg; distilled water, 800 ml; (pH 5.2). The antimicrobial agents were prepared in aqueous solutions and added after the medium was autoclaved.

b) TBM-C: Carrot juice (extract from 200 g autoclaved roots made up in 1 liter of water), 970 ml; agar, 20 g; yeast extract, 2 g; pentachloronitrobenzene (PCNB), 1 g; oxgall, 1 g; nystatin, 50 mg; chloramphenicol, 250 mg; K penicillin G, 60 mg; (pH 5.2). The antimicrobial agents were added to 30 ml of distilled water and kept on a shaker for about 1 hour, and the mixture was brought to 50 C in a water bath and added to the autoclaved medium.

c) TB-CEN2: Carrot juice (extract from 100 g blended and filtered carrot in 100 ml of water (unautoclaved); metalaxyl, 80 mg (instead of etridiazol 80 ml); nystatin, 400 mg (250,000 units); streptomycin sulfate, 500 mg, chlorotetracycline HCl, 30 mg;

$CaCO_3$, 1 g; agar, 15 g; distilled water, 900 ml; (pH 5.3). Carrot juice and aqueous solution of antibiotics were added to the autoclaved medium.

INOCULUM PREPARATION:

C. elegans was isolated from carrots with symptoms of black root rot by surface sterilizing infected carrot pieces with 10% bleach [0.625% sodium hypochlorite (NaOCl)], washing them in sterile distilled water, and incubating on V-8 agar (V-8 juice, 150 ml; agar, 15 g; ampicillin, 100 mg; distilled water, 850 ml) at 25 C. Colonies of *C. elegans* were grown on V-8 agar for two weeks at room temperature (25 C). Phialospores were harvested by flooding the colonies of *C. elegans* grown on this medium with 100-200 ml of sterile distilled water and adjusting the spore concentration with a haemocytometer.

COMPARISON OF MEDIA:

a) Dilution plating from pure spore suspensions: Spore suspensions were prepared according to the above stated procedure, and up to 10^{-3} dilutions were made in distilled water blanks. A small volume (0.5 ml) of the suspension from each dilution was plated onto all three media. The dishes were incubated at room temperature for 5-12 days. Observations were made on the number of colonies developing and the size of individual colonies.

b) Soil dilution from artificially inoculated soil: Soil samples originating from commercial carrot fields were screened using Yarwood's carrot root disc method (see Chapter 3). Soil from one field which did not have any detectable *C.elegans* propagules was selected for this study. To 28 cm^3 of soil, two ml of a phialospore suspension (1.32×10^6 spores/ml) was added and the soil was thoroughly mixed.

Since the initial inoculum level used was high (9.5 x 10^5 spores/cm^3), one cm^3 of soil was placed in 9 ml of sterile water dilution blanks. Soil suspensions were then diluted further (up to 10^{-4}) and 0.5 ml of the suspension from each dilution was plated onto each of the three media. The number of colonies developing was rated after 7-12 days of incubation at room temperature.

c) Soil dilution from natural soil: Four commercial carrot fields located in the Cloverdale area of the Fraser Valley of B.C. that had a history of yielding carrots with black root rot, were chosen. Each field was randomly sampled by taking 50 g of soil from 12-15 different locations and bulking them to make a composite sample/field. Samples were brought back to the laboratory and stored at 4 C for 1-10 days prior to use. For dilution plating, a volume of 5 cm^3 of soil was transferred into 45 ml of sterile distilled water (10^{-1}). The suspension was mixed thoroughly, and further dilutions (up to 10^{-3}) were made. From each dilution, 0.5 ml of soil suspension was evenly plated onto each medium. The plates were incubated at room temperature (25 C) for 7-14 days.

MODIFIED TBM-RBA MEDIA:

Repeated evaluations of all of the three previously described media showed that TBM-C had fewer fungal contaminants and, therefore, had higher recovery of colonies of *C. elegans*. However, *Pythium*, *Fusarium*, *Penicillium*, *Aspergillus*, *Trichoderma*, *Mucor* and various bacteria were major contaminants in all of the three media. Recovery of *C. elegans* was further enhanced by evaluating various modifications of the TBM-C medium. Carrot roots (100, 200, 300 or 400 g/liter) were either blended and filtrated through cheesecloth or cooked and mashed and incorporated into the media before autoclaving.

In addition, different fungicides (per liter) were added to reduce the growth of contaminants. The fungicides tested were metalaxyl (at 10, 20, 30, 35 or 45 mg a.i.), dichloran (at 1, 2, 3, 4, 5 or 6 mg a.i.), benomyl (at 2 mg), rose bengal (at 25 mg) and ampicillin (at 100 mg). Vitamin E acetate at 0.5% had been reported to induce chlamydospore germination (Papavizas and Adams, 1969). Vitamin E acetate was added (at 0.5%, w/v) to the media, when natural field soil was used. The pH of the media was also adjusted (to 4, 4.5, 5, 5.5 or 6) using 1N H_2SO_4 or KOH.

After determining the optimum level of all the ingredients for the new TBM-RBA medium through several comparative studies (data not presented), the new medium (TBM-RBA) was compared with the carrot baiting assay and the other three media that were used in the preliminary studies. For this purpose, pure spore suspension, artificially inoculated soil, and naturally infested soil were used in the experiments as described above .

COMPARING TBM-RBA WITH CARROT BAITING:

A spore suspension (17 x 10^6 phialospores/ml) of *C. elegans* was made from a two-week-old culture grown on V-8 agar at 25 C. The spore suspension was diluted to 170 spores/ml. The moisture content of the field soil was determined by drying several 20 g soil samples at 80 C for two days and re-weighing them. A 25 cm^3 (15.8 g wet soil; 6.69 g dry weight;) sample of moist soil (57.6% moisture content) was inoculated with 3 ml of the diluted spore suspension (510 spores/25 cm^3). The minimum number of spores that can be detected by TBM-RBA without a replication plate from naturally infested soil is 20 CFU/cm^3 or 74 spores/g oven dry weight, when the soil moisture content is 57.6 % (personal observation). Therefore in this study, each soil sample had around 76 spores/g dry weight.

From this inoculated soil, 5 cm^3 was transferred into 45 ml of sterile distilled water. In addition, a 10^{-2} dilution was made. From both dilutions, 0.5 ml of soil suspension was plated onto 8 plates of TBM-RBA media as well as onto carrot root discs (2 discs/ml). The experiment was repeated once.

RESULTS:

COMPARISON OF MEDIA:

a) Dilution plating from pure spore suspension: Colonies of *C. elegans* developed within 5-6 days on TBM-C and on VDYA-PCNB media, whereas the rate of colony growth was slower on TB-CEN2 medium. Therefore, during the evaluation of media after 7 days, colonies on TBM-C and VDYA-PCNB were much darker and easier to identify. The colonies formed on VDYA-PCNB were much larger (8-11 mm diameter) than on TBM-C (5-7 mm) and were comparable to those on TB-CEN2 (7 -10 mm). The colonies on TBM-C were defined, distinct, and were easier to count. Recovery of *C. elegans* was 90- 95% on TBM-C and VDYA-PCNB, whereas the recovery rate on TB-CEN2 was lower. Also, some fungal contamination was observed on TB-CEN2 media.

b) Recovery of *C. elegans* from artificially inoculated soil: TB-CEN2, which contained unautoclaved carrot juice, was highly contaminated by microorganisms present in the organic soil type used throughout the experiment. The major contaminants were various bacteria, *Mucor* and to a lesser extent, *Fusarium*, *Trichoderma* and *Pythium*. Recovery on TBM-C and VDYA-PCNB were comparable at lower dilutions (10^{-2}, 10^{-3}) but recovery was highest on TBM-C at a 10^{-4} dilution, when a higher initial concentration of spore suspension was used to inoculate the soil.

Penicillium was the major contaminant on VDYA-PCNB and *Fusarium* was the major contaminant on TBM-C. However, other contaminants such as *Pythium* and *Trichoderma* were also found on these media.

c) Recovery from naturally infested soil: No *C. elegans* colonies were isolated on VDYA-PCNB or TB-CEN2 media when naturally infested soil (presence of *C. elegans* propagules was confirmed with carrot disc assay) was used in this study. However, colonies of *C. elegans* developed on TBM-C media twice (from two fields) during these experiments.

MODIFIED TBM-RBA MEDIA:

The highest recovery of *C. elegans* was observed in this study with the ingredients and rates indicated in Table 1. Vitamin E acetate did not enhance the recovery of *C. elegans* when added to this medium. The addition of benomyl at 2 mg a.i/L eliminated the recovery of *C. elegans*.

A comparison of TBM-RBA, TBM-C, VDYA-PCNB and TB-CEN2 media for recovery of *C. elegans* is shown in Tables 2-4 and in Figs. 3 and 4. In Table 2, it can be seen that recovery from a pure spore suspension was 87.8% on TBM-RBA. These results are also shown in Fig. 3. In Table 3, recovery from artificially inoculated soil was 100% on TBM-RBA. These results are also shown in Fig. 4. In these experiments, a higher initial concentration of spore suspension was used and therefore definite colonies appeared only at 10^{-4} dilution, when both pure spore suspension and inoculated soil was used. TB-CEN2 had the least number of colonies developing in these experiments. The lowest level of contamination was observed on TBM-RBA medium by comparison. *C. elegans* was recovered from naturally infested field soil only on TBM-RBA and TBM-C media (Table 4). TBM-RBA was the only medium on which *C. elegans* was recovered from samples 2, 3, and 4.

Table 1: Ingredients of TBM-RBA per liter. This medium was developed in the course of this research.

Ingrediets	Amount (a.i.)/liter
Agar (Anacheim brand)	20 g
Carrot juice*	500 ml
Yeast extract	2 g
Oxgall	1 g
PCNB	1 g
(Pentachloronitrobenzene)	
Nyatatin	60 mg
Chloramphenicol	250 mg
Ridomil (metalaxyl)	40 mg
Botran	2 mg
Ampicillin	200 mg
Penicillin	60 mg
Rose bengal (optional)**	25 mg
pH 5.0	

* Extract from 200 g carrot roots after blending and filtering with 350 ml of distilled water.

** If rose bengal is added, the medium is known as TBM-2RBA.

Table 2: Growth of *Chalara elegans* colonies from pure spore suspensions, when plated onto each of four different media.

Medium[a]	Colonies/plate at 10^{-4} dilution	CFU/ml [b]	% recovery [c]
TB-CEN2	43	8.6×10^5	65
VDYA-PCNB	44	8.8×10^5	67
TBM-C	56	1.12×10^6	84.8
TBM-RBA	58	1.16×10^6	87.8

a TB-CEN: Specht, and Griffin, (1985);

VDYA-PCNB: Papavizas, (1964);

TBM-C: Maduwesi, Sneh, and Lockwood, (1976).

b Initial concentration was 1.32×10^6.

c Rated after 7 days of incubation at 25 C.

Table 3: Recovery of *Chalara elegans* colonies from artificially inoculated field soil when plated onto four different media.

Medium	colonies/plate at 10^{-3}	CFU/cm^3 [a]	% recovery [b]
TB-CEN2	-	-	-
VDYA-PCNB	42	8.4×10^4	93
TBM-C	44	8.8×10^4	97
TBM-RBA	50	1×10^5	100

a Initial concentration was 0.9×10^5.

b Rated after 7 days of incubation at 25 C.

Table 4. Recovery of *Chalara elegans* colonies (CFU/cm^3) from four naturally infested field soils located in the Cloverdale area of the Fraser Valley of B.C.

Medium	CFU/cm^3 of soil *			
	Sample 1	Sample 2	Sample 3	Sample 4
TB-CEN2	-	-	-	-
VDYA-PCNB	-	-	-	-
TBM-C	10	-	-	-
TBM-RBA	60	60	20	40

* Average of 2 plates/ sample.

Figure 3. Comparison of TBM-RBA, TBM-C, VDYA-PCNB and TB-CEN2 media, when pure spore suspension of *Chalara elegans* was plated onto each medium. *Chalara elegans* colonies are grey. Photograph was taken after 8 days.

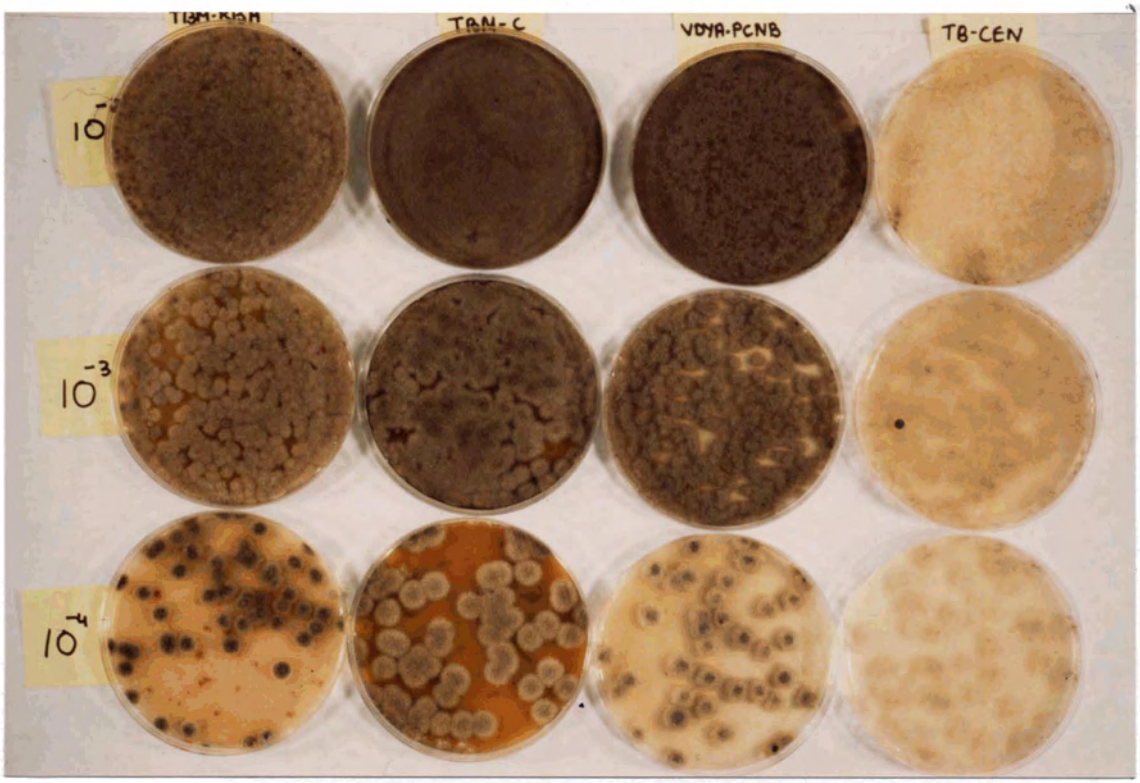
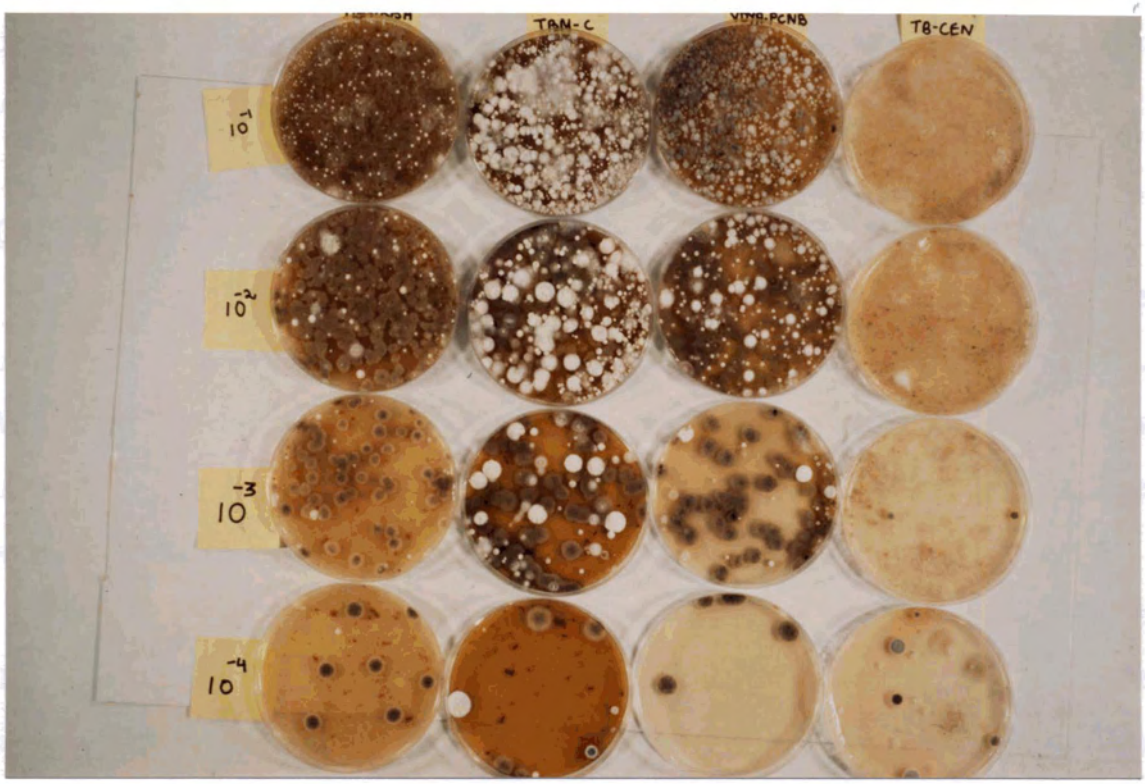

TAN-C
VDYA-PCNB
TB-CEN
10^{-3}
10^{-4}

Figure 4. Comparison of TBM-RBA, TBM-C, VDYA-PCNB, and TB-CEN2 media, when soil that was artificially inoculated with *Chalara elegans* was plated. *Chalara elegans* colonies are grey and the *Fusarium* colonies are white; TB-CEN2 was covered with *Pythium*. Note the low level of fungal contamination on TBM-RBA at 10^{-1}


TAN-C
VDNB-PCNB
TB-CEN
10^-1
10^-2
10^-3
10^-4

COMPARING TBM-RBA WITH CARROT BAITING:

Carrot discs were a more sensitive assay for detecting *C. elegans* than TBM-RBA (Table 5). The minimum number of spores detected by a single TBM-RBA Petri plate would be 20 CFU/cm^3 of moist soil (or 74 CFU/g oven dry soil at 57.6% moisture content). Therefore, to detect 1 CFU/cm^3 from a soil sample, at least 20 plates should be used. It should be noted that on carrot root discs, the population level was over estimated.

DISCUSSION:

The TBM-RBA medium developed in this study was better than all of the other media reported by previous investigators for recovery of *C. elegans* from soil. When natural soil was used, recovery of *C. elegans* on the other media was very low or not possible due to contamination by other fungi. This was probably due to the high microbial activity seen in the organic (muck) soils compared to mineral soils.

Even though peeled and surface-sterilized carrots were used to prepare the TB-CEN2 medium, some contamination was observed even when pure spore suspensions were plated. The low recovery rate on TB-CEN2 in this assay could also be due to the use of organic soil, which has very high populations of resident microorganisms. Another explanation could be because etridiazol, which eliminates Oomycetes, was unavailable , Ridomil (metalaxyl) was used for the same purpose. Etridiazol could have eliminated contaminants in TB-CEN2 media as used by Specht and Griffin, whereas Ridomil may not have. However, Ridomil successfully eliminated *Pythium* and other Oomycetes in the TBM-RBA media even at low concentrations (35 mg/L). Because carrots have natural infections by *C. elegans*, using unautoclaved carrot juice is not recommended in any medium.

Table 5. Comparison of TBM-RBA medium with carrot baiting assay, when artificially inoculated soil containing 20 CFU/ cm^3 was plated.

Medium	colonies* at 10^{-1}	CFU/cm^3	% recovery
TBM-RBA	0.87	17.4	87
Carrot baiting	1.6	32	160

* Average number of colonies of *C. elegans* from 8 replicate plates of TBM-RBA, or from 8 carrot discs.

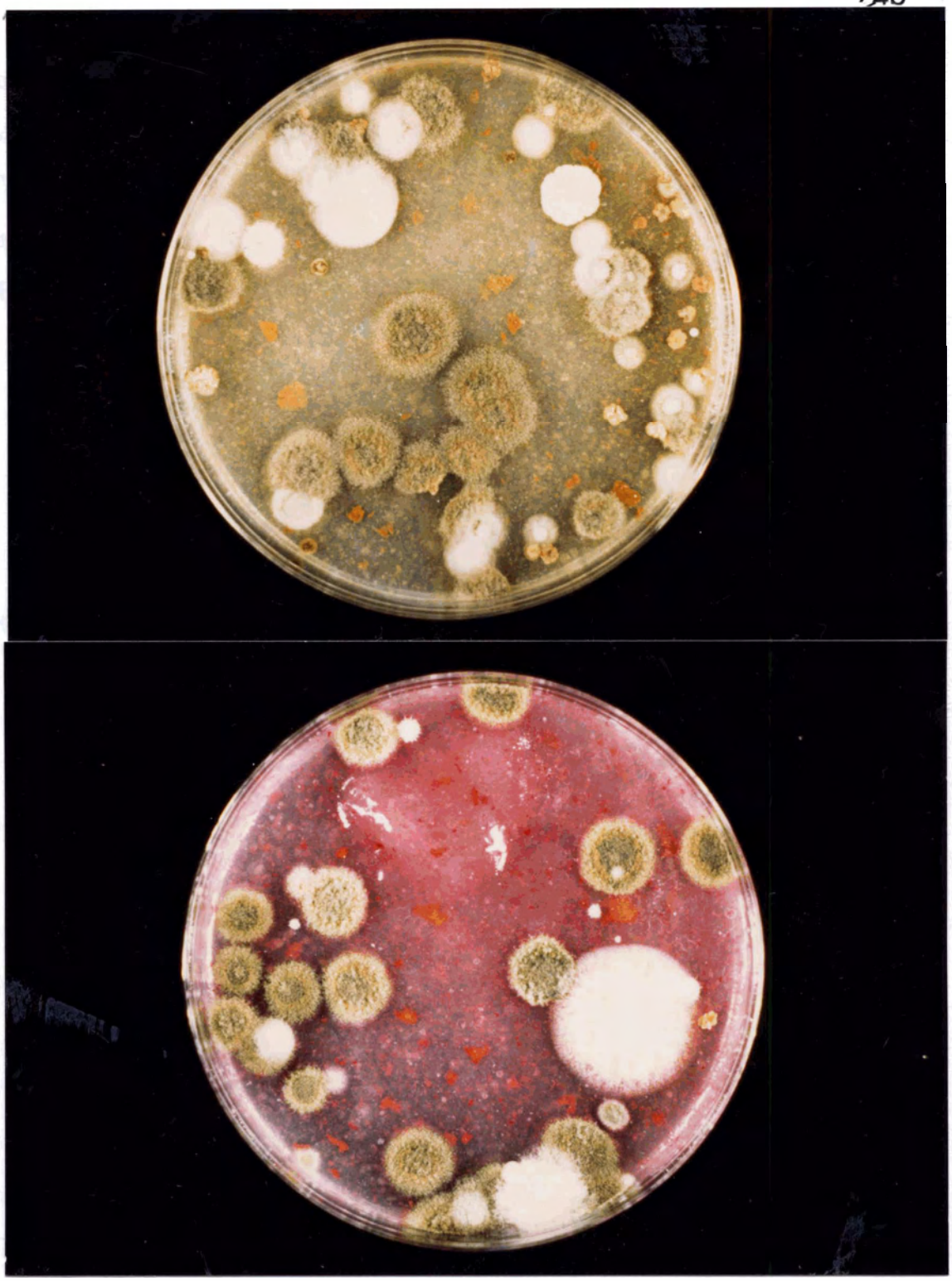
Rapid growth of contaminants as well as larger colonies of *C. elegans* on VDYA-PCNB medium could be attributed to the presence of V-8 juice and glucose in this medium. Addition of carrot juice (which is lower in nutrients) instead of V-8 juice may be the reason why TBM-C achieved recovery of some colonies of *C.elegans* from natural soil. The *C. elegans* colonies on TBM-RBA were very dark and distinct and therefore easy to identify and enumerate. The colonies that originated from artificially infested soil (phialospores) were much larger than the colonies developing from natural soil, which probably originated from chlamydospores. This could be due to the germination rate of phialospores being faster than the germination rate of chlamydospores. Therefore, phialospores may have germinated and established larger colonies on this medium before the other contaminants could grow, whereas the colonies from chlamydospores could have originated at the same time or later than the contaminants, giving rise to smaller colonies.

Eliminating *Fusarium* in TBM-RBA is still challenging. When benomyl was added to eliminate *Fusarium* at a concentration of 2 mg/liter, it also eliminated *C. elegans*. Other fungicides, such as thiram or mancozeb, should be evaluated for this purpose.

The best and consistent recovery of *C. elegans* was obtained when 5 cm^3 of soil was diluted in 45 ml of water and 0.5 ml was plated onto the TBM-RBA medium, especially when naturally infested soil was used. The use of a large volume of soil, instead of 1 cm^3 in 9 ml may have enhanced the probability of recovery.

If the bacterial contamination on the medium is very high, rose bengal can be added (25 mg/L) to enhance the selectivity of the medium (Fig 5). Colonies of *C. elegans* are more visible on the medium with rose bengal.

Figure 5. Comparison of TBM-RBA (new semiselective medium developed for *Chalara elegans* in this study) and TBM-2RBA media (same medium with rose bengal to reduce bacterial contamination). The *Chalara elegans* colonies are grey and are darker and more visible on the TBM-2RBA medium.



This medium should be incubated in the dark since rose bengal is sensitive to light (Martin, 1950). Although TBM-RBA was less sensitive than the carrot baiting assay (Table 5), it provided consistently better results for the quantitative estimation of *C. elegans* from muck soil, which has high levels of different soil microflora. The TBM-RBA medium was therefore used in further quantitative studies (see Chapter 3).

CHAPTER 3

DISTRIBUTION, INOCULUM DENSITY AND SPATIAL PATTERN OF *C. ELEGANS* IN SOILS OF THE LOWER FRASER VALLEY OF BRITISH COLUMBIA:

INTRODUCTION:

Determination of the distribution of a plant pathogen among commercial fields, the inoculum density of the pathogen, and the spatial pattern of inoculum are essential to the understanding of disease problems. Such studies can be useful for determining whether specific control measures are applicable, or whether measures such as pathogen avoidance (Punja et al., 1985) are advisable.

Campbell and Noe (1985) stated: " The spatial pattern of a specific organism or set of organisms provides an opportunity to characterize organismal attributes from a static sample in time or from a series of samples over time. The characterization of a spatial pattern thus provides a tool for the development of plausible biological and environmental hypotheses to account for the association among organisms."

The distribution of *Chalara elegans* in nature has been intensively studied by Yarwood (1981) using a carrot root disc assay. The fungus was found to be as abundant in some virgin areas as in cultivated soils. It can be found associated with over 148 plant species in different regions of the world (Yarwood, 1981).

Previous population and/or inoculum density studies of *C. elegans* have been mainly conducted in tobacco fields in different areas of the U.S.A. and in Ontario, Canada (Rittenhouse and Griffin, 1985; Specht et al., 1987; Anderson and Welacky, 1988; Meyer and Shew, 1991).

In some of these studies, inoculum level was shown to positively correlate with disease development on crops such as tobacco, bean, cotton, and pea (Lucas, 1955; Anderson and Welacky, 1988; Specht and Griffin, 1988; Meyer et al., 1989). However, other factors such as soil pH, soil temperature, soil chemistry, host resistance, and cultural practices (Lloyd and Lockwood, 1963; Specht et al., 1987; Anderson and Welacky, 1988; Meyer and Shew, 1991) also were found to have a great influence on disease development. Inoculum density of *C. elegans* is not related to the field's history of black root rot. In addition, inoculum density levels may also differ largely even between small distances (about 3.6 m) within a field (Rittenhouse and Griffin, 1985).

Most of the previous spatial pattern studies of soilborne fungi have indicated the aggregation or clumping of soil populations of microorganisms (Nicot et al., 1984; Campbell and Noe, 1985; Punja et al., 1985). In addition, Rittenhouse and Griffin (1985) showed that the distribution of *C. elegans* in tobacco field soil had a clumped spatial pattern. In the Lower Fraser Valley of B.C., *C. elegans* was previously isolated from eight out of twelve carrot fields sampled in 1984-1985 (L. MacDonald and D.J Ormrod, 1985).

Within the Fraser Valley of B.C., commercial fields are not continuously planted to carrots. Fields may be planted to other vegetable crops, such as lettuce, celery, potato, or onions, in rotation with carrots. By sampling commercial carrot fields, as well as other vegetable fields, information can be obtained to better understand the distribution of *C. elegans* in these fields. The objectives of this study were to:

a) determine the distribution of *C. elegans* in carrot and other vegetable production fields;

b) determine the inoculum density of *C. elegans* in commercial carrot fields; and

c) determine the spatial pattern of *C. elegans* in one carrot field which was sampled intensively over two growing seasons (1990-1991).

MATERIALS AND METHODS:

DISTRIBUTION OF *C. ELEGANS* IN THE LOWER FRASER VALLEY OF B.C.:

In each of 1990 and 1991, 30 carrot and vegetable production fields were randomly sampled during May-July. In each field, 15-20 soil samples (each around 80-100g of soil) were randomly collected and bulked into 15 x 20 cm polyethylene bags. These bags were tagged with the identity of the field, brought to the laboratory and assayed immediately or stored at 4 C in the cooler prior to examination. Assays were conducted within one week. The presence of *C. elegans* was determined using the carrot root disc assay described in Chapter 2. The areas close to the commercial crop where weeds grew, were also tested for the presence of *C. elegans*.

INOCULUM DENSITY OF *C. ELEGANS* IN CARROT FIELDS:

Soil samples were collected from 24 commercial carrot fields in 1990 and 1991. Samples were collected in the same manner as for the distribution studies above. The samples were analysed immediately or stored in a cooler at 4 C and examined within 4 weeks. After thoroughly mixing each bulked soil sample, a 5 cm^3 volume of soil per sample/field was placed in a 9 ml sterile distilled water blank (10^{-1}), and further dilutions were made. Each dilution was plated onto TBM-RBA medium (see Chapter 2) and incubated at 25 C. The number of propagules/cm^3 of soil was calculated for each field.

SPATIAL PATTERN OF *C. ELEGANS*:

From the inoculum density studies above, two fields (A and B)) located in Cloverdale, B.C. were selected to determine the spatial pattern of *C. elegans*. Field A had a history of black root rot development on carrots and was planted to carrots in 1989 and 1990. Field B had never been planted to carrots until 1990. In July 1990, samples were collected from fields A and B along two rows. Samples comprised of 80-100 g were taken to a depth of 20 cm at 3.0 m (10 ft) intervals along the row, placed in a polyethylene bag and tagged with the sampling distance and row number. In field A, 20 samples/row were collected, whereas in field B, only 10 samples/row were obtained. These samples were first analysed using the carrot root disc assay and then using TBM-RBA medium. These initial studies indicated that the inoculum density was much higher in field B and therefore the population of *C. elegans* (propagules/cm^3) was estimated using the TBM-RBA medium, which gives a quantitative result over 20 propagules/cm^3 of soil.

A portion (55 x 2.4 m^2) of field B was divided into eighteen 6.1 x 1.2 m^2 quadrats. About 80-100 g of soil was collected from the center of each quadrat (18 samples in total) using a hand trowel (Fig. 6). Samples were placed in polyethylene bags and labelled with the sample location. In the laboratory, the soil samples were analysed individually using TBM-RBA medium as described for the inoculum density studies. Samples were obtained at four times during 1990 and three times in 1991 from approximately the same location within the quadrat. The mean number of propagules/cm^3 of soil and the variance to mean ratio (Campbell and Noe, 1985) were calculated for each sampling date.

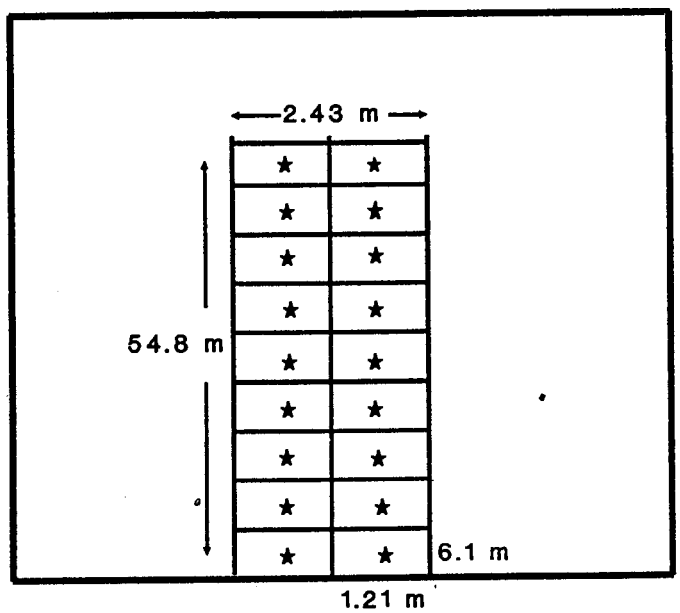


Figure 6. Sampling pattern used in field B. Samples were collected from the center of each quadrat, which measured 6.1 x 1.21 m^2, at various times during 1990-1991.

RESULTS:

DISTRIBUTION OF *C. ELEGANS* IN THE FRASER VALLEY OF B.C:

Among the 30 commercial fields sampled in 1990 and 1991, 65% of the fields were found to be infested with *C. elegans*. Fields planted to celery, lettuce and potato were found to be infested with *C. elegans*. Weeds harbored *C. elegans* as well.

INOCULUM DENSITY OF *C. ELEGANS* AMONG CARROT FIELDS:

C. elegans was found to be present in 15 out of 24 carrot fields sampled. In most of the fields, the inoculum density was <10 propagules/cm^3 (approximately <40 propagules g dry weight at 57% moisture content). The range of inoculum densities among the commercial carrot fields was 0-560 propagules/cm^3 (Table 6). The range of inoculum densities and the corresponding number of fields in each category are shown in Table 6. Only two fields had extremely high inoculum densities of *C. elegans* (>100 propagules/cm^3).

SPATIAL PATTERN OF *C. ELEGANS*:

The highest inoculum density during the sampling years of 1990 and 1991 was observed during the month of September, when the inoculum density ranged between <10-410 propagules/cm^3 (Table 7). The lowest inoculum level was found during the months of June and July and the inoculum density ranged between <10-60 propagules/cm^3 (Table 7). Mean number of propagules/cm^3 of *C.elegans* ranged from 18.8-68.8 from August 1990 to July 1991. The lowest and the highest mean number of propagules/cm^3 were obtained in the months of July 91 and May 91, respectively. The variance to mean ratio was always >1.0, which indicated the aggregation or clumping of *C. elegans* inoculum in soil.

Table 6. Range of inoculum densities of *Chalara elegans* in commercial carrot fields sampled in 1990-1991 using TBM-RBA medium.

Inoculum density (Propagules/cm^3)	Number of fields
0	9
1-10	12
10-50	1
50-100	0
>100	2

Table 7. : Inoculum levels of *C. elegans* (propagules/cm^3) in field B sampled during 1990-1991 at different sites.

Sample		Propagules/cm^3						
Location (m)		16 Aug 90	30 Aug 90	11 Sept 90	26 Sept 90	23 May 91	20 Jun 91	24 Jul 91
RowI:								
0	A1	20	20	10	10	40	50	-
6.1	A2	40	-	30	-	220	60	40
12.1	A3	50	-	30	20	20	-	-
18.3	A4	60	40	30	10	190	20	-
24.4	A5	20	-	40	30	100	30	10
30.5	A6	150	20	130	90	130	10	60
36.5	A7	260	10	30	10	120	10	60
42.6	A8	10	180	40	40	70	-	20
48.7	A9	50	70	150	110	60	60	40
Row II:								
0	B1	-	-	20	10	10	-	-
6.1	B2	-	-	40	70	50	-	-
12.1	B3	-	-	-	410	10	-	-
18.3	B4	30	50	30	30	10	10	-
24.4	B5	-	70	60	40	40	20	-
30.5	B6	-	50	60	110	50	-	-
36.5	B7	30	10	30	40	10	10	-
42.6	B8	10	10	70	30	80	10	40
48.7	B9	210	-	170	115	30	40	20
No.of samples		18	18	18	18	18	18	18
Range		10-260	10-180	10-170	10-410	10-220	10-60	10-60
Mean		53.6	31.3	54.1	65.5	68.8	20	18.8
Variance /mean ratio		104.8	60.8	41.6	134.2	55.7	18.9	20.9
crop present		C	F	L	L	P	P	F

C= Carrot; F= Fallow, after ploughing; L= Lettuce; P= Potato; -= <10 propagules/cm^3

The fluctuations in population levels of *C. elegans* during the sampling period at two different sites are shown in Figures 7 and 8. The overall inoculum density was higher in row I than in row II.

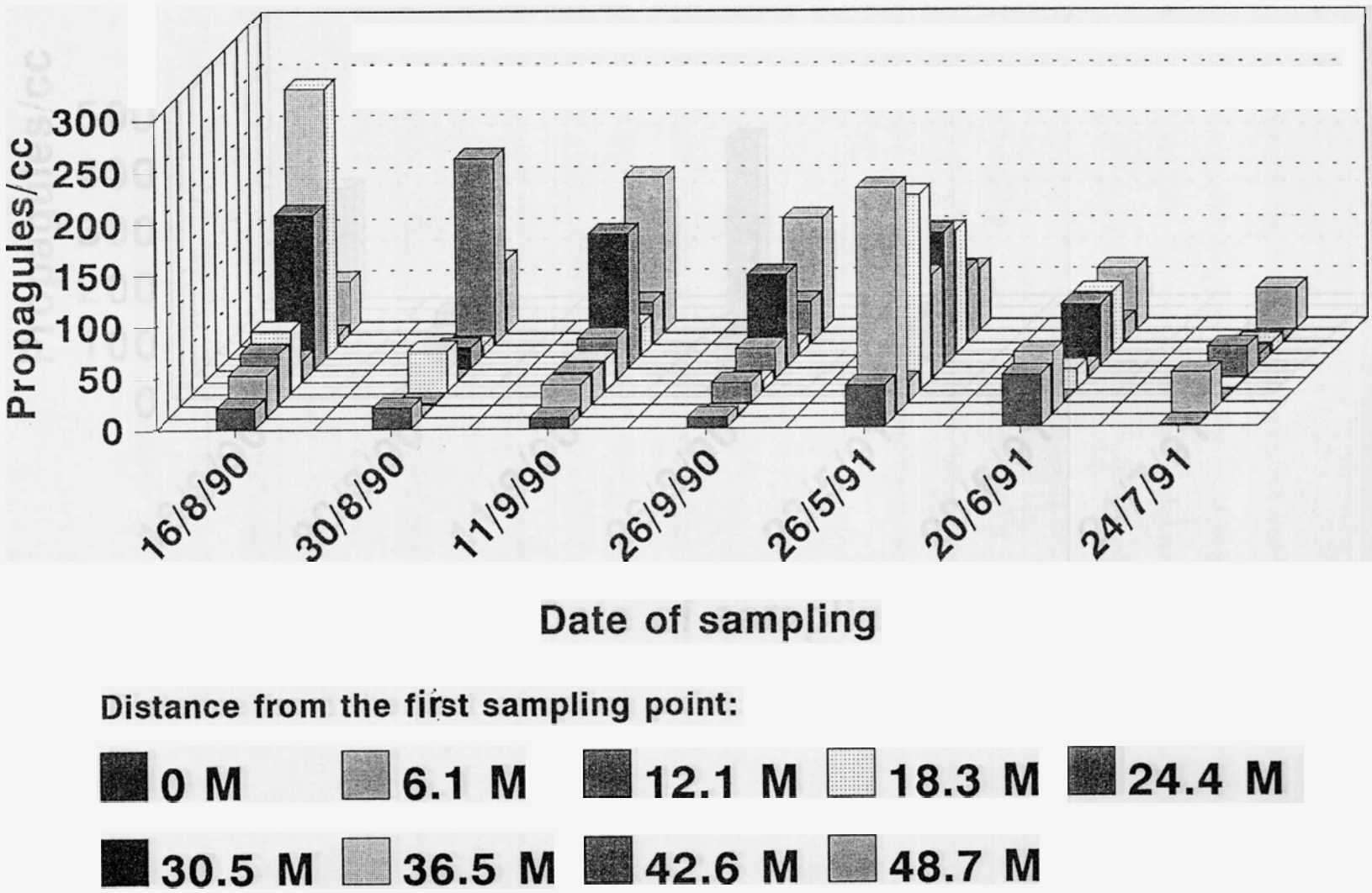


Figure 7. Distribution of *Chalara elegans* in Field B in 1990-1991. Samples were collected at 6.1 m intervals along row I, and were plated onto TBM-RBA medium to determine propagules/cm^3 for each sample.

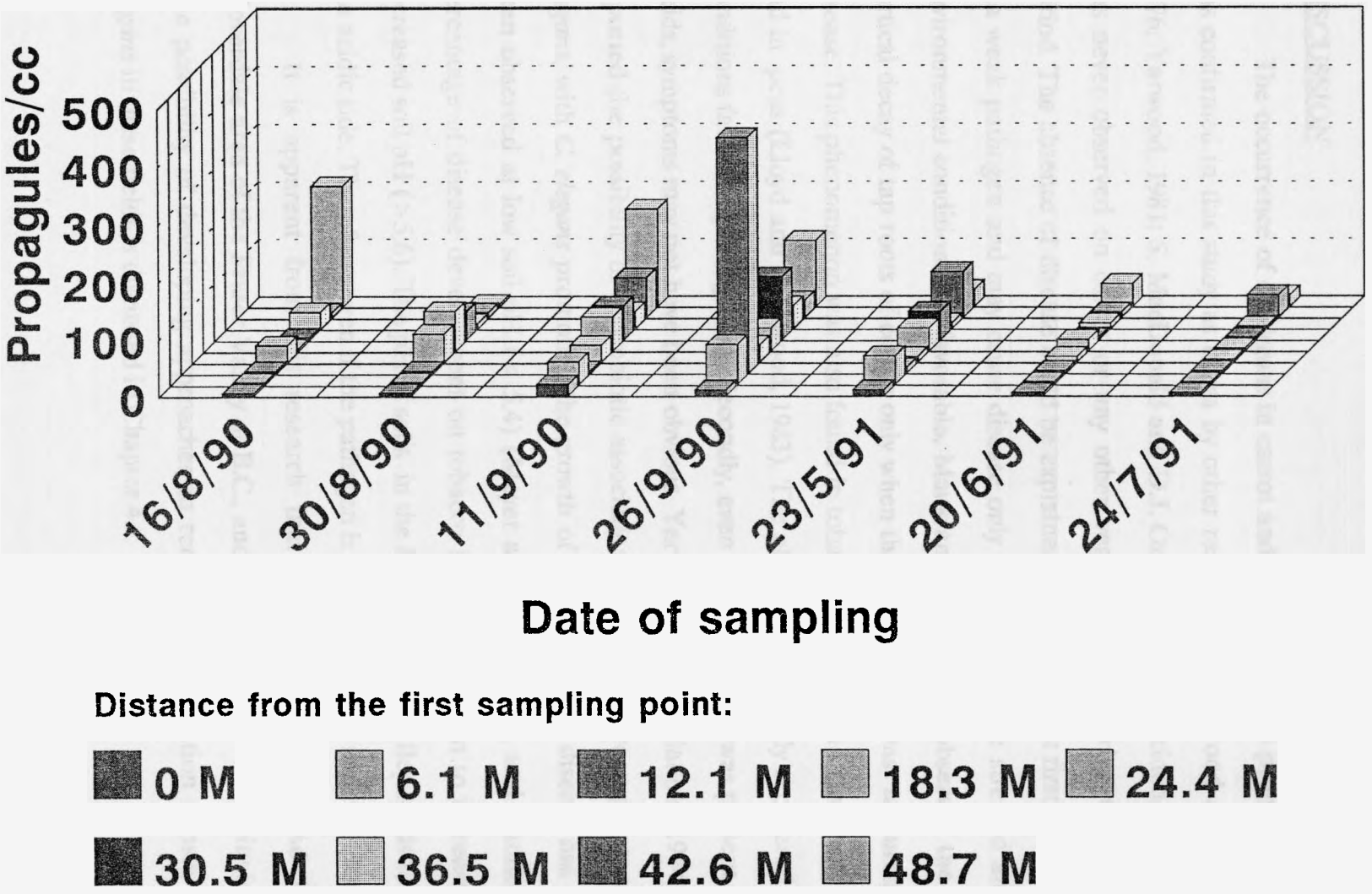


Figure 8. Distribution of *Chalara elegans* in Field B in 1990-1991. Samples were collected at 6.1 m intervals along row I I, and were plated onto TBM-RBA medium to determine propagules/cm^3 for each sample.

DISCUSSION:

The occurrence of *C. elegans* in carrot and other vegetable production fields was confirmed in this study as it has by other researchers (Yarwood and Levkina, 1976; Yarwood, 1981; S. MacDonald and D.J. Ormrod, 1984). Incidence of disease was never observed on carrot or any other vegetable crop during the sampling period. The absence of disease could be explained by the fact that firstly, *C. elegans* is a weak pathogen and may cause disease only if the plants are stressed and the environmental conditions are favorable. Mauk and Hine (1988) observed the black cortical decay of tap roots of cotton only when the temperature was favourable for disease. This phenomenon was also found in tobacco (Johnson and Hartman, 1919) and in peas (Lloyd and Lockwood, 1963). The plants subsequently recovered when conditions favoured plant growth. Secondly, even if some disease was present in the fields, symptoms may not have been obvious. Yarwood and Karayiannis (1974) have reported the possibility of a symbiotic association occurring between plants and *C. elegans*, with *C. elegans* promoting the growth of plants. Thirdly, disease has rarely been observed at low soil pH (< 5.4) (Meyer and Shew, 1991), and stunting and percentage of disease development on tobacco have been shown to increase with increased soil pH (>5.6). The pH of soils in the Lower Fraser Valley tends to be on the acidic side. Therefore, even if the pathogen is present, disease may not develop.

It is apparent from this research that *C.elegans* is widespread in the Cloverdale area of the Fraser Valley of B.C., and that the inoculum density is high. The possibility of developing approaches to reduce the population density of *C. elegans* in these fields is discussed in Chapter 4.

During wet and cool months (September, May) the mean population density of *C. elegans* was fairly high (Table 7); therefore, cool and wet weather, which is typical for the Fraser Valley of B.C., may support the development of *C. elegans*. In contrast, the inoculum density was lower during the months of June and July, when the weather was warm and dry. Temperature and moisture may therefore have an influence on the populations of *C. elegans* in nature. It is also appeared that the populations were increased following a lettuce crop, and reduced after a fallow period. This observation needs to be confirmed in future studies.

Many other factors, such as cultural practices (ploughing, crop rotation), may also influence the population levels of *C. elegans*. The highest inoculum density was observed when the field was planted to lettuce (Table 7). After harvest and ploughing, the population of *C. elegans* appeared to decrease. During normal cultivation practices, these factors (temperature, moisture, ploughing, crop rotation) cannot be separated. Therefore, it would be difficult to conclude precisely which of these factors influenced the fluctuations in the populations of *C. elegans* observed in this study. Any one of these factors or a combination of two or more factors may influence the decrease or the increase of the populations of *C. elegans* in nature.

The clumped spatial pattern of *C. elegans* in soil is consistent with the findings of other investigators working with soilborne fungi (Leach and Davey, 1938; Campbell and Noe, 1985; Punja et al., 1985). Thus, as for most soilborne plant pathogens, *C. elegans* has an aggregated or clumped spatial pattern. This conclusion was made by examining the variance to mean ratio. This finding is similar to the pattern observed by Rittenhouse and Griffin (1985) in tobacco fields. By mapping inoculum levels at different sites, they obtained a visual pattern of aggregation. In the present study, it was observed that the inoculum level is higher in some places than others, and that it changes with time.

Such information is useful for predictive studies, for site selection, and for developing control measures to reduce black root rot development. However, one should be cautious in sampling a limited number of sites and concluding the mean inoculum density of the field from such samples.

CHAPTER 4

FACTORS INFLUENCING SURVIVAL OF PHIALOSPORES OF *CHALARA ELEGANS* IN ORGANIC SOIL:

INTRODUCTION:

In most of the previous reports on survival of *C. elegans* in natural soil, it has been shown that the fungus persists mainly as chlamydospores. Tsao and Bricker (1966) plated soil that was naturally infested with *C. elegans* from citrus rhizopheres on a semiselective medium. They directly observed that the colonies of *C. elegans* originated mostly from chlamydospores, and colonies were never observed to originate from endoconidia or mycelial fragments. Patrick, et al. (1965) and Tsao and Bricker (1966) believed that the endoconidia (phialospores) of *C. elegans* were short lived. However, Stover (1950) reported that a low percentage of phialospores survived for a long period in artificial culture. In cotton soil from Tulare County, California, the phialospores were found to be viable even after 7 months (Linderman and Toussoun, 1967). Papavizas (1968) reported the death of a majority of phialospores in natural soil within 1-4 weeks, but a small number could survive as long as 10 months. Lysis and survival of the phialospores was greatly influenced by the soil type and moisture content. When moist, sandy soil was inoculated with phialospores, 100% were lysed after 125 days (about 18 weeks), whereas in dry sandy soil, only 30% had lysed after 301 days (43 weeks). Likewise, lysis of phialospores in moist and dry, clay loam was 20% and 30%, respectively, after 125 days (18 weeks) (Schippers, 1970).

Several authors have investigated survival and inoculum density changes of *C. elegans* over time with different host and nonhost plants (Bateman, 1963; Lloyd and Lockwood, 1963; Papavizas and Adams, 1969; Reddy and Patrick, 1988;), at different soil temperatures (Lloyd and Lockwood, 1963; Rothrock, 1991) and with different soil amendments (Papavizas and Adams, 1969; Reddy and Patrick, 1988 Kendig and Rothrock, 1991). It has been shown that planting host crops, such as beans (Bateman, 1963; Papavizas and Adams, 1969; Reddy and Patrick, 1988) and cotton (Rothrock, 1991) increased the population of *C. elegans*, mainly in the rhizophere. In contrast, nonhost plants, such as corn, wheat (Bateman, 1963) rye (Reddy and Patrick, 1988) and hairy vetch (Kendig and Rothrock, 1991) were found to reduce the pathogen populations. Survival of *C. elegans* was significantly lower at 24 C and 28 C than at 16 C (Rothrock, 1991). When alfalfa residues were added to soil at the time of inoculation with *C. elegans*, germination of both phialospores and chlamydospores was stimulated, but the germ tubes subsequently lysed. After four or more days of incubation, soil with alfalfa amendment had a higher level of fungistasis than an unamended soil (Papavizas and Adams, 1969). Amending soil with rye (Reddy and Patrick, 1988) and hairy vetch (Kendig and Rothrock, 1991) also showed similar effects. Antagonistic bacterial populations increased when rye was added to the soil (Reddy and Patrick, 1988).

It is clear that chlamydospores can survive longer than phialospores regardless of the soil type. However, survival of phialospores differs with different soil types. The factors influencing survival of phialospores in organic soil and the length of survival are unknown. Therefore, studies were conducted to determine the influence of soil moisture content, the presence or absence of host and nonhost plants, and the influence of soil amendments on survival of phialospores of *C. elegans*.

Information on factors influencing survival may be useful in developing a strategy to reduce populations of *C. elegans* in the organic soils of the Fraser Valley of B.C.

MATERIALS AND METHODS:

SOIL CHARACTERISTICS:

Soil was collected during May 1990 from a field in Cloverdale, B.C. (Paul Garvin Farm) which had black root rot history. Soil was collected from an area where no *C. elegans* propagules had been found (tested with carrot root discs and TBM-RBA medium). The moisture content of this soil was determined by weighing 20 g of soil in duplicate, and drying them at 80 C for 24-48 hr. From this, the average percentage moisture content of the soil was calculated.

The level of soil nutrients, such as ammonium, nitrate, phosphate, potassium, sulphate, calcium, magnesium and aluminum were determined and the soil pH, organic matter content and salinity were measured by Norwest Lab, Langley, B.C. A soil moisture retension curve was developed using a pressure membrane extractor (PME). The PME consists of a cellulose membrane supported by a fine mesh screen. The soil samples are placed on the membrane and subjected to air pressure. When the air pressure inside the chamber is increased above atmospheric pressure, the higher pressure inside the chamber forces water through the minute pores in the cellulose membrane. At equilibrium (when there is no water flow from the soil sample through the membrane), there is an exact balance between the air pressure in the extractor and the soil suction (and therefore the moisture content), in the soil samples. Soil samples collected from Paul Garvins' field were brought to the laboratory, mixed well and twelve sub-samples were placed on the membrane within twelve small rubber rings and compacted.

The sub-samples were saturated with water overnight, so that all the pores in each sub-sample were filled with water. The following day, the PME was tightly sealed and air pressure was applied. At equilibrium, samples were taken out and oven dried at 80 C for 24-48 hr and the moisture content of each was determined. The level of pressure applied, which is equal to the soil matric suction, was plotted against moisture content to obtain a moisture retension curve.

FACTORS INFLUENCING SURVIVAL OF PHIALOSPORES OF *C. ELEGANS*:

a) Soil moisture and crop plant: The soil described above was used throughout all of the experiments on spore survival. The soil was allowed to air dry for two days to obtain an initial moisture content of 44%. The experiments were conducted at two different soil moisture levels:

(a) Declining moisture content; no water was added throughout the experiment.

(b) Constant moisture content; sterile distilled water was added during the experiment to keep the soil moisture content at 55±5 %. The actual moisture content was determined by weighing the soil samples (2-3 g each) before and after drying to 80 C.

Both moisture conditions had three treatments (Fig. 9) imposed, namely:

(i) soil planted to carrot seedlings

(ii) fallow soil

(iii) soil planted to green onion seedlings

An uninoculated control was included.

Each treatment was replicated three times.

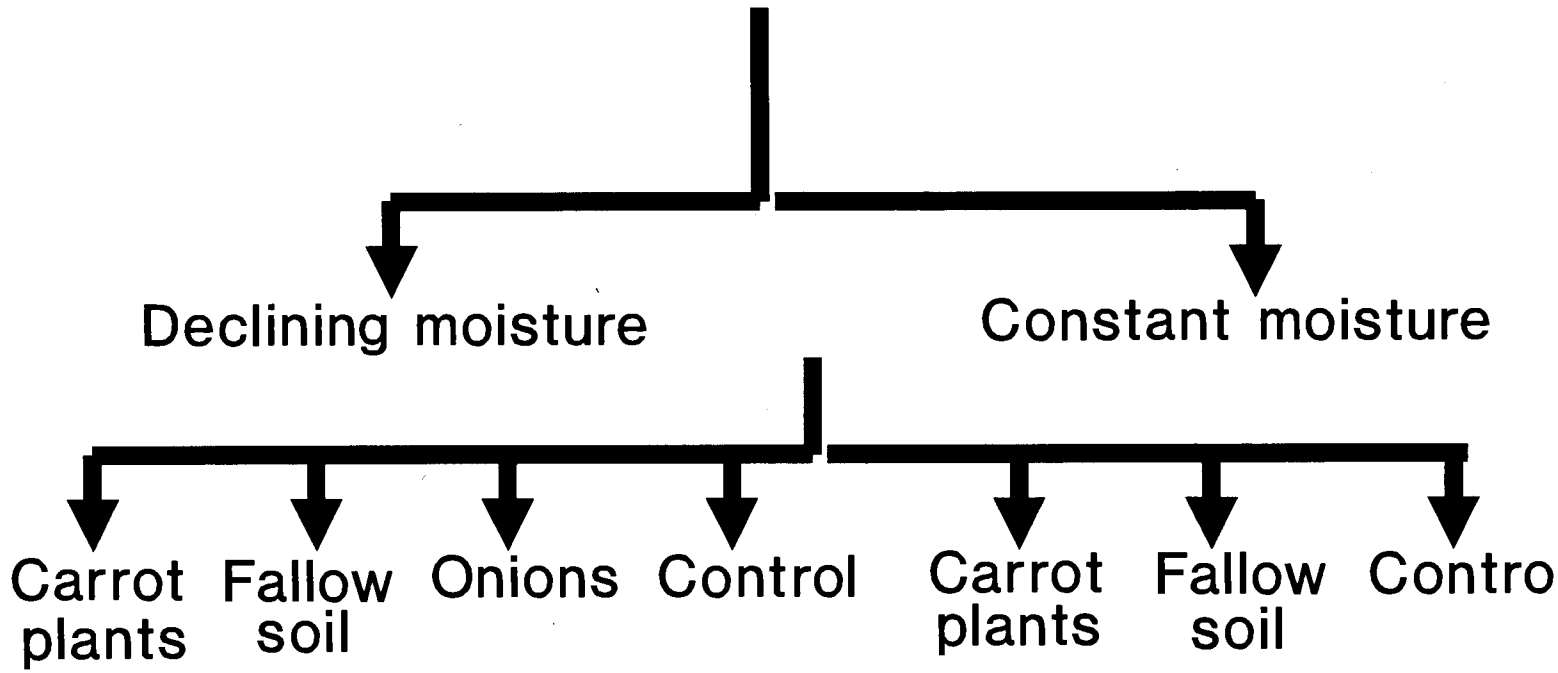


Figure 9. Experimental design of the treatments to determine survival of *phialospores of chalara elegans*. Treatments were 'planted to carrots', 'planted to onions' and 'fallow soil' at two different moisture conditions. Each treatment had three replicate pots.

In each replicate, about 200 cm^3 of soil (about 126-128 g of air dried soil at a moisture content of 44%) was placed in a 250 cm^3 plastic container. The soil was left fallow or planted either to carrots or onions. The carrots and onions (1-2 plants per pot) were planted as 3-month-old seedlings and bulbs, respectively. A 20 ml volume of 6 x 10^5 phialospores/ml suspension was added to all treatments except the uninoculated controls. The soil moisture content increased to 55%. The soil was mixed well on a paper towel to spread the inoculum of *C. elegans* and placed in the plastic containers. Subsequently, the carrot seedlings and onion bulbs were planted into the soil. Controls received 20 ml of sterile distilled water. A 1 cm^3 sample of soil from each treatment was immediately transferred to 9 ml of sterile distilled water (10^{-1}) and up to 10^{-3} dilutions were made. From each dilution, 0.5 ml of soil suspension was plated onto TBM-RBA medium (see Chapter 2), spread well, and incubated at room temperature. After one week, the colonies of *C. elegans* that developed on the medium were counted. Plating from each treatment and determination of propagules/cm^3 of soil per treatment was repeated at 2 week intervals for up to 20 weeks. Time (weeks) versus log propagules/cm^3 of soil was plotted for each treatment.

b) Soil flooding and amendments: In a second set of experiments, the influence of five additional factors were examined, namely:

(i) flooding of soil at low temperature (4 C)

(ii) flooding of soil at high temperature (25 C)

(iii) addition of $CaCO_3$ (1%, w/v)

(iv) soil planted to shallot onions

(v) fallow soil with declining moisture content (for comparison)

The soil was prepared as described in section (a) above. An uninoculated control (vi) was included. For treatments (i) and (ii) distilled water was added and moisture maintained at a level 1 cm above the soil surface. Treatments (iii) and (iv) had the moisture content maintained at 55±5% throughout the experiment by the addition of distilled water. Treatment (v) received no water throughout the experiment. Each treatment had three replicates consisting of a plastic container with 200 cm^3 of the same soil as in the previous experiment. All treatments except the uninoculated control received 20 ml of a 2.4 x 10^5 phialospore/ml aqueous suspension added with a pipette, whereas the control received 20 ml of sterile distilled water. The soil was mixed thoroughly on a paper towel to spread the inoculum. After placing the infested soil in the plastic containers, they were subjected to the respective treatments. A 1 cm^3 sample of soil from each treatment was transferred into 9 ml of sterile distilled water and dilutions were prepared and plated onto the TBM-2RBA as described in the first set of experiments. After incubation of the plates for one week, the colonies were counted and log propagules/cm^3 were calculated. Dilution plating was repeated every other week for up to 20 weeks.

STATISTICAL ANALYSIS:

For both sets of experiments, the mean, standard deviation, and standard error were calculated for each experiment sampling date using the SAS statistical package. The treatments were compared using a two-way ANOVA (Analysis of Variance) with repeated measures on one factor (weeks) using the SAS program. To compare the effect of moisture (constant moisture and declining moisture), two-way ANOVA with repeated measures on one factor (weeks) was conducted. The F-value was assessed at $P \geq 0.01\%$ confidence level.

RESULTS:

SOIL CHARACTERISTIC:

The results from the soil analysis conducted by Norwest labs are given in Table 8. The pH of wet soil and air dried soil (same field) were 5.2 and 5.16, respectively, which indicates that the soil used for the survival experiment was moderately acidic. The organic matter content was very high (80.6% for wet and 75.6% for air dried soil). The level of calcium was very high, and was the highest of all of the mineral elements found in the soil (Table 8).

The moisture retention curve for the organic soil used is shown in Fig. 10. Field capacity (moisture content at soil suction between 0.1-0.2 bars) for the soil was around 57-61%. At 3 bar pressure, the moisture content was 42%, similar to that of air-dried soil.

FACTORS INFLUENCING SURVIVAL OF PHIALOSPORES OF *C.ELEGANS*:

a) Soil moisture and crop plant: Soil moisture content at the beginning of the experiment was about 55%. After air drying the soil for two days before the experiment, the moisture content decreased to 44%, and increased to 60% after the addition of 20 ml of spore suspension or water. The moisture content was maintained at $55 \pm 5\%$ throughout the experiment for the required treatments.

In the uninoculated control soil, no *C. elegans* colonies developed at any time. For all treatments (planted to carrots, onions, or fallow), the number of spores decreased significantly ($P \geq 0.01$) from the initial inoculum level, and after 19 weeks, a low percentage of viable phialospores were detected with the TBM-RBA medium. No significant difference in survival was observed between the 'fallow soil' and 'planted to carrots' treatments at both declining and constant moisture conditions (Figs. 11, 12).

Table 8. Results of soil analysis conducted by the Norwest Lab, Langley, British Columbia for soil used in survival experiments of *Chalara elegans*.

Soil components	Wet soil[a] (ppm)	Air dried soil[b] (ppm)
Elements		
Ammonium	55	44
Nitrate	127	117
Phosphate	172	160
Potassium	474	419
Sulphate	53	35
Calcium	9149	7078
Magnesium	818	797
Aluminum	1.6	1.6
Characteristics		
pH	5.2	5.16
E.C (salinity)	0.64	0.57
Organic matter	80.6	75.6

a Soil was collected from Paul Garvins' field that had about 55% moisture content.

b Soil was subjected to air drying for 2 days. The moisture content was 44%.

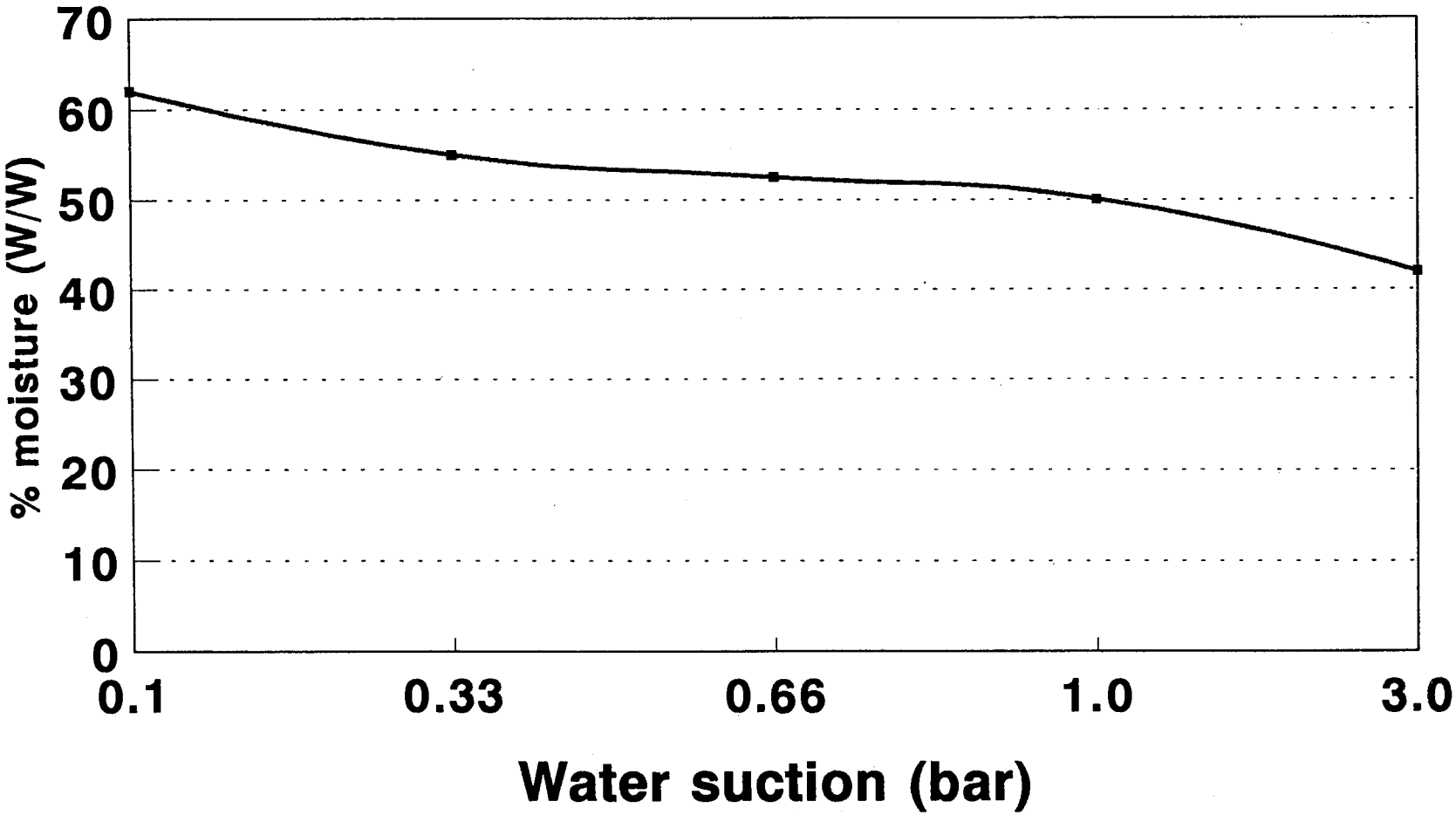


Figure 10. Moisture retention curve of organic soil that was used to study survival of phialospores of *Chalara elegans*.

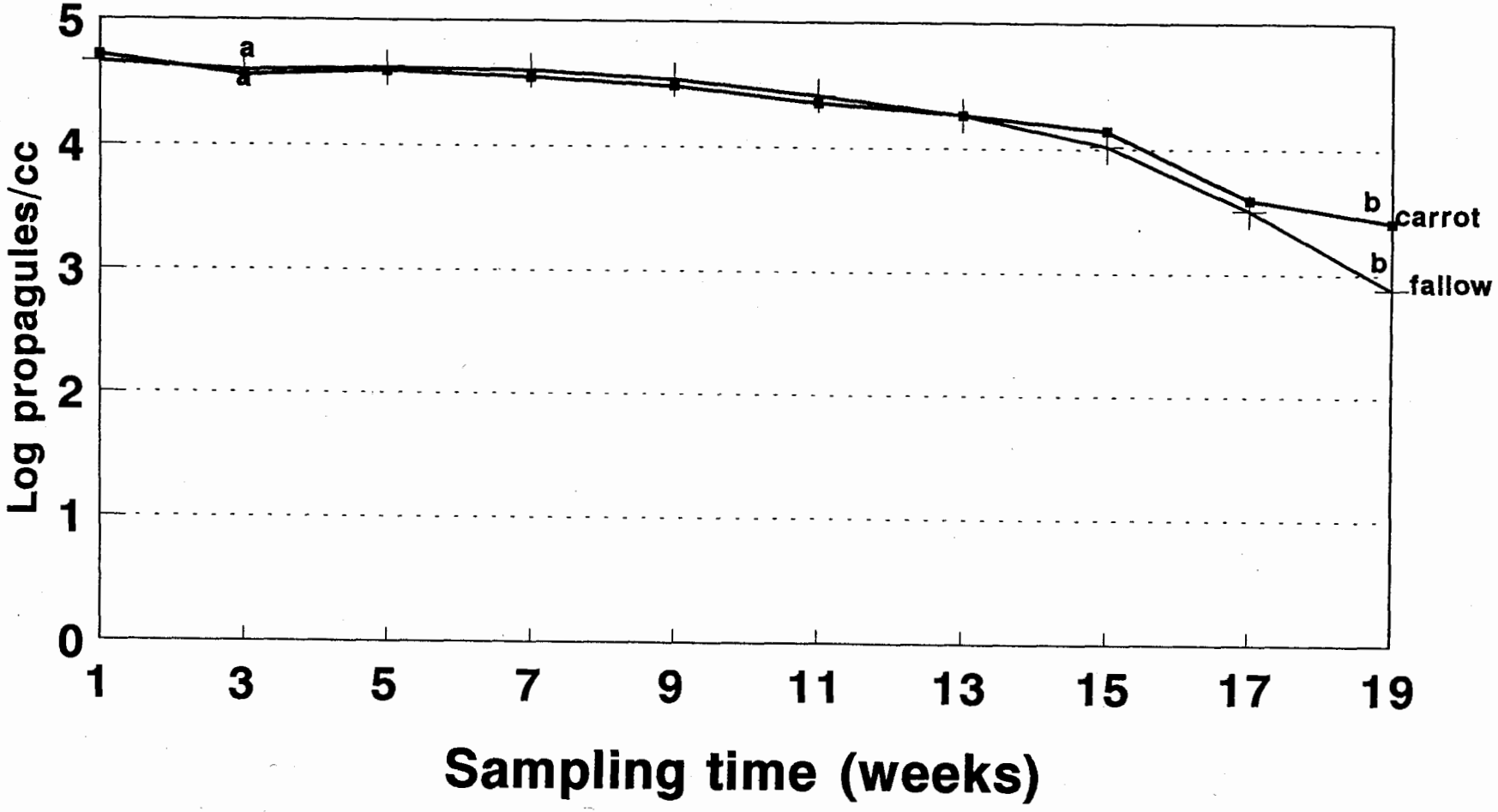


Figure 11. Survival of phialospores of *Chalara elegans* in the organic soil that was planted to carrots or left fallow under constant moisture conditions. Significant differences (P≥0.01) between treatments are indicated by different letters.

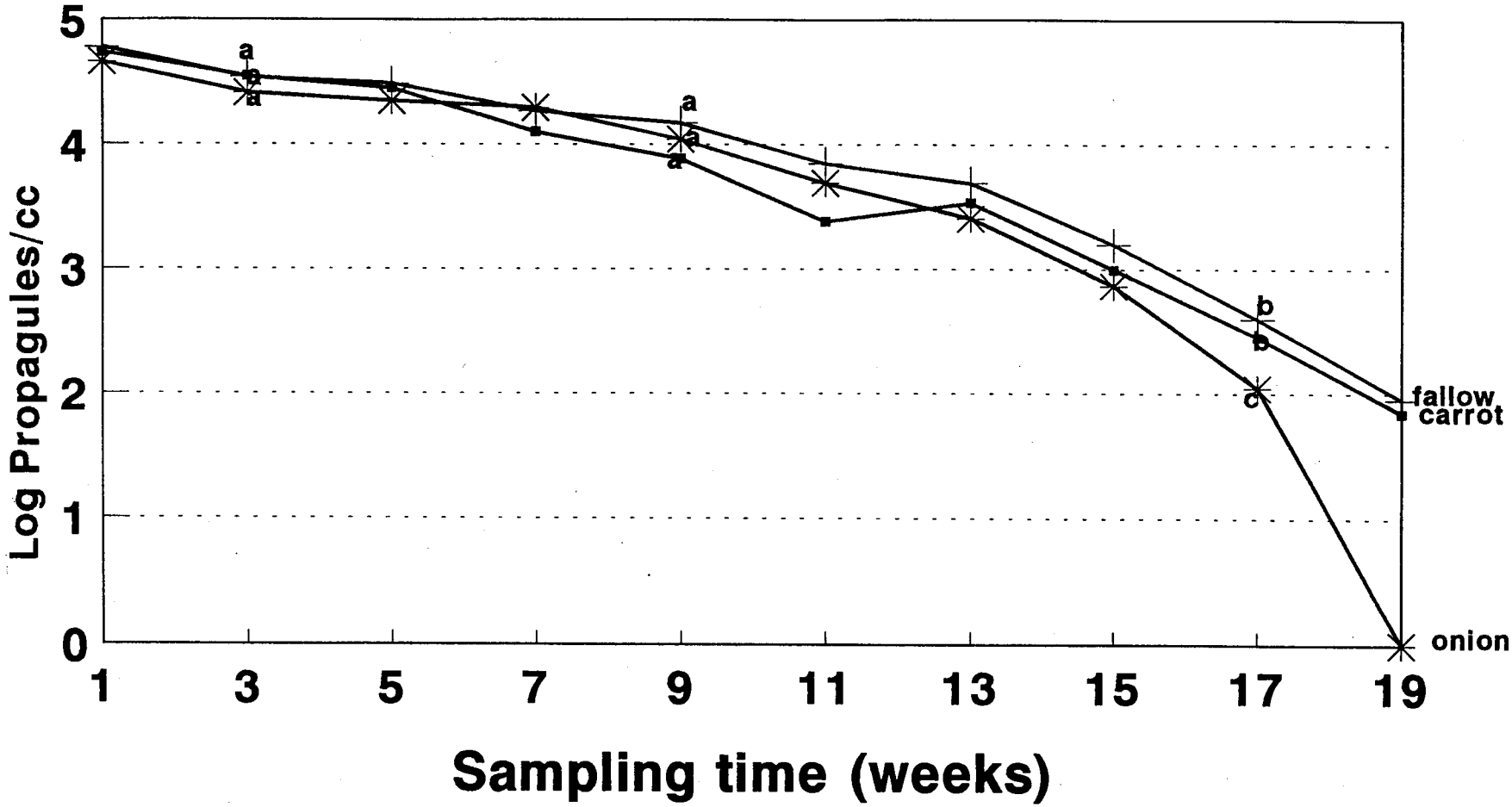


Figure 12. Survival of phialospores of *Chalara elegans* in soil that was planted to carrots, onions or left fallow under declining moisture conditions. Significant differences ($P \geq 0.01$) between treatments are indicated by different letters.

However, after 17 weeks, the population of *C. elegans* decreased significantly ($P \geq 0.01$) in the pots planted to onions when compared with fallow soil or the pots with carrot plants (Fig. 12).

Survival of *C. elegans* at constant moisture conditions (55±5%) was higher than at declining moisture conditions at 25 C after 7 weeks. The moisture content of 'declining moisture condition' soil was 35% at the end of the experiment (Fig. 13).

b) Soil flooding and amendments: No colonies of *C. elegans* were found on any plates from the uninoculated control throughout the experiment. The population of *C. elegans* was lower than the original inoculum level for treatments 'flooding at 4 C','addition of $CaCO_3$' and 'fallow soil' only by the 20^{th} week and was still detectable in the soil. However, for treatment 'flooding at 25 C' the initial population decreased significantly ($P \geq 0.01$) by the 3^{rd} week and for treatment 'planted to onions' by the 5^{th} week compared to the initial inoculum. The highest population of *C. elegans* was maintained when the soil was flooded at 4 C, and the lowest population was maintained when the soil was flooded at higher temperature (25 C). When a comparison between treatments was made, the population of *C. elegans* in the soil that was flooded at 25 C decreased significantly ($P \geq 0.01$) by the 3^{rd} week than in any other treatment. Likewise, a significant ($P \geq 0.01$) reduction in the population level was observed in the pots planted to onions on the 5^{th} week compared with low temperature flooding, $CaCO_3$ amended soil, or fallow soil. On the 20^{th} week, the population was still significantly higher in the pots flooded at 4 C than the other treatments (Fig. 14). No significant difference in the spore population in soil was observed between treatments that had $CaCO_3$ and the fallow soil throughout the experiment.

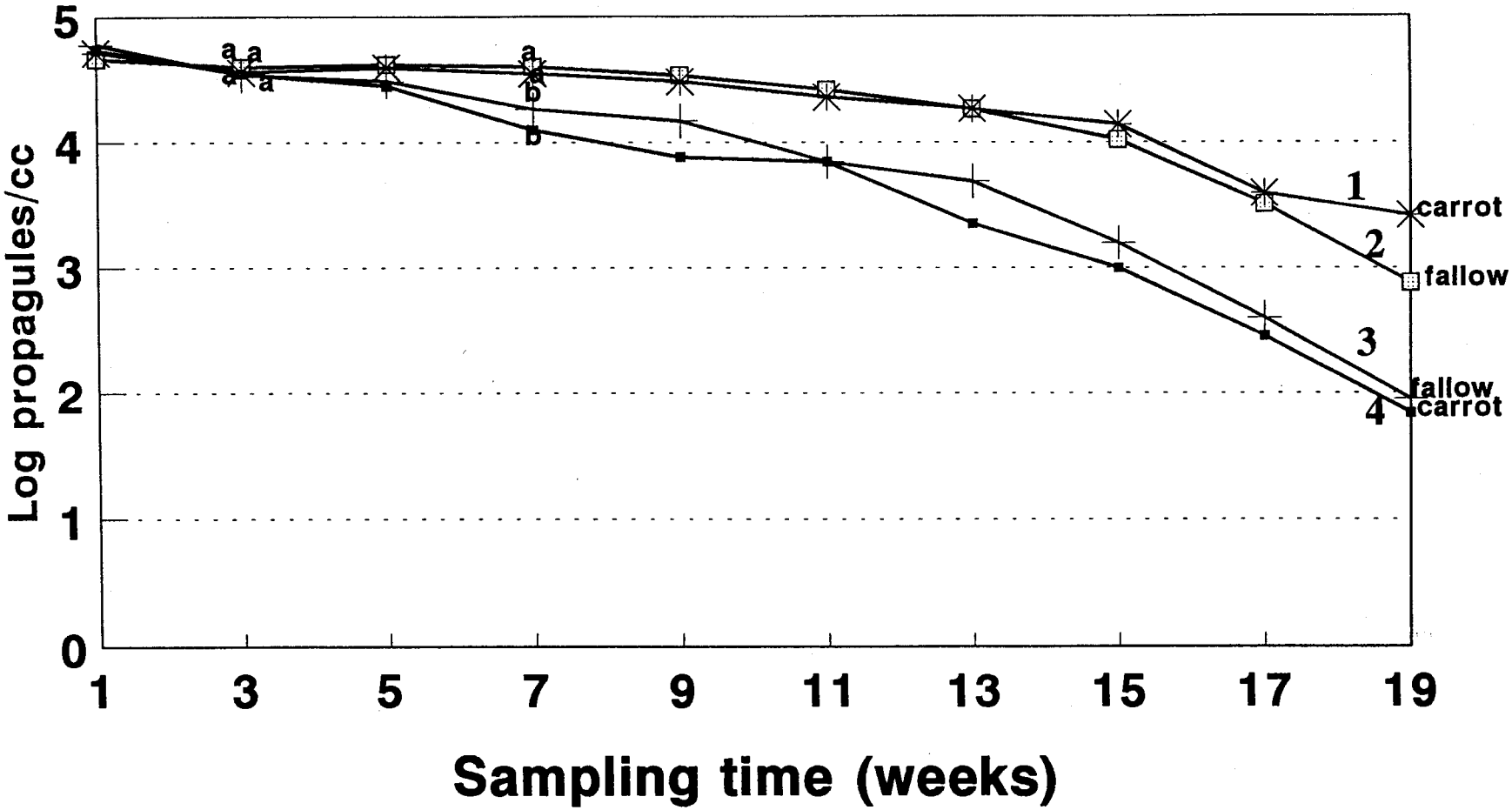


Figure 13. Survival of phialospores of *Chalara elegans* in organic soil at constant moisture (lines 1 and 3) and declining moisture conditions (lines 2 and 4). Treatments were either ' planted to carrots' or 'fallow soil'. Significant difference (P≥0.01) between conditions are indicated by different letters.

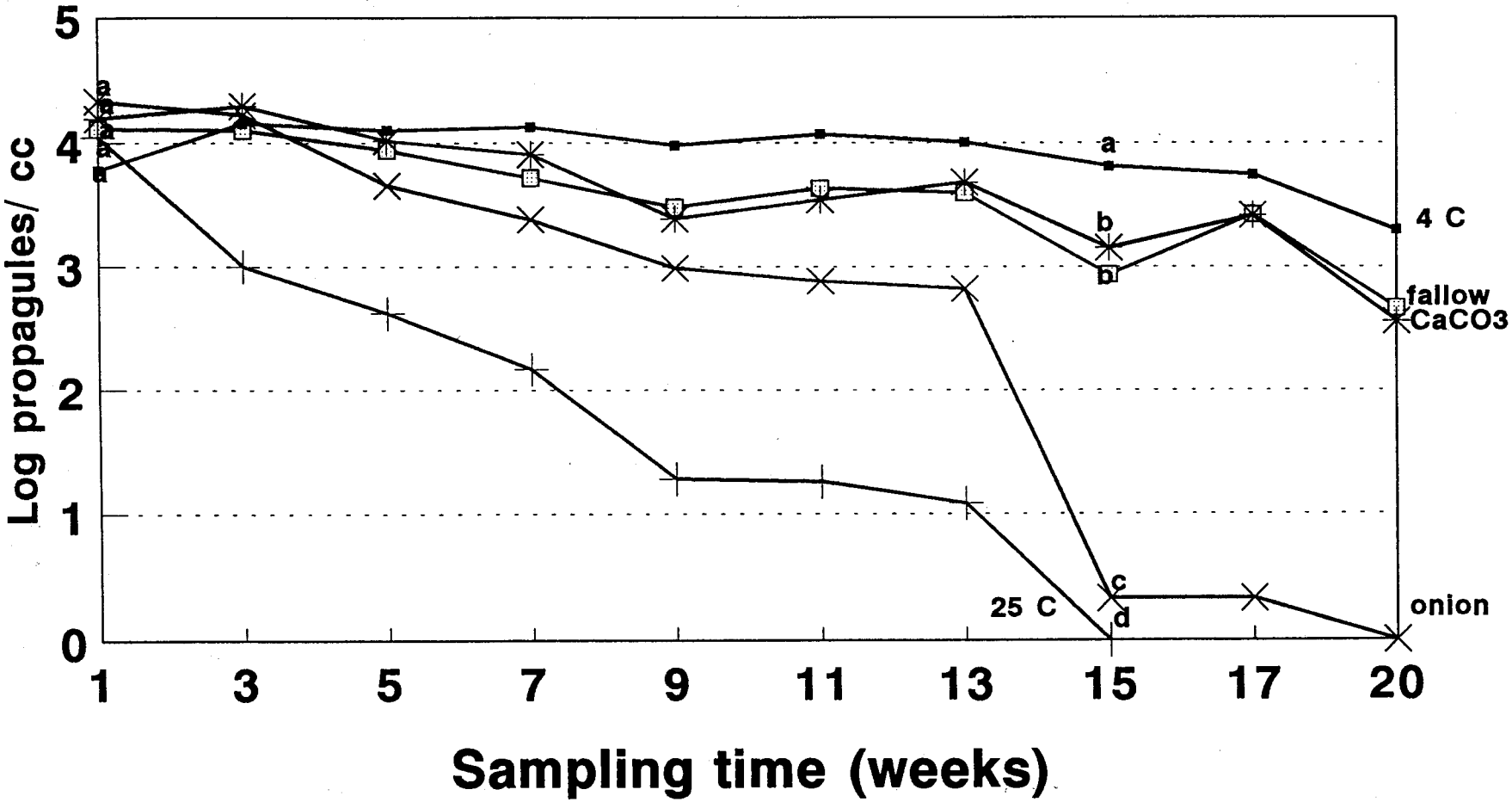


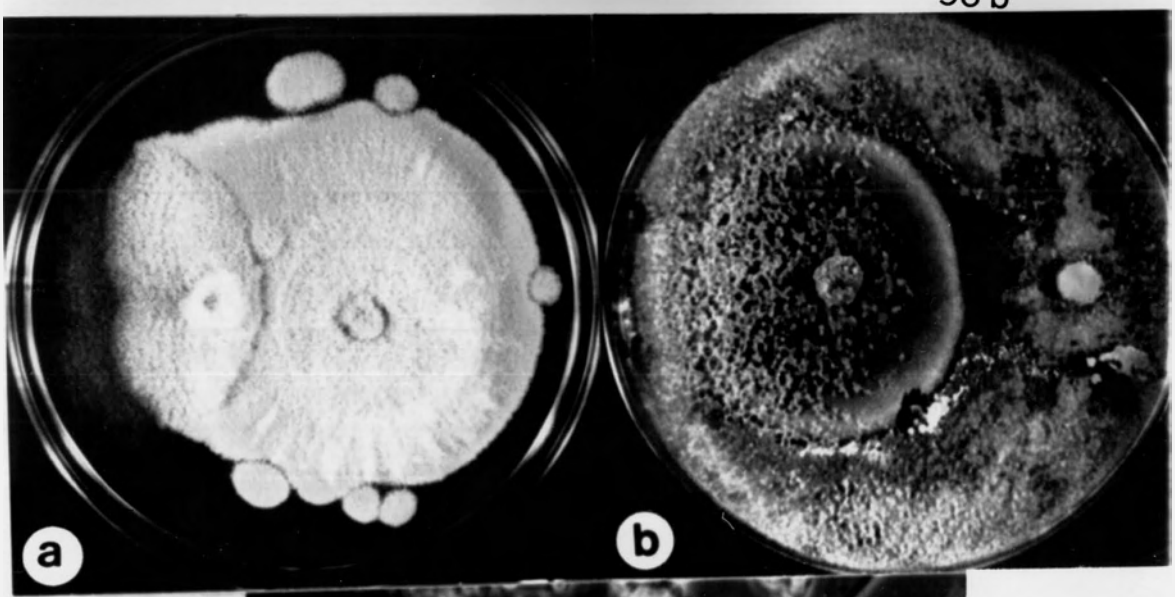
Figure 14. Survival of phialospores of *Chalara elegans* in organic soil that was flooded (at 4 C, 25 C), planted to shallot onions, had $CaCO_3$ or left fallow. Significant differences ($P \geq 0.01$) between treatments are indicated by different letters.

DISCUSSION:

The soil analysis indicated that the soil used in these experiments has a high organic matter and calcium content with a low soil pH. At low pH (5.0-6.0) *C. elegans* grows well in culture on V-8 medium (personal observation). Lucas (1955) observed that the optimum growth for *C. elegans* was at pH 3.9-6.2. In a recent study (Meyer and Shew, 1991), analysis of soils from Western North Carolina, U.S.A, indicated that the suppressive nature to *C. elegans* was found to be dependent upon the interrelationships of soil pH, base saturation and exchangeable aluminum (Meyer and Shew, 1991).

In the first series of experiments, the presence of carrot plants did not enhance the survival or increase the population of *C. elegans*. This is not difficult to explain since *C. elegans* does not infect the carrot plants in the field and therefore, there would be no increase in the population level. When green onions were used in the experiment, the population decreased significantly after 17 weeks. Although the effect of onions in reducing the population level of *C. elegans* is gradual, onions could be used in a crop rotation scheme to decrease *C. elegans* populations. It should be noted that green onions were used in the first experiment, while in the second experiment, shallot onions were used. When soil from the onion pots was plated on media, an increase in the levels of antagonistic fungal populations was observed. Three morphologically distinct *Penicillium* spp. and a *Trichoderma* sp. were isolated from the soil planted to onions. When one of the *Penicillium* species was paired in culture with *C. elegans*, an inhibition zone was observed, which indicated possible in vitro production of antibiotics against *C. elegans* (Fig. 15a), and the culture was over grown by *Penicillium* spp. When *Trichoderma* sp. was paired in culture with *C. elegans*, no inhibition zone was observed, and the culture was over grown by *Trichoderma* sp (Fig. 15b).

Fig. 15. Antagonism of *Penicillum* and *Trichoderma* to *Chalara elegans* in paired culture.
a) A *Penicillium* sp. isolated from onion soil, inhibiting growth and growing over *Chalara elegans*.
b) *Trichoderma* sp. isolated from the onion soil, growing over *Chalara elegans*.
c) Mycelium of *Trichoderma* growing within the hyphae of *C. elegans*.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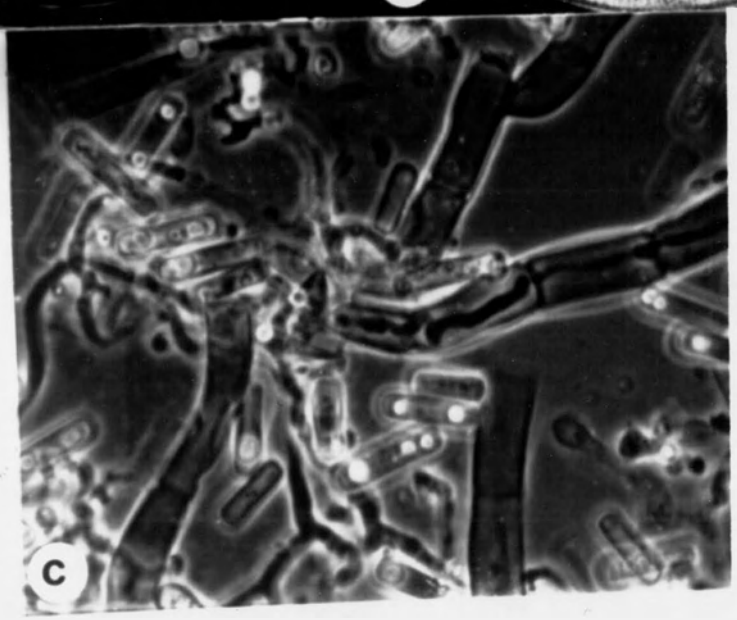
c

Under the microscope, the *Trichoderma* was observed to destroy mycelium and conidia of *C. elegans*. The chlamydospores appeared unhealthy and were lightly pigmented. The *Trichoderma* penetrated the mycelium (Fig. 15c) of *C. elegans* and grew inside the mycelium and phialides of *C. elegans*. Globose, hyaline chlamydospores of *Trichoderma* were found within the mycelium and phialides of *C. elegans*.

An additional advantage to using onions in rotation with carrot is the potential reduction of onion white rot, caused by *Sclerotium cepivorum*, which is a major disease on onion in the Fraser Valley. When organic soil was planted to carrots in Ontario, sclerotial populations of *S. cepivorum* decreased significantly (Banks and Edgington, 1989). Therefore, rotating carrots with onions may decrease both *C. elegans* and *S. cepivorum* populations.

Soil that was flooded and maintained at a low temperature did not reduce the survival of *C. elegans*. In the Fraser Valley, commercial carrot and vegetable fields are frequently flooded during the winter season (November-March), when the soil temperatures are low. High populations of *C. elegans* in the Fraser Valley soils might not be affected by the low temperature flooding of the fields during the winter seasons. However, high temperature flooding was shown to decrease the population dramatically.

While calcium was shown to increase the mycelial growth of *C. elegans* in buffered potato dextrose broth (Lucas, 1955), the addition of $CaCO_3$ to the soil did not have any significant effect on the population of *C. elegans*. Meyer and Shew (1991) hypothesized that low soil calcium might be one of the mechanisms of disease suppression in the soils they tested, because calcium is an important nutrient for many fungi, including *C. elegans*.

Although soil suppressiveness was eliminated by raising the soil pH in their tests, it was not nullified by raising soil calcium only, and therefore calcium deficiency was probably not the mechanism of disease suppression in these soils. However, a high level of calcium in the organic soils of the Fraser Valley may not be detrimental to the survival of *C. elegans*.

When shallot onions were used in the experiment, there was a significant decrease in the population of *C. elegans* and at an earlier stage than when green onions were used. While the mechanism for reduced populations is not clear, the increase in antagonistic fungal populations could be one of the mechanisms responsible. It is also possible that volatile compounds produced from onion roots may decrease the inoculum levels of *C. elegans* in the soil.

In the field, crop rotation with onions or flooding soil at higher temperatures for 1-2 weeks may reduce the populations of *C. elegans* significantly. By reducing the populations in the field, it may be possible to reduce the probability of black root rot development on carrots in the future.

CHAPTER 5

MODE OF INFECTION AND DISEASE DEVELOPMENT OF *C. ELEGANS* ON CARROT:

INTRODUCTION:

Previous histopathological studies of infection of host tissue by *C. elegans* have been reported for several plant species, such as tobacco (Conant, 1927; Stover 1950), bean (Christou, 1962; Pierre and Wilkinson 1970), cotton (Mathre et al. 1966; Mauk and Hine, 1988;), citrus (Tsao and Van Gundy, 1962) and holly (Wick and Moore, 1983). Although the diseases are caused by the same organism, the host-parasite interactions may or may not be similar. For example, in tobacco, hyphae entered the roots through wounds but not by direct penetration (Conant, 1927). In contrast, Stover (1950) reported direct penetration of tobacco roots by *C. elegans*. Hyphae were shown to penetrate tobacco roots and grow intra- as well as inter-cellularly (Stover, 1950). Conant (1927) reported that 50% of the lesions in tobacco occurred at the origin of branch roots. In tobacco, resistance to the pathogen was found mainly in the epidermis, at the root tips and in the zone of elongation. Periderm formation on the roots of tobacco was the most important method of resistance (Conant 1927). In contrast, Jewett (1938) could not observe periderm formation as a resistance mechanism in most of the tobacco plants studied.

Direct penetration by *C. elegans* was observed in bean (Christou, 1962), citrus (Tsao and Van Gundy, 1962), cotton (Mathre et al. 1966) and holly (Wick and Moore, 1983).

Phialospores and chlamydospores produced germ tubes, which penetrated the host tissue within 12 and 48 hr, respectively, at 24 C. An appresorium was produced by both spore types and disappeared after penetration of cotton roots (Mauk and Hine, 1988). During infection of cotton roots, the pericycle was not colonized by the fungus (Mauk and Hine, 1988). Also, numerous chlamydospores were produced within the infected cells in cotton as well as in the other plants. Occasionally, cork cambium tissues walled off the pathogen (Mathre et al. 1966). Bean plants can resist penetration by the fungus by developing a thicker epidermis. Also, Pierre and Wilkinson (1970) correlated cell division in bean roots with resistance, but also believed that a chemical by product was responsible for resistance.

In citrus, root cap and hypodermis tissue were resistant to penetration by *C. elegans* (Tsao and Van Gundy, 1962). In holly plants, the pathogen did not rot the roots. Intact nuclei in infected cells were observed in holly as well as in the other plants. In addition, in holly, when secondary organisms were absent, cell wall and middle lamellae were not macerated. Barrier formation below wounding was the main resistance mechanism in holly, and in mature plants, pericycle activity resulted in resistance of the older root tissue (Wick and Moore, 1983).

Infection by postharvest pathogens on carrots might take place in the field, as in gray mold caused by *Botrytis cinerea* (Sherf and MacNab, 1986), rot caused by *Mycocentrospora acerina* (Davies et al., 1981) or cottony soft rot caused by *Sclerotinia sclerotiorum* (Dennis, 1983; Sherf and MacNab, 1986).

Most of the postharvest diseases on carrots appear during long-term storage. Only a few pathogens are capable of causing diseases on carrot plants in the field as well as in storage, as in the case of seedling disease and crown rot caused by *Rhizoctonia solani*. Most of the postharvest pathogens do not cause disease on carrot plants in the field (Sherf and MacNab, 1986).

Inoculum in soil or plant debris is the major source by which infection in most postharvest diseases on carrots occur (Davies et al., 1981; Goodliffe and Heale, 1975; Sherf and MacNab, 1986; Wall and Lewis, 1980). The pathogen can penetrate the carrot tissue by mechanical pressure or by solvent action through enzymes as in *Sclerotinia sclerotiorum* (Sherf and MacNab, 1986) or mainly through wounds caused by harvesting practices (Dennis, 1983; Sherf and MacNab, 1986). Disease can occur on any part of the roots, as in *Botrytis cinerea*, *S. sclerotiorum* and *Rhizoctonia carotae* (Dennis, 1983; Goodliffe and Heale, 1975) or on certain regions as in *Mycocentrospora acerina* (Davies et al., 1981).

Little is known about the mode of infection and histopathological studies regarding black root rot on carrots. Information on how the fungus infects and the importance of wounding can provide useful information for disease control. Studies were conducted to determine the development and mode of infection on carrots by *C.elegans*. Such information could be very useful in understanding resistance mechanisms in carrot against this disease.

MATERIALS AND METHODS:

DISEASE DEVELOPMENT IN THE FIELD:

To determine whether carrot seedlings or carrot roots become infected in the field, 10-20 seedlings and mature roots were hand-harvested periodically during May to October, 1990 from different commercial carrot fields. These carrot samples were brought back to the laboratory, washed gently under tap water and incubated on moist paper towels in plastic containers at room temperature (25 C). Observations on the development of black root rot were made after one week.

INFLUENCE OF WOUNDING:

To determine the importance of wounding in the development of black root rot on carrots, a field which had a high population of *C. elegans* was chosen. About 20 mature carrots were hand-harvested. Precautions were taken so that no abrasions, damage or wounding occurred. Another 10 carrots were damaged by rubbing roots against each other during the hand harvesting, and all 30 carrots were packed with soil separately in polyethylene bags. The carrots were brought to the laboratory and washed in tap water. Among the 20 undamaged carrots, 10 carrots (unwounded) were incubated in plastic containers on wet paper towels for a week at room temperature in light. The other 10 carrots were subsequently wounded during washing. These carrots as well as the carrots which were wounded in the field were incubated in the containers separately. Observations of symptoms of black root rot development were made after one week of incubation at 25 C.

INFECTION DURING COMMERCIAL PROCESSING:

To determine where infections by *C. elegans* take place during carrot processing at the packing plant in the B.C. Coast Vegetable Co-operative Association, five carrots/replicate (two replicates in total) were collected from different locations during processing (Fig. 16), packed in polyethylene bags, brought to the laboratory and incubated at 25 C as in the previous experiment. Observations of symptoms of black root rot development were made one week later. These samples were obtained 8 times during July-September, 1991.

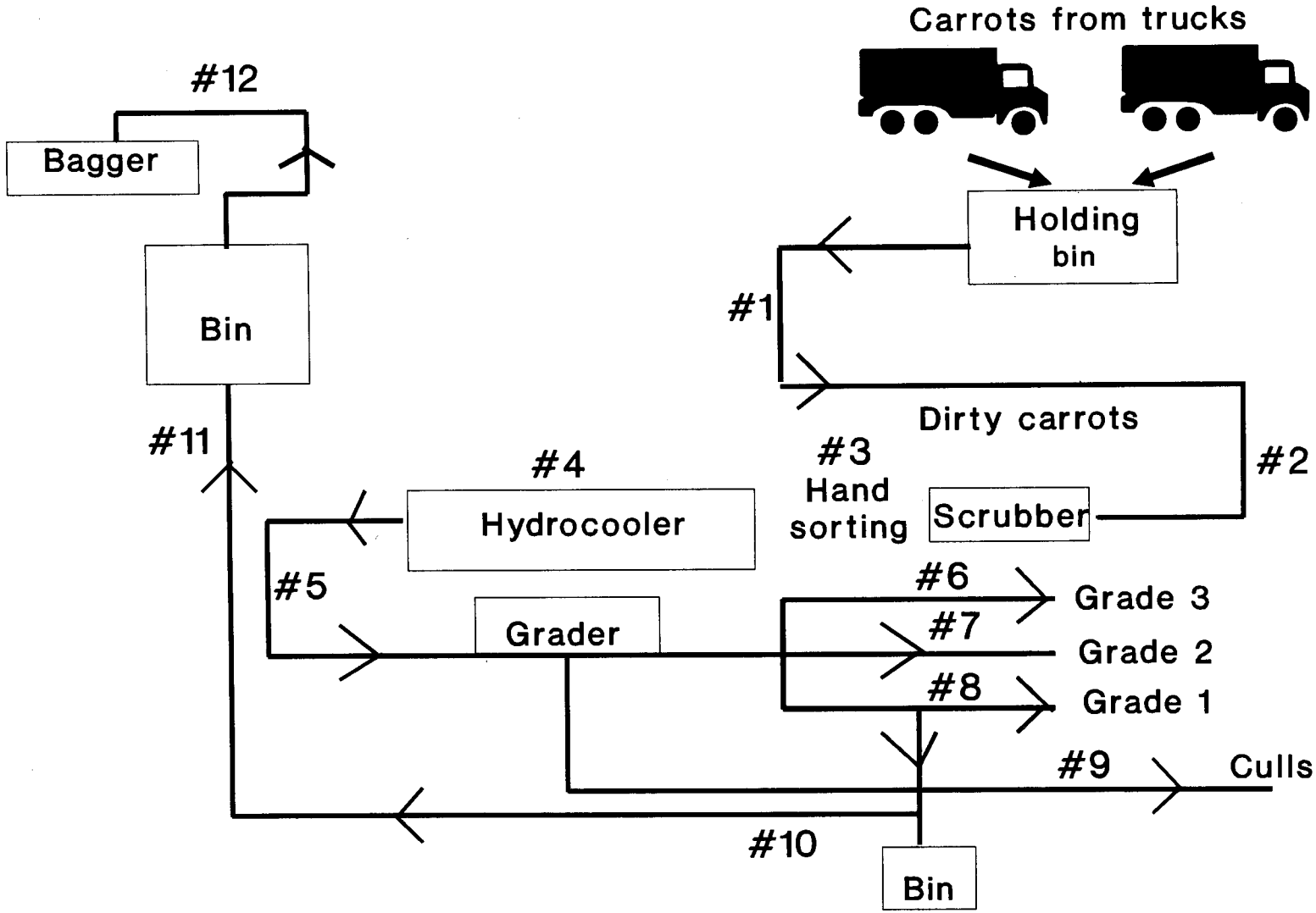

Figure 16. Sites of carrot sampling to determine black root development in the processing plant of the British Columbia Vegetable Co-operative Association, Richmond, B.C., (1991).

LIGHT AND SCANNING ELECTRON MICROSCOPY:

The mode of infection of *C. elegans* was studied using light and scanning electron microscopy. Carrots were hand harvested without wounding, brought to the laboratory, and washed carefully without damaging the periderm of the roots. *C.elegans* was grown on V-8 agar and the phialospores as well as chlamydospores (Mathre and Ravenscroft, 1966) were harvested from two-week-old cultures. Wounded and unwounded carrots were inoculated with either phialospores or chlamydospores. After 24 hr and at 2, 5, and 7 days, 3 mm^2 blocks of infected tissue were cut out from each sample and fixed for either light microscopy or scanning electron microscopy.

For light microscopy studies, the sections were fixed in FAA (1% formalin acetic acid) for at least 24 hr. The sections were washed well with distilled water and then dehydrated through a 10-100% ethanol series. Dehydrated sections were left in 1:1 xylene:100% ethanol mixture and moved to xylene for 2 hr. The sections were washed in two changes of 1:1 xylene:wax mixture and then left in the melted wax at 30 C for at least one hour before embedding in the wax. The embedded tissue was sectioned at 10 μm thickness with a rotary hand microtome. Thin layers of sections were placed on a drop of water on glass slides with albumin fixative. Twenty slides, each with twelve thin sections were made from each block. The slides were left on a slide warmer at thermostat setting 5 (Chicago Surgical and Electrical Co.), to allow the thin sections to dry, expand and adhere to the glass. After 24 hr, the wax was removed by washing in xylene and samples were hydrated with 100%-70% alcohol before staining. The sections were stained with 1% fast green for 30 minutes and dried for 5 minutes on the warmer, washed in xylene and mounted in Permount. The slides were examined with a Zeiss compound microscope and photographs were taken with a Contax 167 MT camera.

For scanning electron microscopy, 3 mm^2 pieces of tissue were obtained from all treatments as well as naturally infected carrots from retail stores. The sections were first fixed in 3% gluteraldehyde in 0.2 M phosphate buffer solution (monobasic sodium phosphate + dibasic sodium phosphate; pH 7.2) for 1 hr and then fixed in 1% Osmium tetroxide. Sections were dehydrated in 10-100% ethanol series at 10% increments, each for 15 minutes. The samples were critical point dried with CO_2, and mounted on stubs with double side sticky cellotape. The specimens were gold coated with using the NRC 3115 vacuum evaporator and examined with an ETEC-model U scanning electron microscope.

RESULTS:

DISEASE DEVELOPMENT IN THE FIELD AND INFLUENCE OF WOUNDING:

Undamaged, hand harvested carrots or carrot seedlings did not develop black root rot or any related symptoms throughout the sampling period. However, when the carrots were wounded during harvesting or washing, black root rot developed on all 20 wounded carrots in 5-7 days.

INFECTION DURING COMMERCIAL PROCESSING:

The percent disease development on carrots at different sites of sampling (Fig. 16) in the processing plant is shown in Table 9. A low level of infection was observed on the machine harvested carrots. After hydrocooling and chlorine treatment, the disease incidence tended to decrease. Disease increased significantly due to wounding from grading and packing practices.

Table 9. Percentage disease development on carrots at different sites of sampling in the processing plant.

	Date of sampling				
Sample No; Location	Jul/18	Aug/2	Aug/14	Aug/22	Aug/19
#1 Belt from holding bin	++	++	-	++	++
#2 Belt prior to scrubber	+++	++++	-	++++	+
#3 Belt leading to cooler	0	++++	++++	+	+
#4 Hydrocooler water	0	_	++++	++	+
#5 Belt after hydrocooler	+	+	0	0	+
#6 Grading belt #3	0	++++	-	+	0
#7 Grading belt #2	++	++++	-	+	+
#8 Grading belt #1	++	++++	-	++++	++
#9 Cull line belt	++++	-	++++	++++	++++
#10 Belt to large bin	+++	-	++++	++	++
#11 Belt to bin before bagger	++++	-	++++	++++	++++
#12 Belt to bagger	+++	-	++++	++++	++++
#13 Packed bag with carrots	++	_	++++	++++	++++

0 = 0 % infection.

+ = 1 - 25 % infection.

++ = 25 - 50 % infection.

+++ = 50 - 75 % infection.

++++ = 75 - 100 % infection.

During the grading process, carrots were observed to drop for about 1.5 m, causing damage to the periderm, and therefore, disease development was also very high after the carrots were graded.

LIGHT SCANNING AND MICROSCOPY:

Light microscopic observations revealed that wounding was essential for the fungus to successfully develop. No infection or very little disease developed on unwounded carrots where the periderm was undamaged (Fig. 17a). In those instances when disease developed on unwounded carrots, it developed only after 7 days and at a high inoculum level ($>10^4$ spores was required), which is not usually found in the fields. On wounded carrots the phialospores germinated within 24 hr and chains of phialospores were produced within 48 hours. After 7 days the infection spread only to a depth of 4-8 cell layers of the cortex (Fig. 17b). The infected cells were packed with intracellular hyphae (Fig. 17c). Aerial chlamydospores were produced after 3-5 days of infection (Fig. 17d). By 7 days chlamydospores were produced within the upper epidermal cells. No intracellular phialospores were observed at any time. Germinating phialospores produced 1 or 2 germ tubes (Fig. 18ab, 19a) within 24 hr near the end of the spore. After infection, phialospores are extruded out in mass (Fig. 18c) from phialides that are produced (Fig. 18d) on the epidermis of the carrot tissue. Some germtubes branched immediately after they were produced, and one penetrated the host cell, while the other produced the aerial phialides, which produces the phialospores (Fig 19d). Chlamydospores were produced as 5-10 spores in a chain (Fig. 20a,b) Occasionally, a single aerial conidiophore branched to produce a phialide on one side and chlamydospores on the other side (Fig. 20c,d).

Fig. 17. Observations on development of *Chalara elegans* on carrot, using light microscopy.
a) An unwounded carrot 7 days after infection.
b) A wounded carrot 7 days after infection.
c) Hyphae inside the infected carrot cells.
d) Chlamydospores and phialospores produced on the epidermis of infected carrot tissue.

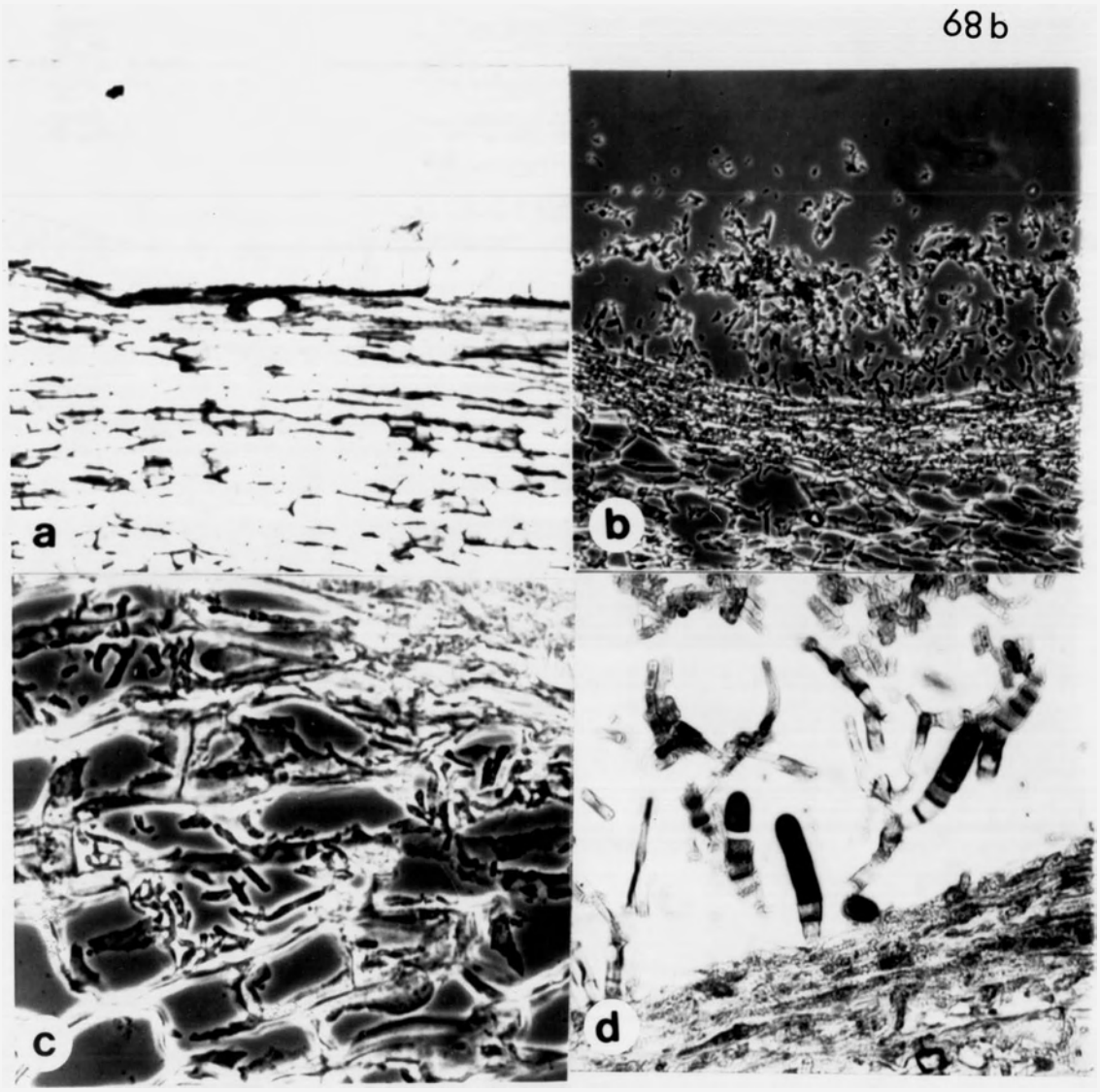
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Fig. 18. SEM of *Chalara elegans* infection and development on carrot.
a) Germination of phialospore at one end to produce germination tube (bar = 4μm).
b) Germination tube produced at the center of a phialospore, and infection hypha (bar = 4μm).
c) Phialospores in mass (bar = 4μm).
d) Mass of phialides. Some phialospores are attached to the phialides (bar = 8μm).

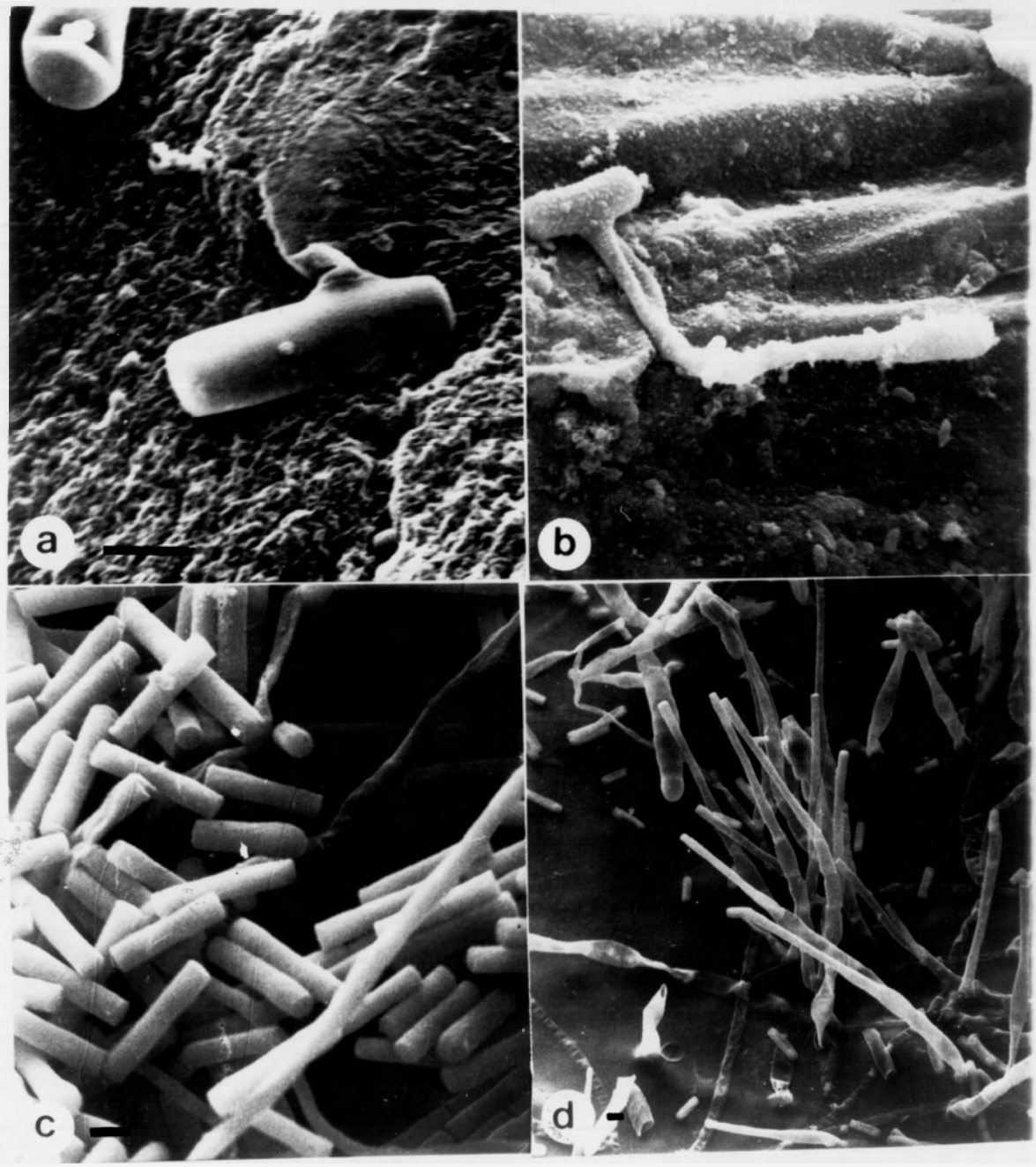
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Fig. 19. SEM of infection process of *Chalara elegans* on carrot.
a) Germinating phialospores with germination tubes (bar = 4μm).
b) Germinating phialospore: Germination tube is penetrating the carrot tissue (bar = 4μm).
c) Aerial phialide extruding a phialospore (bar = 4μm).
d) Germination tube of phialospore branching to produce aerial mycelium and hyphae (bar = 4μm).

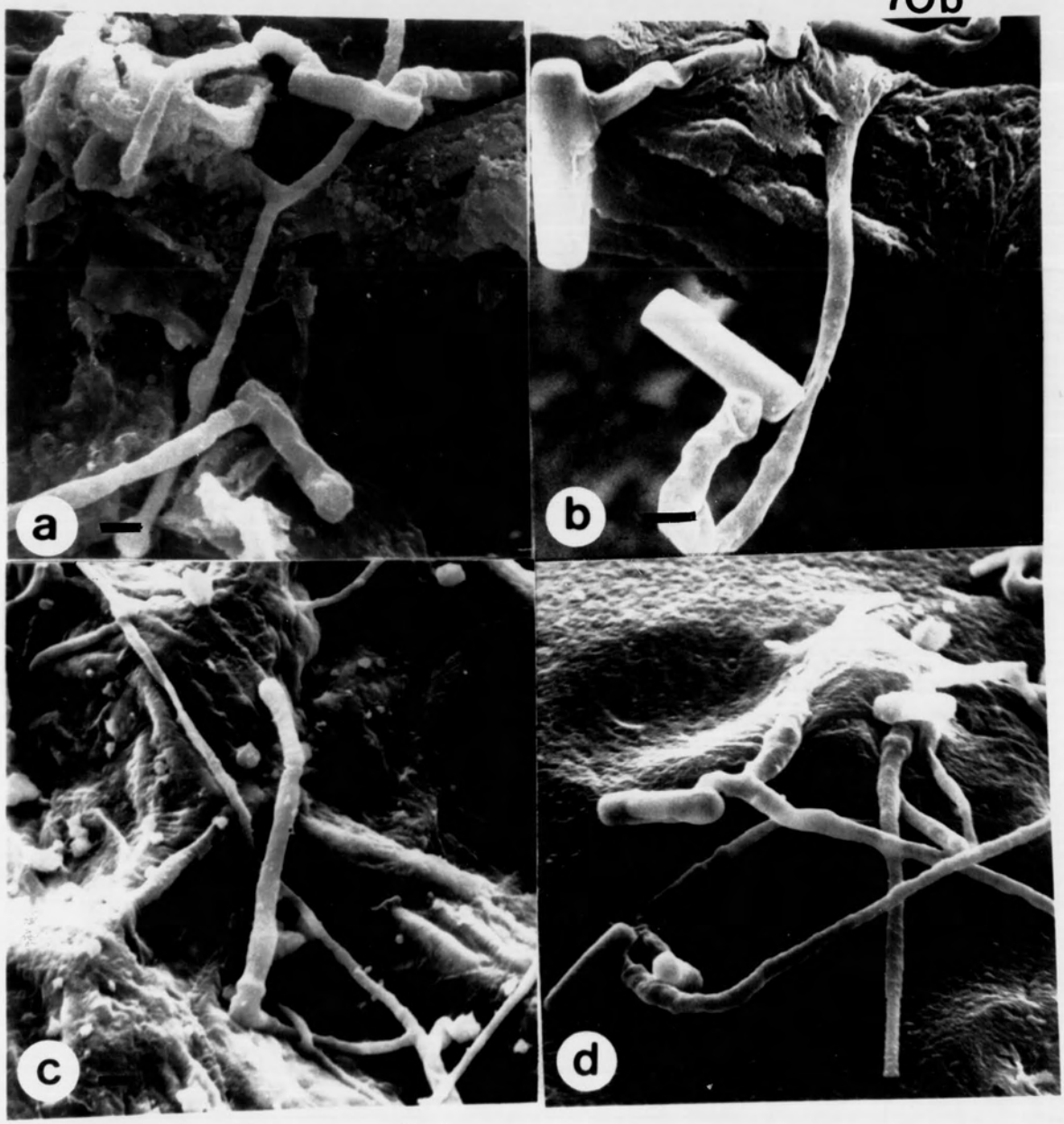
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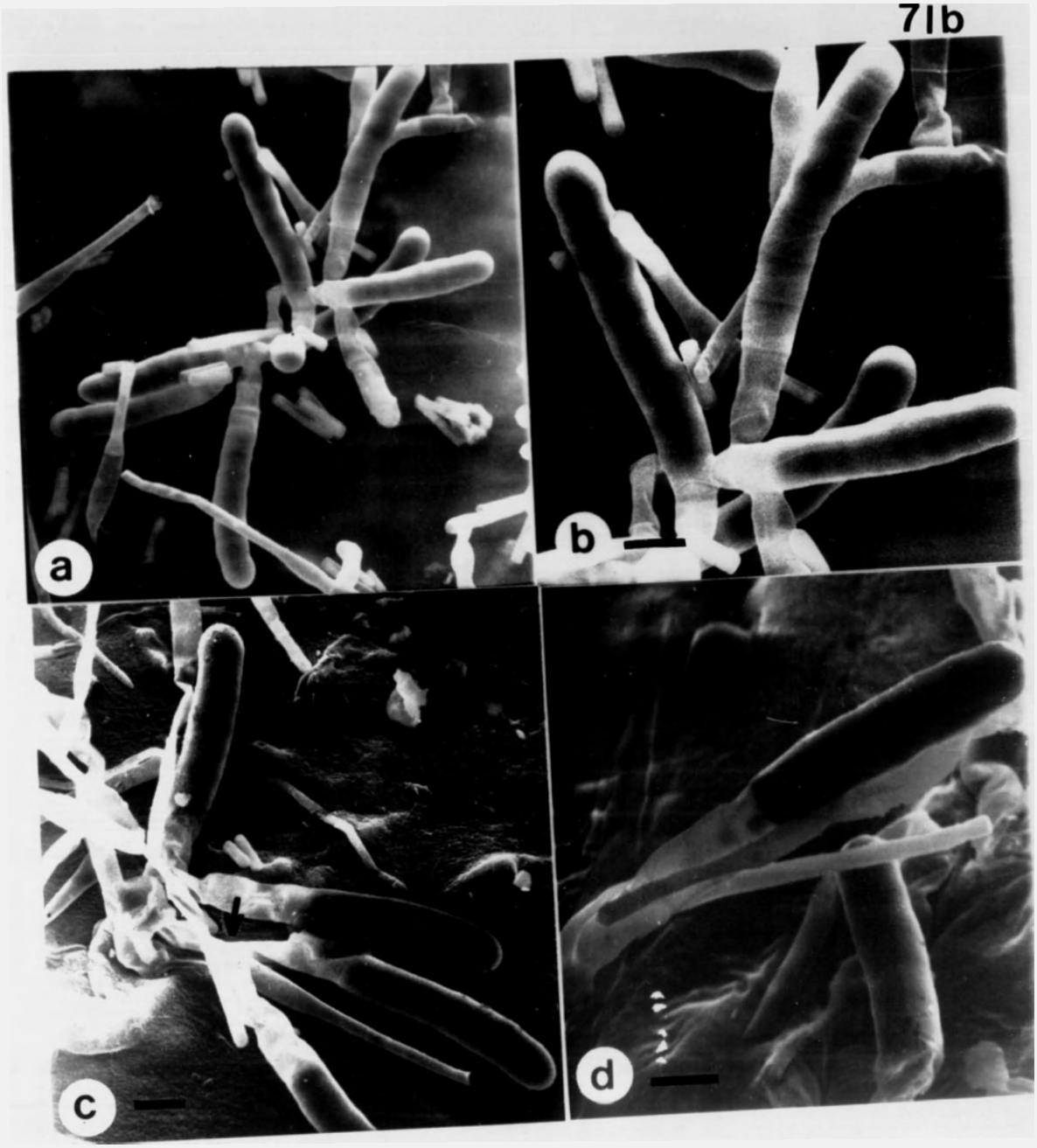
Fig. 20. SEM of development of chlamydospores of *Chalara elegans* on carrot.
a) Chlamydospores and phialospore (bar = 11μm).
b) Enlargement of chain of chlamydospores showing the segmentation. Each segment is considered an individual chlamydospore (bar = 11μm).
c) Chlamydospores (arrow indicates the same conidiophore producing chlamydospores and the phialides (bar = 11μm).
d) Chain of chlamydospores and phialides, produced from the same conidiophore (bar = 11μm).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DISCUSSION:

Under field conditions, *C. elegans* does not cause black root rot on carrot seedlings or carrot plants. Yarwood and Karayiannis (1974) proposed that *C. elegans* may be one of the few organisms which can be pathogenic to a higher plant in one situation and symbiotic in another situation. When carrot seedlings and carrot plants were artificially wounded and inoculated with a high inoculum level (*C. elegans* agar plugs were placed close to seedlings and carrot plants were inoculated with 10^7 phialospores/ml), wounded carrots showed 100% disease, whereas the unwounded seedlings and plants showed no disease and only a few blackening symptoms. In the field, it is possible that carrot plants can be wounded by insects, machinery or natural breaking due to growth. However, when some carrots with growth cracks were incubated in the laboratory, they failed to develop disease symptoms. It is not clear why black root rot does not occur under natural conditions. It may be that growing carrots in the field produce some kind of inhibitory chemical eg. phytoalexins, against *C. elegans* or that wound healing is fast enough to exclude the fungus.

Wounds incurred during harvesting and processing are the major means for the pathogen to enter the carrot roots and to become established within the tissue. This is similar to what Conant (1927) reported on tobacco, or as seen in other storage diseases. Phialospores germinated within 24 hours and penetrated the tissue, whereas germination of chlamydospores occurred later. Following establishment the pathogen produced numerous chains of aerial endoconidia, which could infect other wounded sites. After 3 days, aerial chlamydospores were produced, which could play a role in infecting healthy carrots mainly through contact. After 7 days, chlamydospores were produced within the cells on very short conidiophores. These chlamydospores were mostly curved.

The periderm of carrot roots is probably the most important resistance mechanism against *C. elegans*. Thick walled periderm cells can protect carrots from becoming infected by *C. elegans*. When this tissue was removed by wounding, carrots became susceptible to the pathogen. Although the wounded carrots developed disease, the mycelium and discolored cells were restricted to the upper 5-7 cell layers. It was observed that the wounded and infected cells divided rapidly and thickening of the cell wall occurred in order to contain the fungus. In addition, there may be inhibitory biochemical compounds produced by carrot tissue against *C. elegans*. Hampton (1962) reported that changes in phenolic and fungitoxic compounds occur in carrot root tissue when infected by *C. elegans*. The resistance mechanism of carrot to *C. elegans* in tissues might warrant more studies in order to develop resistance to black root rot.

Wounding of the carrots during harvesting and processing practices is difficult to avoid. Usually, the harvested carrots are brought to the Co-operative with organic soil containing the *C. elegans* inoculum. Therefore, where possible, organic soil should be removed by washing before the carrots are brought to the B.C. Co-operative. Otherwise, there is always a potential for the disease to develop. Although chlorine and hydrocooling reduced the disease, the infections occur later in the packing plant. When the conveyor belts were tested with carrot root discs, the highest number of colonies developed from the belt after the grading process. This is probably due to the accumulation of spores at the later stages of the processing line as well as the increased number of wounds on carrots at this stage. When carrots taken from later stages of processing were stored at 4 C, however, the disease did not develop in 7 days. If these carrots were later incubated at ideal conditions (high humidity, 25 C), which is similar to the environment found in retail shops, the carrots developed black root rot.

At present, the B.C. Coast Vegetable Co-operative Association processing plant has implemented cleaning often and the conveyor belts are scrubbed and sprayed with chlorine, in attempts to reduce the inoculum. However, the plant may require additional changes to reduce wounding of carrots and reduce black root rot further.

GENERAL SUMMARY AND RECOMMENDATIONS FOR FUTURE RESEARCH.

The TBM-RBA medium developed in this study for the quantitative estimation of *C.elegans* can detect an inoculum level of 20 propagules/cm^3. If 20 replicate plates are used, a lower inoculum level (one propagule/cm^3) can be detected. Even though the medium is not as sensitive as carrot root discs, it is superior to all of the other media reported in earlier studies for the quantitative estimation of *C. elegans* in muck soils.

Surveys conducted in 1990 and 1991 indicated that *C. elegans* is prevalent in muck soil in most of the farms sampled in the Cloverdale, B.C. area. When 12 fields were surveyed in 1984 by S. MacDonald and D.J. Ormrod, *C. elegans* was isolated from 8 fields. Population levels among the infested fields sampled in the present research were high (< 10-560 propagules/cm^3). Therefore, avoidance of infested fields is not an effective control method.

As shown in the survival studies, the phialospores of *C. elegans* can survive for more than 20 weeks in muck soils. Therefore, phialospores cannot be disregarded in any survival studies, or as a potential source of inoculum.

The presence of carrot plants did not have any significant influence on the population of *C. elegans* in soil. During machine harvesting, since not all the carrot roots are pulled out, a certain percentage of the roots are left in the soil and allowed to decompose. This practice might increase the populations of *C. elegans* in the soil. Therefore, in future studies, the significance of decomposing carrots on the populations of *C. elegans* in the fields should be addressed.

The effect of soil pH, soil chemistry, other non-host plants (eg: rye, wheat, corn) and different soil amendments on the survival of *C. elegans* in organic soils should be studied, since such studies can be valuable to develop methods to decrease the populations of *C. elegans* in the fields.

Flooding the fields at higher temperatures for 1-3 weeks or rotation with onions may reduce populations of *C. elegans*. If feasible, harvested carrots should be washed immediately by the growers before they are sent to the B.C. Vegetable Co-operative Association packing plant. Since wounding is essential for black root rot development, care should be taken to minimize the injuries during harvesting and packing processes. If necessary, the packing plant should be modified to minimize the extent of wounding, especially during grading of carrots. In addition, chlorine should be applied at a level of 80 μg/ml and the carrots should be hydrocooled as is currently practiced. These control strategies should decrease or may even eliminate development of black root rot.

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