Studies of Components for a Potential Integrated Control system for *Plasmodiophora brassicae*.

Lisa Victoria Page (M.Sc)
Department of
Bioscience and Biotechnology
University of Strathclyde

Dissertation submitted to the University of Strathclyde for the degree of Doctor of Philosophy

September 2001

Declaration.

I declare that the research presented in this thesis was conducted by myself under supervision. I also certify that no part of this thesis has been submitted previously for the award of a degree to any University.

Lisa V. Page.

Acknowledgements.

I would first like to thank my Supervisor Prof. Dixon for all his help, support and discretionary use of a red pen. My thanks go to Hydro Agri and the Ministry of Agriculture, Fisheries and Food for funding this research. Thanks also to Miles Harriman of Hydro Agri for his many visits and unwavering enthusiasm for the research work and to Tore Frogner for his advice from afar.

From Strathclyde University I would like to thank Dr Burge for being a helpful advisor and Irene, Doreen, Dennis and Mick who helped me not only to find my way around Royal College but also miraculously found equipment and supplies no matter how short the notice. Thanks also go to the Strathclyde orienteering club who made a valiant effort to keep me sane throughout my PhD.

Thanks are also due to David, Ultan, Lyn and Glyn for being great mates whilst I was at Auchincruive and beyond. I also owe George a debt of thanks for his enormous help in doing the tractor driving and heavy lifting involved in the field trials.

Last and by no means least I would like to thank my Mom and Dad for keeping me on the straight and narrow, and encouraging me to keep going without their help I would never have got this far, and my husband Roger for putting up with the stress, tears and tantrums.

Thanks everyone you were great.

Abstract

The soil inhabiting organism *Plasmodiophora brassicae* infects brassica crops causing millions of pounds of damage each year. The result of infection is extensive galling of the root system and eventually plant death. Current control measures are limited and the variability of the pathogen and its lifecycle makes finding new controls difficult. It was therefore considered that if several measures could be identified which reduced *P. brassicae* infection then they could be used in combination, reducing the possibility that the pathogen could evolve to overcome the controls.

This research therefore aimed to find a set of control measures, and where possible a mode of action, which could be used in combination or alone to reduce *P. brassicae* infections.

Calcium nitrate was identified as an effective control measure which affected several stages of *P. brassicaes* lifecycle (Fig:I) as well as the predominant pathogen race. The type of growth medium used was also found to affect the extent of infection and to influence the pathogen population.

Soils from two areas were identified as suppressive to *P. brassicae* and the nature of their suppression was determined to be due to both biotic and abiotic factors. The identification of these suppressive soils may lead to the development of a bio-control or it may be possible to encourage these soils to develop in other areas.

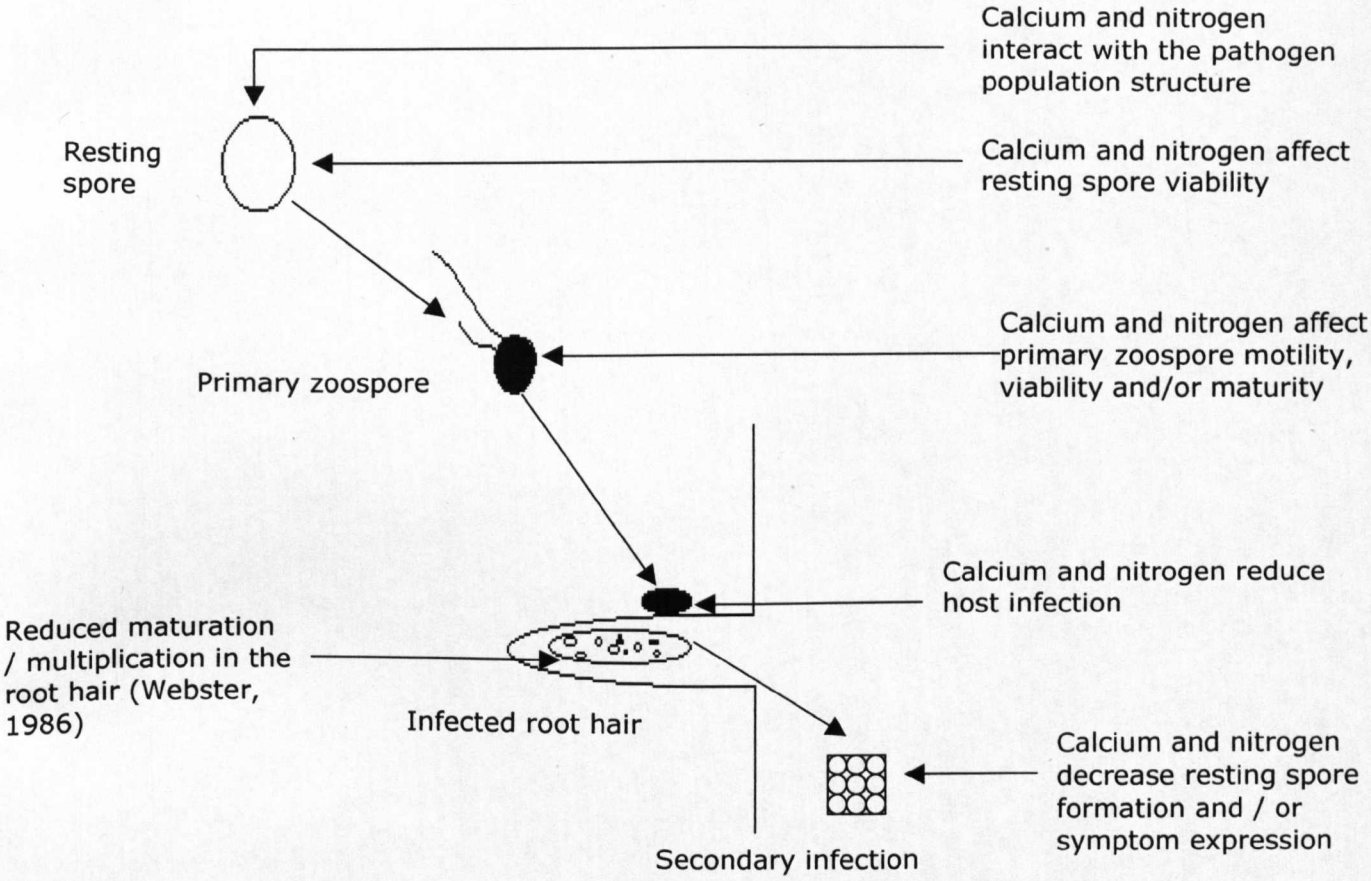
Some more "holistic" control measures were also investigated. Applications of calcified seaweed were found to be at least as effective in decreasing clubbing as calcium carbonate. Applications

of chitin and seaweed extract however were found to have no effect on *P. brassicae* infection in this instance.

The research within this thesis has identified several control measures and answered some questions about *P. brassicae*. It has however, also raised more questions and identified areas which require more research.

Fig I: Synthesis of Suppression of *Plasmodiophora brassicae.*

Suggested interactions between *P. brassicae* calcium and nitrogen.



Other Influences of calcium and nitrogen

1. Possible effects on antagonistic organisms
2. Possible effects on host resistance mechanism
3. Alteration in *P. brassicaes* physical and chemical environment

Contents

Chapter One.

Literature Review.

Literature review.

Introduction

This literature review aims to give an overview of the life cycle of *Plasmodiophora brassicae*, its effects on the host, physiological specialisation, biology and control.

What is *Plasmodiophora brassicae* ?

There is debate as to what *Plasmodiophora brassicae* is. The organism is placed in the order Plasmodiophorales, family Plasmodiophoraceae (Braselton, 1995). Woronin (1878) identified *Plasmodiophora brassicae* as a protist which resembled the Myxomycetes but differed in the absence of a true sporangial membrane. *Plasmodiophora brassicae* has subsequently been re-classified several times (Buczaki, 1983a). The movement *of P. brassicae* through the Proteomyxa of the Protozoa, Mycetozoa, Chytridiales, Archimycetes and many other classifications is catalogued by Karling (1968) and Buczaki (1983a). Currently, the Plasmodiophorales are classified under the order Protozoa. The arguments as to the positioning of the *P. brassicae* were well thought out and were logical but there always seemed to be an un-co-operative element of the Plasmodiophorales which disagreed with the classification. This debate exemplifies the intriguing problems associated with the study of *P. brassicae*.

The Plasmodiophorales.

Members of the Plasmodiophorales are characterised by the formation of naked multi-nucleate plasmodia during the lifecycle (Agrios, 1988). Plasmodiophorales are all obligate parasites (Tommerup and Ingram, 1971).

The order Plasmodiophorales consists of nine genera and thirty five species of organisms which parasitise algae, fungi and higher plants (Karling, 1968). The Plasmodiophorales include several economically important plant pathogens e.g. *Spongospora subterranea* (powdery scab of potatoes), *Polymyxa betae* (vector for beet necrotic yellow vein virus which affects sugar beet), *Polymyxa graminis* (a pathogen of cereal roots) and *Plasmodiophora brassicae* (causing clubroot of crucifers) (Karling, 1968).

Plasmodiophora brassicae.

Plasmodiophora brassicae was first identified by Woronin in 1873 following a competition in Russia to find the cause of "cabbage hernia" (Clubroot). The competition was initiated because of the devastation being caused to brassica crops, the staple diet in Eastern Europe (Zadoks & Schein, 1979).

It is considered that *P. brassicae* originated in Southern Europe or the Western Mediterranean although it has not been found in wild brassicae populations (Dixon, 1981). *Plasmodiophora brassicae* is thought to have been subsequently transported to other areas by

cultivated turnips (*Brassica rapa*) and is now a common problem world-wide (Dixon, 1981).

***Plasmodiophora brassicae* races.**

Cook and Schwartz (1929) postulated that the variation in *P. brassicae* resting spore size may be due to the

> "splitting of *Plasmodiophora brassicae* into several species which are in all probability only host varieties"

Physiological specialisation of *P. brassicae* was later demonstrated by Honig in 1931 (Buczaki *et al.* 1975). Honig discovered differences in the virulence of inocula derived from several clubs and even within clubs from one plant. The races of *P. brassicae* are distinguished by their virulence to specific hosts. In order to produce resistant crops there is a need to identify individual races and determine the range of the resistance available. Buczaki *et al.* (1975) devised the European Clubroot Differential set (ECD) for this purpose. More recently, Jones *et al.* (1982a) and Voorips (1995) have attempted to use the ECD series for the diagnosis of using a single spore inoculation technique but this system has not yet been accepted on a wide scale.

The existence of physiological races is important as populations of *P. brassicae* are very variable not only between fields but also within fields leading to macro and micro geographical variation (Anon 1982). This can lead to the performance of control measures being variable.

Hosts of *Plasmodiophora brassicae.*

The host range of *P. brassicae* extends to all cruciferous plants, including economically important crops such as cabbage (*B. oleracea* var. capitata), swede (*B. napus*), oilseed rape (*B. napus, B. rapa*), and kale (*B. oleracea var* acephala) as well as weeds such as charlock (*Sinapis arvensis*) and shepherd's purse (*Capsella busa-pastoris*). It is generally considered that *Brassica rapa* vegetables are more susceptible to infection than *B. oleracea* types (Yoshikawa & Buczaki, 1978).

Primary stages of infection have been found in non-cruciferous plants. This was observed by Webb (1949) who grew the grass *Holcus lanatus* in a field infected with *P. brassicae* to determine whether *P. brassicae* could maintain itself in the absence of a suitable cruciferous host. The root hairs of *H. lanctus* were found to be infected by *P. brassicae* but there was no subsequent gall development. Similar infections have been observed in the root hairs of perennial ryegrass (*Lolium perenne*) again without gall formation (Macfarlane, 1952; Karling, 1968).

There is no evidence of gall formation in non-cruciferous plants suggesting that *P. brassicae* is unable to complete its life cycle within such hosts. It is not clear however, whether *P. brassicae* is able to maintain the population by the continuous release of secondary zoospores which infect other non-cruciferous hosts until a suitable cruciferous host is available (Macfarlane, 1955). This would explain why crop rotations and the use of break crops are sometimes ineffective in reducing *P. brassicae* infection in succeeding brassica crops.

Knowledge of the lifecycle of *P. brassicae* is therefore very important in developing effective clubroot control strategies. In order to control the pathogen it is necessary to identify stages of the lifecycle which may be influenced by control measures and whether the treatment can be used to attack *P. brassicae* at more than one stage. In identifying the lifecycle stage affected by a control the optimum application time can be determined.

Lifecycle of *Plasmodiophora brassicae.*

The *Plasmodiophora brassicae* lifecycle has been observed for over 100 years and it is still not completely understood. A preliminary lifecycle was published by Woronin in 1878. In 1929 Cook and Schwartz determined that the lifecycle consisted of two phases, one taking place in the root hairs the other in the root cortex. The root hair stage of infection was extensively studied by Samuel and Garrett (1945). A more detailed lifecycle covering both phases of infection, was published almost 100 years later by Tommerup and Ingram in 1971. In 1972 a revised lifecycle was released by Ingram and Tommerup, and is the most likely lifecycle for *P. brassicae*. Infection processes occur in two stages, primary and secondary development. Primary infection begins with the release of a zoospore from the resting spore and progresses through a number of stages prior to gall formation.

Primary infection.

Resting Spores.

Plasmodiophora brassicae resting spores are extremely robust and are able to remain viable after exposure to a wide range of environmental conditions. The resting spores are spherical (3-5 μm diameter) and may remain viable for at least 20 years (Buczaki *et al.*, 1979). Spores do germinate spontaneously and it is considered that the half life of *P. brassicae* resting spores is 3.6 years. It would therefore take 18 years, in the absence of a suitable host, for a field population to decrease to 3 % of the original spore population (Wallenhammar, 1996). This is in line with Buczaki's statement (1979).

Whilst resting spores are located inside the host plant gall the spores surface appears granular and covered in spines. Prior to release to the external environment a fibrous layer is deposited onto the spore surface which gives the spores their robustness (Buczaki, *et al.*, 1979). Through microscopic observation a small area in the resting spore coat which is thinner than other areas has been observed. It is considered that this area provides a point of emergence for the zoospore contained within (Buczaki *et al.*, 1979).

Resting spores are induced to germinate by the presence of root exudates (Suzuki *et al.*, 1992), wet acid soils and a soil temperature of around 25°C. Exudates from some plants e.g. tomatoes decrease spore germination (Yoshikawa *et al.*, 1978).

Once the spore germinates a single, motile, biflagellate, uninucleate protoplast called a primary zoospore (Fig. 1) is released.

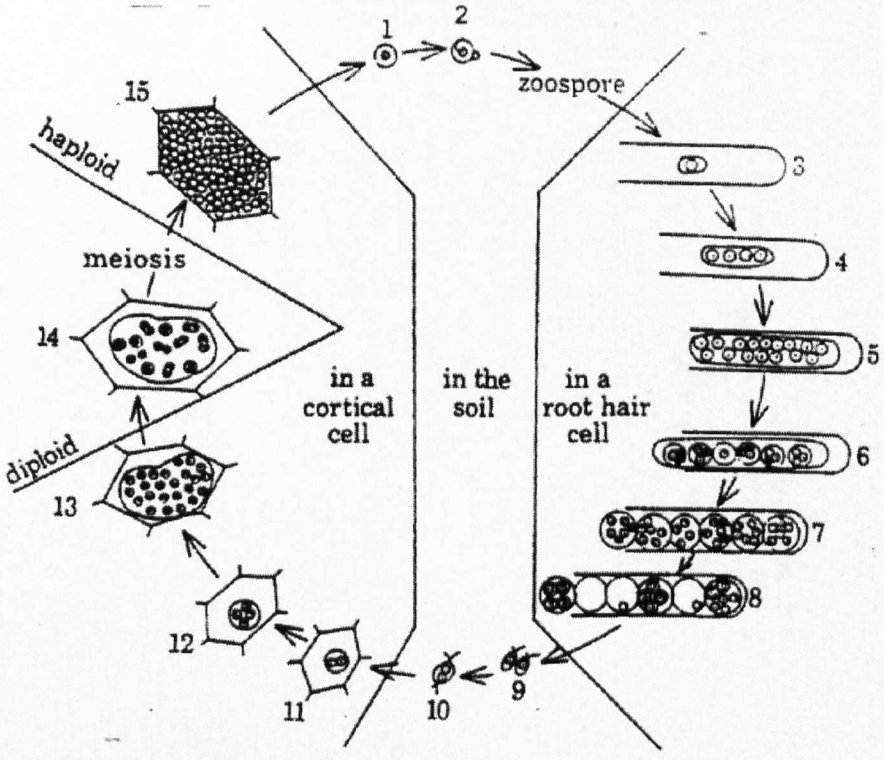
Primary zoospores.

The primary zoospores measure approximately 2-4μm in diameter (Tommerup and Ingram, 1971). The zoospore has two anterior flagella which are unequal in length. These propel the zoospore through the soil moisture to a susceptible host root hair, where it encysts (Ingram and Tommerup, 1972). (Fig 1). Infection of the host follows.

Figure 1. Detailed life-cycle of *Plasmodiophora brassicae.*

From Ingram & Tommerup (1972).

Where 1^{o} = primary & 2^{o} = secondary stages of infection.



Primary Phase

1. Resting spore
2. Resting spore germination
3. Infected root hair containing uninucleate plasmodium (n)
4. Synchronous mitotic divisions of nuclei in the primary plasmodium(n)
5. Multinucleate primary plasmodium(n)
6. Cleavage to yield zoosporangia(1-6 nuclei per zoosporangia (n))
7. Mitosis of zoosporangial nuclei
8. Cleavage of the cytoplasm to yield uninucleate zoospores (n) which are released through a pore
9. Free zoospores (n)
10. Plasmogamy (n+n)

Secondary Phase

11. Binucleate secondary plasmodium (2n)
12. Mitotic divisions of plasmodial nuclei
13. Multinucleate secondary plasmodium (n+n+n....)
14. Karyogamy within the secondary plasmodium (2n)
15. Cleavage of the plasmodial cytoplasm to yield haploid resting spores (n)

Root hair infection.

A detailed study of infection processes was made by Williams *et al.* (1971) using the host *B. oleracea* cv Jersey Queen. They concluded that a primary zoospore may collide several times with a root hair before becoming attached. The zoospore attaches to the root hair on the posterior side.

Once attached the zoospore flattens and the flagella coil around the zoospore which at this stage is filled with ribosomes, lipid bodies, mitochondria and various vesicles.

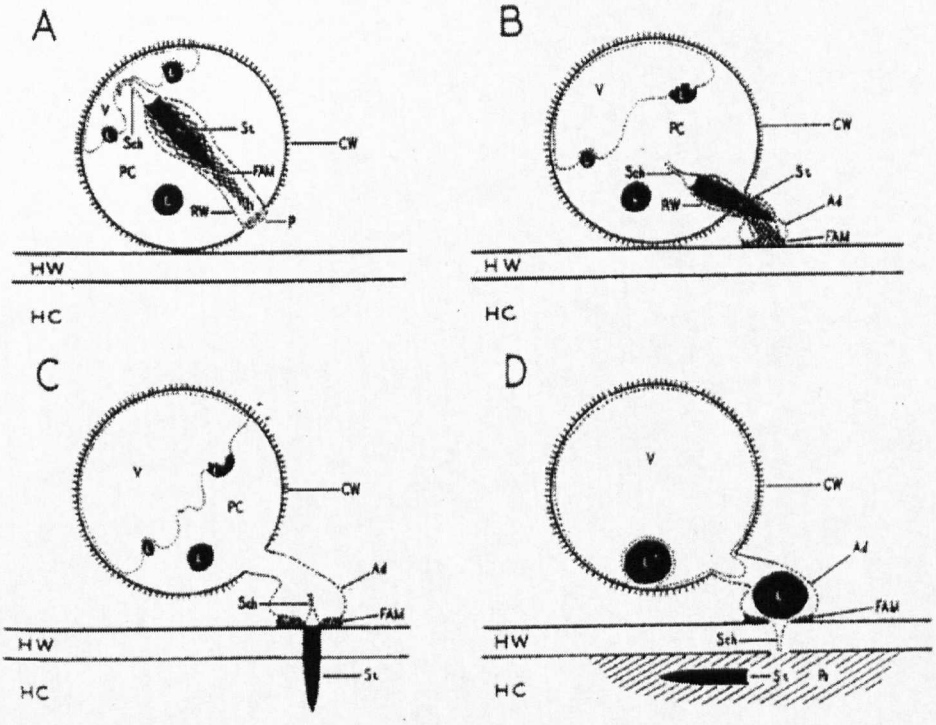
The flagella are retracted into the zoospore body and then absorbed. The zoospore body "rounds up" and along a tubular vesicle, the "rohr" develops (Fig 2) which is bound by a cyst plasma membrane.

The open end of the rohr is directed towards the host cell wall. Contained within the rohr is a sharp pointed rod, "the statchel". The vacuole enlarges filling half of the cyst and the end of the rohr swells forming an adhesorium, which enables the cyst to attach onto the root hair.

Pressure is created inside the cyst by the enlargement of the cyst vacuole and this forces the statchel through the adhesorium and into the host cell wall. The zoospore protoplasm is injected into the host cell upon evagination of the rohr forming a primary plasmodium within the host root hair.

The infection process is considered mechanical as no enzymes have yet been detected. This is one of the best understood sections of the *P. brassicae* lifecycle.

Figure 2. Diagram illustrating infection processes of a primary zoospore.
From Aist and Williams (1971)



Ad: Adhesorium, CW: cyst wall, FAM: Fibrillar adhesive material, HC: host cytoplasm, HW: host wall, L: lipid body, N: nucleus, P: rohr plug, Pa: papilla, PC: parasite cytoplasm, R: rohr, RW: rohr wall, Sch: Schlauch, St: statchel, V: cyst vacuole, VG: vesicle with granular matrix.

(A) Cyst vacuole has not yet enlarged
(B) Vacuole enlarges and small adhesorium appears
(C) Statchel punctures host wall
(D) Penetration has occurred and hot protoplast has deposited a papilla at the penetration site

Primary plasmodium.

The uninucleate plasmodium undergoes several mitotic divisions resulting in a multinucleate plasmodium (Ayers, 1944). The plasmodium enlarges and the number of nuclei increases until the root hair is filled by the pathogen. More than one infection can occur in a root hair but the infections progress through the lifecycle independently (Webster, 1986). Infections may also occur in waves i.e. a root hair may be undergoing primary infection whilst secondary infection is occurring in the tap root.

The plasmodium undergoes sporogenesis producing 4^{10} zoosporangia each approximately 80 μm in diameter (Tommerup and Ingram, 1971). Each possesses a single nucleus approximately 30 μm in diameter (Ingram and Tommerup, 1972). The zoosporangia release a second generation of motile spores, secondary zoospores. Ayers (1944) observed the passage of the secondary zoospores through a pore in the zoosporangial membrane which was in contact with the cell wall into the soil environment. Samuel and Garrett (1945) observed these released secondary zoospores infecting the main root system resulting in cortical infection.

Further research by Tommerup and Ingram (1971) in line with observations by Buczaki and Clay (1984) found that whilst some zoospores were released into the soil most went into the root hair lumen where they underwent plasmogamy. Plasmogamy is the fusion of two zoospores to produce a single binucleate zoospore approximately 6 μm in diameter.

Secondary zoospores released into the soil environment may also be responsible for isolated galls on the lateral roots (Buczaki, 1977) but secondary zoospores released into the root hair lumen were responsible for most gall formation (Dobson and Gabrielson, 1983. Mithen and Magrath (1992) however observed myxamoeba in the root cortex of *Arabidopsis thalliana* during maturation of secondary zoospores within root hairs. They concluded that it is possible for small plasmodia and myxamoeba to move into cortical cells without having to mature into secondary zoospores.

Secondary zoospores released into the lumen move to the root hair base using pseudopodia which aid their movement through the chain of evacuated zoosporangia to the root. Once the zoospores reach the root hair base they encyst in a manner similar to primary zoospores upon attachment to a root hair (Aist and Williams, 1971; Buczaki and Clay, 1984). These then move into the root cortical cells. This leads to secondary infection of the host which is thought to be responsible for the galling of host roots (Dobson & Gabrielson, 1983; Buczaki, 1988).

Secondary Infection

Cortical cell infection.

Upon entering the cortical cells the secondary zoospores form binucleate plasmodia. These undergo 12 mitotic divisions to produce multi-nucleate secondary plasmodia (Ingram and Tommerup, 1972) (Fig 1). The secondary plasmodia spread in all directions through the root cortex from the initial point of infection

and small plasmodia are responsible for disruption in the phloem and cambium regions of the host.

Undifferentiated host tissues are most susceptible to infection by secondary plasmodia as they are rapid growing and offer minimal resistance. They are therefore the first areas in the cortex to become infected. Once plasmodia become multi-nucleate, karyogamy takes place (nuclei associate in pairs) and then meiosis immediately prior to sporogenesis (Buczaki and Moxham, 1980). The resulting plasmodia cleave, producing haploid resting spores which are released by decomposition of galled tissue (Ingram and Tommerup, 1972).

Gustaffson *et al.*, (1986) observed secondary invasion in oilseed rape roots similar to that described by Ingram & Tommerup (1972) in *B. rapa* roots. Gustaffson *et al.* (1986) also observed that as the pathogen passed through the cortical cells hypertrophy (excessive growth due to enlargement of individual cells (Dixon, 1984) and hyperplasia (excessive growth due to increased cell division of cells (Dixon, 1984)) occurred inducing gall formation. These observations concur with those of Ingram & Tommerup (1972), Buczaki (1977) and Mehorta (1980) who considered that gall formation is caused by secondary infection.

Host symptoms.

Physical changes

The most useful diagnostic symptom in host plants infected by *P. brassicae* is the formation of irregular galls on the roots of the host (Agrios, 1988). These galls or clubs, which are described as the "finger and toe" effect by Anderson (1853), may form on the lateral roots of the host or on the main tap root. Buczaki (1988) stated, however, that a single gall on the main tap root is more serious than the formation of galls on the lateral roots as this would interfere with the root cortex functions of water and nutrient transport. Infected plants are also prone to wilting even when adequate water is available, indicating that the hosts water transport mechanism is in some way being compromised (Brokenshire, Channon & Wale, 1984). Plants may also become stunted and the foliage often turns purple (Anon, 1984).

The time taken for the gall to form is also important in determining how long the host will survive once gall formation is initiated (Buczaki, 1988).

Galls have also been observed growing on the aerial parts of some plants such as on the hypocotol of swedes (*Brassica rapa*) (Dixon, 1981) and on the aerial shoots of water cress (*Nasturtium spp*) (Buczaki, 1988).

Cellular and biochemical host changes.

Apart from galling symptoms observed on the roots of infected hosts, invasion by *P. brassicae* has a number of other effects on

host plants. Although secondary infections cause the most serious damage to host roots, primary infections can cause root hairs to become distorted. Williams, Aist & Aist (1971) observed that root hairs of cabbages infected with *P. brassicae* may be stunted and / or distorted as did Samuel and Garrett (1945). They also noted that root hairs which were less than 12 hours old at the time of infection were more likely to become clubbed.

Upon secondary infection, however, more detrimental changes occur within the root cortex which eventually lead to gall formation. Once a secondary plasmodium forms within a host cell the nucleus of the host cell has been observed to increase in size. Williams *et al.* (1971) considered this to be a fundamental part of a successful parasitic establishment. Infection by secondary plasmodia or secondary zoospores in cambium cells, cortical xylem, parenchyma and xylem rays exhibit pathological proliferation followed by hypertrophy (Slepyan & Minina, 1979).

The structure of lateral roots in the region of gall formation is disrupted more markedly than that of the main tap root. Invasion of the medullary rays leads to distortion of the cells (Kunkel, 1918) resulting in severe disruption of the hosts nutrient and water supply, causing infected plants to wilt (Carlile, 1988). Secondary infection also leads to an alteration in the partitioning of carbon in host plants resulting in the gall becoming an additional carbon sink (Evans and Scholes, 1995).

Kunkel (1918) observed that cells some distance away from the infection became swollen and suggested that a substance may be released from infected cells either by the host or the pathogen which diffuses into surrounding healthy tissue. In contrast to this

Williams (1966) stated that healthy cells adjacent to infected cells did not exhibit hypertrophy and appeared identical to cells from un-infected hypocotols. However, the possibility that the pathogen could produce a small diffusable molecule which would stimulate adjacent cells to divide abnormally was not discounted by Williams (1966).

Williams (1966) examined sections of young clubs and found that infection by *P. brassicae* induced cellular, nuclear and nuceolar enlargement and noted that nuclear and nuceolar hypertrophy appeared to be closely related to cellular infection and that the parasite may alter or control the host cell nucleic acid metabolism.

This is not the only biochemical change which has been observed in infected cells. Hypertrophied cells of host plants were found by Williams (1966) to contain up to 16 times more DNA than uninfected cells. Cytokinins have also been found to play a role in the formation of galls. Infected turnip roots have been reported by Dekhuijzen & Overeem (1971) to contain 10 to 100 times higher levels of cytokinin than healthy roots. In further studies it was found that callus tissues infected with *P. brassicae* grew on media without the addition of cytokinins or auxins unlike healthy tissue which needed to be supplemented with both hormones. This suggested that the infection induces an increase in auxin production.

It is not clear whether the pathogen provides these growth regulators or whether the pathogen stimulates the host to produce an excess of these chemicals (Ingram, 1969). It is more likely that the pathogen stimulates host production as Butcher, El-Tigani & Ingram (1974) found indole glucosinolates and glucobrassin

become de-compartmentalised within galled tissue, suggesting that the host is stimulated by the pathogen to increase production of auxins and cytokinins. A similar increase in auxin production has been observed in the green islands produced by cereal rusts (Dekhuijzen & Overeem, 1971) where it is argued that the increase in production is from the host.

To produce this wide range of effects upon the host *P. brassicae* must first infect the host, and there are a wide range of environmental factors which influence infection.

Factors influencing infection by *Plasmodiophora brassicae*.

The level of infection and the extent of pathogen invasion is in part dependent upon a range of factors which can increase or decrease the damage caused to the host by the pathogen, these factors include soil moisture, temperature, pH, inoculum level, host resistance and nutrients. These factors will now be considered in more detail.

Soil moisture.

Soil moisture is important to clubroot infection as primary zoospores require moisture to swim from the resting spore to the host (Watson, 1967). Resting spores also need to absorb water before being able to germinate (Macfarlane, 1970). It has often been noticed that clubroot is more severe in waterlogged or poorly drained areas of infected fields (Anon, 1982). This is in line with the work of Thuma, Rowe & Madden, (1983) who found a direct relationship between increased soil moisture content and the level of primary infection i.e. as the moisture content of the soil is

increased the level of infection increased. The occurrence of clubroot in soils of high pH was observed by Colhoun (1953a) where there was a high soil moisture content. Work by Monteith (1924) illustrated that at low soil temperatures infection was aided by a high soil moisture content. Infections have been seen to occur at soil temperatures as low as 12°C in association with high soil moisture content (Anon, 1984).

Hamilton and Crête (1978) stated that the soil type also played an important role in determining the soil moisture requirements. They found that a moisture content of only 9% was required in mineral soils whereas a soil moisture content of 60% was needed in organic soils.

Soil temperature.

Monteith (1924) investigated the effects of soil temperature upon clubroot infection. Plants of the cabbage cv, Copenhagen Market, were grown in cans of clubroot infected soil maintained at a range of temperatures by soil temperature tanks. Monteith (1924) found that uniform clubs only developed on the plants in soil maintained at higher temperatures and concluded that clubroot requires a soil temperature above 12°C for infection to occur, and that the optimum temperature was between 20 and 25°C.

Work by Colhoun (1953a) confirmed that the optimum temperature for infection was 23°C but suggested that the minimum temperature for infection may be as high as 18 or 19.5°C. Buczaki, *et al.* (1978) showed that the optimum temperature for infection was within the range of 20-24°C. Temperatures of 50°C however,

have been reported to be needed to kill *P. brassicae* resting spores by Porter & Merrimen (1985) during an investigation into the effectiveness of solarisation as a control measure.

Buczaki *et al.* (1978) suggested that the influence of soil temperature may be greatest during the second week of infection. During this study it was also noted that light intensity may play a role in influencing infection particularly during the second and third week of infection.

Inoculum concentration.

The effect of inoculum concentration upon infection is an important factor. Macfarlane (1952) stated that the number of root hair infections increases with increasing resting spore concentration. Samuel & Garret (1945) found that the level of root hair infection increased with increasing resting spore density and that at high alkalinity (pH 7.2) infection only occurred at the higher resting spore concentrations of 10^6 and 10^7. At a pH of 6.2 however, infection occurred at all spore concentrations. This was supported by the work of Colhoun (1953b) who also concluded that the level of clubroot infection increases with increasing resting spore concentration.

Soil pH.

The optimum pH for clubroot infection is approximately 6.0 (slightly acidic soils) (Macfarlane, 1952). Alkaline soils are known to reduce clubroot development, and it is recommended for good control of clubroot that the pH of the soil be raised to between 7.3

and 7.5 (Anon, 1984). However care must be taken when liming due to the wide variability of soil pH within a small area and because different soil types respond differently to the application of lime. It is important that lime is incorporated well into the soil as even micro-sites within the soil where the pH of the soil is not high enough will allow infection (Dobson *et al.*, 1983).

There is debate as to whether the alkalinity of the soil is responsible for decreased infection or whether this is partly due to the increase in calcium ions (Dixon & Webster, 1988). Earlier work by Samuel & Garrett (1945), in which soil alkalinity was altered using nutrient solutions which did not contain calcium, suggested that it was the direct effect of pH which decreased the level of infection.

A soil pH greater than 7.2 was found by Myers & Campbell (1985) to decrease primary infections due to abortion of primary thalli before the release of secondary zoospores. At a soil pH greater than 8.0, thalli that did develop zoospores were misshapen and aborted and no clubs developed. This supports the suggestion that secondary zoospores are necessary for gall development. Infection can occur in alkaline soils if the soil inoculum levels are sufficiently high (Colhoun, 1953a; Karling 1968).

Control measures used to prevent *Plasmodiophora brassicae* infection.

Plasmodiophora brassicae is very difficult to control and a variety of measures are used to prevent infection due to the pathogens longevity in the soil and its ability to overcome host plant

resistance. The controls may be used independently or in combination.

Resistance to *Plasmodiophora brassicae.*

Some species within the brassicas are more resistant to clubroot than others. It is generally considered that *Brassica rapa* vegetables, for example, turnips are more susceptible than *B. oleracea,* and within the *B. oleracea* cabbage is considered to be more resistant than cauliflower or broccoli (Yoshikawa & Buczaki, 1978).

There have been many attempts to try to improve the resistance of crops to *P. brassicae* but due to the variability of the *P. brassicae* population no cultivar is currently resistant to all strains of the pathogen (Macfarlane, 1955; Karling, 1968). Problems are encountered in resistance breeding because of the small number of sources of resistance and the recessive and often complex inheritance of the resistance along with the genetic variability of the pathogen (Voorips, 1995). He suggested the presence of resistance within a host could conceivably block or hamper pathogen development, but even a very low survival rate of *P. brassicae* during the root hair stage would allow infection of the root cortex. This leads to pathogen races which are able to complete their lifecycle in a resistant host, reproducing whilst other pathogen races fail, resulting in a higher percentage of the population being capable of overcoming host resistance in the next season.

Where resistance has been achieved it has often been short lived. The cabbage cultivar, Badger schipper, which possessed *P.*

brassicae resistance, only lasted three years due to the selection of genotypes within the pathogen population which were able to overcome this resistance (Seaman & Larson, 1963). Galls may develop on resistant cultivars due to the presence of a compatible pathogen genotype at a low level within the *P. brassicae* population (Dekhuijzen, 1979; Williamson, 1987).

Husbandry

A number of general measures can be taken to prevent contamination of an area and to decrease the level of *P. brassicae* infection in affected areas.

In order to prevent contamination of an area, machinery and clothing used in contaminated areas should not be used on uncontaminated land. This in practice is difficult: if a farm has only half its land contaminated it is not feasible to own two sets of machinery, but machinery should be disinfected before being used in different areas to minimise the risk of contamination.

The majority of vegetable brassica crops are bought as module transplants which are then transplanted into fields. It is recommended by the ADAS that modules should only be bought from reputable sources. Reputable sources however cannot guarantee clubroot free modules as the compost they are using may be infected or, as in a case in Sweden, the modules were placed outside a glasshouse to harden but became infected during a storm which blew contaminated soil over the area (Wallenhammer, Per Comm, 1998).

Manure from animals fed on infected brassicas should not be used to fertilise fields as the *P. brassicae* resting spores can survive digestion. This means that manure from animals can act as an inoculation source (Anon, 1982).

In infected areas some husbandry methods can decrease the incidence of *P. brassicae*. Liming (the application of calcium carbonate) is the oldest control method used in the fight against clubroot (Fletcher *et al.*, 1982). It is recommended that calcium carbonate should be applied at a rate sufficient to raise the soil pH to 7.3-7.5 (Anon, 1984). Hamilton and Crête (1978) reported however that liming to pH 7.0 can decrease the crop yield as the availability of other nutrients can become limited.

The effectiveness of calcium carbonate applications is not guaranteed (Monteith, 1924). This is due to a number of factors including the spore load, soil moisture and soil temperature (Colhoun, 1953a).

The application of calcium carbonate is also a factor as it is difficult to apply across a whole area and get a uniform pH. Some areas may still have a relatively low pH. Dobson *et al.* (1983a) also reported that micro pores may be present around the root system which are unaffected by the calcium carbonate application and allow the disease to develop. Hanseler (1937) reported that 2-3 years of calcium carbonate applications were required before the disease was reduced. Colhoun (1953a) however reported that lime was effective in decreasing *P. brassicae* infection provided the spore load was not excessive after only one application of calcium carbonate.

There is great debate as to whether raising the soil pH is responsible for the decrease in *P. brassicae* infection or the presence of calcium ions. This argument will be discussed under the title of calcium in the section on the effects of nutrients.

Crop rotation is an old method employed in attempts to decrease infection. A rotation period of four years between cruciferous crops should be used to reduce inoculum. A break of 18 years would be required, however to eradicate the inoculum (Wallenhammer, 1996). During the break years an effort to remove all cruciferous weeds is also needed. The presence of other plants however may maintain the *P. brassicae* population as has been previously discussed. Bremer (1924) suggested that in the absence of a susceptible host an acid soil would increase the rate of spore decay. The ability to maintain an acid soil would however be dependent upon the crop being grown and its requirements.

Soil solarisation has been explored as a means of control for *P. brassicae.* Porter and Merriman. (1985) found that heating infected soil to 50°C decreased the incidence of clubroot. The effectiveness of this technique was however dependent upon the location, soil type, soil moisture, soil temperature and the inoculum level.

The method is therefore very difficult to use and in areas of the UK which are *P. brassicae* infected, obtaining soil temperatures of 50°C prior to planting is not possible. In later experiments Porter *et al.* (1991) found that *P. brassicae* resting spores could survive for more than 28 days at 45°C in dry soil but died at 40°C after 14 days in moist soil demonstrating how an alteration in one factor can greatly influence the effectiveness of *P. brassicae* infection.

The use of catch crops has also been recommended. Macfarlane (1952) observed that fewer infections occurred on cabbage seedlings after crucifers or ryegrass had been grown. Catch crops are sown into an infected area, prior to the crop being planted, and destroyed before gall formation. The presence of these catch crops encourages the germination of resting spores. The plants become infected and are then destroyed before the pathogen completes its lifecycle ensuring that there is no return of innoculum to the infected area. This reduces the number of resting spores in the area immediately prior to planting. In Japan some success has been reported in planting Daikon prior to brassica crops being grown (Murakami *et al.*, 2000).

A more recent development in husbandry control of *P. brassicae* is put forward by Ekeberg *et al.* (1997). In a number of field experiments Ekeberg established that a reduction in the amount of soil tillage decreased the level of *P. brassicae* infection. The experiment compared using deep tine, shallow tine and minimum tillage cultivation, the yields increased by 23%, 52% and 59% respectively. The effects were deemed to be caused by a change in cultivation practice as the application of calcium carbonate increased the yield but did not influence the effect of tillage. The soil pH was 0.1-0.3 units lower with decreased tillage which was considered insufficient to have any effect on disease incidence.

Work by Chellemi *et al.* (1998) on the use of minimum tillage as an alternative to fumigation with methyl bromide highlighted some of the problems with the system. In their research the failure to remove broad leaf weeds resulted in the tomato crop being infected with *Meloidogyne* spp. The incidence of tomato spotted wilt was also 100 % higher in the minimum tillage plots. The yield of the

alternative system was therefore lower than that of plots treated with methyl bromide.

The decreased input cost of the minimum tillage system resulted in the profit from the alternative plots being 568 US$ per hectare higher than the methyl bromide system. These research projects illustrate how a decreased input system may decrease disease in some systems and increase it in others, but that the overall profit of such a system should be considered before the idea is discounted.

The control of *P. brassicae* by husbandry therefore requires an integrated approach which takes into account characteristics such as soil pH, moisture and temperature of the region.

Chemical control of soil borne diseases

Chemical control of soil borne diseases, especially for clubroot, is notoriously difficult. Ensuring that the soil is treated uniformly is only one of the problems associated which chemical control of clubroot. Many chemicals used to control *P. brassicae* are general soil sterilants such as methyl bromide and mercury based compounds. With many of these compounds being either already banned, or about to be banned there has been renewed interest in chemical control, but many of these chemicals have also had adverse environmental effects.

Chemical control of *Plasmodiophora brassicae.*

There has been little success in developing a chemical control for *P. brassicae* which has a uniform effect in all conditions due to the

variability of the pathogen and its ability to rapidly develop resistance. The development of chemical controls for any plant pathogen is a long process with huge development and registration costs. These factors have not however deterred researchers from looking for potential controls.

Mattusch (1979) recommended two main points which should be considered when developing a chemical control for *P. brassicae*.

1. "The main parameter must be the assessment of yield as the number of marketable cabbage heads.
2. There has been little success in finding materials able to protect the crop throughout the growing period. The effectiveness of the materials must be seen, therefore, from the standpoint of protecting plants from attack during the first 4-5 weeks. The fungicide must be able to bring the plants into a position of developing sufficient healthy root during early development. "

These two statements take into account one of the main problems with chemical control assessment, even if a control does not completely eliminate *P. brassicae* infection it may still reduce the extent of clubbing and increase yield making its use viable. Potential chemicals should therefore be judged on the resulting profit to a grower through an increased yield and not solely on its ability to decrease infection.

With the removal of mercury based chemicals from the market including mercurous chloride (calomel) which was used for many years as a clubroot control and thiophanate-methyl which was determined as a potential control by Buczaki and Stevenson (1981)

there is a need for replacement chemical controls (Humpherson – Jones, 1983).

The potential of several other chemicals has been investigated with some degree of success. The main chemicals which have been examined are, flusulfamide, trichloramide and PCNB (penta chloro nitro benzene). More recently epoxydon and non-ionic surfactants have also been shown to reduce clubroot. Each of these chemicals will now be considered individually.

Pentachloronitrobenzene (PCNB)

Pentachloronitrobenzene was shown to be active against *P. brassicae* by Smeilton in 1939. Further studies showed that the effects of PCNB *on P. brassicae* in the UK were inconsistent (Chanon 1959). In Japan however where Quintozene is the most widely used PCNB (Yoshikawa, 1978) it has been shown to be superior to calcium hydroxide and calcium cyanamide applications in decreasing *P. brassicae* infection. Miller (1979) reported the effectiveness of using Brassicol a 75% PCNB fungicide in decreasing the extent of infection in crops in Malaysia when it was applied to the planting holes of module transplants.

The mode of action of PCNB is not clear. Naiki and Dixon (1987) conducted a range of experiments on the effects of Quintozene on *P. brassicae* and concluded that PCNB does not effect spore viability but it does effect the early stages of *P. brassicae* infection.

Gabrielson (1979) compared the effectiveness of PCNB with calcium carbonate and a combination of PCNB and calcium carbonate. The results showed that applications of calcium

carbonate with PCNB had the greatest effect in reducing *P. brassicae* infection and that by using calcium carbonate with PCNB the number of seedlings per plot at emergence was increased. Gabrielson (1979) concluded that the effect of combining PCNB and calcium carbonate was additive and not synergistic.

Robak (1979) compared the effects of several PCNB containing fungicides and found that the most effective were those which acted systemically for the control of *P. brassicae*. The effectiveness of the fungicides was also enhanced when they were applied as a root dip in combination with peat moss and methyl cellulose.

PCNBs are detrimental to the environment and so other chemicals have been sought resulting in the discovery of trichlamide (Ohmori *et al.*, 1982).

Trichlamide.

Trichlamide is a salicyl amide derivative and has been described as a soil applied fungicide with activity against *P. brassicae* (Dixon and Wilson, 1984a). The effectiveness of trichlamide has been reported by Dixon and Wilson (1984a,b) and Buczaki (1983).

Buczaki (1983) reported that the use of trichlamide resulted in a decrease in the percentage of diseased plants but without a significant effect on the symptoms expressed on infected plants or the percentage of plants dying due to infection. Dixon and Wilson (1984a) however stated that in experiments where trichlamide was applied at a rate of 45 kg a.i. ha^{-1} there was a decrease in symptom expression and an increase in yield. At a lower application rate (30 kg a.i. ha^{-1}) trichlamide was still effective and

despite the higher level of disease the heads weights were higher. This may indicate that at higher rates of application trichlamide is phyto-toxic.

Trichlamide is used extensively in Japan for the control of clubroot.

Mode of Action.

Naiki and Dixon (1987) found that trichlamide had a major effect on *P. brassicae* infection seven days after plants became infected through pot experiments. They therefore concluded that trichlamide effects the pathogen during secondary development and must attack *P. brassicae* in the hosts' cortical tissue.

Benomyl (Methyl I (butylcarbomoyl) benzimidazol - 2- ryl carbamate) (MBC)

Benomyl was described by Delp and Klopping (1968) as a protective and eradicant fungicide with systemic activity. Jacobsen and Williams (1969) reported the effectiveness of benomyl against *P. brasssicae*. Benomyl is one of the chemicals used as a mercury replacement (Naiki and Dixon, 1987). The use of these chemicals is now more restrictive due to the development of resistance in pathogens such as eyespot (*Pseudocercosporella herpotrichoides*).

Mode of Action.

The experiments of Naiki and Dixon (1987) established that Benomyl increases in activity with time and that it effects both primary and secondary stages of infection. Benomyl is also known to have the ability to bind to tubulin, a protein known to be involved in the mitotic phase of cell division (Wallenhammer,1999)

Flusulfamide.

Flusulfamide was recommended as a control measure *for P. brassicae* by Shimotori *et al.* (1996). It is a sulphonamide and it has been evaluated in UK trials. In field trials where flusulfamide has been incorporated into the top 10 cm of cropping beds prior to planting the level of *P. brassicae* infection decreased by 78%. The field inoculum potential was also decreased (Dixon *et al.* 1998).

Flusulfamide is currently used in Japan and is being registered in the UK (Dixon, pers. comm).

Mode of Action.

Hildebrand *et al.* (1998) suggested that the mode of action of flusulfamide is to suppress resting spore germination by adsorbing to the resting spore walls. Further work in Japan has established that flusulfamide is ineffective once the infection has reached the cortical stage but is effective when applied to infected soil prior to planting. Staining revealed that the resting spores remained viable following treatment with flusulfamide suggesting that whilst resting spore germination is suppressed spore viability is unaffected (Tanaka *et al.*, 1999).

Non-ionic Surfactants.

Surfactants are used widely with other pesticides to improve the wettability of leaves. Humpherson-Jones (1993) compared the effectiveness of two sodium dioctyl sulphosuccinate surfactants with three fungicides. The surfactants and fungicides were applied separately and in combination. Pour treatments with the surfactant Agral and a combined soak / pour treatment with dichlorophen reduced disease severity and increased yields by 250% and 97% respectively. Hildebrand (1998) also reported the effectiveness of non-ionic surfactants in decreasing host infection by *P. brassicae*. In the absence of *P. brassicae* though applications of the surfactants were phyto-toxic and so there is a need to correlate the level of infection with the surfactant application rate. Agral is recommended by the Horticulture development Council (HDC) for the control of *P. brassicae* (Dixon, pers. comm).

Mode of Action.

The mode of action has not yet been established but it is likely to be due to the lytic effect of the surfactants on *P. brassicae* zoospores (Hildebrand, 1998).Although surfactants are effective and cheap they have a persistent nature and toxicity to aquatic ecosystems (Hildebrand, 1998).

Other chemicals.

Fluazinam is an anti fungal compound, which has been assessed for its effectiveness against *P. brassicae* in Japan (Suzuki *et al.*, 1995) and Australia (Porter *et al*, 1998). Fluazinam is not used in Europe due to manufacturers restrictions.

Cyanoimidazole is a systemic broad spectrum fungicide which is effective against oomycetes (*Phytophthora and Pythium*). Mitani *et al.* (1998) found cyanoimidazole to be more effective than flusulfamide. It affects the respiratory chain of the pathogen.

Research into epoxydon (5-hydroxy-3-(hydroxymethyl)-7-oxolate bicyclo [4.1.0] hepta-3-en-2-one) may pave the way for a new range of agrochemicals. Epoxydon was obtained from a culture of *Phoma glomerata* (No. 324 = JCM 9972) from the leaves of *Viola spp*. Comparisons of the effectiveness of epoxydon with several anti-auxins indicated that the anti auxin 2, 3, 5 tri-iodo benzoic acid was the active ingredient (Arie *et al.*, 1998).

This is an example of how research into a biological control can result in the production of a useable agrochemical. More research into biological controls which can be translated into agrochemical production will probably follow and this may become the new route for the production of agrochemicals as in the case of Strobilurins.

In conclusion there is no reliably effective chemical control currently available in Europe for the control of *P. brassicae* and therefore husbandry and nutrient applications remain the main method of control.

These examples of chemical control highlight how many stages of the *P. brassicae* lifecycle may be affected by a chemical and how variable the results from similar research can be.

Biological Control.

Biological control was defined by Garrett (1965) as

"any conditions under which, or practice whereby, survival or activity of a pathogen is reduced through the agency of any other living organism (except man himself), with the result that there is a reduction in the incidence of the disease caused by the pathogen"

Biological control not only includes individual organisms which reduce disease but also husbandry methods such as intercropping, flooding, rotations, and the application of manure (Lockeretz, 1983). These measures which influence the biological components of the habitat are referred to as "indirect biological control" (Campbell, 1989).

The bio-control of soil-borne pathogens has concentrated mainly on the introduction of bacteria which are deleterious to pathogens by producing antibiotics or out competing them (Baker, 1986) This is known as "direct biological control" (Campbell 1989). These bacteria are generally referred to as antagonists (Weller, 1988). Antagonists are often isolated from soils which are naturally suppressive to a particular pathogen e.g. *Pseudomonas fluorescens* may be responsible for the decline in the amount of Take All (*Guammanomyces graminis*) after four years of continuous cropping (Campbell, 1989).

Direct biological control is summarised by Odunfa & Oso (1979)

"Soil micro-organisms may stimulate, inhibit or suppress the growth of soil borne pathogens and may also affect root respiration

(Katznelson & Rouatt, 1957), mineral nutrient uptake (SubbaRao, Bidwell & Bailey, 1961) and plant growth (Pidoplichko, Moskovets & Zhdanova, 1965)"

Many potential biological control agents have been identified for plant disease control from plant tissue or suppressive soil samples. These control pathogens on agar or in plant tissue samples e.g. tuber slices (Rhodes *et al.*, 1986). It is notoriously difficult however to get soil micro organisms to grow in a laboratory environment let alone to encourage them to act in their role as bio-control agents (Renwick *et al.,* 1991).

Despite these problems several biological controls are commercially available including *Agrobacterium radiobacter* strain 84, which controls *Agrobacterium tumefaciens* (crown gall), *Bacillus subtilus* A13 a peanut seed treatment, *Bacillus subtilus* for control of apple canker (*Nectria galligena*), *Peniophora* for the control of heart rot of pines (*Heterobasidion annosum*) and *Trichoderma* spp. used for soil borne disease control (Weller, 1988 and Campbell 1989).

The inconsistent performance of direct biological control is a major obstacle to it's wide spread use (Weller, 1988) and no direct biological controls are currently available for use against *P. brassicae.* The occurrence of suppressive soils may however help to identify potential organisms or groups of organisms, which would suppress *P. brassicae* upon application to infected soils. Due to the variability of the pathogen, though it is unlikely that a single bio-control agent would be sufficient to provide field control of the pathogen.

The majority of research has concentrated on isolating specific biological control agents for specific plant pathogens. Reports of research in this area describe isolation techniques and the results of screening processes. The common thread of the results is that in the laboratory, with time, it is possible to identify antagonistic organisms, but that when placed in a field environment the effectiveness is lost. The isolation techniques used do not take into account the soil ecosystem as a whole, hence the majority of controls fail in the field. Isolates obtained on agar may be identified because the medium or environmental conditions particularly suit them. In the soil however these organisms may be less competitive. For research into biological control to be successful attention needs to be moved away from the laboratory and into the field.

The future of biological control may therefore not lie with specific control agents, which require extended periods of time to be isolated and are unreliable in the field environment but with a more indirect method of biological control such as suppressive soils.

Suppressive soils.

Several examples of suppressive soils are currently well documented. The occurrence of take all decline after prolonged wheat monoculture is the classic example of a suppressive soil. The occurrence of take all decline has been extensively researched but is still far from fully understood (Hornby, 1979, Rouxel, 1991). It is considered that during the years of continuous monoculture there is an increase in antagonists to *Guammanomyces graminis* in the soil microbial population (Hornby, 1979). Soils suppressive to

Streptomyces scabies (Common Scab of Potatoes) have also been investigated (Menzies,1959).

Soils suppressive to *Fusarium* wilt of muskmelon have been identified in the Chateaurenard region of France. Transferring samples of suppressive soils to conducive soils decreased the incidence of wilt (Alabouvette, 1986) and this is considered to be a characteristic of suppressive soils. During the identification of the agent responsible for the suppressive quality of the soils 400 micro-organisms were isolated and tested, of which only several non-pathogenic strains of *Fusarium oxysporum* were found to reduce the levels of *Fusarium* wilt (Larkin *et al.*, 1996).

This is probably part of the reason why large companies are not interested in screening for biological controls. Biological control agents are not widely available for the control of plant diseases in a field environment due to these difficulties (McQuilken,1995).

Soils suppressive to *Plasmodiophora brassicae.*

Some soils suppressive to *P. brassicae* have been identified. In some instances the soils did not remain suppressive after sterilisation, indicating that a biological agent was not responsible and that the physical/chemical properties of the soil were responsible for the reduction in disease intensity (Jaw-Fen and Wen-Hsui, 1986, Hseih and Jaw-Fen, 1986, Tsushima *et al.*, 1996 and Workhu and Gerhardson, 1996).

Further studies have identified soils which are suppressive to clubroot and lose their suppressiveness upon sterilisation, indicating the action of a biotic factor (Murakami *et al.* 1997).

Djatnika (1991) isolated microbes from suppressive soils, one isolate of which could reduce *P. brassicae* infection from 90.3% to 46.7% in glasshouse trials. In field trials the isolate was not as effective in decreasing *P. brassicae* infection and its performance was further affected by field conditions which proved to be unfavourable to the suppressive soil isolate. Rouxel (1984, 1988) studied the receptiveness of soils in Brittany to *P. brassicae* and concluded that although biotic suppressive soils were present their performance was influenced by the soil pH. This indicated the presence of a specific type of microflora but the bio-control agent was not identified.

Work by Takahashi (1994) also identified suppressive soils. In Takahashi's research the viability of *P. brassicae* spores incubated in natural and autoclave sterilised soils in the absence of host roots was compared. The addition of glucose to the natural soil increased the number of non-viable spores indicating a biotic nature to the soil suppressiveness, but again no specific micro-organisms were identified.

Kroll *et al.* (1984) showed that where soil containing *P. brassicae* was inoculated with isolates from the root surface of radishes, and the extent of infection was measured. Some organisms increased infection whilst others decreased infection.

Six bacterial strains were identified by Kroll *et al.* (1984) which decreased the incidence of *P. brassicae* in glasshouse environments. The addition of the antibiotic ampicillin to a liquid growth medium used for growing radish plants resulted in a significant increase in the level of *P. brassicae* infection indicating the involvement of bacteria in the reduction of *P. brassicae*

infection. It was also suggested that as *P. brassicae* is an obligate parasite the bacterial isolates may have an indirect influence upon the host rather than the pathogen.

Elsherif and Grossman (1991) screened the antagonistic abilities of 141 *Pseudomonas* spp. against a range of pathogens including *P. brassicae*. Nineteen isolates reduced the level of *P. brassicae* infection with varying degrees of efficiency depending on the mode of application. Einhorn *et al.* (1991) compared several screening methods both *in vitro* and *in vivo* to identify antagonists to *P. brassicae* and reported on their usefulness.

More specific bio-control agents have been identified for the control of *P. brassicae*. Tsushima *et al.* (1997) tested the efficacy of six fungal isolates and found that four of them were effective in decreasing clubbing when applied as a root dip. Root colonising fungi have also been isolated from Chinese cabbages grown in a variety of soils. Two isolates from the *Heteroconium chaetospira* were effective in sterile and non-sterile soil. The hyphae of these two fungal isolates covered the root surface and extensively colonised the inner cortical tissues (Narisawa *et al.*, 1998). These isolates appeared to be promising biological control agents, but once again their effectiveness in a field environment has not been established

An alternative to isolating specific organisms from a suppressive soil is to encourage the microflora as a whole in the hope of producing a suppressive soil. One method in which to do this is to green manure soils (green plant material is ploughed into the soil prior to the planting of a crop). Green manuring is a more indirect form of biological control and is widely used especially in organic

farming (Parry, 1990). The effect of green manuring is therefore not immediate in the case *of Pythium spp.* a period of two weeks is required between manuring and planting (Lampkin, 1990).

Work by Millard and Taylor (1927) established that applying a green manure to trenches prior to potato planting decreased the incidence of *Streptomyces scabies* (Common Scab). Later experiments using a green manure of soybeans confirmed these findings and in addition an increase in the population of *Bacillus subtilus* was observed (Weller, 1988).

In the case of *P. brassicae* a more economical method of green manuring may be to use green composts in modules or in crop rows rather than building up the microflora across the entire field area. This method would also encourage a broad micro-organism population which would be more difficult for *P. brassicae* pathogen races to become resistant too.

Green composts.

Hadar and Mandelbaum 1992 have investigated using green composts for pathogen control. Example of its effectiveness include the reduction of *Sclerotinia minor* on lettuce (Lumsden *et al.*, 1983) and *Pythium ultimum* and *Rhizoctonia solani* on legumes (Schüler *et al.*, 1989). Composts such as tree bark (Stephens and Stebbins, 1985), hemlock bark (Kai et al, 1990) liquorice roots (Hadar and Mandelbaum, 1986) and grape marc (Gorodecki and Hadar,1990) have also been shown to decrease disease. The control of disease has been attributed not only to an increased microbial population but also an increase in the availability of nitrogen, phosphorous, magnesium and calcium.

Experiments using composts from municipal waste have shown that the these composts effectively eradicated *P. brassicae* as well as reducing the extent of other soil borne disease. No specific micro organism was identified (Hedges, 1996)

A suppressive compost is defined as

"An environment in which disease development is reduced although the pathogen is introduced in the presence of a susceptible plant"

The suppression is due to re-colonisation of the compost by antagonistic micro-organisms during the cooling phase of composting (Hadar and Mandelbaum, 1992).

More conventional composts however may also help to reduce plant disease. Sphagnum peat composts have generally been considered to be conducive to disease, but they may also be useful in the fight against disease. Research into the effect of these composts on *Pythium* root rot of poinsettia has shown that these composts varied in their suppressiveness depending on how far the peat was decomposed, with the least decomposed peat being the most suppressive (Boehm and Hoitink, 1992).

A different way in which to use composts may be to employ them in a process known as root camouflage. Gilbert *et al.* (1994) suggested the theory of root camouflage to explain how altering the root environment can alter the level of disease incidence. The research on this hypothesis was stimulated by Timonin *et al.* (1940) who determined that the number of bacteria in the rhizosphere is higher in non-rhizosphere soils. Research into the

bacteria in the rhizosphere of tobacco and flax plants which varied in their resistance to black root rot and Fusarium wilt showed that the roots of susceptible cultivars had higher densities of *in-vitro* culturable bacteria.

Gilbert *et al.* (1994) reviewed experiments by West and Lochead (1940) in which it had been established that the micro-flora in the rhizosphere of resistant plants were an intermediate between those found in suppressive soils and the bulk soil. In combination with research of their own they concluded that disease severity is greatest when the rhizosphere population differed greatly from the soil community. Disease control may therefore be possible by raising transplants in composts with a microflora similar to the soil environment into which they will be planted. This is of importance especially in crops such as vegetable brassicas where seedlings are raised in compost modules and then planted into the bulk soil.

When using composts however the hazards from the compost contents e.g. other pathogens, (human or plant), heavy metal content should be considered before they are used (Mc Quilken, 1995).

Factors which influence the suppressiveness of a soil or a compost include soil texture, pH, moisture content, organic matter content and mineral or nutrient composition. Suppressiveness may therefore be encouraged by altering these factors (Broadbent & Baker, 1974, Boehm & Hoitink, 1992). The addition of physical and chemical factors which enhance the soil suppressiveness are also likely to enhance the capacities of micro organisms for biological control (Campbell, 1989). Therefore the addition of organically derived factors, such as chitin and seaweed extracts, to the soil

environment can alter the suppressive quality of a soil and help to decrease the level of disease. These methods provide a more holistic approach to disease control.

Seaweed extracts

Seaweed has been applied to fields as a nutritional supplement and soil conditioner for centuries (Blunden, 1991). An advantage of using seaweed over compost or manure is that it does not contain weed seeds or fungal spores. It is also high in potash and lower in phosphate than farmyard manure which suits crops such as potatoes, cabbages and grass (Stephenson, 1968).

The use of seaweed extracts is associated with an increase in seed germination (Senn and Skelton, 1969) and root growth (Finnie and van Staden, 1985, Crouch and van Stadden, 1991, Blunden and Wildgoose, 1977). Cogram (1994) confirmed this using wheat but the increase in root growth was only recorded in plants in the presence of a natural soil microflora or *Pseudomonas* spp. The root growth increases and increased seed germination are considered to be due to the presence of cytokinins in the seaweed extracts (Wilczek and Ng, 1982, Finnie and van Staden, 1985) Increases in crop yield of wheat, grass, vines, celery and tomatoes have also been reported (Stephenson, 1976).

Seaweed extracts have been reported to decrease the incidence of plant diseases such as grey mould of strawberries (*Botryis cinerea*), turnip powdery mildew (*Erysiphe polygoni*) damping off of tomatoes (Stephenson, 1965) and take all (*G. graminis*) (Cogram,1994).

Walsh (1997) observed an increase in extracellular activity when liquid seaweed extract was applied to potting compost and a direct relationship between the microbial population and concentration of seaweed extract. The growth of both bacteria and fungi was increased by liquid seaweed applications but the growth of bacteria was favoured.

The application of seaweed extracts to a soil environment may therefore promote soil suppressiveness by increasing the soil micro-flora populations and hence the level of enzyme activity and it may be possible to use this suppressiveness to control *P. brassicae*.

Chitin

The study of chitin is becoming very popular due to the interests of molecular biologists in chitinases and their role in plant defence mechanisms (Shirashi, 1999). Since the spore walls of *P. brassicae* are composed, in part, of chitin the potential exists for control by employing techniques which may enhance chitinolytic activity in soils harbouring the pathogen.

Applications of chitin to soil have successfully reduced soil-borne plant pathogens. *Fusarium oxysporum ssp. pisi* (pea wilt) was reduced by 82% when chitin was applied to the soil several weeks prior to planting. An increase in the actinomycete population was also noted. Soil amendments with chitin have also been shown to decrease the incidence of *Rhizoctonia solani* and *Sclerotium rolfsii* infections (Sneh *et al.*, 1971). Applications of 0.1 % liquid chitosan (a derivative of crab shell chitin) solution to grape plants decreased the level of infection of the pants by mildew (Gorbatenko, 1996)

The application of chitin to soil increases the number of chitinase producing organisms e.g. *Trichoderma spp.* Chitinases are produced by such organisms to degrade their food source but they are also known to attack the cell walls of fungal pathogens which are composed of chitin (Delacruz *et al.*, 1993).

Trichoderma harzianum strains have been found to decrease a range of pathogens including Fusarium wilt of cotton and muskmelon (Ordentlich *et al.*, 1991). *Aeromonas caviae* has also been shown to decrease *Sclerotium rolfsii* in a greenhouse environment and its action is linked to its chitinolytic ability (Inbar and Chet, 1991). This again indicates that raising the level of chitinolytic enzymes can result in a decrease of disease incidence.

There are several ways in which chitin may be effective. Applications of chitin affect host plants directly, making them more resistant to pathogen attack. Further research has shown that seed treatments of chitin result in an associated expression of the plant defence mechanisms indicating that chitin can elicit host plant resistance (Benhamou *et al.*, 1994). Chitinases are also produced by some plants as a defence mechanism (Punja and Zhang, 1993).

Chitin also influences the root growth of perennial ryegrass (*Lolium perenne*) in pot experiments. Grass grown in chitin amended soil had a significantly lower root:shoot ratio bought about by an increased availability of nitrogen (Sarathchandra *et al.*, 1996). If this effect is universal then the reduced root growth means that there is less root area vulnerable to attack. In the case of *P. brassicae* additions of chitin to infected soil would result in a multi-pronged attack upon the pathogen. An increase in chitinolytic

enzymes would erode the viable resting spore population, host plant resistance would be stimulated and there would be a reduction in the root area available for infection.

In the case of *P. brasssicae* a control measure which has multiple modes of action is a distinct advantage. Most methods of control fail due to the ability of the pathogen to develop resistance.

The complete control of *P. brassicae* is therefore unlikely to be obtained solely through direct biological control despite the isolation of several potential microbes. A combination of using composts as "root camouflages" with individual bio-control agents and nutritional supplements has a greater potential success. Research into the ways in which this may be achieved is therefore needed along with the identification of UK soils suppressive to *P. brassicae*.

The manipulation of the nutrient environment through the use of fertilisers may also help to control *P. brassicae* through this more holistic approach to disease control.

The influence of nutrients upon plant disease.

The availability of nutrients in the root environment may influence the occurrence and severity of disease. High levels of phosphorous enhance the resistance of wheat plants to Take All (Cook and Reis, 1981). In contrast, high levels of phosphorous increased the susceptibility of *Aster novi-belgii* to vascular wilt (*Phialophora asteris)* and high levels of potassium increase the incidence of Aster wilt and tomato wilt (*Verticellium dahliae*) (Burge and Isaac,

1977). Increases in potassium also increased the susceptibility of both asters and tomatoes to their respective wilt diseases.

Much research has investigated the control of plant pathogens using macro and micro-nutrients. For example, Keniath and Loria (1996) studied the effects of several nutrients including sulphur, calcium potassium and iron, on the infection of potatoes (*Solanum tuberosum*) by common Scab and Huber (1996) undertook a similar study into the effects of nutrition on the incidence of Take All.

Reviews of research into the role of nutrition in the control of plant disease have been published by Graham (1983) and Huber (1980) and in a collection of papers edited by Engelhard (1996).

Research investigating the control of *P. brassicae* has mainly focused on the use of boron, calcium and varying forms of nitrogen.

Boron and *P. brassicae.*

The ability of boron levels to influence the infection levels of *P. brassicae* has been recognised for more than fifty years (Dixon, 1991). Boron is associated with auxin metabolism, cellular differentiation and the biosynthesis of lignin in higher plants (Lewis, 1980).

O' Brien and Dennis (1936) recommended the use of boron in the form of Borax or "Terravit" to reduce *P. brassicae* infections in heavily limed soils, as boron becomes deficient in high pH soils. Rhode (1953) observed a decrease in *P. brassicae* when Borax was

applied at 20 kg ha^{-1}. Experiments by Palm (1963), Antonova *et al.* (1974), Vytskii (1979), Utkina *et al.* (1980), Ill'ina and Shekunova (1981) and Dixon *et al.* (1983, 1984b, 1985) observed reductions in *P. brassicae* when boron was used alone and in combination with other nutrients and fungicides.

Dixon and Webster (1988 and 1991a) investigated the effects of boron upon the infection of Chinese cabbage seedlings by *P. brassicae* in solution culture where nutrient levels and pH levels could be maintained. The results from this research indicated that the application of boron reduced the rates of reproduction of *P. brassicae* in the root hairs. Work on field grown plants treated with boron by Craig and Dixon (1993) showed that the rate at which *P. brassicae* develops in the root hairs was retarded and resulted in a reduction in the level of pathogen development.

It is unclear though whether the influence of boron in host-parasite interactions between *P. brassicae* and root hair and epidermal cells is direct, i.e. an interference with the functioning of fungal metabolism or via changes in plant cytoplasm (Dixon, 1991).

The research reported here however concentrates on the effects of calcium and nitrate individually and in combination upon the infection process of *P. brassicae*.

<u>The use of calcium in plant disease control.</u>

Calcium is needed in plants for carbohydrate removal, neutralisation of cell acids, cell wall deposition and formation of pectates in the middle lamella (Huber, 1996). Calcium is also required in a wide range of cellular functions and acts as a

secondary messenger and in regulating mitosis, cytokinesis and cytoplasmic streaming (Hepler and Wayne, 1985). A review of the involvement of calcium in plant functions is given by Hepler and Wayne (1985). A further review of the role of calcium in higher plants is provided by Roux and Slocum (1982).

Shear (1975) listed more than 30 disorders in plants caused by calcium deficiency. These disorders develop despite an adequate supply of calcium in the soil being available (Bangerth, 1979). The high calcium content of most soils in comparison with plant demand, implies that mass flow of calcium from the soil solution to the root surface is the pre-dominant transport process (Barber and Ozanne, 1970). Bangerth (1979) provides a detailed review of the absorption processes of calcium and its subsequent involvement in plant functions.

Calcium reportedly decreases the level of infection by several plant pathogens. Infiltration of Golden Delicious apples (*Malus domestica Borkh*) with calcium resulted in a decrease in rot caused by *Penicillium expansum* (Conway and Sams, 1983). The addition of calcium to fertiliser used for cucumber (*Cucumis sativus*), aubergine (*Solanum melongena*) and pepper (*Capsicum annuum*) resulted in a decrease in the levels of grey mould *(Botrytis cinnerea*). The effect of calcium on cucumber infection was however linked with the amount of nitrate present and the humidity (Elad *et al.*, 1993). Calcium also decreased grey mould on roses (Elad, 1988, Volpin and Elad 1991) and anthracnose (seedling blight) caused by the pathogen *Colletotrichum dematium* in soybean (Muchovej *et al.*, 1980).

Calcium is found in high concentrations in plant cell walls (Ressignol *et al.*, 1977) and maintains membrane integrity and cell wall production (Baydoun and Northcote, 1981, Simon 1978). Calcium binds to pectin within the cell walls providing mechanical strength (Demarty *et al.*, 1984).

There are also examples of calcium affecting plant pathogens directly. Elad *et al.*, (1992) observed a reduction in grey mould which was associated with the level of calcium making plants less susceptible to degradation enzymes by *B. cinerea*. In contrast however, Wiseniewski *et al.* (1995) determined that an addition of calcium (calcium chloride), to the germination medium resulted in decreased spore germination and germ tube growth by *B. cinerea* and *Candida oleophila*.

The effect of calcium on *B. cinerea* may be two fold, but if the germination ability is reduced then there is a lower innoculum pressure to be resisted and hence a lower level of enzymes.

Calcium affects the swimming patterns of *Pythium aphanidermatum* zoospores and their ability to infect host plants (Donaldson and Deacon, 1993). High levels of calcium affected the motility of *Phytopthora parasitica* zoospores (von Broembsen and Deacon, 1997).

Calcium and the control of *Plasmodiophora brassicae.*

The use of calcium carbonate is one of the earliest known control methods for *P. brassicae* (Campbell *et al.*, 1985). The mode of action though is still a matter for investigation. Naumov (1927) undertook experiments where seedlings were grown in soil with

high calcium carbonate content before being transplanted to *P. brassicae* infected soil. No differences in the level of infection between seedlings treated with calcium carbonate and those which received no treatment was found. This lead to the conclusion that calcium carbonate does not affect host – plant metabolism.

There is also debate whether soil alkalinity is responsible for decreased infection or whether this is partially due to increased calcium ions (Dixon & Webster, 1988). Early work by Samuel & Garrett (1945), in which soil alkalinity was altered and nutrient solutions deficient in calcium were used, confirmed that there was a direct effect of pH on *P. brassicae* which decreased inoculum potential. Work by Macfarlane (1952) suggested that control of clubroot through liming was due to increased soil alkalinity. More recent work, however has illustrated that increased calcium availability is also important.

Fletcher *et al.* (1982) undertook field trials which examined the ability of calcium carbonate to decrease clubroot disease and whether calcium or pH was the controlling factor. *Plasmodiophora brassicae* infested plots were treated with either calcium carbonate or sodium carbonate. Results showed that although the soil of both treatments was of similar pH, calcium carbonate treated cabbages had significantly lower incidence of clubbing, again indicating that the controlling factor was calcium and this was independent of soil alkalinity.

These results agree with those of Hamilton and Crête (1978) who carried out similar experiments in pots rather than field plots. In support of this Dixon and Webster (1991) found that the application of calcium sulphate to Chinese cabbage plants infected

with *P. brassicae* reduced the level of clubbing despite being associated with an increase in rhizosphere acidity.

Myers and Campbell (1985) undertook a series of experiments using nutrient solutions of varying pH values and calcium concentrations. The level of clubbing was less in plants grown at pH 7.2 compared with 6.2, the effects were greatest when the calcium level was raised by increasing the level of calcium nitrate to 2.5mM. Raising the level of calcium using calcium chloride had the next greatest effect upon disease infection. Myers and Campbell (1985) concluded that at more acidic pH values calcium incorporation into the roots was less efficient. This was confirmed by assessing the level of calcium in root tissue. Webster and Dixon (1991b) also observed an increase in calcium uptake with alkaline pH values confirming that calcium absorption increases as the alkalinity increases.

Following field experiments, which measured the extent of clubroot disease over several years after the application of calcium carbonate, Campbell *et al.* (1985) postulated that the mode of action of calcium carbonate in affecting *P. brassicae* is an effect of calcium, which is enhanced by an alkaline pH. Other work on the effects of calcium and pH, reviewed here appears to support their hypothesis.

The role of calcium in suppressing *P. brassicae* is beginning to be understood. Solution culture experiments where the pH and / or the calcium concentration could be altered allowed Webster and Dixon (1991) to study these factors as the influences on the infection processes of *P. brassicae*. The results revealed that elevated levels of calcium inhibited the release of secondary

zoospores from primary sporangia growing in the host root hairs. Lower calcium concentrations resulted in an inhibition of sporangial development. Alkaline pH decreased the number of infections and the maturation rate (Dixon and Webster, 1991).

Suzuki *et al.* (1992) established that an abiotic factor was present in the root exudates of plants and was particularly high in the exudates of susceptible Chinese cabbages. This factor was also present in exudates from resistant plants. Further research indicated that a second stimulating factor was involved in the germination of resting spores. Yano *et al.* (1991) analysed the levels of calcium ions in spore suspensions prepared with de-ionised water. This established that the release of calcium ions from germinating spores induces that process in neighbouring spores.

These findings agree with observations of zoospore release from cysts of *Pythium aphanidermatum* and *Phytophthora parasitica*. Donaldson and Deacon (1992) showed that solutions containing calcium ions, calcium ion entry blockers and flux inhibitors that calcium ions were essential for the release of zoospores from cysts of *P. aphanidermatum.*

The level of calcium was found to be instrumental in the release of zoospores from germination cysts of *Phytophthora parasitica* (von Broembsen and Deacon, 1996). The release of zoospores from un-germinated cysts after a two hour period was inhibited by calcium. This response was attributed to lost of signal transduction. Calcium was released from the cysts and then reabsorbed as described by Irving *et al.* (1984) and Iser *et al.* (1989). This theory explains why

the level of calcium ions in the experiments by Yano *et al.* (1991) increased prior to *P. brassicae* resting spore germination.

The resting spores used by Yano *et al.* (1991) were prepared from galled material and were not induced into germinating by the presence of root exudates other than those released from the parent galls. Apparently two factors are involved in the stimulation of *P. brassicae* resting spores. Host plant exudates stimulate resting spore germination which in turn release a second stimulating factor which is considered to be calcium encouraging further germination. The application of calcium to soil prior to planting may stimulate *P. brassicae* resting spore germination. The primary zoospores might die before they find a host plant.

These observations go some way towards explaining the occurrence of soils which are suppressive to *P. brassicae* but not due to biotic factors. Such soils have been identified in Taiwan. These soils had a pH greater than 7.4 and a calcium content of 1210 ppm (Hseih and Wang, 1986). The latter used suppressive soil samples with a pH 7.3 and a calcium content of 1460 ppm. When the suppressive soil was diluted with conducive soil the pH was altered to 6.7 and the calcium content to 1210 ppm. Suppressiveness was lost when acidified with sulphuric acid but returned when sodium carbonate was applied. Acidification of the soil increased the level of calcium by the dissolution of calcium compounds. As a result the availability of calcium decreased due to the higher concentrations of hydrogen ions.

The potential of calcium and nitrogen as control measures for *P. brassicae.*

Nitrogen fertilisers are known to influence the occurrence of diseases and as has already been discussed calcium can also decrease disease. It is therefore possible that applications of fertilisers containing both calcium and nitrate may provide a certain level of disease control. In relation to *P. brassicae* one such fertiliser, calcium cyanamide has been extensively studied and a review of the research findings follows.

Calcium cyanamide

Calcium cyanamide ($CaCN_2$) has been used for many years as a nitrogenous fertiliser and for its additional herbicidal and pesticidal properties (Humpherson-Jones *et al.*, 1992). In the European Union calcium cyanamide is registered as a fertiliser and not as a pesticide. It contains 20% nitrogen and 50% calcium hydroxide (CaO) (Klasse 1996).

The activity of calcium cyanamide against *P. brassicae* infection was recognised as long ago as 1828 by Kindshoven. Karling (1968) cites 17 studies carried out between 1928 and 1963, only three of which failed to demonstrate any control. Further interest was generated by the production of calcium cyanamide in a granular form which made it far easier to use.

Mattusch (1979) showed a reduction in the extent of clubbing using calcium cyanamide when compared to ammonium sulphate. In other research Mattusch (1978) compared the effectiveness of calcium cyanamide to that of lime and found that whilst lime

decreased the number of unmarketable heads by 50%, calcium cyanamide decreased the number by 70%. Dixon and Wilson (1983) found that the calcium cyanamide was not as effective as Dazomet in decreasing the level of infection but the reduced cost of calcium cyanamide made its use more economic.

Various research has investigated both the mode of action and application rates and timings of calcium cyanamide.

Active breakdown product and mode of action.

The mode of action of calcium cyanamide has long been a topic for discussion. Calcium cyanamide degrades to release free cyanamide and di-cyanamide ions which are both phyto- and fungi-toxic (Mattusch, 1979).

Walker (1935) stated that urea was a product of the hydrolysis of calcium cyanamide and that urea was responsible for decreasing *P. brassicae* infection. In experiments, where calcium cyanamide was compared in its effectiveness to a corresponding quantity of hydrogen cyanamide, calcium cyanamide was found to be more effective. It was therefore concluded that *P. brassicae* control by calcium cyanamide was due to a combined calcium and cyanamide effect (Venter, 1979) and not due to a urea effect. In 1996 however Klasse again stated that hydrogen cyanamide was the active breakdown product. It is possible that this author's review may have overlooked the information given by Venter.

The mode of action has been established by Naiki and Dixon (1987). In experiments, resting spores were incubated in solutions of calcium cyanamide, benomyl, quintozene and trichlamide for 1,

5, 10 and 15 days prior to their use to inoculate Chinese cabbage seedlings. The most dramatic decrease in infection was exhibited in resting spores which had been incubated in calcium cyanamide for 1 day. Although the pathogenicity of calcium declines over prolonged periods the experiment indicates that calcium cyanamide decreases the viability of resting spores. It is not clear though, whether calcium cyanamide has any other effects on *P. brassicae*.

In another experiment calcium cyanamide has been found to increase the resistance of some cultivars of swede as well as decreasing the inoculum density (Williamson, 1989). It has therefore been suggested that, as well as decreasing the viability of resting spores, there is an additive relationship between increased cultivar resistance and the concentration of calcium cyanamide.

Application rates and timings.

A wide range of application rates have been used in research on calcium cyanamide e.g. Walker and Larson (1935) used 448-897 kg ha^{-1}; Renard (1935) 300-500kg ha-1; Shirama (1955) 1130-1500 kg ha-1; Zavra and Rod (1967) 600-1200 kg ha-1 and Horiuchi *et al.* (1982) and Naiki and Dixon (1987) 1000 kg ha-1.

The effectiveness of calcium cyanamide was found to increase with increasing concentration. Lower application rates were used in later research due to the use of modular transplants. This decreased the phytotoxic effects of calcium cyanamide. Phytotoxic effects were reported by Dixon and Williamson (1984) and Williams and Dyce (1989) at higher application rates.

This is in contrast with the work of Humpherson-Jones *et al.* (1992) in which no phytotoxicity was observed at rates of 1500-1600 kg ha-1. Dixon and Wilson (1984) established that there was no advantage in increasing the application rate of calcium cyanamide above the recommended 1000kg a.i. ha-1. In contrast Humpherson-Jones found an increased disease control at rates of 1500- 1600 kg ha-1. The reason for this may be linked to the environmental conditions at different test sites or the inoculum potential in the soil.

Klasse (1996) stated that a high soil humidity was needed to release, dissolve and distribute the ions produced by the degradation of calcium cyanamide. It is therefore possible that in the experiments of Dixon and Wilson (1984) the soil humidity was acting as a limiting factor to the efficacy of calcium cyanamide. Unless every experiment on clubroot was to be carried out at the same site it is difficult to compare research results in detail.

This illustrates how difficult it is to interpret information about *P. brassicae* as the level of infection can be influenced by many factors before a chemical is even applied. It is however recommended that application rates greater than 1000 kg ha-1 should be avoided to prevent phytotoxicity and to increase profit margins Dixon and Wilson (1984).

The optimum time of application of calcium cyanamide has also long been debated. Naiki and Dixon (1987) stated that applying calcium cyanamide too early resulted in a loss of effect and if applied to late phyto-toxic effects were observed. As module plants are less sensitive than bare root transplants they recommended that applications should be made seven days before planting.

In addition to the successful studies of calcium cyanamide the fertiliser calcium nitrate has also been considered as a possible control measure for *P brassicae*.

Calcium nitrate

Calcium nitrate increases diseases such as *Gaeumannomyces graminis* (take all of wheat) (Smiley and Cook, 1973), and *Steptomyces scabies* (common Scab of potato)(Kienath and Loria (1996) when compared with more acidic fertilisers such as ammonium nitrate.

By contrast, calcium nitrate applications decreased the levels of *Fusarium oxysporum* (Engelhard, 1996, Jones *et al.*, 1996) and *Sclerotium rolfsii* (Punja, 1996) on a wide range of hosts, *Erwinia caratova* (soft rot of potato)(Kelman *et al.*, 1996) and *Botrytis cinerea* (grey mould on peppers) (Elad *et al.*, 1993).

The ability of calcium nitrate to increase or decrease the incidence of disease is related to its effects in the soil environment. This material increases the calcium content of the rhizosphere which, has already been recognised as a factor in the control of some plant pathogens. Calcium nitrate also increases the alkalinity of soils (Elad *et al.*, 1993) thereby altering nutrient availability and the balance of microbes. Increased soil alkalinity favours pathogens such as common scab of potatoes but discriminates against pathogens favoured by more acid soil such as *P. brassicae*.

The increase in soil alkalinity resulting from calcium nitrate is due to a greater equivalent uptake of nitrate ions compared with calcium ions (Smiley and Cook, 1973).

While testing the effect of lime particle size on the control of *P. brassicae,* Dobson *et al.* (1983a) also compared the effects of calcium nitrate, potassium nitrate, ammonium sulphate, ammonium sulphate and urea. They found decreased incidence of clubroot in plots where calcium nitrate was used in limed and untreated plots. In limed plots calcium nitrate alone and in combination with potassium nitrate treatments significantly reduced the level of clubbing when rates of 112kg ha^{-1} were used.

Experiments in Scandinavia have indicated that calcium nitrate resulted in decreased incidence of *P. brassicae* infection when compared with the application of ammonium nitrate (Thulsen and Lisbjerg, 1992).

Calcium nitrate appears to have potential for the control of *P. brassicae* as an alternative to liming. The mode of action of calcium nitrate is unknown but possibly the effect results from both an increase in soil alkalinity and availability of calcium. The stage of *P. brassicae* infection most affected by calcium nitrate and hence the optimum time of application also requires substantial investigation.

Summary and Objectives of this Research.

Plasmodiophora brassicae, the causal agent of clubroot, is a complex pathogen whose study seems to raise more questions than answers. Despite over a hundred years of study, questions concerning the lifecycle and position in the phylogenetic tree remain. The incidence of *P. brassicae* remains a world wide problem and control is still not effective by chemical or biological means. Cultural control remains the most viable option although its success is variable.

The ability of *P. brassicae* to segregate and the range of pathogen races is a major obstacle to control by resistant cultivars. The effect of nutrient availability, chemical controls and biological controls upon *P. brassicae* pathogen races including their potential to induce a shift in the pathogen population is unknown.

There is also very little known about the effects of 'holistic approaches' to clubroot control, such as the use of seaweed extracts and chitin.

There are therefore many unresolved problems concerning the interactions between *P. brassicae* and the soil environment.

The aims of this research project were :-

1. Determine the ability of calcium nitrate to decrease the incidence of *P. brassicae* in UK soils and identify relationships between *P. brassicae* and calcium nitrate concentration.

2. Identify the stage of pathogen development most influenced by calcium nitrate.

3. Determine the predominant *P. brassicae* pathogen race present at field sites

4. Establish the influence of calcium nitrate upon *P. brassicae* pathogen races

5. Establish the influence of the growth substrate upon the *P. brassicae* pathogen races

6. Establish whether it would be possible to "camouflage" the roots of module transplants by raising them in non-peat based compost.

7. Examine soil samples from several areas, which have been reported to be suppressive to *P. brassicae* and establish whether the suppressiveness results from biotic or abiotic factors.

8. Compare the effectiveness of calcium carbonate and calcified seaweed in reducing *P. brassicae* infection.

9. Investigate whether applying liquid seaweed extract to compost throughout the growing period would decrease the level of club formation.

10. Determine whether applications of chitin to the growing medium decrease the extent of clubbing.

Chapter Two

General
Methods and Materials.

General Methods and Materials

This Chapter describes methods and materials which are used throughout this research.

Collection and storage of samples.

Inoculum Source.

Plasmodiophora brassicae infected roots were collected from the Auchincruive field site (Grid Ref. 238415,623365*) from untreated areas and those used in previous field trials. Galls were washed in cold water removing the field soil, placed into labelled polythene bags (20 x 40 cm) and stored at -40^{0}C until required. Some additional inoculum was from glasshouse experiments. These were washed and stored in the same manner.

Soil samples.

Soil samples were collected from field sites at Auchincruive (Grid Ref. 238415,623365*), Crail (Grid Ref. 362005,7075600*) and Kings Kettle (Grid Ref. 330930,708335*). All of these sites were infested with *P. brassicae* and had been cropped with vegetable brassica crops for many years. A soil analysis for each site is given in Appendix 1.

Samples were obtained form the top 10 cm soil. Samples taken when crops were being grown were taken from the edges of planted areas preventing crop damage. Soil was placed in labelled

polythene bags (20 cm x 40 cm) and stored at -40^{0}C. This inhibited growth by soil micro flora and fauna whilst in storage.

(* Grid references obtained from streetmap.co.uk)

Preparation of *Plasmodiophora brassicae* resting spore suspensions.

Preparation of basic resting spore suspensions.

The method of MacFarlane (1952) was used to prepare each suspension:-

1. 5 galls were defrosted overnight.

2. The galls were sectioned and placed in a blender (Waring) with 300 ml of de-ionised water (USF CD100 de-ioniser). The material was blended on full speed for thirty seconds and filtered through two layers of cotton cloth. Blending for any longer than thirty seconds heated the spore suspension leading to spore inactivity.

3. The filtrate was centrifuged at 2000g for 10 mins in a Bedman TJ-6 centrifuge.

4. The supernatant was discarded and the pellet re-suspended in distilled water, before being re-centrifuged. The suspension was centrifuged four times removing unwanted plant debris and starch grains.

5. The resulting pellet was resuspended and the concentration of *P. brassicae* resting spores was determined using a Neubaur Improved Haemocytometer.

6. Resting spore suspensions of the required concentrations were prepared by serial dilution and the resting spore concentration re-determined as before.

Use of a Haemocytometer to calculate the number of resting spores ml^{-1}.

A 200 x dilution was prepared with 1 ml of the test resting spore suspension. The diluted suspension was used to estimate the number of resting spores ml^{-1}. The number of resting spores in five large divisions on the haemocytometer grid (made up of 16 smaller squares) were counted at random.

The sum of all five squares (E) was calculated.
The number of spores mm^{-3} = E X 5 X 10
The number of spores ml^{-1} = E X 5 X 10 X 1000
The dilution was then taken into account by multiplying the number of spores ml^{-1} by 200.

This gave the final number of resting spores ml^{-1} present in the original resting spore suspension.

Choice of hosts

Glasshouse and controlled environment experiments.

Brassica rapa var. pekinensis (Chinese cabbage) was used in glasshouse and controlled environments since it is very susceptible to *P. brassica* and has a rapid growth rate. The cultivar Mariko was used (Nickerson Seeds Ltd) since this allowed comparability with previous studies. Mariko also grows well in glasshouses.

Field experiments.

Field experiments used *Brassica oleracea* cv. Castello (Nickerson Seeds Ltd). Again this permitted comparability with previous studies and it is established that Castello is susceptible to *P. brassicae* infection and is well suited to the environment of the field site used.

Experimental environments.

Field environment.

The field site used was situated at the Scottish Agricultural College, Auchincruive. The area is well drained and naturally infested *with P. brassicae*. A soil analysis is given in appendix 1.

Glasshouse environments.

Three glasshouse environments were used during this research. Benches were covered in pea size gravel and heated from below by a coiled electrical cable, the temperature of which was maintained

at 25^{0}C. Plants were illuminated by lamps placed 1m above the bench, radiation reaching the bench had a lux of 9340. The lamps provided a 16 hour photoperiod.

Glasshouse temperature was maintained at 25^{0}c +/- 5^{0}c. During the autumn, winter and spring months this was controlled electronically but in the summer no heat was required and the glass house was kept well ventilated

Controlled environments.

Mercia and Gallenkamp controlled environment chambers were used for some experiments. These chambers were illuminated for a 16 hour photoperiod and the temperature was maintained at 25^{0}C. The light level had a lux value of 280.

Growth rooms.

Two growth rooms were used in this research. Both were fitted with fluorescent lightning positioned 0.5 m above the plants and were illuminated for a 16 photoperiod. The temperature was maintained at 22^{0}C +/- 5^{0}C.

Preparation of experiments.

All treatments were arranged in a randomised block design. The number of plants in each plot and the number of replicates is described in each experiment protocol.

Field experiments.

Ground preparation.

The field was ploughed mechanically and tilled. The ground received a fertiliser treatment of 100 kg ha^{-1} phosphorous, 135 kg ha^{-1} potassium and 200 kg ha^{-1} nitrogen.

A stale seed bed was used for weed control pre-planting with applications of Grammoxone (Paraquat). Post planting a tank mix of Dacthal (Chlorthal-dimethyl 75% w/w) and Ramrod (Propachlor) was applied.

Experimental areas were covered by nets suspended over stakes 2.5 M high to prevent damage to the crops from birds.

The area was divided equally into the required plots using twine and 45cm wooden stakes. Each plot measured 9.6 m^2 and was labelled.

Plot treatment.

Chemical treatments required prior to planting were weighed into labelled polythene bags (20cm X 40cm) and then sprinkled manually over the area. Treatments were manually tilled into the soil and left for two weeks prior to planting.

Planting.

Seed was sown into p250 module trays (Plantpak) filled with the required compost. Insecticide was incorporated into the compost to

protect against cabbage root fly (Burlane (chlorfenvinphos 10% w/w ai)). The seedlings were raised for 5 weeks in a glasshouse environment before being planted into the field area.

Seedlings were planted into the field area using a commercial planter and tractor. Five rows formed each plot with 20 plants per row giving a total of 100 plants per plot. The planted area was then treated with Draza (Methiocarb 4% w/w a.i.) slug pellets.

Trial areas were sprayed with Decis (Deltamethrin) a broad spectrum insecticide, to control the levels of aphids and caterpillars.

The field experiment areas were monitored regularly for damage caused by pests and pathogens throughout the experimental period. No irrigation was required.

Glasshouse and controlled environment experiments.

Filling of Seed Trays and pots.

Seed trays (Ward) and pots (Plantpak) were filled to within 1 cm of the rim with the appropriate growth medium which was pressed firmly into them. Pots and trays were placed into gravel trays (Ward) to allow drainage but prevent contamination of the glass house bench. All treatments were watered 24 hours before sowing to allow the media to re-absorb moisture. The tray and pot sizes were as follows :-

Large trays = 35.56 x 21.59 cm
Small trays = 21.59 x 15.24 cm

Pots = 7cm x 7cm square pots (Plantpak) or 6.25 cm diameter round pots (Ward)

Where field soil samples were used in a glasshouse environment, they were mixed with either gravel or perlite to improve drainage. A 1:1 ratio by volume was used for these preparations.

Experiments were monitored regularly for pests and pathogens. In the event of aphid or caterpillar invasions the plants were sprayed with Tumblebug (Heptenophos and permethrin).

All trays were re-used after washing with Decon 90. All pots were disposed of after use into a landfill site.

Application of treatments.

In the experiments in Chapter 7 the treatments were mixed with the compost prior to sowing. The required number of 7 cm^2 pots were filled with an appropriate compost which was then mixed with the appropriate treatment. This was done by placing the correct treatment mass for each pot into polythene bags (15 cm x 20 cm) with the contents of each pot. The bags were vigorously shaken until the compost and the treatment were thoroughly mixed and replaced in the pot.

When treatments were applied in solution they were poured evenly over the growth media surface at the appropriate time.

Nutrient cultures.

Several methods were used in an attempt to obtain *P. brassicae* infection in a soilless environment. All pots were inoculated with *P. brassicae* by pouring 20ml of a 10^6 resting spores/ ml suspension over their surfaces.

Growth media.

Perlite, vermiculite and acid-washed sand were used as rooting media. Perlite and vermiculite are chemically inert and can be autoclaved.

Sand was washed in acid to remove organic debris.

Preparation of acid washed sand.

Horticultural grade sand, particle size 0.1-0.5mm, was prepared by placing 4 litres of sand into a 10 litre bucket containing 3% hydrochloric acid for seven days. After this time the acid was drained and the sand washed with tap water, supplied at the base of the container for 5 days (Hewitt, 1953). Trays were half filled with sand and placed in an oven at 50^0C until completely dry.

Seed sterilisation.

Seeds were surface sterilised by placing them firstly in 90% ethanol for 1 minute and then 1% sodium hypochlorite for 15 minutes. Finally the seed was washed five times with sterile de-ionised water (Webster, 1985).

Nutrient solutions.

Throughout the course of this research three nutrient solutions were used. Each of the solutions were prepared with de-ionised water and the pH adjusted to 6.4, using molar hydrochloric acid or molar sodium hydroxide and monitored with a Jenway 3320 pH meter.

All nutrient solutions were autoclaved before use in 1l polypropylene bottles. The use of glass bottles was avoided as boro-silicate can dissolve from glass into the solution affecting the nutrient concentration. All autoclaving was carried out in a portable autoclave (Express Autoclaves) for 10 mins at 120^0C and 1.035 bars of pressure.

The mineral content of these nutrient solutions is given in Table 2.1.

Table 2.1. The mineral content of three nutrient solutions.

Mineral	Nutrient solution		
	Hoagland's No. 2 Basal Salt Mixture mg l^{-1}	Modified Hoagland's mg l^{-1}	Kristalon Red mg l^{-1}
Ammonium phosphate monobasic	115.03	115.03	39.2
Boric acid	2.86	2.86	0.19
Calcium nitrate	656.4	656.4	0
Copper sulphate pentahydrate	0.08	0.08	0.08
Ferric tartrate	0.2820	5.32	0.53
Magnesium sulphate heptahydrate	0	246.0	4.5
Manganese chlorate	0.482	240.76	0.3
Molybdenum trioxide	0.016	0.016	0.03
Sodium EDTA	0	42.0	0
PIPES	0	10 mM	0
Potassium nitrate	606.6	606.6	224
Zinc sulphate heptahydrate	0.22	0.22	0.19

PIPES = Piperazine-N,N'-bis(2-ethanesulfonic acid) 1,4-Piperazinediethanesulfonic acid

Hoagland's No. 2 Basalt Salt mixture solution.

Commercially prepared packets (Sigma) were added to de-ionised water. One litre of the resultant solution contained the nutrients given in table 2.1. Plants in this solution suffered nutrient deficiency symptoms (leaves lacked a deep green colour). Hence this solution was compared with modified Hoagland's solution used by Webster (1985) and amendments made.

Modified Hoagland's solution.

This solution had a similar mineral content to that used by Webster (1985). Stock solutions were prepared in 10l batches and placed in

aspirator bottles (BDH). The stock solutions and their additions are given in Table 2.2. All chemicals used were either Ultrapure / Tissue Culture Tested (Sigma) or Analar (BDH).

Table 2.2. Mineral concentrations in stock solutions for the preparation of modified Hoagland's solution and the amount of stock solution needed to be added to 10l of water.

Mineral	Concentration in 1l stock solution(g)	Amount needed to be added to 10l (ml)
Ammonium phosphate monobasic	11.5	100
Boric acid	0.572	50
Calcium nitrate	65.64	100
Copper sulphate pentahydrate	0.016	50
Ferric tartrate	0.266	200
Magnesium sulphate heptahydrate	4.92	50
Manganese chlorate	48.152	50
Molybdenum trioxide	0.0032	50
Sodium EDTA	2.14	200
Potassium nitrate	60	100
Zinc sulphate heptahydrate	0.44	50

10.5g of PIPES buffer was dissolved in 70ml sodium hydroxide and added to the 10l of nutrient solution. This stabilised the pH at 6.4. Some stock solutions were made up in combination those combinations were :-

Ammonium phosphate and Calcium nitrate
Boric acid and molybdenum trioxide
Copper sulphate, zinc sulphate, manganese chloride and magnesium sulphate

Ferric tartrate and Sodium EDTA

Sodium EDTA was added to nutrient solutions as a chelating agent making iron more readily available.

Kristalon red.

This was supplied as a crystalline powder by Hydro Agri (UK) and added to de-ionised water at 0.75g l^{-1}. Kristalon red was used in some experiments as it did not contain a source of calcium nitrate and was therefore suitable for use in experiments which compared the effects of different nitrate sources.

Nutrient culture systems.

Five methods were assessed to produce plants with *P. brassicae* symptoms in a soil free environment.

Method A.

Sterile magenta tubs (Sigma) (340 cm^3) were filled with 4cm of autoclaved vermiculite. One hundred ml of Hoagland's No.2 basal salt nutrient solution (Sigma) was added to each tub. Surface sterilised seed was then sown into each tub. The tubs were placed into plastic crates (30.5 cm x 41 cm) and glass placed over three quarters of the crate top preventing excessive moisture loss. Crates were placed into growth rooms and the nutrient level in the tubs maintained.

This method proved unsatisfactory due to the lack of drainage within the tubs. Plant growth was poor and condensation from the

glass led to leaves being constantly wet, resulting in infections by *Botrytis cinerea* (grey mould).

Method B.

Small gravel trays were lined with black plastic (Nortene) and additional plastic was placed over the top of each tray to prevent algal growth. Four holes were cut into the top piece of plastic through which a $7cm^2$ plant pot (FP8, Plantpak) could be fitted such that the base rested on the gravel tray.

Drainage holes in the pots were plugged with cotton wool forming a wick through which nutrient solution could move by capillary action from a reservoir in the gravel trays.

The pots were filled with acid washed sand and irrigated with 70ml of nutrient solution treatment. Each pot was sown with five surface sterilised seeds of Chinese Cabbage cv. Mariko spaced equidistantly. Four pots, which had been irrigated with a similar nutrient solution were placed into each gravel tray and 250ml of the appropriate nutrient solution was poured into each gravel tray.

Nutrient level was maintained in each gravel tray and the pots were flushed once weekly by placing the pots on a grill and pouring 100ml of sterile de ionised water into each pot and allowing them to drain for 0.5 hours. Each pot then received 70ml of the required solution.

When the treatment nutrient solution was altered during an experiment it was changed after the pots had been flushed with

de-ionised water. This procedure prevented the nutrient levels increasing to toxic levels.

One plant was removed from each pot at weekly intervals for four weeks to monitor root hair infection.

This method was unsatisfactory because evaporation from the sand surface resulted in salt formation and the cotton wool wicks were incapable of replacing the nutrient solution.

Method C.

This method resembled method B except that strips of capillary matting (Netlon) (0.5 cm X 5cm) were pushed through the holes in the base of pots so that 3 cm of the matting was within the pot. This attempted to improve the supply of nutrients to the plants. This method proved more satisfactory for plant growth but no galling developed.

Method D.

Capillary matting was inserted into holes in 7 cm^2 pots as described in method C and they were filled with a 1:1 autoclaved mixture of vermiculite and perlite (Daggs). The pots were placed into 9cm petri dish bases covered with black plastic to prevent algal growth. The pots were sown with five surface sterilised seeds of *B. oleracea* cv. Castello. One plant was removed from each pot at weekly intervals for four weeks for the assessment of root hair infection.

The pots received 70ml of nutrient solution and a similar quantity was placed into the petri dish. The plants were flushed at weekly

intervals and changes in nutrient solution content were made after flushing.

The pots were placed in a growth chamber (LMS) for four weeks. The growth media in the pots did not retain moisture effectively and the reservoir provided by the petri dishes evaporated too quickly leading to poor plant growth and a failure to form galls. However, the use of cv Castello instead of Mariko showed that Summer cabbage types were more suited to a nutrient culture environment since no wilting was apparent and the plants were less susceptible to infection by unwanted pathogens.

Method E

This method resembled method D except that discs of capillary matting, (8.5 cm), were placed into each petri dish. This reduced evaporation by the nutrient solution and improved plant growth but no symptoms formed. It is apparent that nutrient cultures can be used to study primary root hair infection but for reasons unproven it is not possible to easily carry progression to stage 2 of the disease cycle and encourage good development.

These methods and the nutrient solutions were used throughout the research period in a series of experiments, which aimed to investigate how calcium and nitrogen applications affected *P. brassicae.* The methods proved unsatisfactory due to the lack of gall formation in inoculated plants. No results from this area of research are presented.

Disease Assessments and Plant Measurements.

Disease assessment.

Disease development was evaluated either through root hair analyses during plant growth or gall assessment at final harvest.

Root hair analysis.

Plants were removed from the growth media, roots were washed in distilled water and placed into 7ml sterile bijoux bottles (Sterelin) containing acetocarmine stain (BDH). Roots were stored at 4^0C until required. For root hair infection evaluation, roots were removed, washed in distilled water and mounted on glass microscope slides (76 mm x 22 mm) in 70% glycerol. Cover slips (22 mm x 22 mm) were sealed over each root sample using nail varnish.

Samples were observed using an Olympus binocular microscope. One hundred root hairs on each root sample were assessed for *P. brassicae* infection and the determination of the stage of pathogen development (Table 2.3 and Figure 2.1).

The data were used to calculate maturation indices using the formula of Hamilton & Crête, (1978) :-

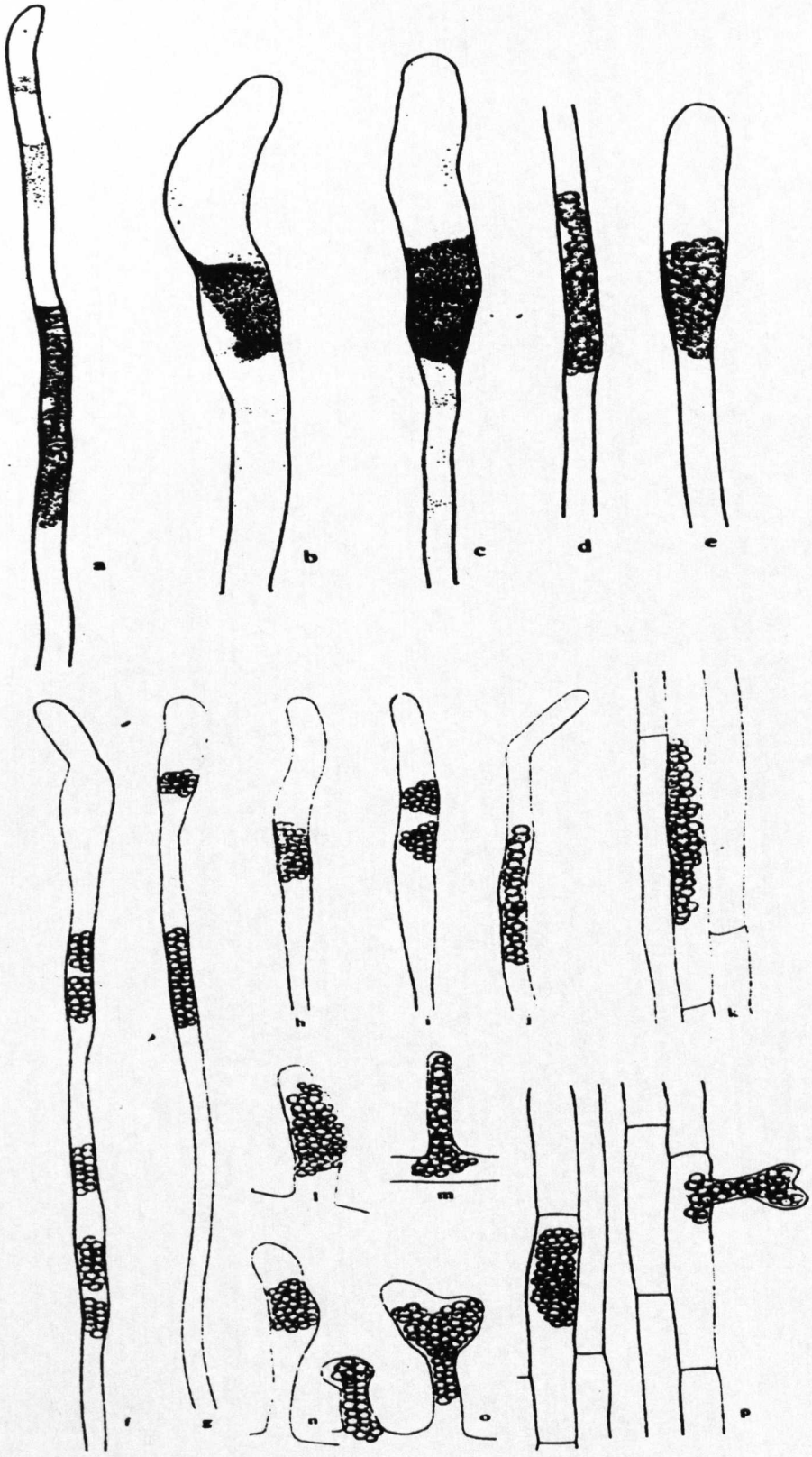
$$D.I = \frac{1a + 2b + 3c + 4d + 5e + 6f}{a + b + c + d + e + f} \times \frac{100}{6}$$

Where a, b, c etc represent the number of root hairs at stage 1, 2,3 etc respectively

Table 2.3. Root hair infection stages.

Infection Stage	Stage in Life cycle of *P. brassicae*.
Stage 1	Primary plasmodia present in root hairs
Stage 2	Primary zoosporangia present in root hairs
Stage 3	Dehisced primary zoosporangia present in root hairs
Stage 4	Secondary plasmodia present in root cortical cells
Stage 5	Secondary zoosporangia present in root cortical cells
Stage 6	Dehisced secondary zoosporangia present in root cortical cells

Figure 2.1. Illustrates the infection stages observed in root hairs. (From Samuel and Garrett, 1949).



a-c = the zoosporangial plasmodium, d-e = differentiation into sporangia, f-g = root hairs containing several sporangial groups, h-p = root hairs with sporangial groups, showing various types of hypertrophy of the root hair, and two infected cortical cells, q-s = emergence of zoospores, some within the cavity of the root hair, but most escaped into the surrounding water, t-u = empty sporangia in root hairs. (Samuel and Garrett, 1949)

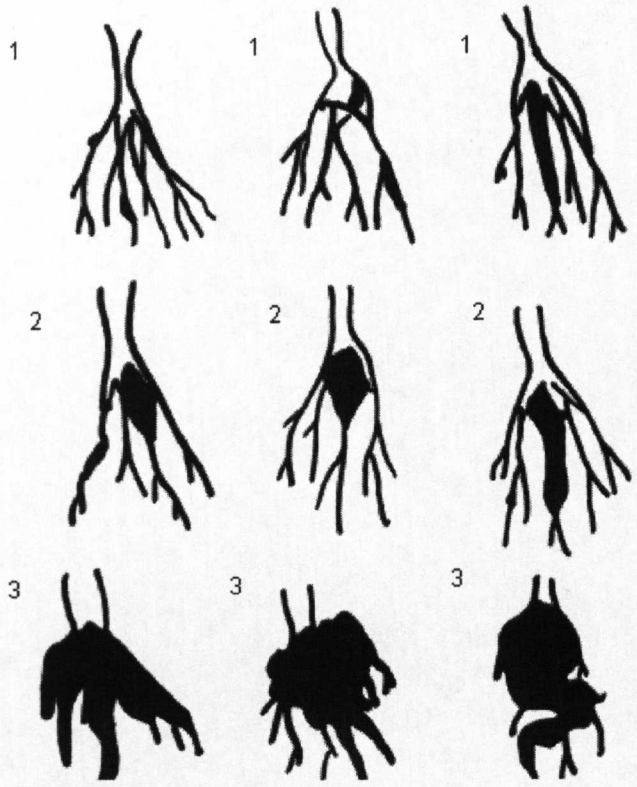
Gall assessment.

The growth media was washed from plant roots using a riddle (0.5 cm^2 mesh) placed over a sink. Each gall was categorised visually using the scale given in Table 2.4 and illustrated in Fig. 2.2

Table 2.4. The evaluation system used to categorise galls (Williamson, 1987).

Category	Gall size seen in plants up to 8 weeks after infection.	Gall size seen in plants up to 15 weeks after infection.
0 Healthy	No visible infection	No visible infection
1 Slight	Very small galls <4mm.	Small galls <5 x 2.5mm Healthy root system.
2 Moderate	Galls <10 x 5mm or 7.5mm diameter.	Galls <15 x 7.5mm or 10mm diameter. Most of fibrous roots healthy.
3 Severe	Galls <20 x 10mm or 15mm diameter.	Galls <30 x 15mm or 20mm diameter. Reduced fibrous root system.

Figure 2.2. Guide to gall categories (Webster, 1985)



Resultant gall categories were used to calculate the disease categories in each treatment using the formula below

$$\text{Disease Category} = \frac{1N_1 + 2N_2 + 3N_3}{T}$$

Where :-

N_1 = the number of plants with galls in category 1

N_2 = the number of plants with galls in category 2

N_3 = the number of plants with galls in category 3

T is the number of plants sampled.

This was then used to calculate the Disease Index by multiplying the Disease Category by 100/3 (Deidrechessen & Sacristan, 1996).

Plant measurements.

To achieve comparisons of the effects of *P. brassicae* infection on host plant growth several measurements were used.

Fresh mass.

Plants were removed from the growth media, washed and divided into root and shoot portions. These were weighed individually (Oxford A3203, accurate to 0.0001g) and the measurements recorded.

Dry mass.

The plant dry mass was obtained by wrapping plant parts in absorbent paper and inserting these bundles into labelled envelopes. The envelopes were placed in a Gallenkamp drying oven (Hotbox, size 2) at 50^0C for four days. To ensure complete dryness envelopes were randomly selected and the contents weighed on day three and re-weighed on day four to ensure no changes in mass had occurred.

Leaf area measurements.

Leaf area was measured by placing leaves upon a delta T leaf area meter and the leaf area obtained automatically.

Chlorophyll fluorescence.

The level of chlorophyll fluorescence of plants grown in field experiments was measured using a plant efficiency analyser to

establish that plants in the experiments were not suffering any undue stress. The chlorophyll fluorescence of 5 randomly selected plants was measured in every plot.

The analyser measures the amount of fluorescence which occurs after the test area of the leaf has been without light for a period of several minutes and is then again exposed. The value given by the analyser is referred to as Fv which is the difference between the maximal fluorescence signal Fm and the low level signal Fo. The fluorescence is a product of electrons in photosystem II being promoted to a higher orbital level. The level of chlorophyll fluorescence can be used to determine whether a plant is under stress by comparing it to the values in the Kautsky curves which illustrate a range of fluorescence values. High values of fluorescence indicate that the plant is under stress (Cameron, 1993). Detailed descriptions of chlorophyll fluorescence can be found in Bolhar-Nordenkampf *et al* (1989) and Bjorkman and Demmig (1987).

Vigour assessments.

Vigour assessments were taken from forty plants in every field plot.
The scale used for vigour assessments was:-

0. Least vigour
1. All leaves senescent
2. Most leaves moderately senescent
3. Most leaves slightly senescent

4. Top leaves slight growth. Stunted growth, plant size approximately ¼ of the size of plants in category 9. Leaves yellow
5. Top section moderate expansion. Yellow leaves. Stunted growth.
6. Top section expanding slowly. No senescence. Green leaves. Slow growth, stunted plants.
7. Most leaves expanding. Plants not stunted.
8. All leaves expanding fast
9. Most vigorous-with healthy deep green leaves.

Assessments of microflora activity.

Preparation of Air dried soil / compost.

Soil samples were spread thinly across a piece of plastic (Nortene) in small seed trays (Wards) and placed in a drying oven (Gallenkamp) at 40^0C for 48 h. Trays were weighed and replaced in the oven for a further 2 h before being re-weighed to ensure there was no further loss of moisture (Walsh, 1998).

Dried soil samples were passed through a 0.85 mm mesh and stored at 4^0C in acid washed universal bottles (30ml, Merck) until required.

Soil Dilution Plates.

One gram of air dried soil or compost was placed in a 30 ml acid washed universal bottle containing 9ml of sterile distilled water. The sample was shaken vigorously on a wrist action flask shaker (Stuart Scientific) for 30 minutes. One ml of the resultant suspension was removed and placed into 9ml of sterile distilled

water in a universal bottle and shaken for a further 10 minutes. This process was repeated until a dilution of 10^{-6} was obtained. The dilutions were plated, 0.1ml per plate, onto tryptic soya broth agar (3g tryptic soya broth, (Sigma), 15g Agar (Merck) / l of distilled water) and malt extract agar (48g malt extract agar, 0.005g streptomycin sulphate / l of distilled water) there were six replicates per treatment (Dhingra & Sinclair 1985). Plates were incubated in a cooled incubator (Gallenkamp) at 24^{0}C for seven days.

The number of distinct colonies per plate was recorded after two and seven days using a colony counter (Gallenkamp). Plates overgrown by large fungal colonies or with areas of antibiosis were disregarded.

European Clubroot Differential Series.

The European Clubroot Differential series is a group of fifteen Brassica genotypes used to determine the *P. brassicae* pathigen racess. The series consists of three groups *Brassica napus, Brassica rapa* and *Brassica oleracea* and each is given a host number (differential number), binary number and denary number. The fifteen hosts are shown in Table 2.5. The resistance or susceptibility of these brassicas to *P. brassicae* determines the pathogen race (Buczaki *et al*, 1975, Toxopeus *et al,* 1983).

Fifteen trays were filled with compost and each placed inside a gravel tray. Fifty seeds of the required host were sown into each tray and after seven days the number of seedlings was reduced to 30. Treatments were maintained in a glasshouse until harvest when the gall development category of each plant was assessed.

The disease category of each host was calculated and used to determine the predominate pathogen race. Hosts with a disease category greater than one were assessed as susceptible. The denary numbers of the susceptible hosts in each group were then summed producing three values which form a label for the pathogen race. For example if Hosts 5, 6, 9, 10, 13 (Table 2.5) were deemed to be susceptible then the pathogen race would be labelled as 16/15/4.

Table 2.5. The European Clubroot Differential Series.

Host Number	Differential host.	Binary No. series	Denary No. series
	20 chromosome group (*Brassica rapa* sensu lato)		
01	ssp. *rapifera* line aaBBCC	2^0	1
02	ssp. *rapifera* line AabbCC	2^1	2
03	ssp. *rapifera* line AABBcc	2^2	4
04	ssp. *rapifera* line AABBCC	2^3	8
05	ssp. *Pekinensis* cv Granaat	2^4	16
	38 Chromosome group (*Brassica napus*)		
06	var *napus* line Dc101	2^0	1
07	var *napus* line Dc119	2^1	2
08	var *napus* line Dc128	2^2	4
09	var *napus* line Dc129	2^3	8
10	var *napus* line Dc130	2^4	16
	18 Chromosome number group (*Brassica oleracea*)		
11	var. *capitata* cv. Badger shipper	2^0	1
12	var. *capitata* cv. Bindsachsener	2^1	2
13	var. *capitata* cv. Jersey Queen	2^2	4
14	var. *capitata* cv. Septa	2^3	8
15	var. *acephala* subvar.laciniata cv.Verheul.	2^4	16

Where a,b and c are resistant genes (capitals implies resistance)

Analysis of results

The statistical analysis of results was carried out using the Genstat computer programme. The analysis of variance command was used to obtain means, standard error of difference (S.E.D.) and least significant difference (L.S.D). Disease indices were transformed using the angular command to allow analysis of the data. This was necessary due to the disease index value being a percentage. The transformation meant that a disease value of 100% became a value of 90 upon transformation.

Root and shoot values which were found to be skewed were transformed using the square root (sqrt) command to allow a normal distribution of data to be obtained which could then be analysed using statistics. Skewed data occurs when some of the values are much larger than the typical values of a data set (Sanders, 1990) transforming the data makes it more uniform and reduces the effect of the outlying data upon the statistical analysis.

Chapter Three.

Studies on the interaction of nitrogen form and *Plasmodiophora brassicae* growth and reproduction.

3. Determination of the effects of calcium nitrate on plants infection and *Plasmodiophora brassicae*.

It has been established that the presence of calcium in the root environment can decrease the reproduction rate of *P. brassicae* in root hairs (Webster, 1985). It is not clear how this effect is achieved, but in reducing maturation of primary sporangia in the root hairs the subsequent level of cortical infection may be reduced.

The aim of the series of experiments discussed here was to determine the effectiveness of calcium nitrate in decreasing *P. brassicae* infection and try to establish possible modes of action. The effects of calcium nitrate were investigated because as has already been discussed (Chapter 1), it has the potential to reduce the extent of *P. brassicae* infection. Nitrates have been shown to decrease the level of disease caused by several plant pathogens (Engelhard, 1996, Jones *et al.*, 1996, Punja, 1996, Kelman *et al.*, 1996, Elad *et al.*, 1993). Calcium nitrate is an alkaline fertiliser and it has already been established that alkaline soils can reduce the level of gall formation (see Chapter 1). In addition calcium has also been shown to reduce gall formation making calcium nitrate a good candidate for *P. brassicae* control. Myers and Campbell (1985) confirmed in their preliminary investigations that in solution cultures calcium nitrate does reduce clubroot disease but their work was not carried out in compost or soil.

3.1. Field investigation into the concentration of calcium nitrate which reduces *P. brassicae* infection of brassicas.

The use of calcium nitrate in a field environment, to reduce gall formation on vegetable brassicas would reduce the need for separate further chemical controls and no extra field preparations would be required for its use. As very few controls are available for *P. brassicae* an addition through husbandry practices would be a valuable asset.

Method and materials.

The investigation was undertaken as described in Chapter 2. The experiment consisted of seven treatments in six replicates. The treatments were, calcium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}, ammonium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1} and a mixture of calcium nitrate and ammonium nitrate at a rate of 300 kg of nitrate ha^{-1} with 150 kg of nitrate coming from each fertiliser.

Soil was sampled from the experimental site prior to treatment, and at seven days post and twenty one days post treatment. A chemical analysis of these soil samples is given in Appendix 1.

In order to ensure the plants were growing well chlorophyll fluorescence measurements were made three weeks after the field trial was planted and vigour assessments were made six weeks after planting. These measurements helped to reduce the possibility that poor plant growth was influencing the experiment and to ensure that plants did not require irrigation, the measurements also helped to ensure that plant growth was

uniform and that there were not areas of the field where modules had established poorly. Chlorophyll fluorescence was used as it is considered a good indicator of general plant health. Vigour assessments were used as an alternative guide as chlorophyll fluorescence.

The experiment was harvested after fourteen weeks.

Results.

Forty plants were harvested from the centre three rows of each plot. The outside rows were avoided during harvest as these plants may have been contaminated by nutrient leaching from surrounding plots, these plants acted as a guard to reduce any leached fertilisers affecting the main experiment. The gall categories of the plants were recorded and the trimmed heads of the cabbages weighed.

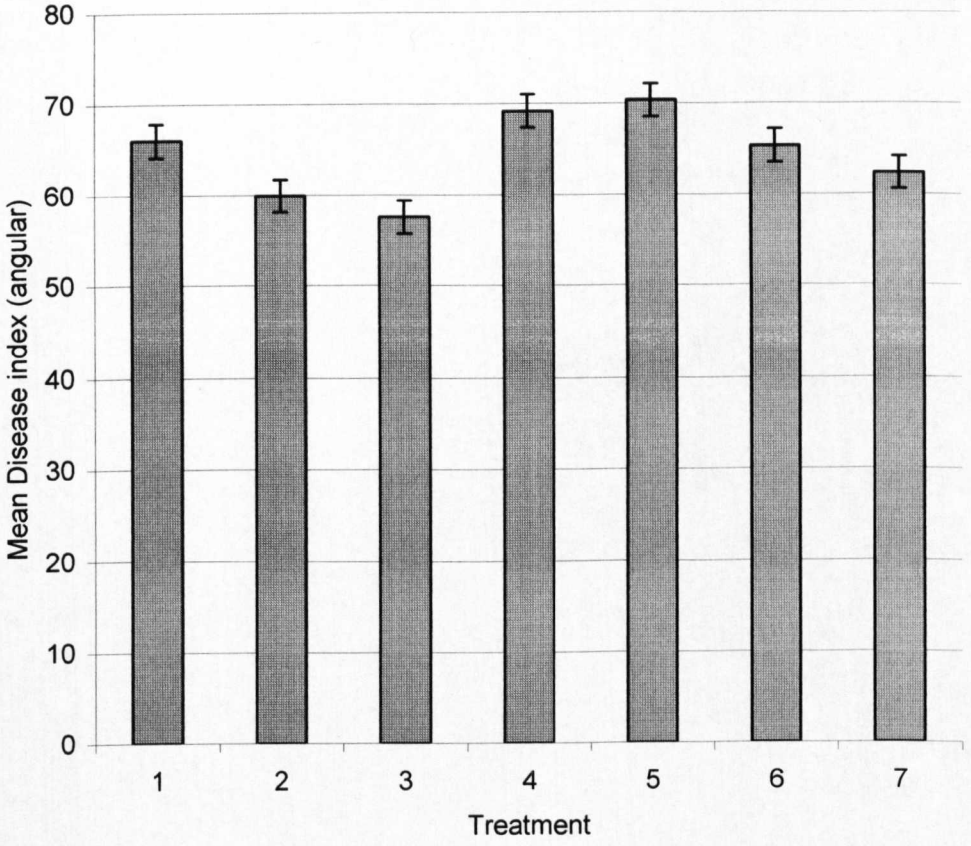
Table 3.1. Disease indices and mean treatment masses of plants from plots treated with calcium and ammonium nitrate.

Treatment	Disease Index (%) (angular)	Mean treatment head mass (kg)
Calcium nitrate at 200 kg of nitrate ha^{-1}	66b	18.12ab
Calcium nitrate at 300 kg of nitrate ha^{-1}	59.88 a	20.62b
Calcium nitrate at 400 kg of nitrate ha^{-1}	57.53 a	21.33b
Ammonium nitrate at 200 kg of nitrate ha^{-1}	69.16bc	14.23a
Ammonium nitrate at 300 kg of nitrate ha^{-1}	70.33c	15.57a
Ammonium nitrate at 400 kg of nitrate ha^{-1}	65.32bc	19.34b
Mix of calcium and ammonium nitrate at a rate of 300 kg of nitrate ha^{-1}	62.32ab	18.14ab
S.E.D	3.614	1.964
L.S.D	7.380	4.012

Values with the same letter are not significantly different using the L.S.D value.

The mean disease indices of each treatment are shown in Graph 3.1.1 and Table 3.1. The graph illustrates that with an increasing concentration of nitrate the level of galling is decreased. When comparing the level of disease at the individual rates of nitrogen applied it can be seen that the level of disease was always lower in plots treated with calcium nitrate. The lowest disease categories

Graph 3.1.1. Mean Disease Indices of plants treated with different fertilisers in a field experiment.



s.e.d = 3.614

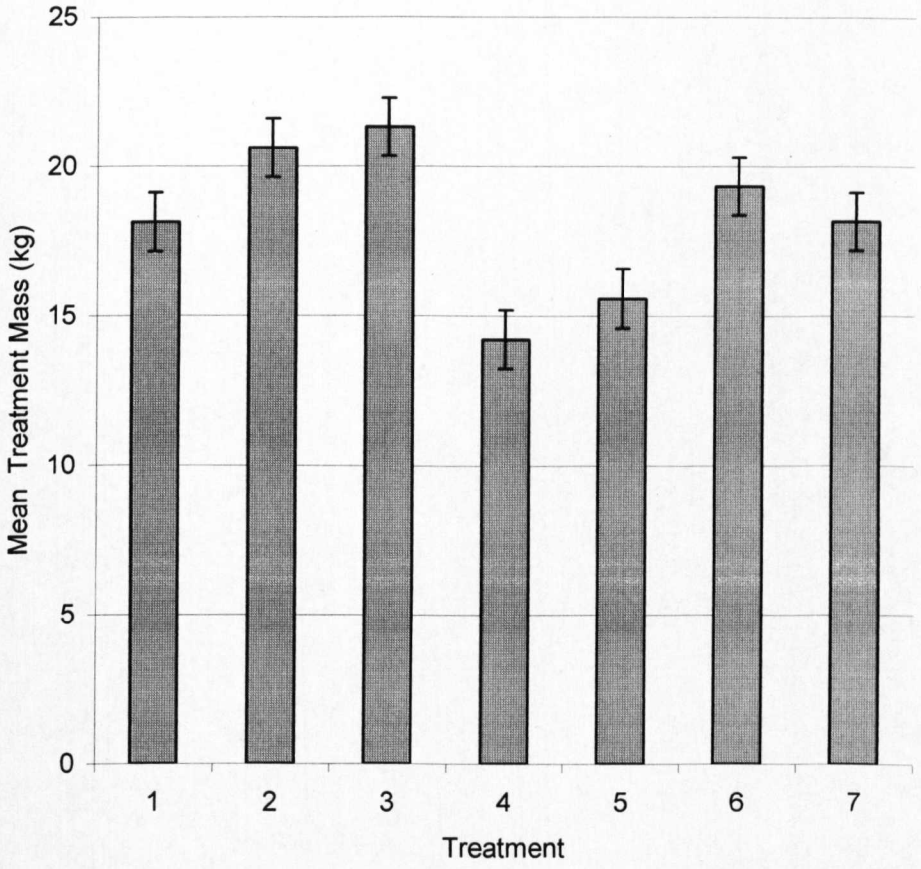
Treatments

1, 2, and 3 = Calcium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}

4, 5 and 6 =Ammonium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}

7 = Ammonium nitrate mixed with calcium nitrate at 300 kg of nitrate ha^{-1}

Graph 3.1.2. Mean plot head masses of plants treated with different fertilisers in a field experiment.



Standard error of difference = 1.964

Treatments

1, 2, and 3 = Calcium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}

4, 5 and 6 =Ammonium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}

7 = Ammonium nitrate mixed with calcium nitrate at 300 kg of nitrate ha^{-1}

were observed in plants from plots treated with calcium nitrate at a rate of 300 and 400 kg ha^{-1}.

The mean head mass for each treatment is given in Table 3.1 and illustrated in Graph 3.1.2, from which it can be seen from the graph that the mean masses of plants treated with calcium nitrate are significantly greater than the masses of plants treated with 200 and 300 kg of nitrate ha^{-1} from ammonium nitrate.

The mean masses of plants treated with 400 kg of nitrate ha^{-1} from calcium nitrate, were significantly higher than plants treated with ammonium nitrate with the exception of plants treated with 400 kg of nitrate ha^{-1} of nitrate from ammonium nitrate.

The mean mass of plants treated with a mixture of calcium nitrate and ammonium nitrate at a rate of 300 kg of nitrate ha^{-1}, was significantly higher than plants treated with 200 and 300 kg of nitrate / ha^{-1} from ammonium nitrate alone. This indicated that the addition of calcium nitrate, even in combination with ammonium nitrate, was associated with an increase in plant mass.

Conclusions

The results indicate that applications of calcium nitrate to a field infested with *P. brassicae* can decrease the extent of gall formation and increase produce mass when applied at a rate between 300 and 400 kg ha^{-1}. It would therefore appear that the nitrate form did influence plant growth and decrease disease development in this experiment. It is not clear however whether the host plant resistance to the pathogen is affected by calcium nitrate or the pathogen itself.

3.2 Glasshouse investigation into the interactions between calcium nitrate, *P. brassicae* and time.

The experiment aimed to determine whether the effects of calcium nitrate on the level of *P. brassicae* infection were influenced by the time of application. If applications made prior to transplanting are more effective it would suggest that calcium nitrate may be affecting *P. brassicae* resting spores.

Experiment 3.1 established that treating the field soil, and hence *P. brassicae* resting spores, with calcium nitrate one week prior to planting decreased the level of clubroot. The experiment did not however indicate whether the level of disease would be decreased by extending the time between calcium nitrate application and planting, hence extending the time *P. brassicae* resting spores are exposed to calcium nitrate prior to a suitable host being presented.

This experiment aimed to determine whether the level of clubroot can be decreased further if the soil is treated with calcium nitrate three weeks prior to planting.

Method and materials.

Soil samples which had been taken from random plots of all the treatments in the field trial prior to treatment, one week after treatment and three weeks after treatment in the manner described in Chapter 2, were removed from storage and allowed to return to glasshouse temperature.

The experiment consisted of 21 treatments in three replicates arranged in a random block design. The soil samples were mixed

with perlite and placed in small seed trays as described in chapter 2. Twenty, seven-day-old Chinese cabbage seedlings cv. Mariko raised in modular peat compost (Levington's F1) were transplanted into each tray. The treatments were therefore soil samples of :-

Calcium nitrate and ammonium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1} and a mixture of calcium nitrate and ammonium nitrate at a rate of 300 kg of nitrate ha^{-1} sampled prior to treatment, seven days post treatment and 14 days post treatment.

The plants were harvested five weeks after planting.

Results.

Ten plants were randomly harvested from each tray. It was not possible to harvest all of the plants as some had not established after transplantation leading to losses, ten plants per tray was chosen as a harvest figure in order to over come this.

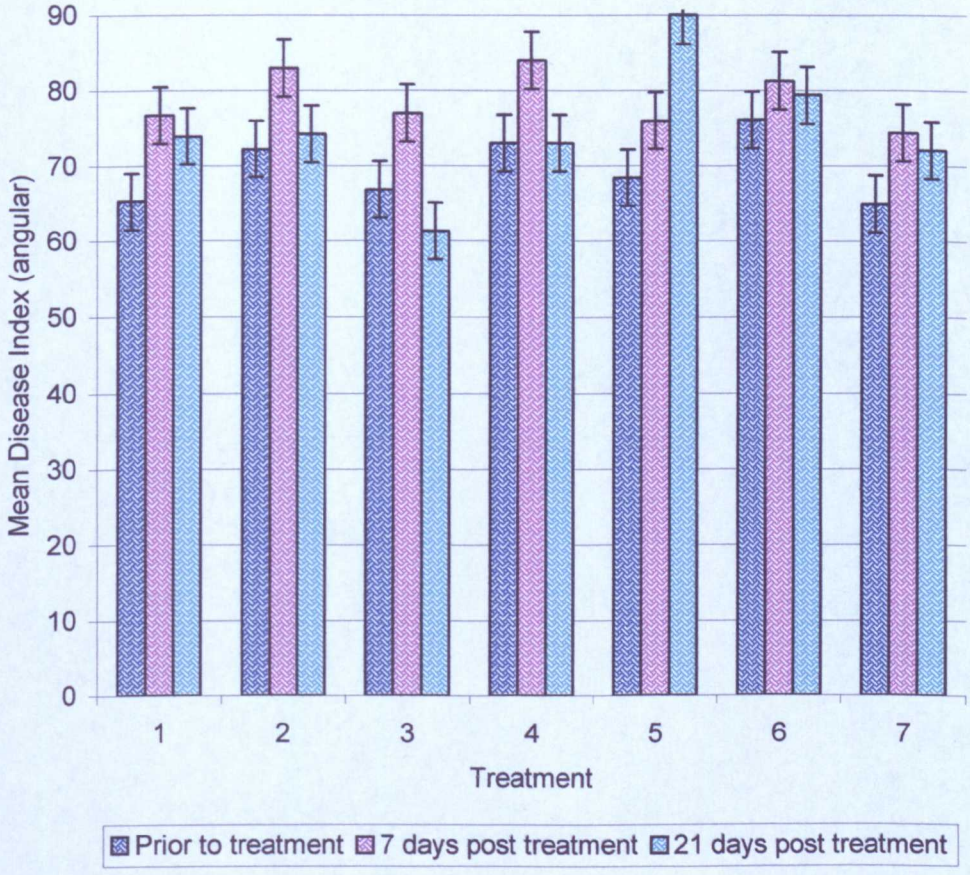
Table 3.2.1. Disease indices (angular) of plants grown in calcium nitrate treated soil.

	Prior to treatment	7 days post treatment	21 days post treatment
Calcium nitrate at 200 kg of nitrate ha^{-1}	65.2a	76.7ab	73.9a
Calcium nitrate at 300 kg of nitrate ha^{-1}	72.2a	83b	74.2a
Calcium nitrate at 400 kg of nitrate ha^{-1}	66.8a	77b	61.3a
Ammonium nitrate at 200 kg of nitrate ha^{-1}	73a	84b	73a
Ammonium nitrate at 300 kg of nitrate ha^{-1}	68.4ab	76ab	90b
Ammonium nitrate at 400 kg of nitrate ha^{-1}	76.1a	81.3b	79.4b
Mix of calcium and ammonium nitrate at a rate of 300 kg of nitrate ha^{-1}	64.9a	74.4a	72.1ab
S.E.D	7.57		
L.S.D	15.53		

Values with the same letter are not significantly different using the L.S.D value.

Graph 3.2.1 illustrates the general trend that plants grown in pre-treated soil have a slightly lower disease index. The lowest disease index, however was found on plants grown in soil treated with calcium nitrate at a rate of 400 kg of nitrate ha^{-1} and collected 21 days after treatment. The highest disease index was found in plants grown in soil collected 21 days after treatment with

Graph 3.2.1. Mean disease categories (angular) of plants treated with different fertilisers at three different times.



s.e.d = 7.57

Treatments.

1, 2, and 3 = Calcium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}

4, 5 and 6 =Ammonium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}

7 = Ammonium nitrate mixed with calcium nitrate at 300 kg of nitrate ha^{-1}

ammonium nitrate at a rate of 300 kg of nitrate ha^{-1}. The disease indices were transformed using angles to allow statistical analysis.

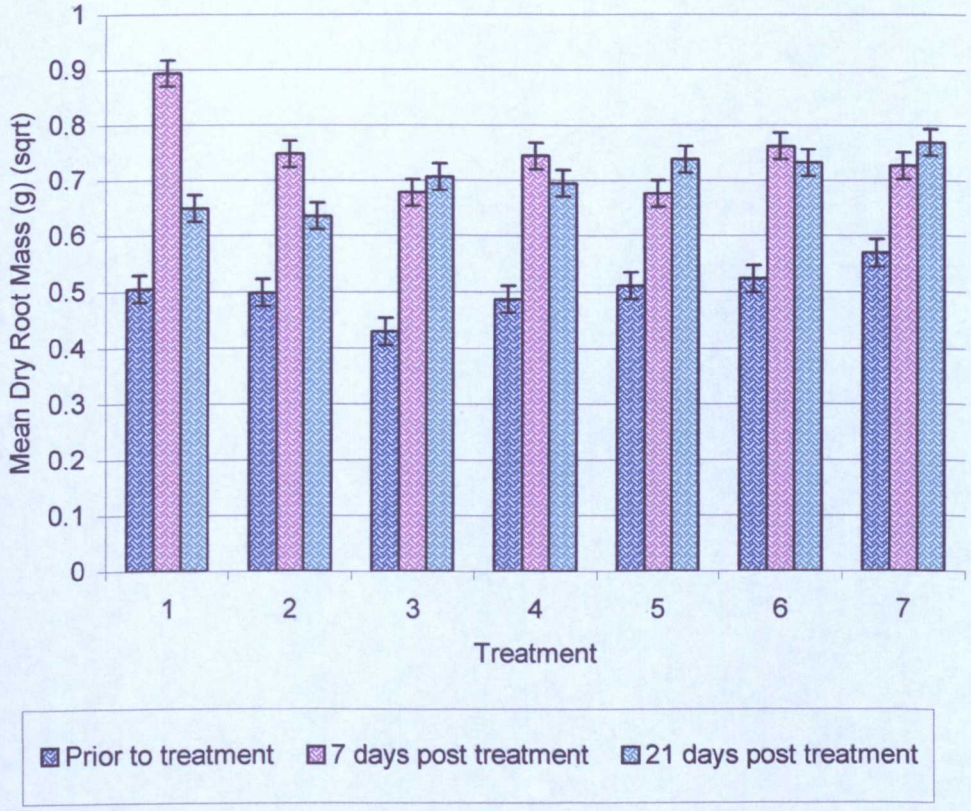
Table 3.2.2. Mean dry root (sqrt) (g) masses of plants grown in calcium nitrate treated soil.

	Prior to treatment	7 days post treatment	21 days post treatment
Calcium nitrate at 200 kg of nitrate ha^{-1}	0.566a	0.8456ac	0.8484a
Calcium nitrate at 300 kg of nitrate ha^{-1}	0.556a	0.9716b	0.9129ab
Calcium nitrate at 400 kg of nitrate ha^{-1}	0.4935a	0.9251bc	0.8987a
Ammonium nitrate at 200 kg of nitrate ha^{-1}	0.5360a	0.8126a	0.9338a
Ammonium nitrate at 300 kg of nitrate ha^{-1}	0.5378a	0.7994a	0.8459a
Ammonium nitrate at 400 kg of nitrate ha^{-1}	0.5605a	0.8123a	0.9559b
Mix of calcium and ammonium nitrate at a rate of 300 kg of nitrate ha^{-1}	0.6697b	0.8375ac	1.1377c
S.E.D	0.05098		
L.S.D	0.10016		

Values with the same letter are not significantly different using the L.S.D value.

The dry root mass was transformed using the square root due to the data being skewed. The mean dry root masses are given in Graph 3.2.2 and Table 3.2.2 illustrate that plants grown in soil

Graph 3.2.2. Mean dry root masses of plants treated with three different fertilisers at three different times.



s.e.d = 0.048

Treatments

1, 2, and 3 = Calcium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}

4, 5 and 6 =Ammonium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}

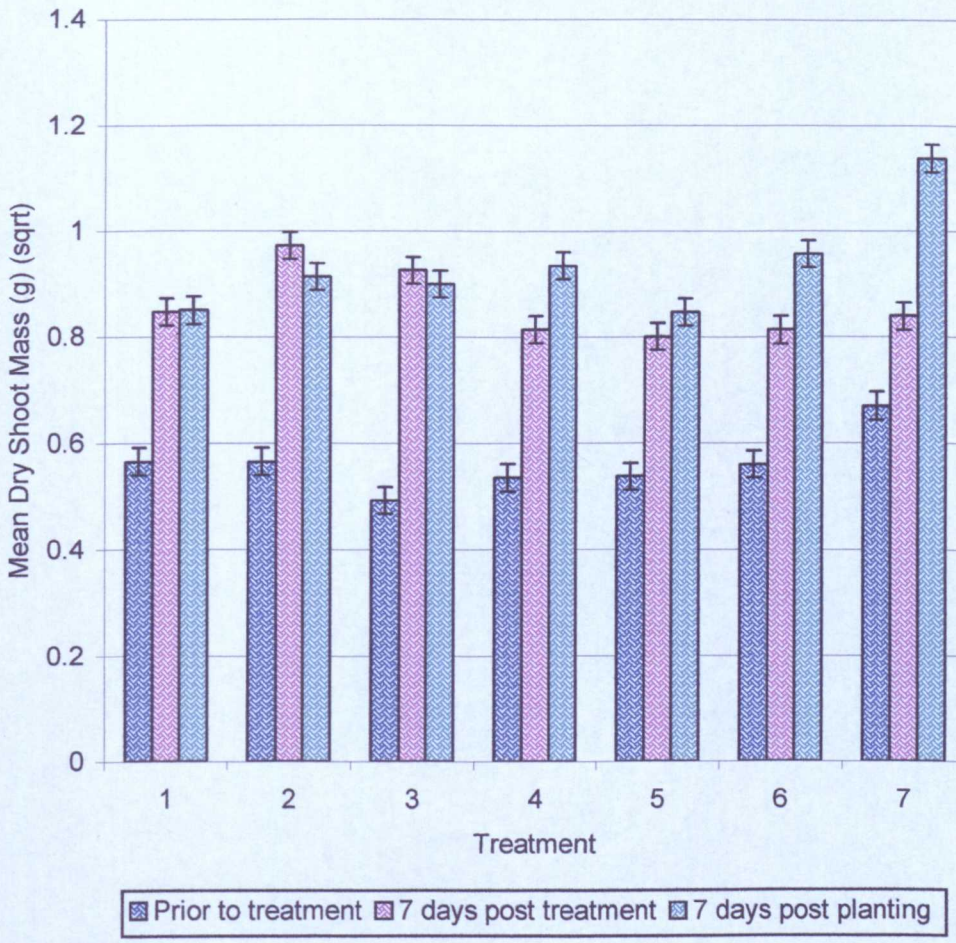
7 = Ammonium nitrate mixed with calcium nitrate at 300 kg of nitrate ha^{-1}

samples collected prior to fertiliser application consistently had lower dry root masses. The highest root masses occurred on plants which were grown in soil collected 7 days after treatment and treated with ammonium nitrate at a rate of 400 kg of nitrate ha^{-1} from ammonium nitrate. Higher root masses are generally associated with more extensive galling and as was illustrated in Graph 3.2.1 the mean disease indices were lower for plants grown in untreated soil.

Table 3.2.3. Mean dry root (sqrt) (g) masses of plants grown in calcium nitrate treated soil.

	Prior to treatment	7 days post treatment	21 days post treatment
Calcium nitrate at 200 kg of nitrate ha^{-1}	0.5056a	0.8938f	0.6484d
Calcium nitrate at 300 kg of nitrate ha^{-1}	0.4979a	0.7478e	0.6346d
Calcium nitrate at 400 kg of nitrate ha^{-1}	0.4295a	0.6777d	0.7065d
Ammonium nitrate at 200 kg of nitrate ha^{-1}	0.4872a	0.7437e	0.6944d
Ammonium nitrate at 300 kg of nitrate ha^{-1}	0.5114a	0.6764d	0.7379e
Ammonium nitrate at 400 kg of nitrate ha^{-1}	0.5231b	0.7619	0.7325e
Mix of calcium and ammonium nitrate at a rate of 300 kg of nitrate ha^{-1}	0.5696c	0.7266d	0.7692e
S.E.D	0.04805		
L.S.D	0.09442		

Graph 3.2.3. Mean dry shoot masses of plants treated with three different fertilisers at three different times.



s.e.d = 0.051

Treatments.

1, 2, and 3 = Calcium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}

4, 5 and 6 =Ammonium nitrate at rates of 200, 300 and 400 kg of nitrate ha^{-1}

7 = Ammonium nitrate mixed with calcium nitrate at 300 kg of nitrate ha^{-1}

Values with the same letter are not significantly different using the L.S.D value.

Table 3.2.3 and Graph 3.2.3 give the mean dry shoot masses of each treatment which were transferred using the square root function due to the data being skewed. Plants raised in soil samples collected before the soil was treated had the lowest shoot masses. Plants treated with calcium nitrate had a higher shoot mass than plants treated with ammonium nitrate.

Conclusions

The application of calcium nitrate prior to planting decreased the level of infection when compared to equivalent ammonium nitrate applications. The most effective application being calcium nitrate at 400 kg of nitrate ha^{-1} applied 21 days prior to planting. The higher shoot masses of plants grown in treated soils illustrate the effects of fertilisation upon Chinese cabbage. The addition of fertiliser to the soil resulted in increased shoot growth which in turn may have led to increased galling. Gall production relies upon a supply of carbohydrates from the shoots of infected plants i.e. with an increase in shoot size the production of carbohydrates increases leading to the production of bigger galls. The joint application of both fertilisers resulted in increased galling and increased plant growth possibly due to the acidifying effect of the ammonium nitrtae. This experiment therefore illustrated the effects of fertilistion upon Chinese cabbage as well the effects upon *P. brassicae* infection.

3.3. Determination of whether treating brassica seedlings prior to their exposure to *P. brassicae*, with low concentrations of calcium nitrate decreases the extent of host galling.

These experiments were designed to determine whether treating brassica plants with calcium whilst they are growing in module plug trays provides the plants with protection against *P. brassicae* infection upon transplantation to inoculated compost.

If applying calcium nitrate to plants prior to their transplantation into *P. brassicae* infected soil were to decrease the level of clubroot, it would suggest that the reduction in disease is due to the effect of calcium nitrate upon the host rather than its effect on *P. brassicae*. Ammonium nitrate was also used in this experiment so that it would be possible to see if any reduction in galling was due to fertilisation of the plants rather than a specific effect of calcium nitrate.

3.3.1. Comparison of the effects of treating brassica seedlings with calcium nitrate or ammonium nitrate upon *P. brassicae* infection.

Method and materials.

Chinese cabbage cv. Mariko was sown in to twelve p450 modular plug trays each consisting of fifteen plugs filled with Arthur Bower's seed and potting compost and placed in a glasshouse. Each tray was irrigated with 75 ml of one of the treatment solutions as required. The treatments were :-

1. Sterile de-ionised water
2. 32.7 kg of nitrate ha^{-1} provided by calcium nitrate (the equivalent of 15 mel of calcium)
3. 32.7 kg of nitrate ha^{-1} provided by ammonium nitrate.

mel = milligram equivalents / l, The formula used to calculate this is given in Appendix 1.

The concentration of 15 mel calcium was chosen as this was a concentration used by Webster (1985) which retarded the maturation of *P. brassicae* in host root hairs. Low concentrations of calcium were used as it can be toxic to plants in high concentrations especially very young seedlings.

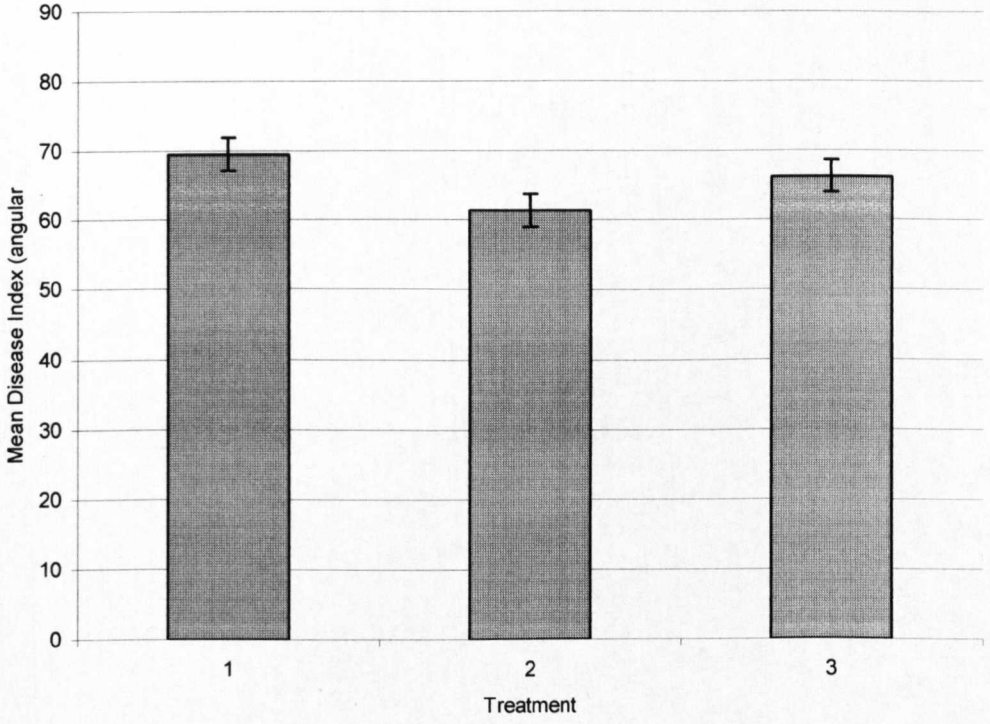
Ten plants from each modular tray were transplanted into small seed trays filled with Arthur Bower's Seed and Potting Compost and inoculated with 100 ml of a 10^6 resting spores /ml suspension of *P. brassicae* as described in chapter 2.

Results.

The plants were harvested five weeks after transplantation.

Graph 3.3.1 and Table 3.3.1 illustrate the mean disease indices. The disease index of plants treated with calcium nitrate was the lowest. Plants treated with ammonium nitrate had a mean disease index lower than the infected control plants but not as low as plants treated with calcium nitrate.

Graph 3.3.1. Mean disease indices of plants treated with different fertilisers prior to transplantation to *P. brassicae* infested compost.



□s.e.d = 4.72

Treatments

1 Sterile de-ionised water

2 32.7 kg of nitrate ha^{-1} provided by calcium nitrate (the equivalent of 15 mel of calcium)

3 32.7 kg of nitrate ha^{-1} provided by ammonium nitrate.

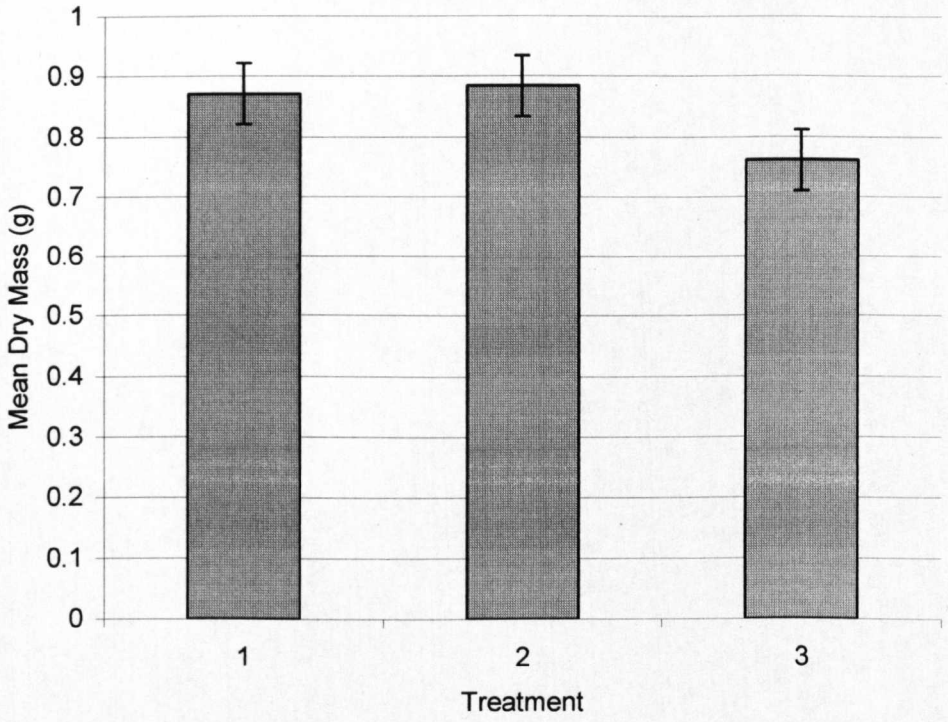
Table 3.3.1. Mean disease indices (angular) of plants treated with calcium and ammonium nitrate prior to transplantation in to P. brassicae infected soil.

Treatment	Disease Index (angular)
Sterile de-ionised water	69.5a
32.7 kg of nitrate / ha^{-1} provided by calcium nitrate (the equivalent of 15 mel of calcium)	61.4a
32.7 kg of nitrate / ha^{-1} provided by ammonium nitrate.	66.3a
S.E.D	4.72
L.S.D	11.56

Values with the same letter are not significantly different using the L.S.D value.

Graph 3.3.2 and Table 3.3.2 illustrate the mean root masses. Plants treated with ammonium nitrate had a lower root mass than both control and calcium nitrate treated plants but the results were not significantly different when compared using the L.S.D value.

Graph 3.3.2. Mean dry root masses of plants treated with different fertilisers prior to transplantation to *P. brassicae* infested compost.



s.e.d = 0.1008

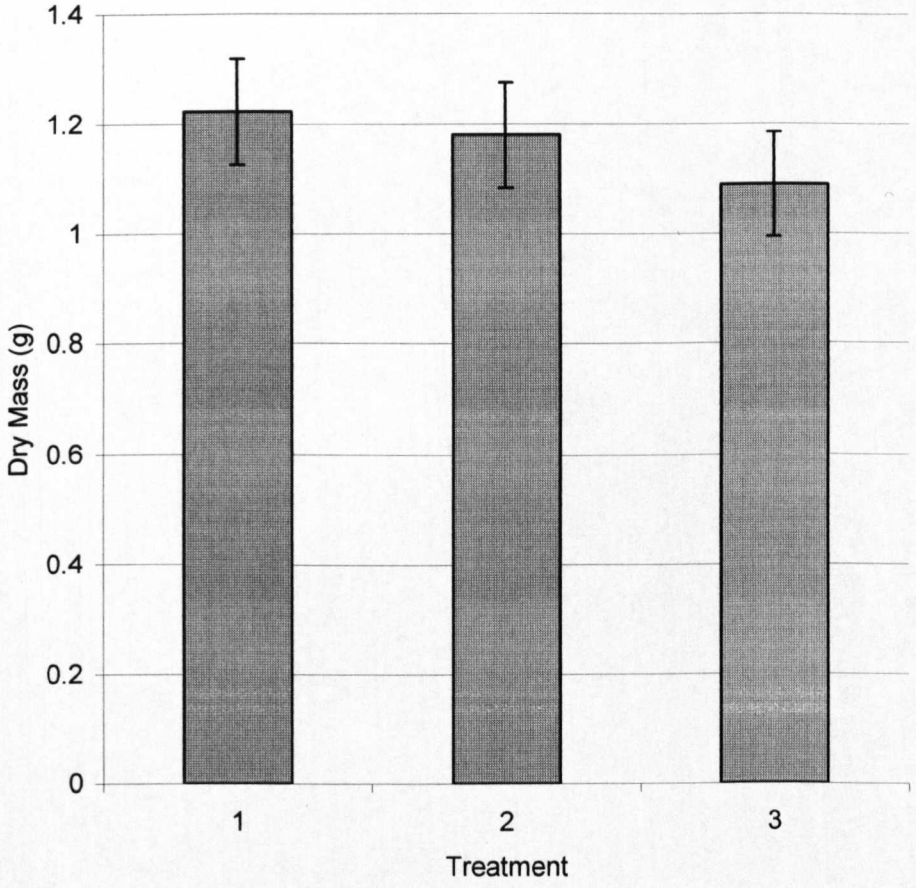
Treatments

1 Sterile de-ionised water

2 32.7 kg of nitrate ha^{-1} provided by calcium nitrate (the equivalent of 15 mel of calcium)

3 32.7 kg of nitrate ha^{-1} provided by ammonium nitrate.

Graph 3.3.3. Mean dry shoot masses of plants treated with different fertilisers prior to transplantation to *P. brassicae* infested compost.



s.e.d = 0.191

Treatments

1 Sterile de-ionised water

2 32.7 kg of nitrate ha^{-1} provided by calcium nitrate (the equivalent of 15 mel of calcium)

3 32.7 kg of nitrate ha^{-1} provided by ammonium nitrate.

Table 3.3.2. Mean dry root mass (g) of plants treated with calcium and ammonium nitrate prior to transplantation in to P. brassicae infected soil.

Treatment	Mean dry root mass (g)
Sterile de-ionised water	0.871a
32.7 kg of nitrate / ha^{-1} provided by calcium nitrate (the equivalent of 15 mel of calcium)	0.884a
32.7 kg of nitrate / ha^{-1} provided by ammonium nitrate.	0.761a
S.E.D	0.1008
L.S.D	0.1998

Values with the same letter are not significantly different using the L.S.D value.

The mean dry shoot masses are shown in Graph 3.3.3 and there were no significant differences between dry shoot masses from the treatments.

Conclusions

When applied to modules calcium nitrate did not reduce *P. brassicae* infection indicating that calcium nitrate is affecting the pathogen rather than promoting resistance in the host plant. Plants treated with ammonium nitrate had a lower disease index and a correspondingly lower root mass, however the shoot mass was not significantly lower than calcium nitrate treated and control plants

suggesting that the galls were smaller due to poorer plant growth rather than any resistance effect.

3.3.2. Further comparison of the effects of treating brassica seedlings with calcium nitrate or ammonium nitrate upon *P. brassicae* infection.

This experiment was aimed at determining whether a lower rate of calcium nitrate to treat brassica seedlings prior to transplantation decreased the level of *P. brassicae* infection. Since high rates of calcium may be toxic to plants and the rate of application was reduced to test the effect on host resistance.

Method and materials.

P450 modular plug trays were cut into sixteen units each containing eight modules. The modules were filled with Arthur Bower's Seed and Potting Compost and sown with Chinese cabbage seed cv Mariko and placed in a glasshouse. The trays were irrigated with one of the following treatments.

1. Sterile de-ionised water.
2. 50 kg ha^{-1} of calcium nitrate (7.75 kg of nitrate ha^{-1}, 2.54 mel calcium nitrate)
3. 75 kg ha^{-1} of calcium nitrate (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate)
4. 100 kg ha^{-1} of calcium nitrate (15.5 kg of nitrate ha^{-1}, 5.2 mel calcium nitrate)

Each plug received 5 ml of the appropriate solution. Two weeks after sowing four seedlings from each unit were transplanted into

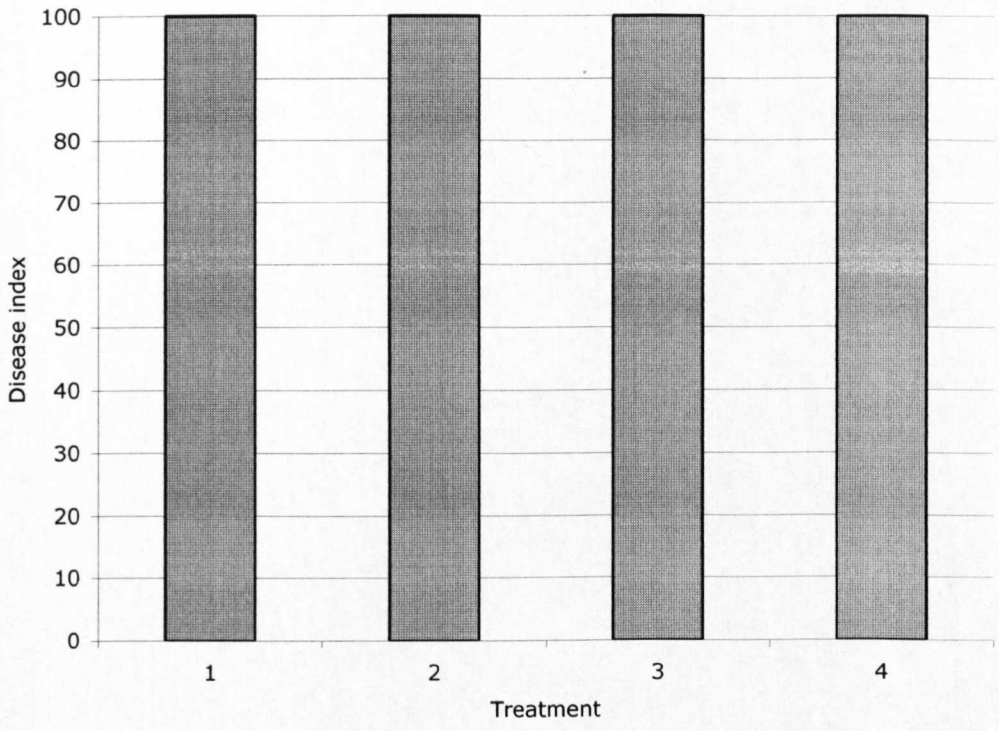
four FP8 pots filled with Arthur Bower's seed and potting compost. Each pot had been inoculated with 30 ml of a 10^6 resting spores ml^{-1} *P. brassicae* suspension in the manner described in Chapter 2. This gave a total of four treatments in four replicates.
The experiment was harvested six weeks after transplantation.

Results.

The mean disease categories for each treatment are given in Graph 3.3.4. Plants from all of the treatments had disease indices of 100 %. Applying these levels of calcium nitrate to the seedlings whilst they are still growing in their modules did not decrease the subsequent level of *P. brassicae* infection.

Graph 3.3.5 illustrates the dry root masses of plants grown in this experiment. The mean root mass of plants treated with calcium nitrate at a rate of 11.62 kg of nitrate ha^{-1} was significantly higher than the masses of the other treatments.

Graph 3.3.4. Mean disease indices of plants treated with different fertilisers prior to transplantation to *P. brassicae* infested compost.



Treatments

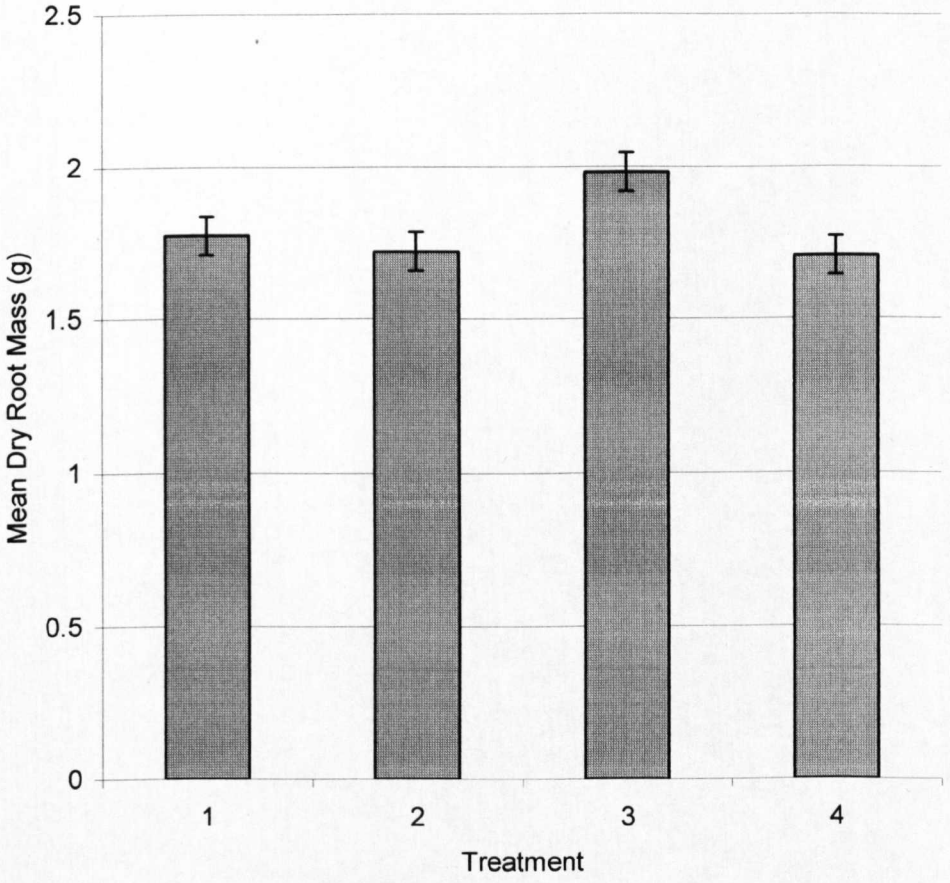
1 Sterile de-ionised water.

2 50 kg / ha^{-1} of calcium nitrate (7.75 kg of nitrate ha^{-1}, 2.54 mel calcium nitrate)

3 75 kg / ha^{-1} of calcium nitrate (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate)

100 kg ha^{-1} of calcium nitrate (15.5 kg of nitrate ha^{-1}, 5.2 mel calcium nitrate)

Graph 3.3.5. Mean dry root masses of plants treated with different fertilisers prior to transplantation to *P. brassicae* infested compost.



Standard error of difference = 0.127

Treatments

1 Sterile de-ionised water.
2 50 kg ha^{-1} of calcium nitrate (7.75 kg of nitrate ha^{-1}, 2.54 mel calcium nitrate)
3 75 kg ha^{-1} of calcium nitrate (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate)
4 100 kg ha^{-1} of calcium nitrate (15.5 kg of nitrate ha^{-1}, 5.2 mel calcium nitrate)

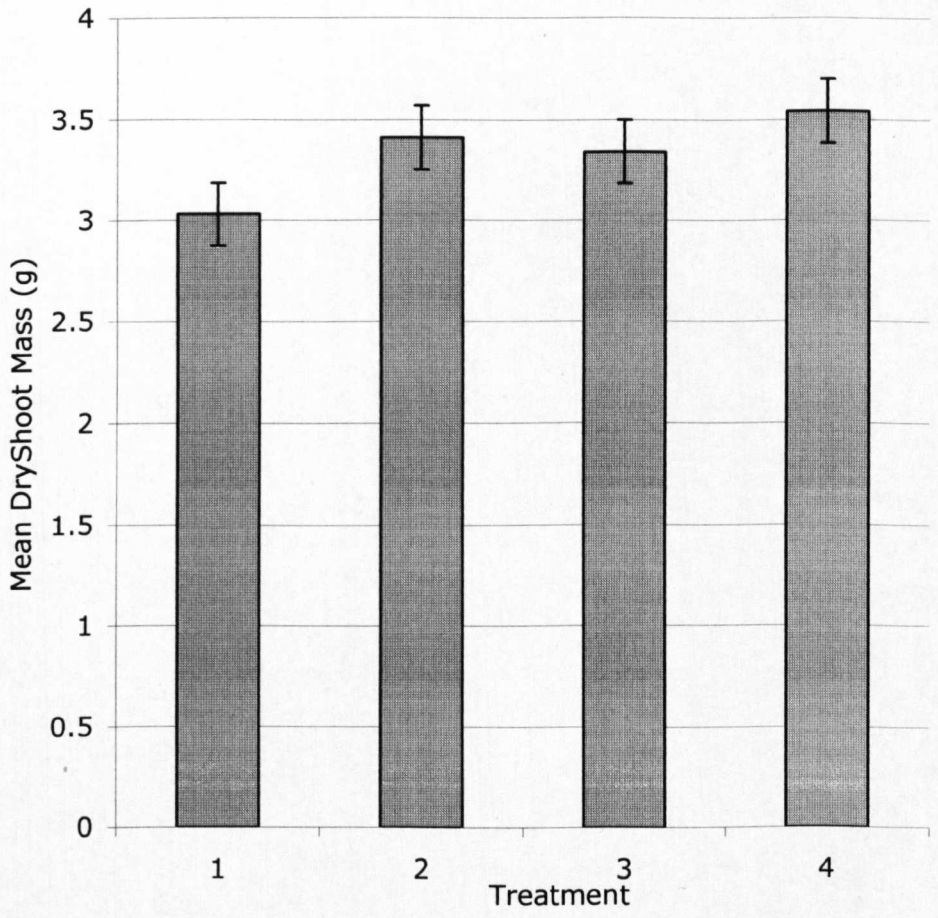
Table 3.3.5. Mean dry root mass (g) of plants treated with calcium and ammonium nitrate prior to transplantation in to *P. brassicae* infected soil.

Treatment	Mean dry root mass (g)
Sterile de-ionised water.	1.773ab
50 kg ha^{-1} of calcium nitrate (7.75 kg of nitrate ha^{-1}, 2.54 mel calcium nitrate)	1.720a
75 kg ha^{-1} of calcium nitrate (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate)	1.982b
100 kg ha^{-1} of calcium nitrate (15.5 kg of nitrate ha^{-1}, 5.2 mel calcium nitrate)	1.706a
S.E.D	0.127
L.S.D	0.2515

Values with the same letter are not significantly different using the L.S.D value.

The mean dry shoot masses, given in Graph 3.3.6 and Table 3.3.6, of plants treated with calcium nitrate at a rate of 15.5 kg of nitrate ha^{-1} were greater than those of other treatments but not significantly so when compared using the L.S.D values. There was no significant difference in mass between calcium nitrate treated plants.

Graph 3.3.6. Mean dry shoot masses of plants treated with different fertilisers prior to transplantation to *P. brassicae* infested compost.



s.e.d = 0.314

Treatments

1 Sterile de-ionised water.

2 50 kg ha^{-1} of calcium nitrate (7.75 kg of nitrate ha^{-1}, 2.54 mel calcium nitrate)

3 75 kg ha^{-1} of calcium nitrate (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate)

4 100 kg ha^{-1} of calcium nitrate (15.5 kg of nitrate ha^{-1}, 5.2 mel calcium nitrate)

Table 3.3.6. Mean dry shoot mass (g) of plants treated with calcium and ammonium nitrate prior to transplantation in to P. brassicae infected soil.

Treatment	Mean dry root mass (g)
Sterile de-ionised water.	3.03a
50 kg ha^{-1} of calcium nitrate (7.75 kg of nitrate ha^{-1}, 2.54 mel calcium nitrate)	3.41a
75 kg ha^{-1} of calcium nitrate (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate)	3.34a
100 kg ha^{-1} of calcium nitrate (15.5 kg of nitrate ha^{-1}, 5.2 mel calcium nitrate)	3.54a
S.E.D	0.314
L.S.D	0.621

Values with the same letter are not significantly different using the L.S.D value.

Conclusions.

From the results in experiments 3.2 and 3.3 it would appear that treating young Chinese cabbage seedlings cv Mariko with calcium nitrate at a rate of 11.62 kg of nitrate ha^{-1} prior to transplantation increased root mass, which may indicate a higher density of gall

material. The application of calcium nitrate did not however reduce the level of plant galling upon exposure to *P. brassicae* infested soil. It would therefore seem that calcium nitrate does not encourage resistance *to P. brassicae*.

3.4. The effect of treating seeds with calcium nitrate prior to sowing on subsequent *P. brassicae* infection.

The aim of this experiment was to establish whether germinating seed in a solution containing calcium nitrate affected the subsequent level of *P. brassicae* infection.

Treating seeds with calcium nitrate was considered as an alternative to treating seedlings prior *P. brassicae* exposure.

The experiment was undertaken to assess whether calcium nitrate can influence the host plant resistance if applied from prior to seed germination. In applying calcium nitrate solely to the seed, the compost micro flora and the resting spores will not be influenced by calcium nitrate. Hence if the level of *P. brassicae* infection in plants germinated in calcium nitrate were to be reduced it would indicate that calcium nitrate influences host resistance to *P. brassicae* infection rather than the pathogen itself.

Method and materials.

One piece of 90 mm No. 1 filter paper was placed into the lid of a 90 mm petri dish (BDH) and two pieces into the base of the dish.

Each dish was irrigated with one of the four treatment solutions. Three and a half ml of the treatment solution was applied to the

paper in the base of the dish and 1.5 ml was applied to the lid (Webster, 1985).

The treatment solutions were :-

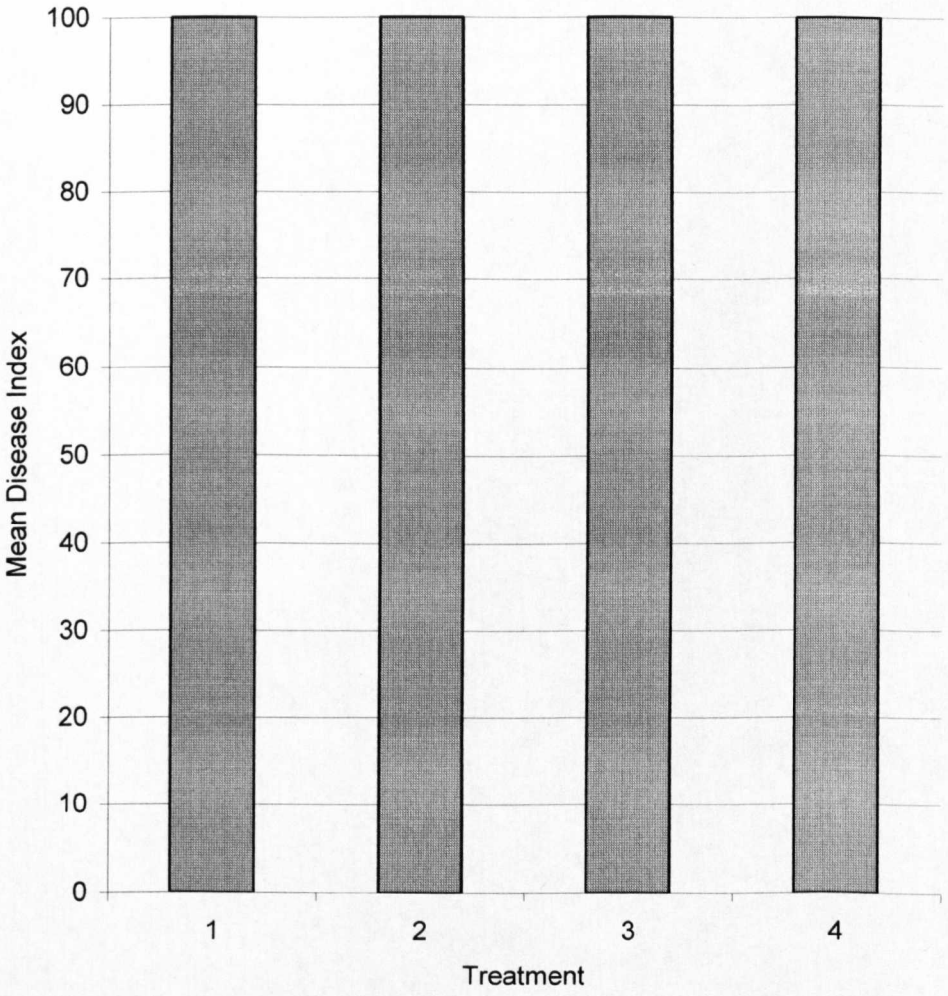
1. Sterile de-ionised water.
2. 50 kg ha^{-1} of calcium nitrate (7.75 kg of nitrate ha^{-1}, 2.54 mel calcium nitrate)
3. 75 kg ha^{-1} of calcium nitrate (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate)
4. 100 kg ha^{-1} of calcium nitrate (15.5 kg of nitrate ha^{-1}, 5.2 mel calcium nitrate)

Twenty five surface sterilised seeds (Chapter 2) were spaced equidistantly in the base of each petri dish. The dishes were incubated in a cooled incubator (Gallenkamp) at 22^0C for 48 h.

The seeds were transferred into FP8 pots filled with Arthur Bower's seed and potting compost and placed into a glass house. Sixteen pots were sown with one seed from each treatment. Only seeds which had started to germinate were used in order to standardise seed quality. The experiment therefore consisted of four treatment replicates each consisting of four pots.

The plants were inoculated with *P. brassicae* seven days after sowing by irrigating each pot with 30 ml of a suspension containing 10^6 resting spores ml^{-1}. The plants were harvested six weeks after inoculation.

Graph 3.4.1. Mean Disease Indices of plants whose seeds were treated with calcium nitrate prior to sowing in *P. brassicae* infested compost.



Treatments

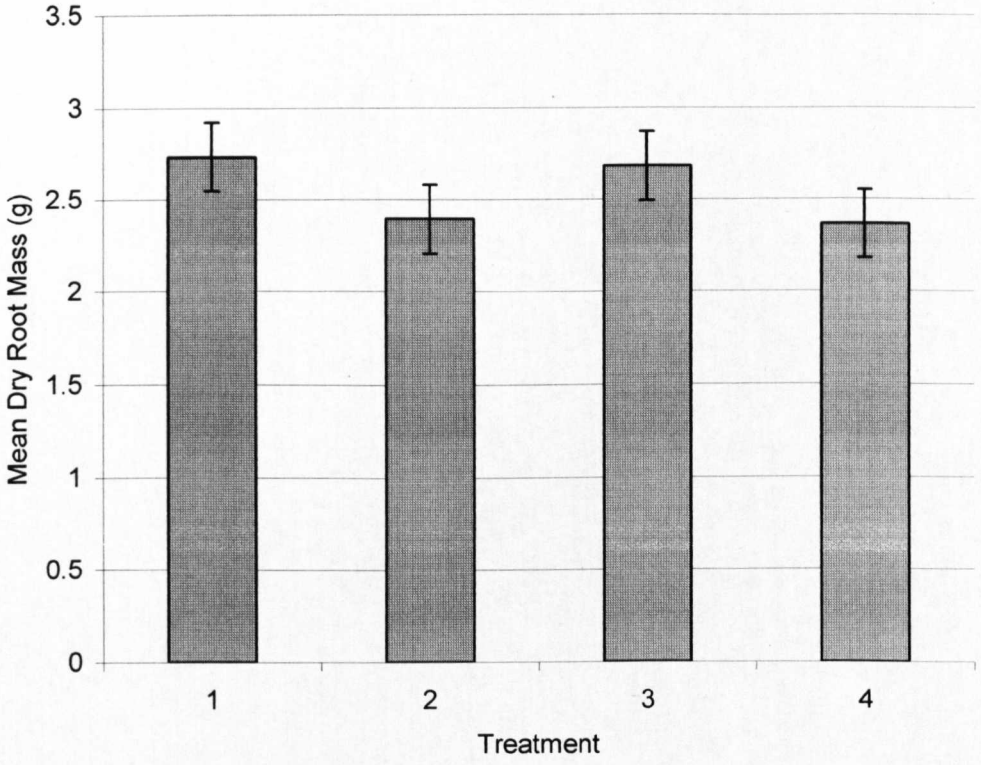
1 Sterile de-ionised water.

2 50 kg ha^{-1} of calcium nitrate (7.75 kg of nitrate ha^{-1}, 2.54 mel calcium nitrate)

3 75 kg ha^{-1} of calcium nitrate (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate)

4 100 kg ha^{-1} of calcium nitrate (15.5 kg of nitrate ha^{-1}, 5.2 mel calcium nitrate)

Graph 3.4.2. Mean dry root masses of plants whose seeds were treated with calcium nitrate prior to sowing i*n P. brassica*e infested .compost

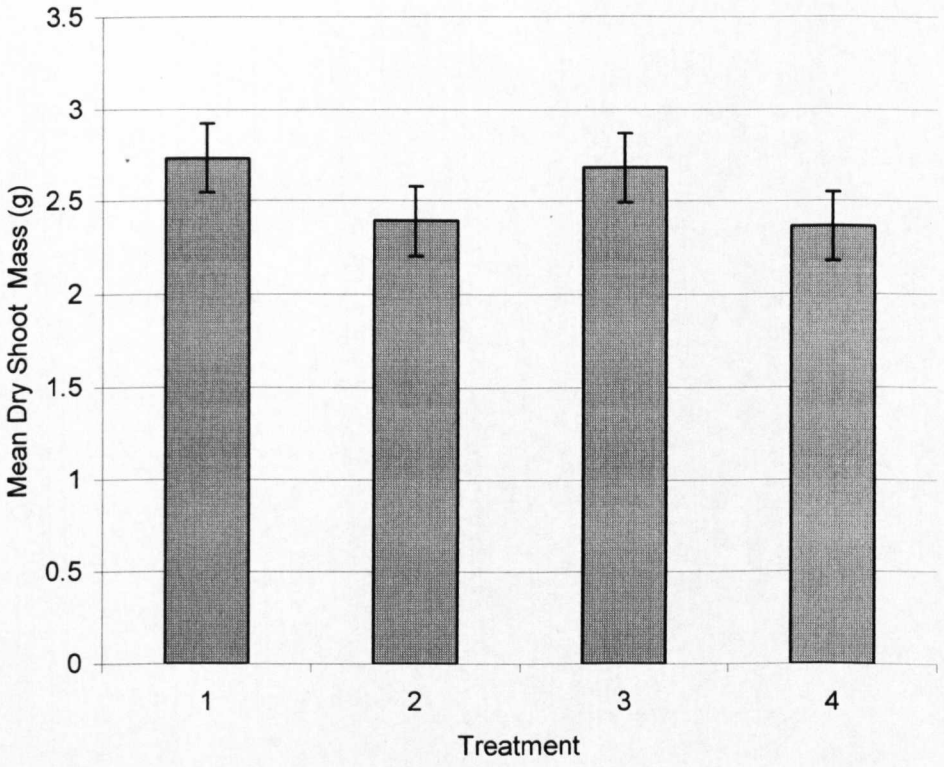


s.e.d = 0.334

Treatments

1 Sterile de-ionised water.
2 50 kg ha^{-1} of calcium nitrate (7.75 kg of nitrate ha^{-1}, 2.54 mel calcium nitrate)
3 75 kg / ha^{-1} of calcium nitrate (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate)
4 100 kg / ha^{-1} of calcium nitrate (15.5 kg of nitrate ha^{-1}, 5.2 mel calcium nitrate)

Graph 3.4.3. Mean dry shoot masses of plants whose seeds were treated with calcium nitrate prior to sowing *in P. brassica*e infested compost



s.e.d = 0.375

Treatments

1 Sterile de-ionised water.
2 50 kg ha^{-1} of calcium nitrate (7.75 kg of nitrate ha^{-1}, 2.54 mel calcium nitrate)
3 75 kg ha^{-1} of calcium nitrate (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate)
4 100 kg / ha^{-1} of calcium nitrate (15.5 kg of nitrate ha^{-1}, 5.2 mel calcium nitrate)

Results.

The mean disease indices are illustrated in Graph 3.4.1 all plants had a disease index of 100%. The treatment of seeds with low levels of calcium nitrate does not decrease the level of clubroot infection.

The mean dry root masses are given in Graph 3.4.2. The graph shows that there was no significant difference in root mass either between treatments or between treatments and the control. There was no significant difference between the dry shoot masses, given in Graph 3.4.3.

Conclusions

The germination of Chinese cabbage seeds cv Mariko in calcium nitrate solutions did not influence future plant growth or decrease gall formation and hence it can be concluded that at these concentrations calcium nitrate does not influence the level of host plant infection by *P. brassicae.*

3.5 Investigation into the effect of calcium nitrate on *P. brassicae* resting spore viability.

The aim of this experiment was to determine whether calcium nitrate can alter the viability of *P. brassicae* resting spores.

Plasmodiophora brassicae resting spores take 36 hours after spore suspension preparation to germinate (Aist and Williams,1971). Therefore if the level of *P. brassicae* infection in host plants is decreased when resting spores are incubated with calcium nitrate for 24 hours prior to being used to inoculate host plants, it would suggest that calcium nitrate is having a direct effect upon resting spore viability and not upon 1^0 zoospores.

If the level of clubroot in plants inoculated with *P. brassicae* resting spores treated with calcium nitrate for 48 hours before use is decreased, whilst the level of infection was not decreased in plants inoculated with resting spores incubated with calcium nitrate for 24 hours, then it would suggest that calcium nitrate is affecting primary zoospores as these begin to be released 36 hours after spore suspension preparation (Aist and Williams, 1971).

If the level of infection is decreased after 24 hours incubation and then decreased further after 48 hours incubation calcium nitrate may be affecting both resting spores and 1^0 zoospores.

Method and materials.

A *P. brassicae* resting spore suspension was prepared from wild type galls and was used to prepare six one litre resting spore suspensions containing 10^6 resting spores ml^{-1} in 1 litre plastic bottles.

Three resting spore suspensions were treated with calcium nitrate at a rate equivalent to 75 kg ha^{-1} (11.62 kg of nitrate ha^{-1}, 3.8 mel calcium nitrate). The three remaining resting spore suspensions remained untreated and will be referred to as controls.

The spore suspensions were used to inoculate seven-day-old Chinese cabbage seedlings cv. Mariko in FP8 plant pots filled with Arthur Bower's seed and potting compost in the following way.

One control and one calcium nitrate treated spore suspensions were used to inoculate 16 seedlings immediately after preparation. The remaining spore suspensions were placed in a dark cupboard at room temperature to encourage resting spore germination (Aist and Williams, 1971).

After 24 hours one control spore suspension and one calcium nitrate treated spore suspension were each used to inoculate sixteen FP8 plant pots.

The remaining spore suspensions were used to inoculate seedlings 48 hours after preparation.

The experiment consisted of six treatments with four replicates each consisting of four plants.

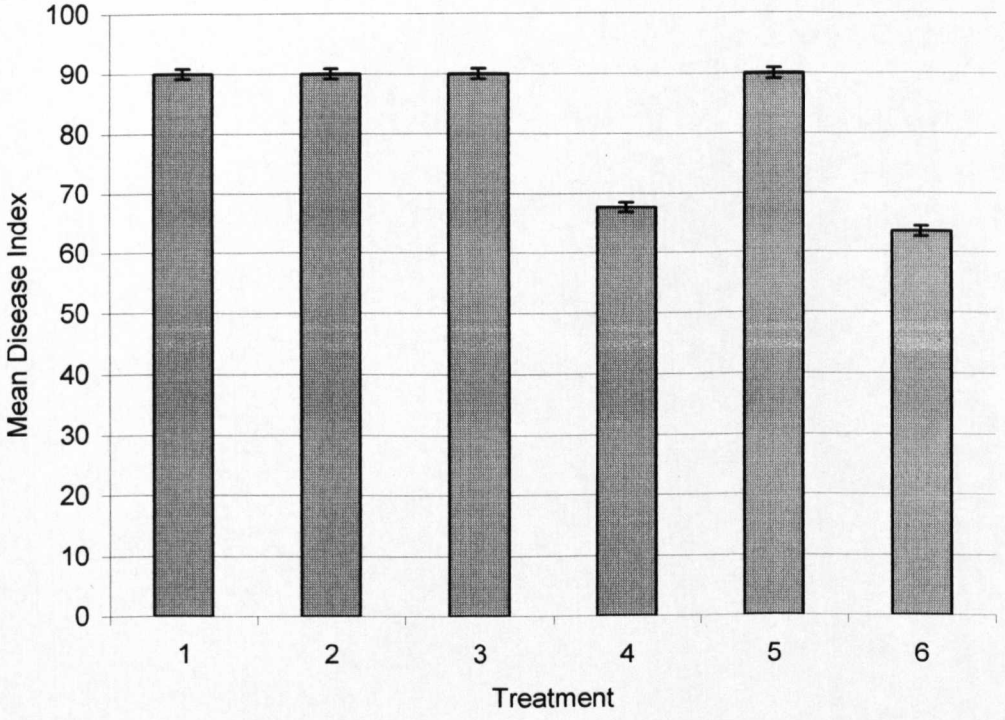
Samples were taken from each spore suspension immediately prior to use to be observed using fluorescence. This method of observation failed, however and no conclusions could be made (Appendix 4).

The plants were harvested six weeks after inoculation.

Results.

The mean disease indices for each treatment are illustrated in Graph 3.5.1 and are presented in Table 3.5.1. The mean disease indices of plants inoculated with resting spore suspensions prepared in calcium nitrate and used 24 and 48 hours after preparation were significantly lower than those plants inoculated with the remaining treatments. The disease index of plants inoculated with calcium nitrate treated spores 48 hours after preparation was in turn lower than plants inoculated with calcium nitrate spores 24 hours after preparation.

Graph 3.5.1. Mean Disease Indices of plants inoculated with *P. brassicae* resting spores treated with calcium nitrate.

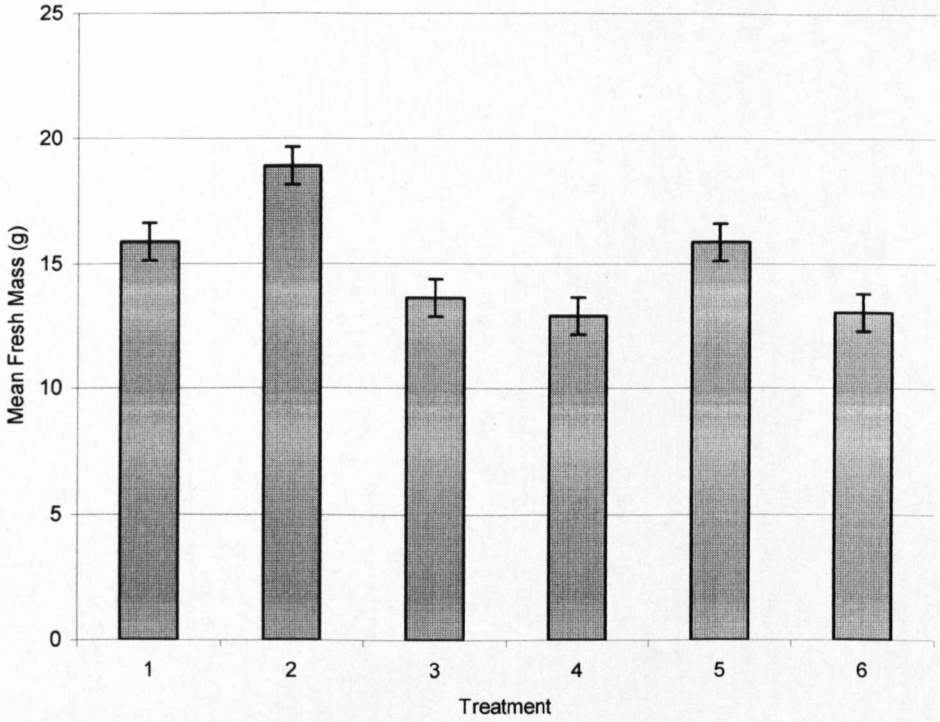


s.e.d = 1.721

Treatments

1 *P. brassicae* resting spores prepared in distilled water and applied immediately.
2 *P. brassicae* resting spores prepared in a calcium nitrate solution and applied immediately.
3 *P. brassicae* resting spores prepared in distilled water and applied after 24 hours.
4 *P. brassicae* resting spores prepared in a calcium nitrate solution and incubated for 24 h prior to use.
5 *P. brassicae* resting spores prepared in distilled water and applied after 48 hours.
6 *P. brassicae* resting spores prepared in a calcium nitrate solution and incubated for 48 hours prior to use.

Graph 3.5.2. Mean fresh root masses of plants inoculated with *P. brassicae* resting spores treated with calcium nitrate.



s.e.d = 1.498

Treatments

1 *P. brassicae* resting spores prepared in distilled water and applied immediately.
2 *P. brassicae* resting spores prepared in a calcium nitrate solution and applied immediately.
3 *P. brassicae* resting spores prepared in distilled water and applied after 24 hours.
4 *P. brassicae* resting spores prepared in a calcium nitrate solution and incubated for 24 h prior to use.
5 *P. brassicae* resting spores prepared in distilled water and applied after 48 hours.
6 *P. brassicae* resting spores prepared in a calcium nitrate solution and incubated for 48 hours prior to use.

Table 3.5.1. Mean disease indices (angular) of plants inoculated with *P. brassicae* spores prepared in de-ionised water or calcium nitrate.

Spore Treatment	Mean disease index (angular)
Sterile de-ionised water used immediately	90a
Calcium nitrate used immediately	90a
Sterile de-ionised water soaked for 24 hours	90a
Calcium nitrate soaked for 24 hours	67.65b
Sterile de-ionised water soaked for 48 hours	90a
Calcium nitrate soaked for 48 hours	63.54c
S.E.D	1.721
L.S.D	3.589

Values with the same letter are not significantly different using the L.S.D value.

The mean fresh root masses for each treatment are given in Graph 3.5.2 and Table 3.5.2. The root mass was significantly decreased in plants inoculated with spore suspensions prepared 24 hours before use and the spore suspension prepared in calcium nitrate 48 hours before use when compared with plants inoculated with the remaining treatments. The mean root mass of plants inoculated with the spore suspension prepared in calcium nitrate immediately after preparation were significantly higher than plants in other treatments.

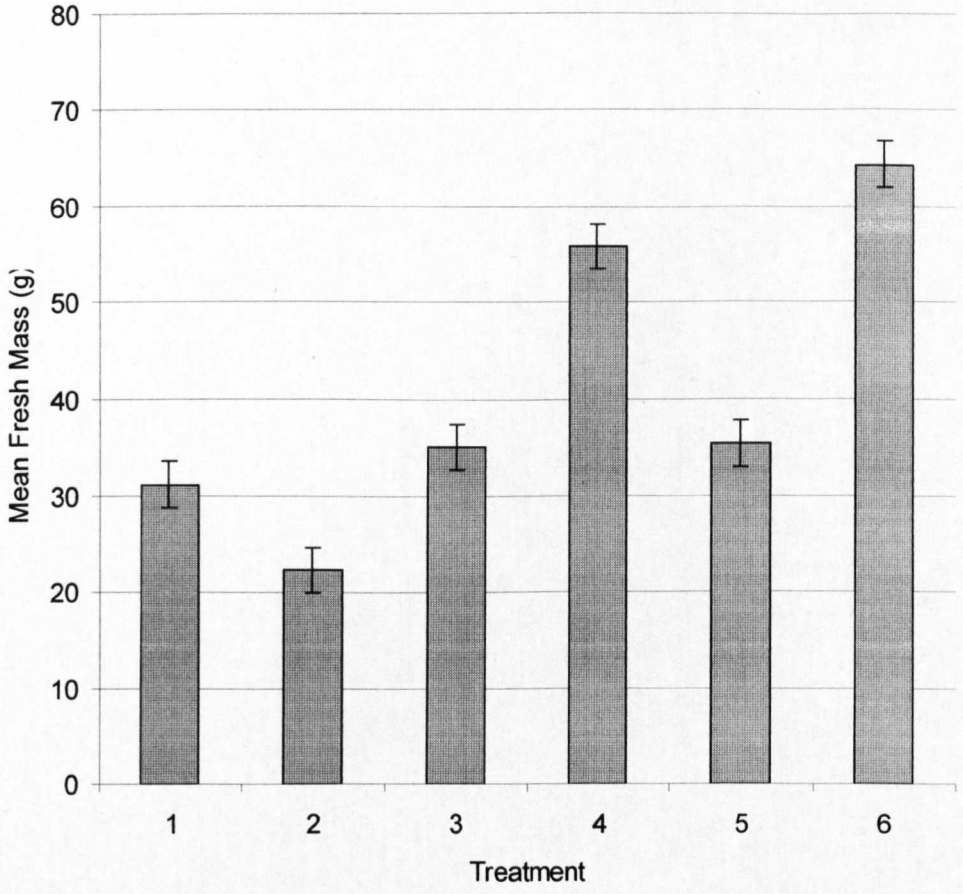
Table 3.5.2. Mean fresh root mass (g) of plants inoculated with *P. brassicae* spores prepared in de-ionised water or calcium nitrate.

Spore Treatment	Mean fresh root mass (g)
Sterile de-ionised water used immediately	15.88a
Calcium nitrate used immediately	18.90b
Sterile de-ionised water soaked for 24 hours	13.64a
Calcium nitrate soaked for 24 hours	12.91a
Sterile de-ionised water soaked for 48 hours	15.88a
Calcium nitrate soaked for 48 hours	13.04a
S.E.D	1.498
L.S.D	2.969

Values with the same letter are not significantly different using the L.S.D value.

The mean fresh shoot masses are illustrated in Graph 3.5.3 and Table 3.5.3. The shoot masses of plants inoculated with a spore suspension prepared in calcium nitrate and applied immediately were significantly lower than the shoot masses of plants inoculated with other treatments. The root mass of these plants was however significantly higher then other treatments. This suggests that the disease had progressed to a stage where the galls were utilising so much carbohydrate that the growth of shoots was retarded.

Graph 3.5.3. Mean fresh shoot masses of plants inoculated with *P. brassicae* resting spores treated with calcium nitrate.



s.e.d = 4.72

Treatments.

1 *P. brassicae* resting spores prepared in distilled water and applied immediately.
2 *P. brassicae* resting spores prepared in a calcium nitrate solution and applied immediately.
3 *P. brassicae* resting spores prepared in distilled water and applied after 24 hours.
4 *P. brassicae* resting spores prepared in a calcium nitrate solution and incubated for 24 h prior to use.
5 *P. brassicae* resting spores prepared in distilled water and applied after 48 hours.
6 *P. brassicae* resting spores prepared in a calcium nitrate solution and incubated for 48 hours prior to use.

Table 3.5.3. Mean fresh shoot mass (g) of plants inoculated with *P. brassicae* spores prepared in de-ionised water or calcium nitrate.

Spore Treatment	Mean fresh shoot mass (g)
Sterile de-ionised water used immediately	31.2ab
Calcium nitrate used immediately	22.3a
Sterile de-ionised water soaked for 24 hours	35.1ab
Calcium nitrate soaked for 24 hours	55.8c
Sterile de-ionised water soaked for 48 hours	35.4b
Calcium nitrate soaked for 48 hours	64.3c
S.E.D	4.72
L.S.D	9.35

Values with the same letter are not significantly different using the L.S.D value.

Conclusions

The decreased gall size and increased shoot growth of plants inoculated with spore suspensions prepared in calcium nitrate and incubated for 24 hours suggests that calcium nitrate has a direct effect upon the resting spores of *P. brassicae*. The disease index of plants inoculated with calcium nitrate and incubated for 48 hours was lower than that of plants incubated in calcium nitrate for 24

hours and the shoot masses were higher. This indicates that not only are the resting spores of *P. brassicae* affected by calcium nitrate but also the 1^0 resting spores.

3.6. The effect of calcium nitrate on gall formation, when applied after *P. brassicae* infection has occurred.

The aim of this investigation was to determine whether calcium nitrate is effective in decreasing the extent of *P. brassicae* infection after the host has already been infected. The previous experiments have indicated that calcium nitrate can affect both *P. brassicae* resting spores and 1^0 zoospores but this experiment would help to determine whether calcium nitrate affects the pathogen during the later stages of disease development.

If the extent of galling is decreased when calcium nitrate is added to the root environment after the host plants have become infected with *P. brassicae* it would suggest that calcium nitrate is able to influence the pathogen lifecycle within the host plant.

Method and materials.

The experiment consisted of four treatments and six replicates with four FP8 plant pots in each treatment replicate. The pots were sown with Chinese cabbage cv. Mariko and placed in a glasshouse. The number of seedlings in each pot was reduced to two after seven days and each pot was inoculated with 30 ml of a 10^6 resting spores ml^{-1} *P. brassicae* spore suspension. The number of seedlings per pot was further reduced to one after five days and a sample of the seedlings removed were assessed for root hair infection.

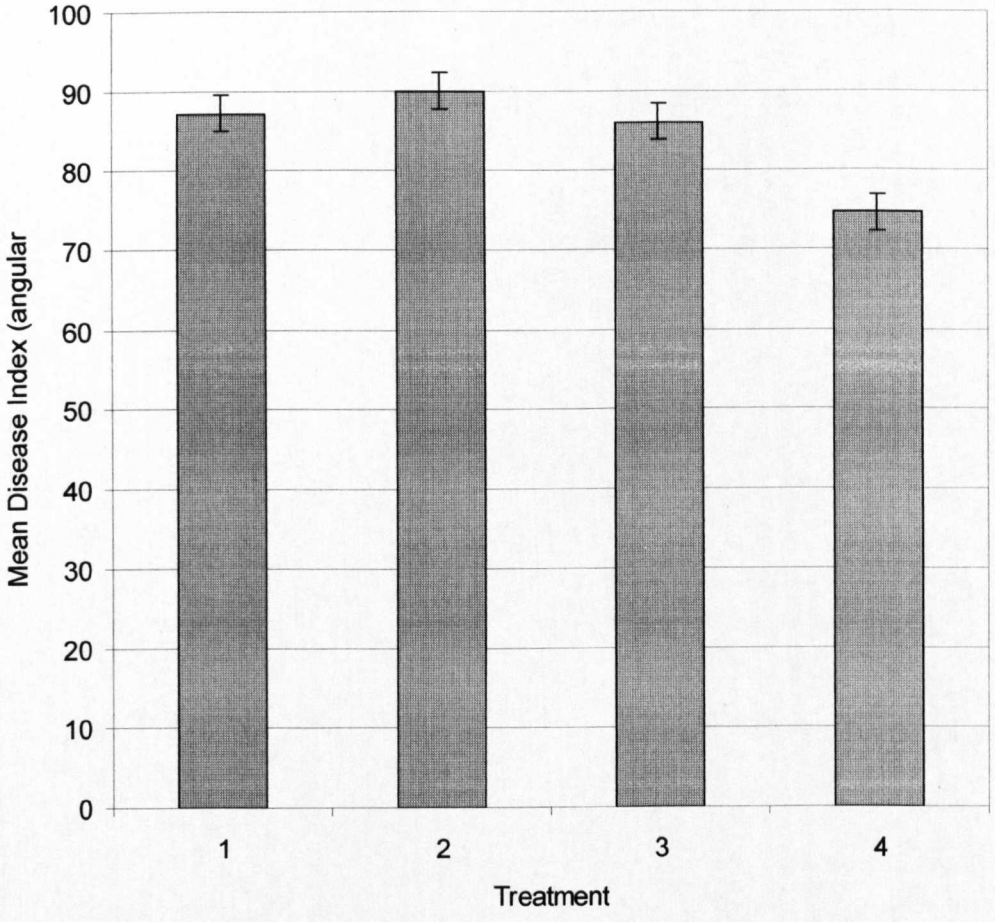
After inoculation with *P. brassicae* the pots were treated with 30 ml of a calcium nitrate solution which provided each pot with 300 kg of calcium nitrate ha^{-1} (279 mel). The pots were treated with calcium nitrate at one of three times, at the time of inoculation, five days after inoculation or ten days after inoculation, the remaining pots remained untreated and these acted as the controls.

The plants were harvested six weeks after inoculation.

Results.

The mean disease indices (angular) are given in Graph 3.6.1 and Table 3.6.1. The results show that the mean disease index of plants treated with calcium nitrate 10 days after being inoculated *with P. brassicae* was significantly lower than that of other treatments.

Graph 3.6.1. Mean disease indices (angular) of plants treated with calcium nitrate at varying times following *P. brassicae* infection.



s.e.d = 4.57

Treatments

1. *P. brassicae* infected controls
2. Calcium nitrate applied at the time of inoculation
3. Calcium nitrate applied 5 days after inoculation
4. Calcium nitrate applied 10 days after inoculation

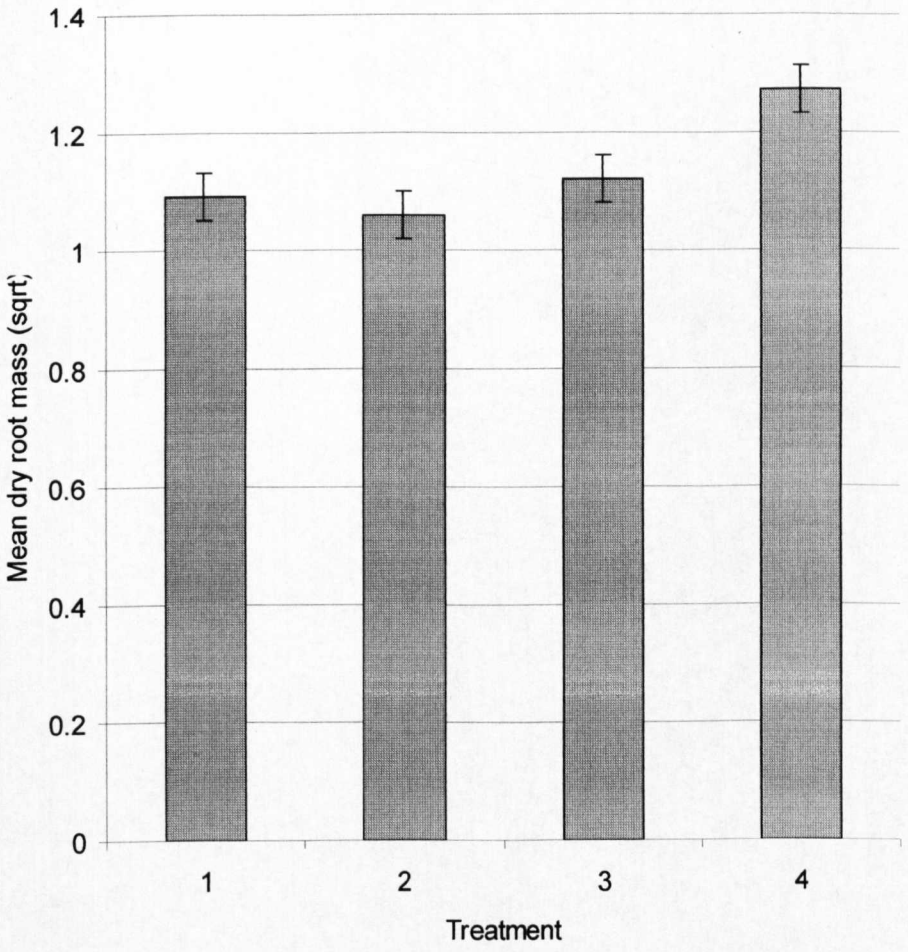
Table 3.6.1. Mean disease indices (angular) of plants treated with calcium nitrate after inoculation with *P. brassicae*.

Treatment	Mean disease indices (angular)
Untreated control	87.2b
Calcium nitrate applied at inoculation	90b
Calcium nitrate applied 5 days after inoculation	86b
Calcium nitrate applied 10 days after inoculation	74.5a
S.E.D	4.57
L.S.D	9.75

Values with the same letter are not significantly different using the L.S.D value

The mean dry root masses (sqrt) of plants treated with calcium nitrate after inoculation with *P. brassicae* are given in Graph 3.6.2 and Table 3.6.2. The results show that plants treated with calcium nitrate 10 days after inoculation had significantly higher dry root masses.

Graph 3.6.2. Mean dry root masses (sqrt) of plants treated with calcium nitrate at varying times following *P. brassicae* infection.



s.e.d = 0.0814

Treatments

1. *P. brassicae* infected controls
2. Calcium nitrate applied at the time of inoculation
3. Calcium nitrate applied 5 days after inoculation
4. Calcium nitrate applied 10 days after inoculation

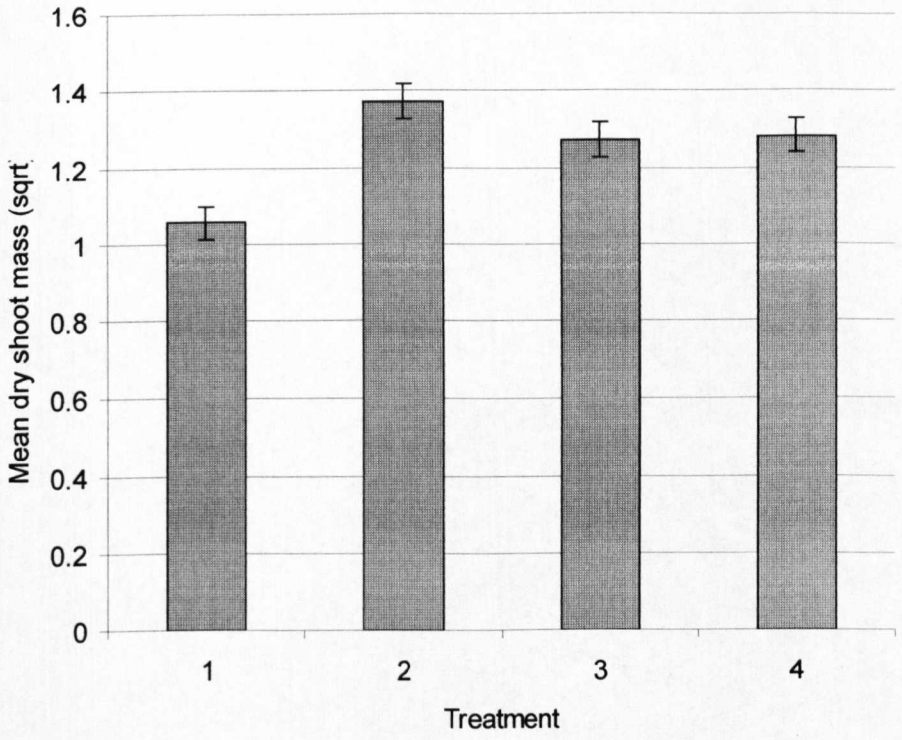
Table 3.6.2. Mean dry root masses (sqrt) of plants treated with calcium nitrate inoculation with *P. brassicae.*

Treatment	Mean dry root masses (sqrt)
Untreated control	1.091a
Calcium nitrate applied at inoculation	1.059a
Calcium nitrate applied 5 days after inoculation	1.121ab
Calcium nitrate applied 10 days after inoculation	1.274b
S.E.D	0.0814
L.S.D	0.1618

Values with the same letter are not significantly different using the L.S.D value

The mean dry shoot masses (sqrt) are given in Graph 3.6.3 and Table 3.6.3. The shoot masses of plants treated with calcium nitrate were significantly higher than untreated plants

Graph 3.6.3. Mean dry shoot masses (sqrt) of plants treated with calcium nitrate at varying times following *P. brassicae* infection.



s.e.d = 0.0899

Treatments

1. *P. brassicae* infected controls
2. Calcium nitrate applied at the time of inoculation
3. Calcium nitrate applied 5 days after inoculation
4. Calcium nitrate applied 10 days after inoculation

Table 3.6.3. Mean dry shoot mass (sqrt) of plants treated with calcium nitrate after inoculation with *P. brassicae.*

Treatment	Mean dry shoot mass (sqrt)
Untreated control	1.055a
Calcium nitrate applied at inoculation	1.372b
Calcium nitrate applied 5 days after inoculation	1.273b
Calcium nitrate applied 10 days after inoculation	1.282b
S.E.D	0.0899
L.S.D	0.1787

Values with the same letter are not significantly different using the L.S.D value

Conclusions

Treating plants with calcium nitrate at a concentration of 300 kg of nitrate ha^{-1} (279 mel) reduced the extent of galling when applied 10 days after inoculation. Plants treated with calcium nitrate five days after inoculation had a level of galling similar to that of untreated controls. Plants treated at inoculation had the highest level of infection. The shoot and root growth of plants treated with calcium nitrate 5 and 10 days after inoculation was significantly higher than plants in the other treatments. The results therefore suggest that calcium nitrate can influence the progression of *P. brassicae* infection up to a point 10 days after inoculation. In fact the results suggest that post planting applications of calcium

nitrate may help to decrease galling further than pre- planting applications. Post inoculation applications of calcium nitrate promoted increased shoot growth regardless of whether the infection was slowed.

Discussion.

The results of the field experiment show that a crop fertilised with calcium nitrate not only has reduced levels of *P. brassicae* infection, when compared to plants fertilised with ammonium nitrate, but also had increased marketable mass. The increase in head mass would increase the profit of a grower regardless of whether the disease was prevented.

The results of experiment 3.2 illustrate the difficulties encountered during this work. The experiment became infected with *Botrytis cinerea* (grey mould) which badly affected some replicates leading to a large standard error of difference, this made it difficult to distinguish between some results. These results however, illustrated a general trend, that the level of infection in calcium nitrate treated plants was lower than in ammonium treated plants. The most effective calcium nitrate application was three weeks prior to being used in the experiment the shoot mass was increased. In this experiment it was not possible to distinguish which was most effective in decreasing *P. brassicae* infection.

In order to determine whether there were alternative ways to use calcium nitrate, the test solutions were applied to seedlings in module trays prior to transplantation. The method of applying fertilisers to the module blocks was used in experiments 3.3.1 and 3.3.2. In both experiments the application of fertilisers to the

seedlings whilst they were growing in their modules failed to significantly decrease the severity of *P. brassicae* infection.

Dixon and Wilson (1985) established that the application of fungicides to propagation module blocks was ineffective unless the land is also treated. In the second experiment (3.3.2) however the shoot masses of plants was significantly higher, the greatest shoot mass being obtained by plants treated with calcium nitrate at a rate of 75 kg ha^{-1}. It is possible that although the concentration of calcium is low it may increase plant growth sufficiently to overcome the losses caused by the disease. Higher concentrations of calcium nitrate may have failed to illustrate this due to the level of calcium being toxic to the plants. A more effective control measure may therefore be to treat seedlings with a low concentration of calcium nitrate prior to transplantation to a contaminated field which has also been treated with calcium nitrate.

In comparison seeds germinated in calcium nitrate failed to show any signs of improved growth and there was no reduction in disease incidence. This suggests that calcium may need to be absorbed by the seedling after germination in order to promote plant growth.

The results of the module experiments and the seed treatment experiments suggest that treatment of plants with low levels of calcium nitrate over a period of time increases plant growth significantly. Disease incidence is reduced when calcium nitrate is placed in the root environment contaminated with *P. brassicae* resting spores. It is unclear whether this is an effect on the germination of *P. brassicae* resting spores or the viability of 1^0 zoospores. This was investigated in experiment 3.5.

Experiment 3.5 confirmed that calcium nitrate has a direct effect upon resting spore viability. Plants inoculated with resting spore suspensions prepared in calcium nitrate and applied 24 hours and 48 hours after preparation had significantly lower disease indices than plants inoculated with untreated spores. Indicating that resting spore viability had been reduced. In turn the disease indices of plants inoculated with calcium nitrate treated spores and applied 48 hours after preparation had a lower disease index than plants inoculated with calcium nitrate treated spores 24 hours after preparation. This implies that 1^0 zoospores may also be affected by calcium nitrate. Although this area needs further examination the findings are in line with those of Donaldson and Deacon (1993) and Von Broembsen and Deacon, (1997, 1996) who found that calcium can affect the swimming pattern of *Phytopthora* and *Pythium* spp., decrease spore germination and reduce the level of infection

As the disease index decreases further after 48 hours it is possible that calcium nitrate also effects 1^0 resting spores. *Plasmodiophora brassicae* resting spores germinate approximately 36 hours after preparation in the absence of light and at room temperature. The level of calcium nitrate used in this experiment was very low when compared to the levels used in the field experiment. Suzuki *et al.*, (1992) however found that the germination of resting spores is regulated by a "germination stimulating factor" thought to be low levels of calcium. The presence of calcium nitrate at low levels may therefore decrease disease incidence by encouraging the germination of resting spores and then affecting the ability of the primary zoospores to infect host plants.

In addition the results of experiment 3.6 indicate that applications of calcium nitrate after infection has established can also lead to a reduction in gall formation.

Calcium nitrate can therefore be used as an effective control measure against *P. brassicae* as illustrated by the field experiment. The remaining experiments illustrate that the addition of calcium nitrate affects resting spores, primary zoospores and disease progression.

Conclusions

1. The use of calcium nitrate in a field environment reduces *P. brassicae* infection and increases the mean plot mass.

2. Calcium nitrate remains able to increase plot mass of cabbages three weeks after application.

3. The application of calcium nitrate to seedlings whilst they are growing in module plug trays does not decrease disease incidence, however the shoot mass increases significantly.

4. Seeds germinated in calcium nitrate solutions did not result in decreased disease incidence or an increased mass.

5. Calcium nitrate directly affects the viability of *P. brassicae* resting spores.

6. Calcium nitrate can reduce the extent of galling even when applied after infection has occurred.

Chapter Four

Investigation of the effects of calcium nitrate and the root environment on *P. brassicae* races.

4. Experiments to determine the affects of calcium nitrate and the root environment upon the predominant pathogen race.

The European Clubroot Differential Series (E.C.D) has been used extensively to study the variability of the predominant *P. brassicae* pathogen race. From the experiments carried out in Chapter 3 and from research carried out by Webster (1985) it is known that calcium nitrate can decrease infection of brassica crops by *P. brassicae*. It is not known, however, how the nutrient environment affects variation in the *P. brassicae* population.

The aim of the current series of experiments was to establish whether changes in the nutrient environment of the root system can alter the balance of *P. brassicae* races infecting the E.C.D series.

Although the pathogen population can vary within a single gall (Crute *et al.*, 1980), the pathogen race identified using the E.C.D series will be referred to as the predominant pathogen race. Untreated galls collected from the Auchincruive field site will be referred to as wild type galls and the spore suspension produced from these galls will be referred to as the wild type suspension.

The E.C.D series hosts used in these experiments will be referred to by their host numbers. A copy of Table 2.5 (Chapter 2) which lists the hosts and the relevant host number is provided at the end of this Chapter for reference. All of the E.C.D tests were carried out in a glass house. A disease category of greater then one was used to designate a host as susceptible to a *P. brassicae* pathogen race.

4.1. Experiment to identify the predominant pathogen race present in a resting spore suspension prepared from untreated galls.

Method and Materials

The E.C.D series was sown into Levington's multi-purpose compost as described in Chapter 2. Seven days after sowing the seed trays were inoculated by pouring 200 ml of a 10^{-6} resting spore ml^{-1} suspension prepared from wild type galls over the surface of each tray. The experiment was harvested six weeks after inoculation and the gall categories of each plant recorded.

Results.

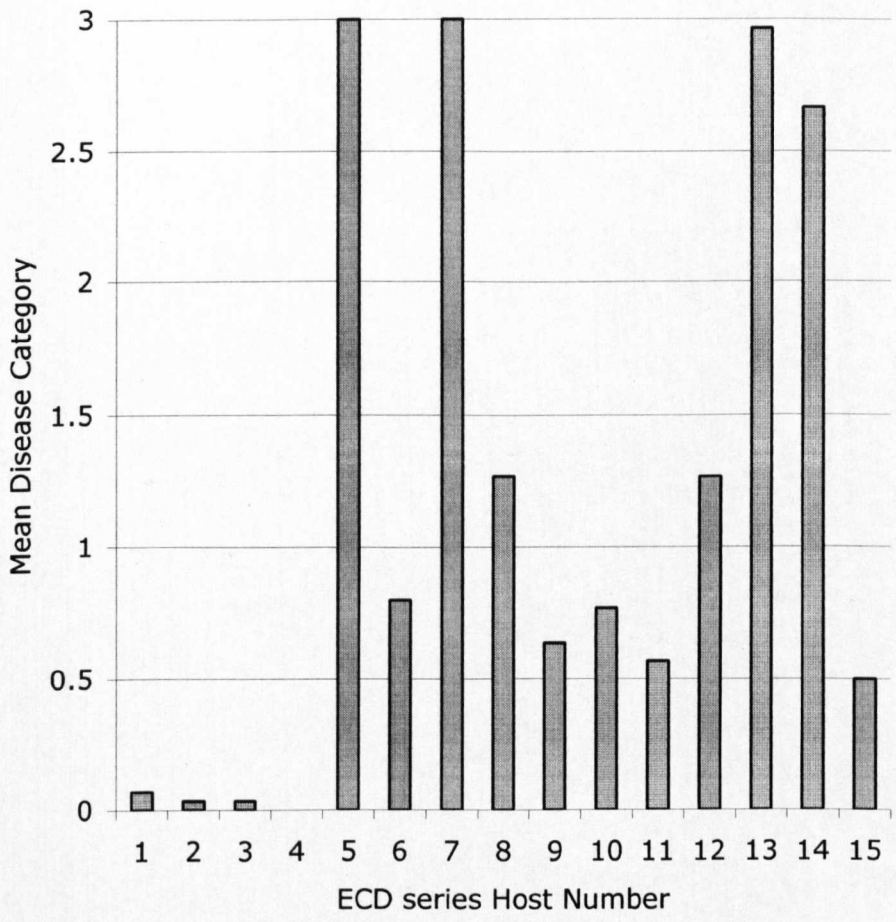
The disease categories for each host were calculated and are illustrated in Graph 4.1. Using a mean gall category of greater than 1 to indicate a susceptible host, the predominant pathogen race of the wild type spore suspension was determined to be 16 / 6 / 14.

4.2. Identification of the predominant pathogen race present in a resting spore suspension prepared from calcium nitrate treated galls.

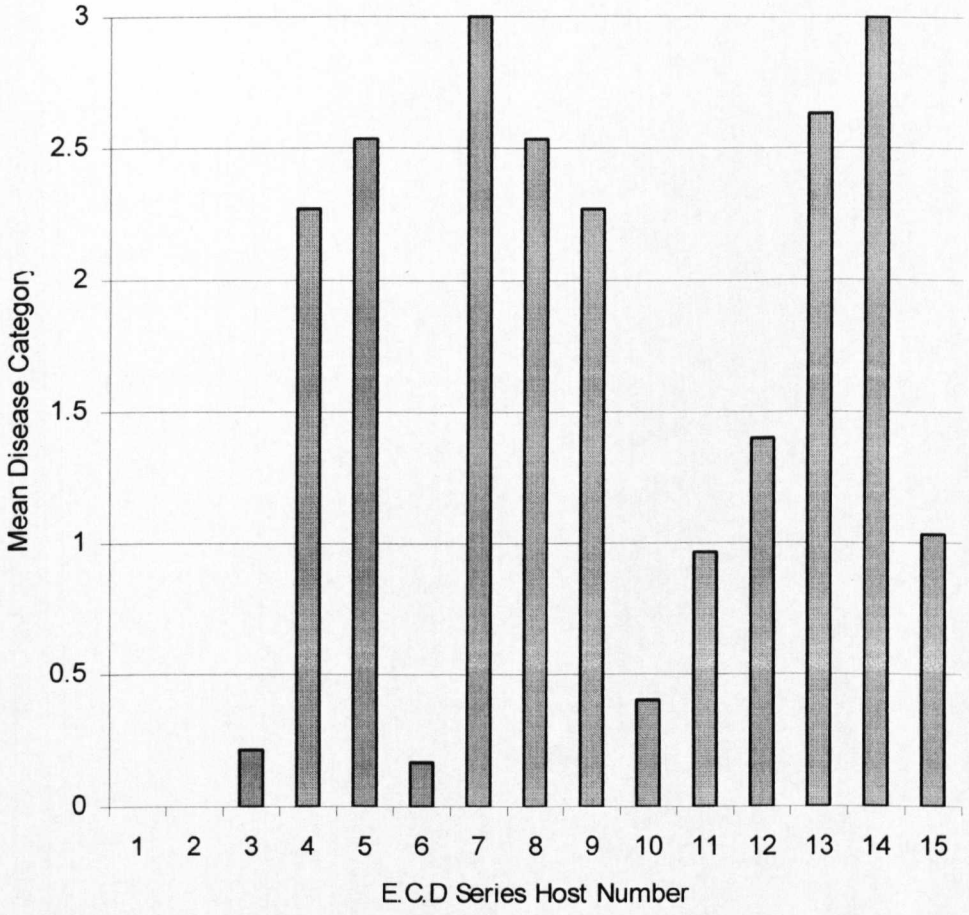
Method and Materials

The E.C.D series was sown into Levington's multi-purpose compost. Seven days after sowing the seed trays were inoculated with 200 ml of a 10^{-6} resting spore suspension prepared from galls collected from field trial plots treated with calcium nitrate (Chapter 3).

Graph 4.1. Mean disease categories of E.C.D series hosts infected with a wild type *P. brassicae* resting spore suspension.



Graph 4.2. Mean disease categories of E.C.D series hosts infected with a *P. brassicae* resting spore suspension prepared from calcium nitrate treated galls.



Results.

The disease categories for each host were calculated and are illustrated in Graph 4.2. Using a mean disease category of greater than 1 to indicate a susceptible host, the predominant pathogen race was determined to be 16 / 12 / 14.

4.3 The effects of two composts upon the predominant pathogen race of a spore suspension derived from calcium nitrate treated gall material.

The presence of nodules on the stems of some E.C.D series hosts in experiments 4.1 and 4.2 was observed (Plate 4.1). It was considered that the nodules may have been caused by an E.C.D host response to a biological agent called "Stimulex" which is incorporated into Levington's multi-purpose compost. It is claimed that "Stimulex" helps to promote plant growth and decrease the incidence of disease.

The purpose of this investigation was therefore to determine whether in the absence of "Stimulex" the predominant pathogen race differs.

Method and Materials

One E.C.D series was sown into Levington's multi-purpose compost and another was sown into Arthur Bowers seed and potting compost. Both E.C.D series were inoculated with 200ml of a 10^6 resting spore suspension extracted from calcium nitrate treated gall material. The plants were harvested six weeks after inoculation and the gall categories of each plant calculated.

Plate 4.1: Nodules formed on ECD host *B. oleracea var. capitata cv. Septa* grown in Levingtons multi-purpose compost.

Results.

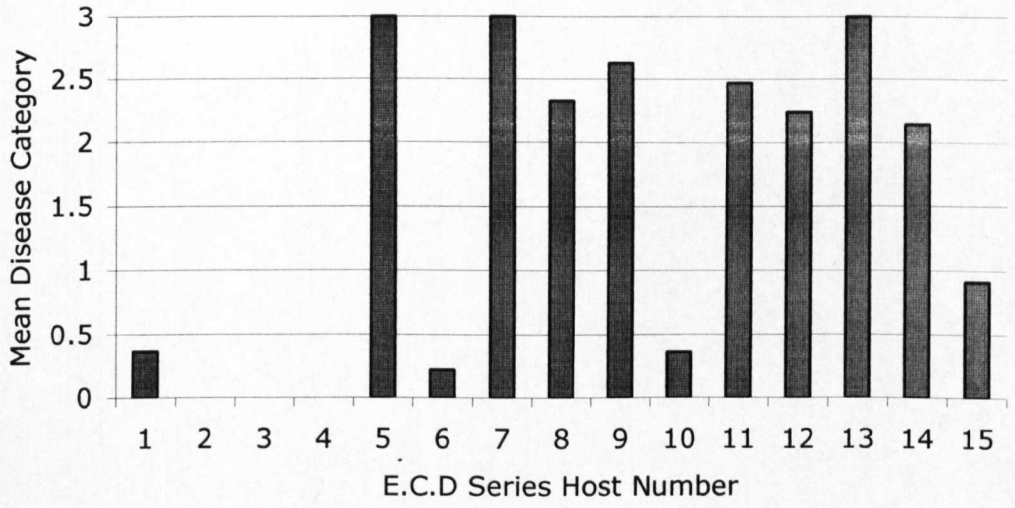
Graphs 4.3.1 and 4.3.2 illustrate the results for both treatments.

Using a disease category of greater than one to indicate a susceptible host the predominant pathogen population for both of the treatments was found to be 16/14/15. This race did however differ from that found in experiment 4.2 where calcium nitrate treated galls were also used to prepare the spore suspension.

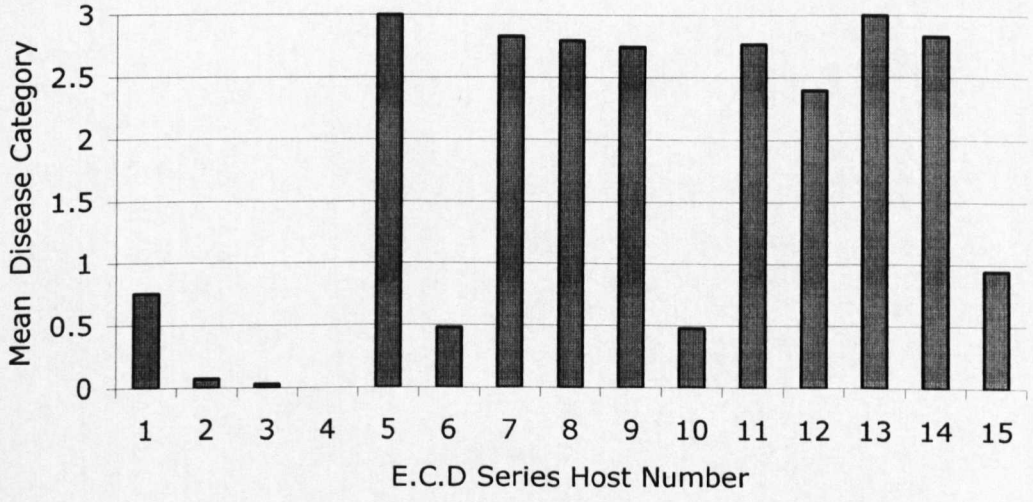
Conclusions

Therefore there was no difference in host responses in the two composts in this experiment

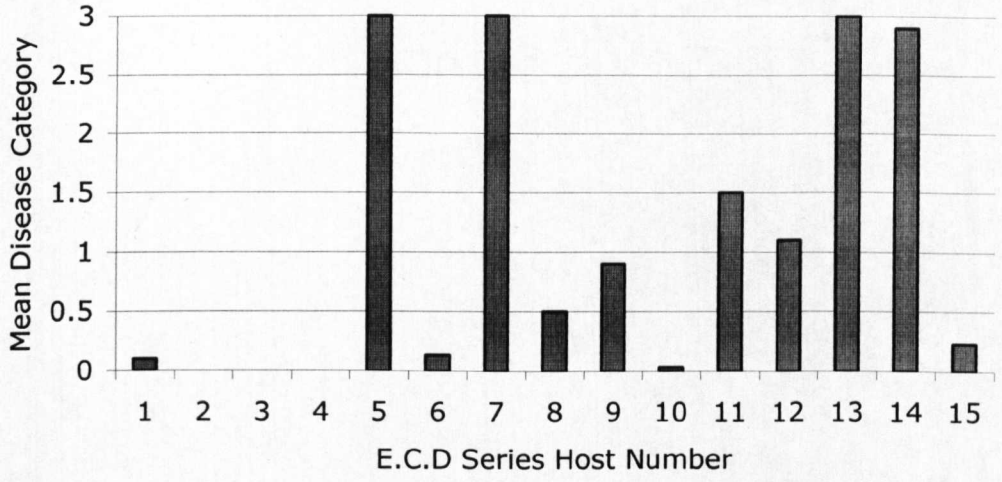
Graph 4.3.1. Mean disease categories of E.C.D series hosts infected with a *P. brassicae* resting spore suspension prepared from calcium nitrate treated galls and raised in Levington's multipurpose compost.



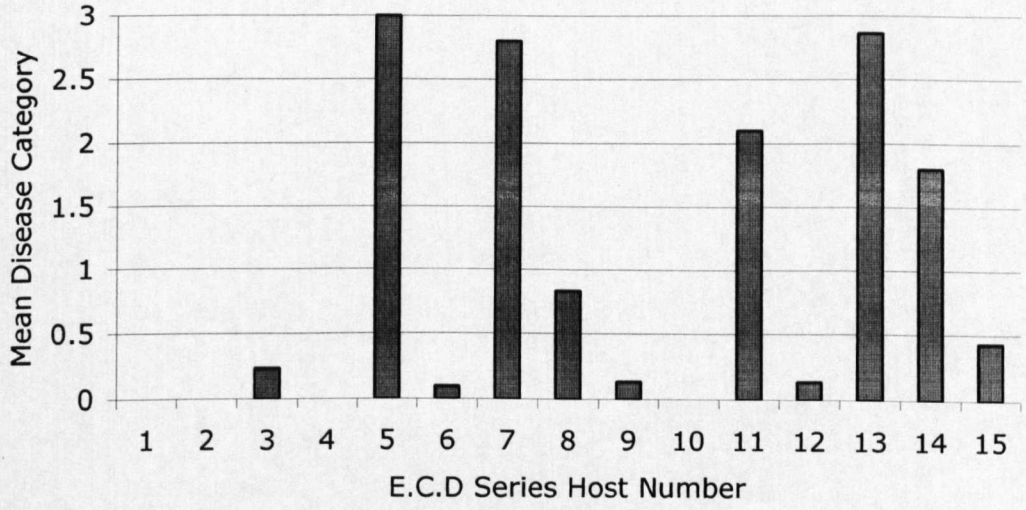
Graph 4.3.2. Mean disease categories of E.C.D series hosts infected with a *P. brassicae* resting spore suspension prepared from calcium nitrate treated galls and raised in Arthur Bower's seed and potting compost.



Graph 4.4.1. Mean disease categories of E.C.D series hosts infected with a *P. brassicae* resting spore suspension prepared from wild type galls and raised in Levington's multi-purpose compost.



Graph 4.4.2. Mean disease categories of E.C.D series hosts infected with a *P. brassicae* resting spore suspension prepared from wild type galls and raised in Arthur Bower's seed and potting compost.



4.4. The effects of two composts upon the predominant pathogen race of a spore suspension derived from wild type gall material.

This investigation aimed to identify whether a variation in the root nutrient environment, bought about by using different composts, affected the predominant pathogen race present in wild type gall material.

Method and Materials

The experiment was carried out in the same way as 4.3, the only difference being that the E.C.D. series were inoculated with a spore suspension prepared from wild type galls.

Results.

Graphs 4.4.1 and 4.4.2 illustrate the mean disease categories for each E.C.D series test.

Using a mean disease category of greater than 1 to identify susceptible hosts the predominant pathogen hosts were identified as 16/12/15 for the E.C.D series raised in Levington's multipurpose compost and 16/2/13 for the E.C.D series raised in Arthur Bower's seed and potting compost.

Conclusions

In this experiment the type of compost used did influence the predominant pathogen race. This is in contrast to the results obtained in experiment 4.4 where the predominant pathogen race was not influenced by the type of compost used. This implies that

the pathogen race, found in calcium nitrate treated spores, is more 'stable'. The calcium nitrate spore suspension may consist of fewer races due to the selection pressure of infecting a plant in the presence of the fertiliser and hence it may be more difficult to influence the remaining pathogen races.

4.5. Determination of the predominant pathogen race in a spore suspension prepared from a second generation of calcium nitrate treated galls.

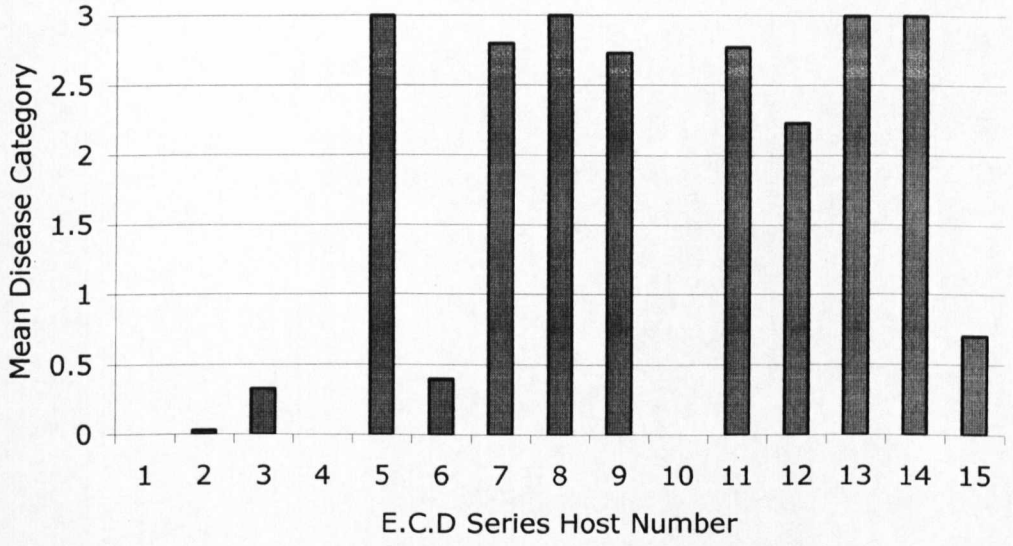
The aim of the experiment was to determine whether in the absence of calcium nitrate the predominant pathogen race present in calcium nitrate treated galls would revert back to the same predominant race present in wild type galls.

Method and materials

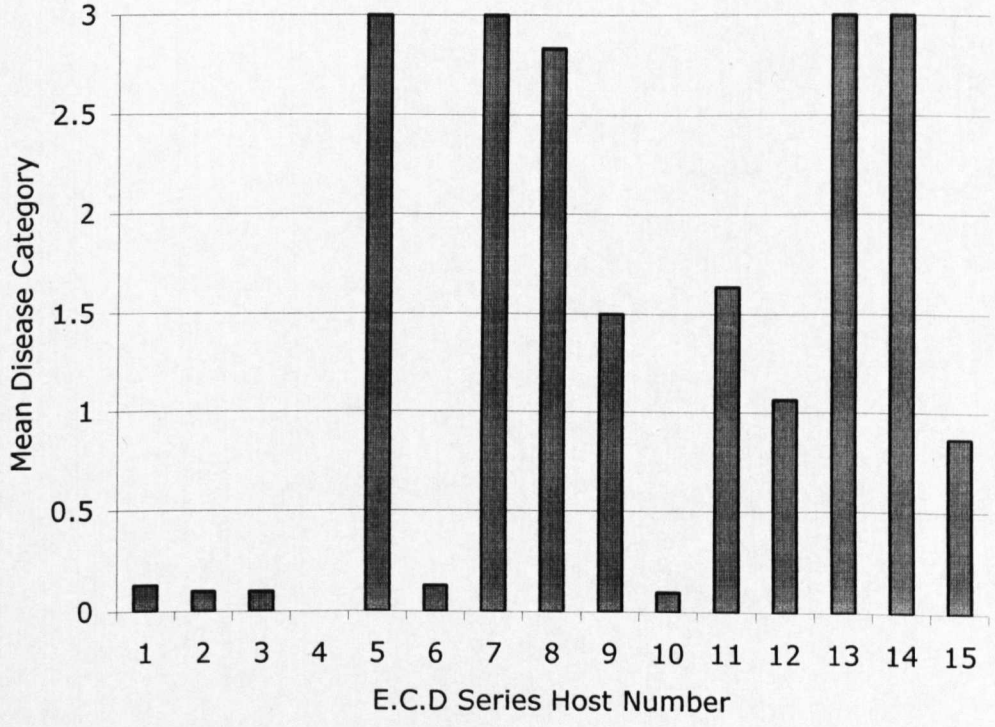
The resting spore suspension for this experiment was obtained from galls collected from Summer cabbage cv. Castello that had been inoculated with a resting spore suspension prepared from calcium nitrate treated galls. These galls were obtained by sowing pots ($7cm^2$), filled with Arthur Bower's seed and potting compost, with two seeds of Castello. The number of seedlings in each pot was reduced to one after seven days.

Each pot was inoculated with 30 ml of a resting spore suspension containing 10^6 resting spores / ml prepared from calcium nitrate treated galls. The plants were maintained in a glass house for six weeks. The galls were removed from the plants, washed and placed in a freezer. These galls will be referred to as 2^{nd} generation

Graph 4.5.1 Mean disease categories of E.C.D series hosts raised in Arthur Bower's Seed and Potting compost infected with a *P. brassicae* resting spore suspension prepared from a second generation of calcium nitrate galls.



Graph 4.5.2 Mean disease categories of E.C.D series hosts raised in Levington's multi-purpose compost infected with a *P. brassicae* resting spore suspension prepared from a second generation of calcium nitrate galls.



calcium nitrate galls. Castello was used as the Brassica host as this was the host for the original calcium nitrate treated galls.

An E.C.D series was sown into large size seed trays filled with Arthur Bower's seed and potting compost. After seven days the trays were inoculated with 200 ml of a resting spore suspension containing 10^6 resting spores / ml. The resting spore suspension was prepared from the second generation of galls obtained from Castello.

The experiment was repeated using Levington's Multi-Purpose compost as a growth medium.

Results.

Graphs 4.5.1 and 4.5.2 illustrate the mean disease categories obtained from E.C.D series hosts raised in Arthur Bower's Seed and Potting Compost or Levington's Multi-Purpose compost six weeks after inoculation.

The predominant pathogen race present in 2^{nd} generation calcium nitrate spores in both composts was determined to be 16 / 14 / 15 using a mean disease category of greater then 1 to identify susceptible hosts.

Conclusions

The predominant pathogen race did not revert back to that found in the wild type spore suspension, in fact it remained the same as that found in calcium nitrate treated galls. This therefore further supports the theory that there is less variation in pathogen races

within the calcium nitrate treated spore suspension and hence there is less opportunity for the race to respond to further changes within the soil environment.

4.6. Does treating the E.C.D series with calcium nitrate prior to inoculation alter the predominant pathogen race in a spore suspension prepared from calcium nitrate treated galls.

The experiment aimed to determine whether treating the soil environment with calcium nitrate would cause the predominant pathogen race present in galls from plants treated with calcium nitrate to alter further.

Method and Materials

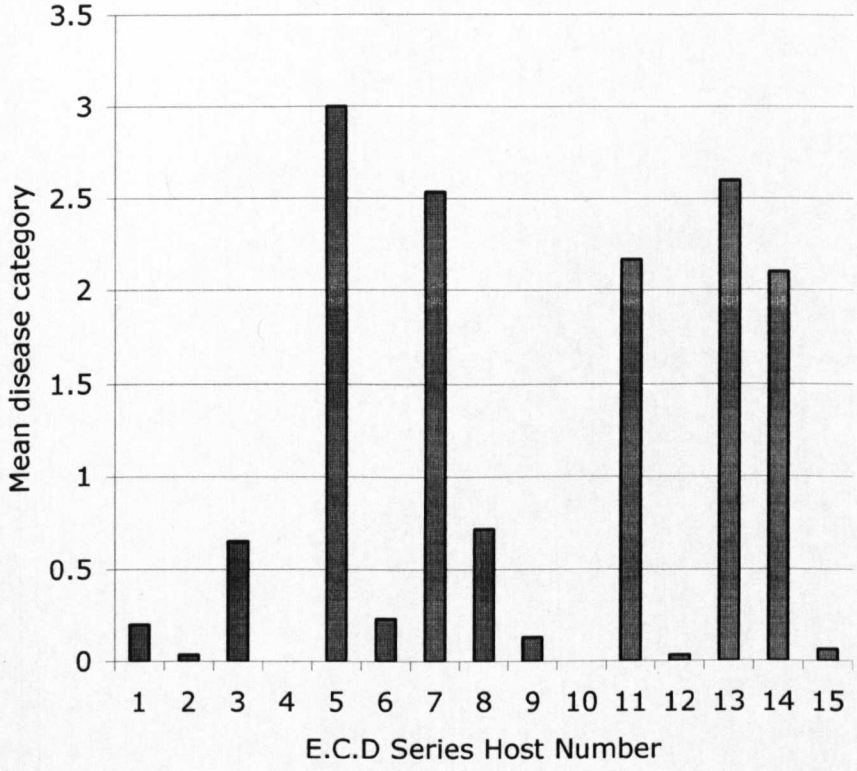
Fifteen full size seed trays were filled with Arthur Bower's Seed and Potting Compost. The trays were treated with calcium nitrate at a rate of 400 Kg / ha. The required mass of calcium nitrate for each tray was weighed and dissolved in 200 ml of water, and the solution poured evenly over the surface of the compost.

The E.C.D series was sown into the treated trays and was inoculated with a 10^6 resting spores / ml suspension prepared from calcium nitrate treated galls.

Results.

The results of these experiment are given in Graph 4.6. The predominant pathogen race of was found to be 16/2/14, using a disease category of 1 to determine susceptible hosts, which differed from races previously found

Graph 4.6. The mean disease categories of E.C.D series hosts treated with calcium nitrate infected with a *P. brassicae* resting spore suspension prepared from calcium nitrate treated galls.



Conclusions

These results suggest that whilst the predominant pathogen race is less able to respond to subtle changes in the soil environment it is still able to respond to the presence of calcium nitrate. It is unknown however, whether, with repeated applications of calcium nitrate the predominant pathogen race selection would result in a more virulent race, which would no longer be affected at all by calcium nitrate, or whether the number of races present in a calcium nitrate treated field would become so limited that other factors would impair its ability to infect the host plant. This would effectively remove the pathogen's ability to infect hosts, rendering it to become a pathogen which is no longer such a major crop threat.

4.7. Investigation into whether the resistance of E.C.D series hosts is affected by the presence of more than one pathogen population.

Experiments 4.1 – 4.6 illustrated that calcium nitrate and the root environment can alter the pre-dominate pathogen race. The following series of experiments was aimed at determining whether plant resistance is affected when resting spore solutions containing a mixture of two pathogen populations is applied to a revised E.C.D series.

The revised E.C.D series consisted of hosts 5, 6, 9, 10, 11, 12, 13 and 15. Hosts 1-4 are resistant to both pathogen races and will no longer be used. The reasons for using these hosts are:-

- Host 5 is universally susceptible.
- Host 6 is known to have vertical resistance to *P. brassicae*.
- Hosts 9 & 11 are indicators of the change in population between treated and untreated galls.
- Calcium appears to enhance the resistance of host 10 but reduces the resistance of host 11 so both these hosts have been retained.
- The level of infection in hosts 12 and 13 is similar for both types of spores.
- The level of infection of host 15 increases when spores from calcium treated plants are used.

4.7.1. The effect of *P. brassicae* resting spores suspensions prepared from mixtures of calcium nitrate treated and wild type spore suspensions on E.C.D series host resistance.

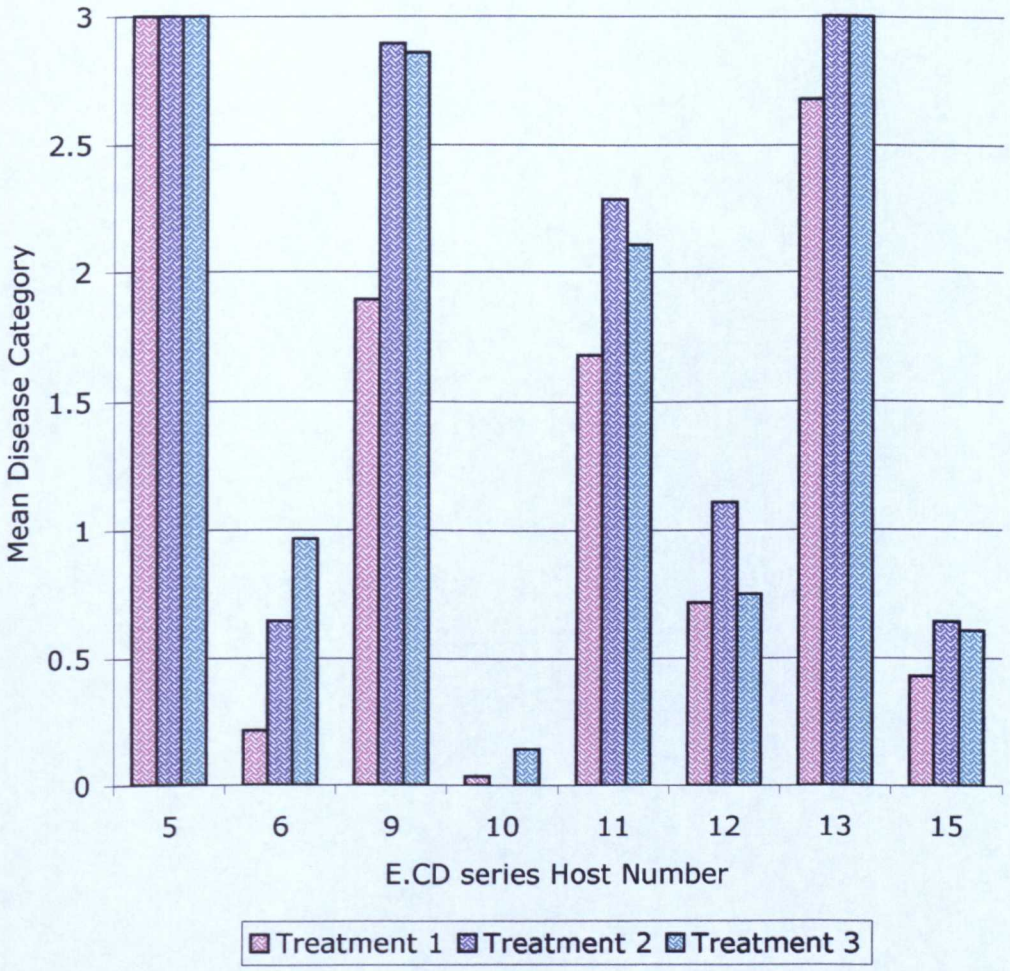
Method and Materials.

The revised E.C.D series was sown into round pots (3.5 cm diameter) filled with Arthur Bowers Seed and Potting Compost. Six seeds of the appropriate host were sown in each pot. The experimental design consisted of seven replicates with three treatments, each treatment replicate contained 2 pots of each host. Seven days after sowing the number of seedlings per pot was reduced to two. Each pot was then inoculated with 20ml of one of the following treatments :-

1. 25% of resting spores from calcium nitrate treated galls and 75% from normal galls.
2. 50% of resting spores from calcium nitrate treated galls and 50% from normal galls.
3. 75% of resting spores from calcium nitrate treated galls and 25% from normal galls.

Resting spore suspensions were prepared separately from calcium nitrate treated and wild type galls. The concentrations of the two spore suspensions was adjusted to 10^6 resting spores / ml with de-ionised water. The two resting spore suspensions were then mixed in the proportions stated for the three treatments.

Graph 4.7.1 Mean disease categories of the revised E.C.D series hosts treated with *P. brassicae* resting spore suspensions prepared from a calcium nitrate treated and wild type galls.



Treatments :-

1. 25% of resting spores from calcium nitrate treated galls and 75% from normal galls.
2. 50% of resting spores from calcium nitrate treated galls and 50% from normal galls.
3. 75% of resting spores from calcium nitrate treated galls and 25% from normal galls.

Results.

Graph 4.7.1 shows the mean disease categories of the revised E.C.D series inoculated with spore suspensions prepared from a mixture of calcium nitrate treated and wild type spores six weeks after inoculation.

Conclusions

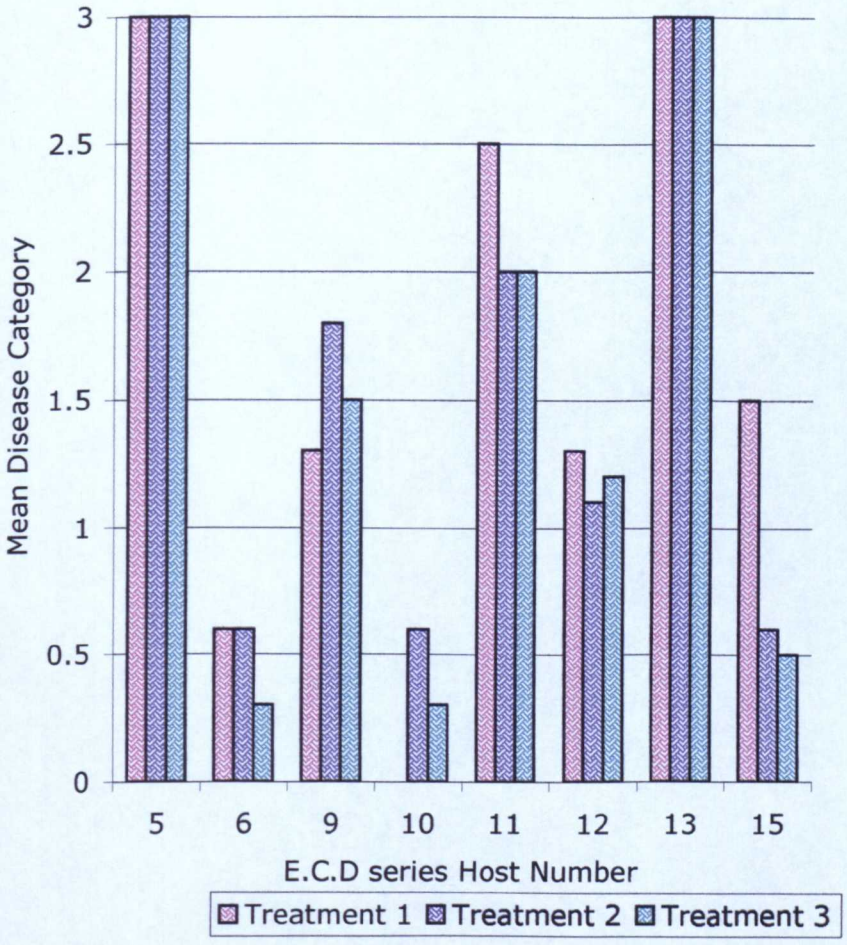
Only the increase in infection of host 12 treatment 2 resulted in a non-susceptible host becoming classed as susceptible. The infection level of hosts 9, 11 and 13 was however higher when treated with spore ratios 2 and 3 indicating that on these hosts at least these spore suspensions were more virulent.

4.7.2 A further investigation into the effect of *P. brassicae* resting spores suspensions prepared from mixtures of calcium nitrate treated and wild type spore suspensions on E.C.D series host resistance.

Method and Materials.

In 4.7.1 plants were grown in 6.25 cm diameter round pots. However this resulted in overcrowding of the pots and it was considered that plant growth was restricted. This experiment was therefore carried out in the same way as described for experiment 4.7.1 except that the revised E.C.D series was sown into 7 cm^2 pots and after seven days the number of hosts per pot was reduced to one.

Graph 4.7.2 Mean disease categories of the revised E.C.D series hosts raised in 7 cm^2 pots and treated with *P. brassicae* resting spore suspensions prepared from a calcium nitrate treated and wild type galls.



Treatments :-

1. 25% of resting spores from calcium nitrate treated galls and 75% from normal galls.
2. 50% of resting spores from calcium nitrate treated galls and 50% from normal galls.
3. 75% of resting spores from calcium nitrate treated galls and 25% from normal galls.

Results.

Graph 4.7.2 shows the mean disease categories of the revised E.C.D series raised in 7 cm^2 pots and inoculated with spore suspensions prepared from a mixture of calcium nitrate treated and wild type spores six weeks after inoculation.

In this experiment treatment 1 increased the infection level of host 15 beyond a disease category of 1 deeming this host susceptible. This is interesting as in previous experiments the infection of this host had been increased by the presence of spores obtained from calcium nitrate treated galls and yet in this experiment the treatment with the least proportion of calcium nitrate spores resulted in the highest level of infection. Treatment 1 also resulted in a higher level of infection of host 1 than the other two treatments. The infection level in host 12 was raised above a disease category of 1 by all the treatments unlike in the previous experiment.

Conclusions

The results differed from those in experiment 4.7.1. Increasing the pot size influenced how the host plants responded to the spore treatments, thus it would appear that the overcrowding of the plants in experiment 4.7.1 had influenced the pattern of infection. From these results however, a clear pattern of infection was not seen other than treatment one appeared to differ in its predominant pathogen race.

4.7.3 A further determination of the effect of *P. brassicae* resting spores suspensions prepared from mixtures of calcium nitrate treated and wild type spore suspensions on E.C.D series host resistance using the revised method.

Method and Materials.

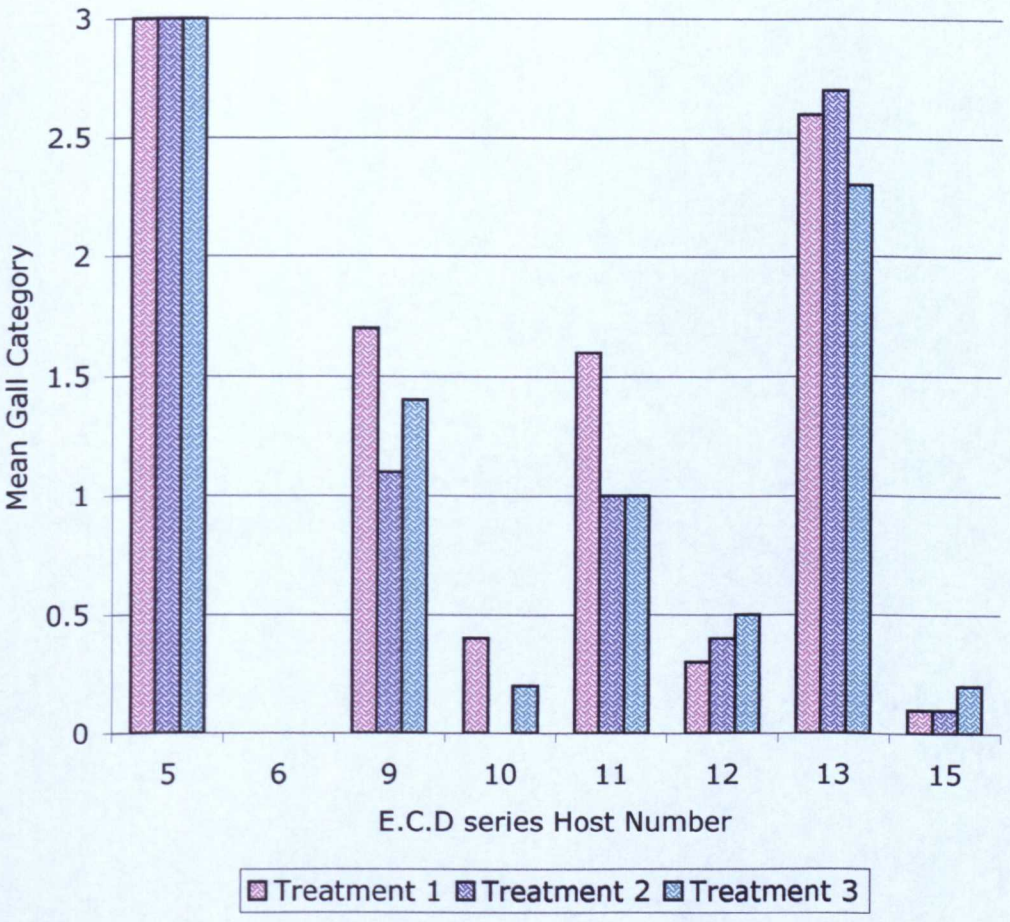
The results of 4.7.2 varied from experiment 4.7.1. However it was not clear whether this was due to changes in the host resistance / pathogen variation or due to the change in the experimental method. The experiment was therefore repeated using the same method and materials as in experiment 4.7.2.

Results.

Graph 4.7.3 shows the mean disease categories of the revised E.C.D series raised in 7 cm^2 pots and inoculated with spore suspensions prepared from a mixture of calcium nitrate treated and wild type spores six weeks after inoculation. The results were compared to those in experiment 4.7.2. Although host 6 had not been designated susceptible in experiment 4.7.2, the level of infection decreased to zero. The level of infection in host 9 differed because treatments 2 and 3 previously caused the highest levels of infection but in this experiment treatment 1 did.

Host twelve had an infection level which would deem it to be resistant to all of the treatments, this is in contrast to the previous results where all three treatments had resulted in the host being classified as susceptible. A difference of results in host 15 also occurred in that the level of infection caused by treatment level

Graph 4.7.3 Mean disease categories of the revised E.C.D series hosts raised in 7 cm^2 pots and treated with *P. brassicae* resting spore suspensions prepared from a calcium nitrate treated and wild type galls (2^{nd} experiment).



Treatments :-

1. 25% of resting spores from calcium nitrate treated galls and 75% from normal galls.
2. 50% of resting spores from calcium nitrate treated galls and 50% from normal galls.
3. 75% of resting spores from calcium nitrate treated galls and 25% from normal galls.

was reduced drastically resulting in this treatment no longer to be classified as causing the host to be classified as susceptible.

Conclusions

The results of this series of experiment highlight how difficult it is to obtain repeatable results when working with *P. brassicae*, as the experiments failed to produce results similar to each other it is not possible to say how the different ratios of the two spore suspensions affect the reduced E.C.D series. It can be concluded, however that the resultant spore suspensions may be very variable and this may be the reason as to why repeatable results were not obtainable. The experimental method may also have influenced the variability as the spore suspensions were prepared 'fresh' for each experiment and it has already been shown that the suspensions can vary simply from batch to batch.

Discussion

The results of these experiments will be discussed in three sections.

Wild type resting spore suspensions.

Resting spore suspensions obtained from wild type galls were used on two occasions to inoculate the E.C.D series (4.2 and 4.4.1). The results of these experiments indicate a different predominant pathogen race present in each suspension. The difference in the pathogen race was due to hosts 8 (*Brassica napus var napus* line Dc128) and 11 (*Brassica oleracea var. capitata* cv. Badger shipper). Host 8 had a greater disease category in experiment 4.1 and host 11 was more highly infected in experiment 4.4.1. Both of these hosts have differential resistance (Jones *et al.*, 1981) and the results of these experiments are a good illustration as to how variable the resistance of these two hosts can be.

The difference however is likely to be due to the changes in the predominant pathogen race. In general the E.C.D series in experiment 4.1 was more highly infected than the E.C.D series in experiment 4.4.1. The differences did not all result in changes to the predominant pathogen race number but there was more infection in the first E.C.D series experiment. Host number 6 (*Brassica napus* var *napus* line Dc101) has a disease category of 0.8 in experiment 4.1 making it very close to being determined as a susceptible host but in experiment 4.4.1 the disease category was reduced to 0.2.

In experiment 4.4.2, where the E.C.D series was sown in to Arthur Bower's seed and potting compost the predominant pathogen race was found to vary from both of the previous *P. brassicae* races. The difference between the Arthur Bower's population and the Levington's population in experiment 4.4.1 was due to differences in the disease categories of host 12 (*Brassica oleracea* var. *capitata* cv. Bindsachsener) Host 12 had a higher disease category in both of the Levington's E.C.D tests and it is therefore concluded that the difference in the Arthur Bower's population is due to the effect of the use of the different composts. The environment is known to influence the *P. brassicae* population (Colhoun, 1958) and as the wild type population appears to be highly variable it is likely that the population is less stable and more easily influenced by environmental effects.

These results highlight the problems encountered by Jones (1981, 1982b) with regards to the variability of the E.C.D series. In an attempt to decrease the variability used a disease category of greater then 1to determine a host as susceptible. This, however, would appear to be of limited use and further improvements need to be made to the system.

Calcium nitrate treated resting spore suspensions.

In experiment 4.2 the predominant pathogen race was determined as 16/14/30 and in experiments 4.3.1 and 4.3.2 the pathogen race was 16/14/15. The difference between these two populations is due to the mean disease category of host 15 (*Brassica oleracea* var. *acephala* subvar.*laciniata* cv.Verheul) being 1.03 in experiment 4.2 and hence its inclusion as a susceptible host . However as the level of disease is only just over the threshold of 1.0 and only

slightly higher than in experiments 4.3.1 and 4.3.2 it is likely that this difference in infection is due to resistance variations in host 15 rather than the differences in the pathogen race.

When compared with the results from the experiments using the wild type spore suspension it can be seen that resting spore suspensions prepared from calcium nitrate treated galls are less variable. However the resting spore suspension prepared from calcium nitrate treated galls are more infective to the *B. rapa* group in the E.C.D series than wild type spores.

In experiments 4.5.1 and 4.5.2 where the spore suspension was derived from a 2nd generation of calcium nitrate treated spores the predominant pathogen races in both composts was found to be 16/14/15, similar to experiments 4.3.1 and 4.3.2. These results suggest therefore that in the absence of calcium nitrate the predominant pathogen population does not revert back to the wild type population. This may be due to a residual level of calcium nitrate being present in the resting spore suspension, however the level of calcium would have been very low and it is unlikely that this very low level could have influenced the pathogen population.

It is more likely that the presence of calcium nitrate in the field situation has selected this pathogen race. Although this race seems to be more pathogenic to *B. rapa* it would seem that it is much less variable than the wild type pathogen. The results from experiments carried out in Chapter 3 show that calcium nitrate does decrease the level of galling and it is therefore possible that the reason for the change in the predominant pathogen race was due to the presence of calcium nitrate providing a selection pressure.

In experiment 4.6, where a spore suspension prepared from calcium nitrate treated galls was used to inoculate host plants grown in calcium nitrate treated soil, the pathogen population was found to be 16/2/14. The difference between this population and that of calcium nitrate treated spores was due to reduced infection in hosts 8 (*B. napus* var *napus* line Dc128) and 9 (*B. napus* var *napus* line Dc1129). This indicates that in a field situation, where the source of inoculum is from previous crops, the continued application of calcium nitrate will continually alter the pathogen population through selection pressure. However, as inoculum can remain viable in the soil for many years there is a continual supply of spores which have not previously been un-exposed to calcium nitrate. This experiment does however provide a further example of the ways in which calcium nitrate can affect *P. brassicae*.

Resting spore suspensions prepared from a mixture of calcium nitrate treated and wild type spore suspensions.

Experiments 4.7.1 – 4.7.3 were aimed at determining whether resting spores obtained from calcium nitrate treated galls are more competitive than resting spores obtained from wild type galls.

A definate pattern of infection was not determined, as it was not possible to obtain clear cut results. This may be due to differences in the interactions between the two resting spores suspensions or due to the spore suspensions being prepared at different times for each experiment. It has already been established that the wild type pathogen populations are highly variable and this may be the reason why it was not possible to get replicable results.

The results of this chapter support the findings of Jones (1981 and *et al.* 1982b) that the E.C.D series is not effective in determining all of the *P. brassicae* populations. In order for the E.C.D series to be more useful, hosts with uniform resistance must be used. A universal system also needs to be developed which determines the level to which a host must be infected before it is deemed susceptible. It was thought that using a mean disease category of greater than one to determine a susceptible host would help to remove some of the variability due to host resistance but it would appear that this measure is ineffective.

A more reliable measure of *P. brassicae* resistance may lie in the use of molecular techniques such as those being developed by Ito *et al* (1999), which use the pathogen DNA. There would, however, be problems due to the apparent variability within galled material collected from even a small area.

Chapter Five

Characterisation of Soils Suppressive to *Plasmodiophora brassicae.*

5. The Characterisation of Soils Suppressive to *Plasmodiophora brassicae.*

Several soils suppressive to *Plasmodiophora brassicae* have been identified in areas that have been continuously cropped with brassicas (Djatnika, 1991 Jaw-Fen and Wen-Hsui, 1986, Hseih and Jaw-Fen, 1986, Murakami *et al.* 1997, Rouxel 1984, 1988 Tsushima *et al.*, 1996 and Workhu and Gerhardson, 1996). The general nature of suppressiveness varies and has been determined as abiotic in some cases and biotic. In other soils an interaction of both these factors may also lead to the development of a suppressive soil.

It has been noted that, in two areas in Fife, Scotland, which have been continuously cropped with brassicas, the level of clubroot has been declining. Soil samples were collected from these two areas with the aim of establishing whether the soils are indeed suppressive to *P. brassicae* and, if so the nature of the suppression.

5.1. Potential suppression of *Plasmodiophora brassicae* by two soil samples.

The aim of this experiment was to compare the ability of soil samples from Crail (grid reference 362005,7075600)* and Kings Kettle (grid reference 330930,708335)* to suppress *P. brassicae* infection compared with soil samples from Auchincruive (238415,623365)*.

(* Grid references obtained from streetmap.co.uk)

Method and Materials.

Samples of soil were collected from Crail, Kings Kettle and Auchincruive following the procedure given in Chapter 2. Samples of the soils were sent to the Scottish Agricultural College in Edinburgh for analysis and the relevant results are given in Table 5.1. The results of the full analysis are given in Appendix 1.

Table 5.1. Soil analysis results for Crail, Kings Kettle and Auchincruive soil samples.

Soil Sample	pH	Extractable Calcium mg/l	Extractable Boron mg/l	Organic Matter %	Total Nitrogen mg/kg
Crail	7.5	3130	2.2	4	1500
Kings Kettle	7.7	3520	2.0	4	1620
Auchincruive	5.4	1440	1.1	6	2430

Soil from each of the sites was mixed with an equal volume of Perlite and placed in small seed trays giving three treatments each

with six replicates. Twenty, seven day old Chinese cabbage seedlings *Brassica rapa* ssp. *pekinensis* cv Mariko, raised in peat compost modules, were transplanted into each tray. After one week the number of plants per tray was reduced to 10

The experiment was carried out in a glasshouse at Auchincruive.

Results.

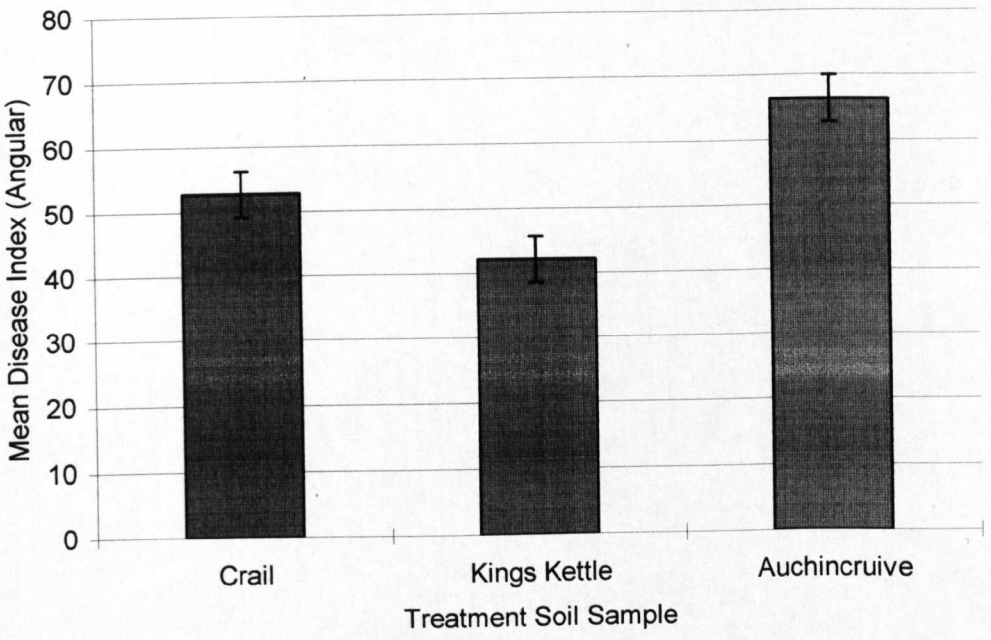
Due to high level of *Botrytis cinerea* (grey mould) infection it was only possible to harvest two replicates of each treatment. The experiment was harvested 50 days after transplantation and the disease indices, dry root and shoot masses were determined (Table 5.2).

Table 5.2. Mean disease indices and mean dry root and shoot masses of plants grown in three *P. brassicae* infected soil samples.

Treatment	Disease Index (Angular)	Dry Root mass (g)	Dry Shoot Mass (g) (sqrt)
Crail	52.7a	0.305a	0.739a
Kings Kettle	42.1a	0.117a	1.038b
Auchincruive	66.3a	0.705a	0.860a
S.E.D	7.12	0.0635	0.0884
L.S.D	30.64	0.1272	0.1770

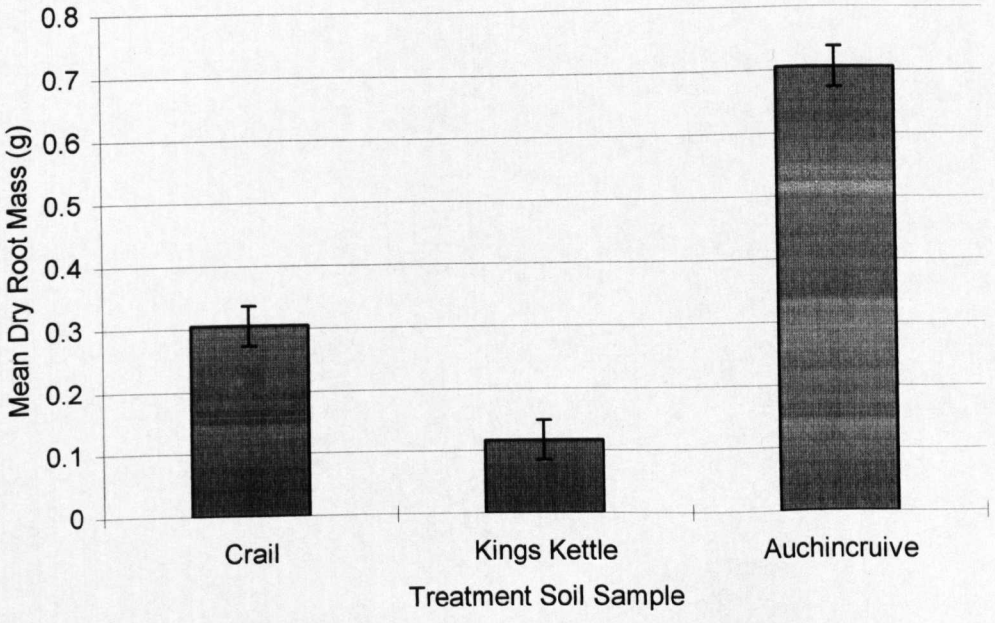
Numbers with the same letters are not significantly different using the L.S.D value.

Graph 5.1. Mean disease indices of plants raised in three *P. brassicae* infected soil samples.



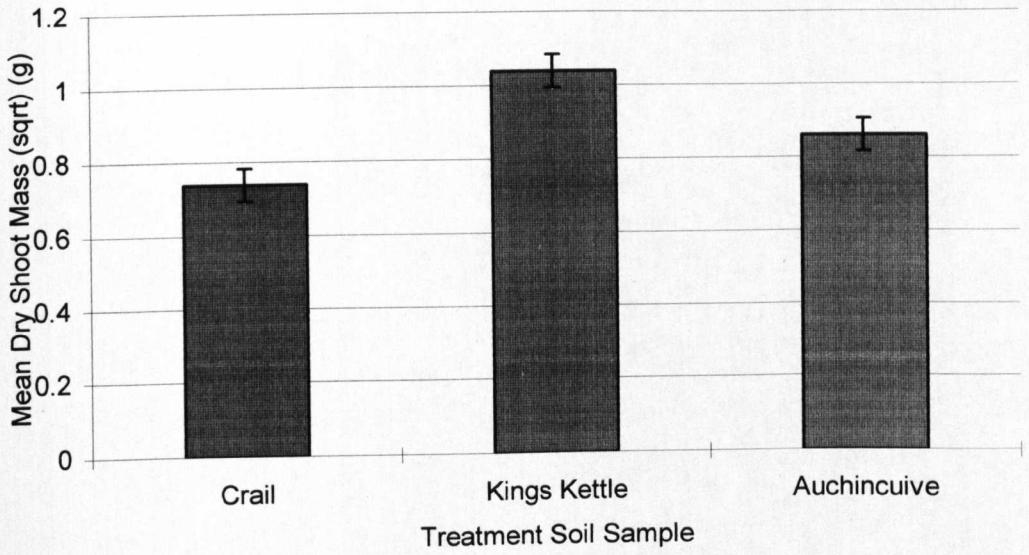
s.e.d = 7.12

Graph 5.2. Mean root masses of plants grown in three *P. brassicae* infected soil samples.



s.e.d = 0.0635

Graph 5.3. Mean dry shoot masses of plants grown in three *P. brassicae* infected soil samples.



s.e.d = 0.0884

The mean disease indices are illustrated in Graph 5.1. The disease levels were lower in the two samples that reportedly suppress *P. brassicae* infection at Crail and Kings Kettle. These differences were not significant when compared using the L.S.D value. The disease levels are however significantly different using the S.E.D values indicating that a real difference in disease levels may have been present but obscured due to the effects of *Botrytis cinerea.*

The lowest mean dry root mass was recorded in plants raised in the Crail soil sample (Graph 5.2, Table 5.1). Again the differences were not significant when compared using the L.S.D, value but did differ when the S.E.D value was used. The results suggest therefore, that plants raised in the Crail soil samples had less densely galled roots.

Plants raised in the Kings Kettle soil sample not only had the highest root mass (although not significantly higher than those of the other two treatments) but also the highest shoot mass (Graph 5.3, Table 5.1). Analysis of the data revealed that the shoot masses of plants raised in Kings Kettle soil were significantly higher than plants grown in the other two soil samples.

Analysis of the soil samples showed that the two soils thought to be suppressive had a more alkaline pH and extractable calcium and boron levels, all of which are associated with reducing the incidence of clubroot.

Conclusions.

The results from the soil analysis (Table 5.1) indicate that the suppressive soils collected from Kings Kettle and Crail have high

levels of calcium and boron in the soil. It is probable, therefore that the suppressive nature of the soil was due to the high levels of these nutrients. This experiment, however, does not discount the possible effects of organic matter or ammonium content. Hseih and Wang (1986) Wang and Hseih (1986) observed that soils with calcium contents of 1210 and 1460ppm and pH's of 7.4 and 7.2 were suppressive to *P. brassicae* infection. The values in both the Crail and the Kings Kettle samples are above these thresholds. It is noted that the calcium content of the Auchincruive sample is also in line with the values which would characterise a suppressive soil, but the acidity of the soil is in contrast to that recorded in the suppressive soils reported by Hseih and Wang (1986) Wang and Hseih, (1986).

The role of the soil micro-flora in decreasing the incidence of clubroot however cannot be discounted and experiment 5.2 was designed to determine whether there was a biotic character to the suppressiveness.

Although the samples from Crail and Kings Kettle decreased the incidence of clubroot the results were not significant. This may have been due to the *Botrytis cinerea* infection which allowed only two replicates to be harvested.

5.2. Further investigations into the suppressive properties of soils collected from Crail and Kings Kettle.

Experiment 5.1 established that the soil samples from Kings Kettle and Crail were associated with *P. brassicae* suppression. The nature of the suppressiveness was not confirmed. The aim of this experiment was to determine whether the microflora of the two samples had a role in the suppressiveness.

Method and materials.

Chinese cabbage seed (*Brassica rapa* ssp. *pekinensis* cv. Mariko) was sown into module trays containing Levington's modular peat compost. Ten days later 20 seedlings were transplanted into each small seed tray which contained one of the following soil treatments :- Crail, Kings Kettle, Auchincruive and autoclaved Crail, Kings Kettle and Auchincruive which had been mixed with an equal volume of perlite to aid aeration and drainage (Chapter 2). The experiment consisted of three replicates of six treatments giving 18 trays.

The autoclaved soil samples were prepared by mixing them with an equal volume of perlite and then placing the mixture into autoclave bags. The samples were autoclaved twice at 120^0C and 1.03 bar pressure for 15 minutes. Immediately following transplantation the soils which had been autoclaved were re-infested with *P. brassicae* by pouring 100ml of a 10^6 resting spores / ml suspension over the surface of each tray. The experiment used a growth room (Chapter 2).

Samples of the treated soil samples were sent to the Scottish Agricultural College in Edinburgh for analysis and the relevant results are given in Table 5.4. The results of the full analysis are given in Appendix 1.

Results

The plants were harvested 50 days after transplantation and the disease indices, dry root and shoot masses are presented in Table 5.3.

Table 5.3. Mean disease indices and dry root and shoot masses of plants grown in autoclaved and non-autoclaved soil samples.

Soil Sample	Mean Disease Indices (Angular)	Mean Dry Root Mass (g) (sqrt)	Mean Dry Shoot Mass (g) (sqrt)
Crail	35.3a	0.232c	0.2124c
Crail (A)	8.0c	0.90a	0.1746c
Kings Kettle	28.4b	0.222c	0.3976a
Kings Kettle (A)	43.7a	0.352b	0.4160a
Auchincruive	35.3a	0.269c	0.3090b
Auchincruive(A)	35.3a	0.279bc	0.3167b
S.E.D	4.88	0.0370	0.02533
L.S.D	10.88	0.0732	0.05012

(A) Indicates a soil which was autoclaved and then inoculated with resting spores.

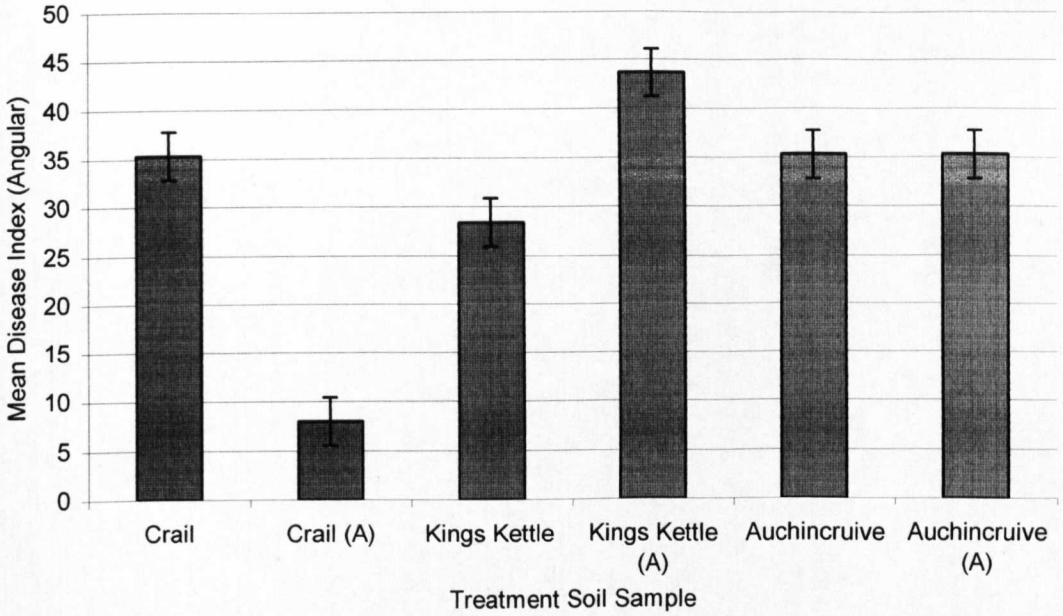
Numbers with the same letters are not significantly different using the L.S.D value.

The mean root and shoot values were transformed using the square root due to the data being skewed.

The mean disease index (Graph 5.4, Table 5.3) of autoclaved Crail soil was significantly lower than all other treatments. In contrast this treatment had the highest root mass which is usually associated with a high level of infection (Graph 5.5, Table 5.3). The shoot mass was significantly lower in the autoclaved Crail sample than all other treatments except the non-autoclaved Crail sample (Graph 5.6, Table 5.3). Low shoot mass is also associated with a high level of clubroot infection. The reason for these conflicting results lies in the effect of the autoclaved Crail soil sample. Plate 5.1 illustrates the phyto-toxic effects of this soil sample.

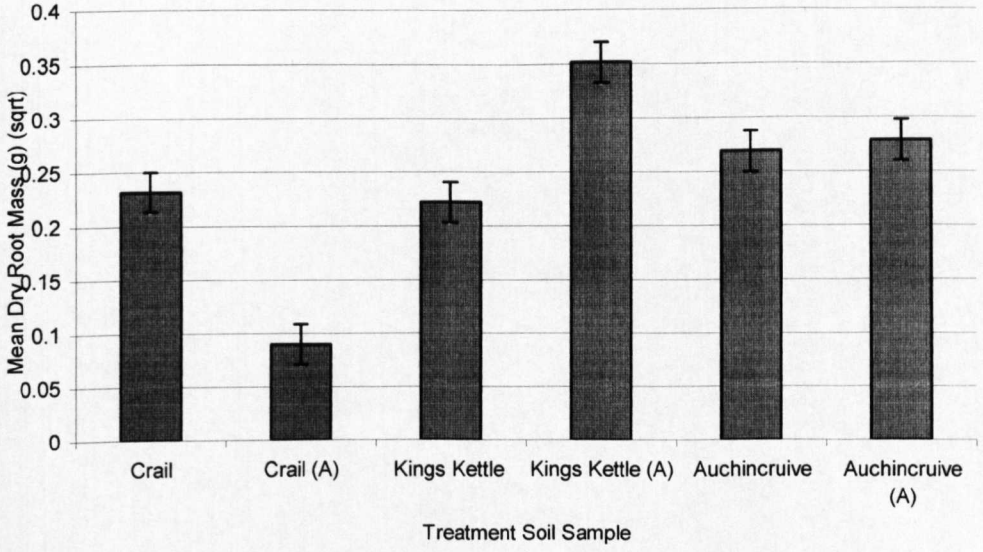
Plate 5.1. Comparison of *Brassica rapa* ssp. *pekinensis* cv Mariko raised in autoclaved and non-autoclaved Crail soil samples.

Graph 5.4. Mean disease indices of plants grown in three autoclaved and non-autoclaved soil samples.



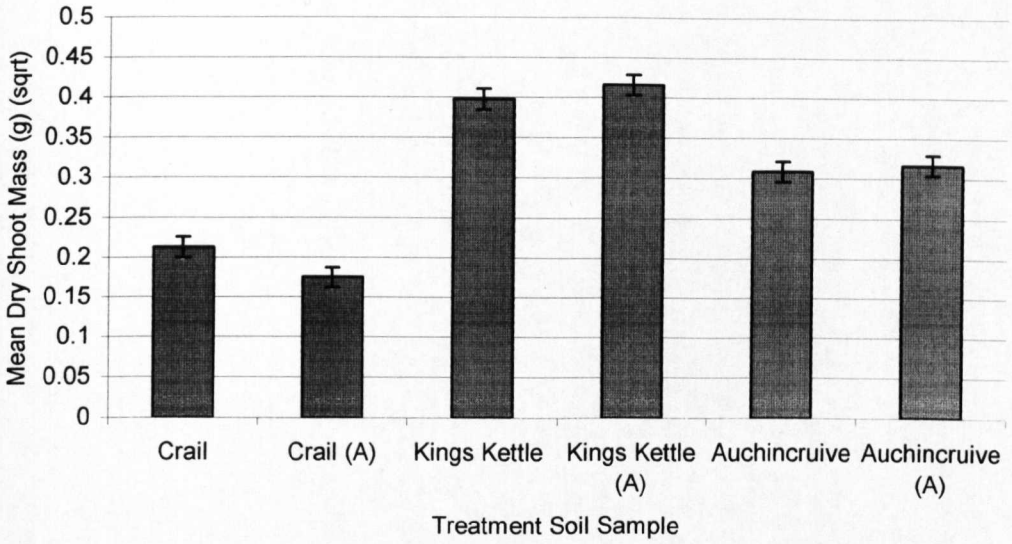
(A) indicates a soil which was autoclaved and then inoculated with resting spores.
s.e.d = 4.88

Graph 5.5. Mean dry root masses of plants grown in three autoclaved and non-autoclaved soil samples.



(A) indicates a soil which was autoclaved and then inoculated with resting spores.
s.e.d = 0.0370

Graph 5.6. . Mean dry shoot masses of plants grown in three autoclaved and non-autoclaved soil samples.



(A) indicates a soil which was autoclaved and then inoculated with resting spores.
s.e.d = 0.05012

The level of clubroot infection increased significantly in the autoclaved Kings Kettle soil sample, compared with the non-autoclaved soil sample (Graph 5.4, Table 5.3). This is characteristic of a soil where suppression occurs as a result of the microflora. The mean dry root mass increased significantly in the autoclaved Kings Kettle soil sample (Graph 5.5, Table 5.3). The mean dry shoot mass increased (Graph 5.6, Table 5.3) but not significantly.

Autoclaving the Auchincruive sample had no effect on the disease index, root mass or shoot mass.

The level of disease in the control sample, Auchincruive, was much lower than had been observed in other experiments and it was considered that the growth environment did not provide optimum conditions for disease development.

Table 5.4. Soil analysis offor autoclaved and non-autoclaved Crail, Kings Kettle and Auchincruive soil samples.

Soil	pH	Extractable calcium (mg / l)	Extractable Boron (mg / l)	Organic matter (%)
Crail	7.5	3130	2.2	4
Crail (A)	7.5	2550	2.2	4
Kings Kettle	7.7	3520	2.0	4
Kings Kettle (A)	7.7	3680	1.7	4
Auchincruive	5.4	1440	1.1	6
Auchincruive (A)	5.8	1160	0.7	5

(A) Indicates a soil sample which has been autoclaved.

Autoclaving the soil samples affected the level of extractable calcium. The level of extractable calcium was decreased in the Crail autoclaved samples, but remained above the level determined by Hseih and Wang (1986) Wang and Hseih (1986) as suppressive. The level did increase slightly in the autoclaved Kings Kettle sample. The level of boron in the Kings Kettle and Auchincruive samples was decreased by autoclaving. The pH of the samples was unaffected by autoclaving.

The level of extractable calcium in the autoclaved Crail sample was reduced and thus it is unlikely therefore that the level of calcium was responsible for the phyto-toxicity. It is not known whether the level of calcium available to plants was also reduced.

Conclusions

Autoclaving the soil samples showed that the suppressive nature of the Kings Kettle soil may be due either in whole or in part to the soil microflora as the level of *P. brassicae* infection increased upon autoclaving of the soil sample. The point of autoclaving was to remove the soil microflora from the host disease interaction. Thus if the level of disease increases it indicates that the soil microflora had been playing a role in suppressing *P. brassicae*.

The suppressive microflora may be encouraged to colonise the soil samples by the high level of calcium and boron or there may be an interaction between the biotic and abiotic factors.

The autoclaving of the Crail soil sample led to a phyto-toxic effect making it difficult to determine whether the suppressiveness of the sample was due to biotic or abiotic factors. The samples were

collected from commercial brassica growing areas and so would have been treated with a range of chemicals. It is therefore possible that upon autoclaving pesticide residues may have been released or converted to toxic radicals.

Because of the problem of phyto-toxicity caused by autoclaving the Crail soil, the following experiment further investigated the suppressive nature of the soils with the addition of nutrient supplements which would possibly overcome the phyto-toxic effects.

Experiment 5.3. Further investigation to determine whether the suppressive properties of soils collected from Crail and Kings Kettle are due to biotic factors.

This extended experiment 5.2 and aimed to identify the suppressive nature of the soil samples and investigate the interactions between biotic and abiotic factors. This experiment however involved the application of nutrients to the trial plants to try to overcome the effects caused by autoclaving of the Crail soil sample.

Method and Materials

The experiment used similar methods and materials as 5.2 with the addition that the plants received two treatments of Baby Bio fertiliser at the commercially recommended rate. A detailed analysis of Baby Bio is given in Appendix2.

Results

The plants were harvested 50 days after transplanting of the seedlings. The disease indices and dry root and shoot masses were recorded and are presented in Table 5.5.

Table 5.5. Mean disease indices and dry root and shoot masses of plants grown in autoclaved and non-autoclaved soil samples.

Soil Sample	Mean Disease Index (Angular)	Mean Dry Root Mass (g) (sqrt)	Mean Dry Shoot Mass (g) (sqrt)
Crail	47.8c	0.62b	1.198a
Crail (A)	4.6d	0.076a	0.331d
Kings Kettle	37.9c	0.353d	0.706bc
Kings Kettle (A)	48.2c	0.6bc	0.840b
Auchincruive	85.4a	0.680a	0.571c
Auchincruive(A)	65.8b	0.751a	0.726bc
S.E.D	6.36	0.0664	0.0812
L.S.D	14.18	0.1318	0.1614

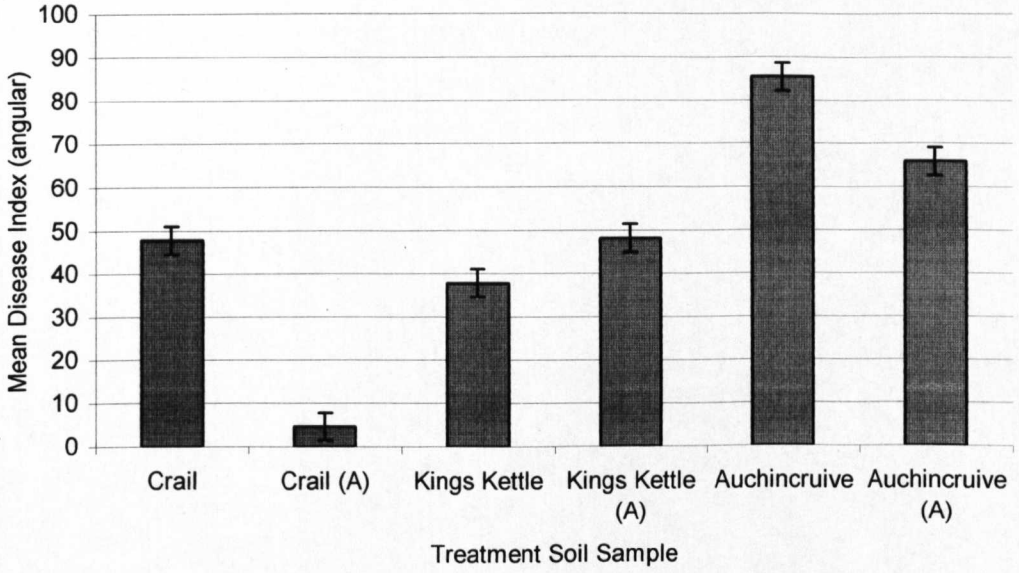
(A) Indicates a soil which was autoclaved and then inoculated with resting spores.

Numbers with the same letters are not significantly different using the L.S.D value.

The mean dry root and shoot masses were converted to square roots due to the data being skewed.

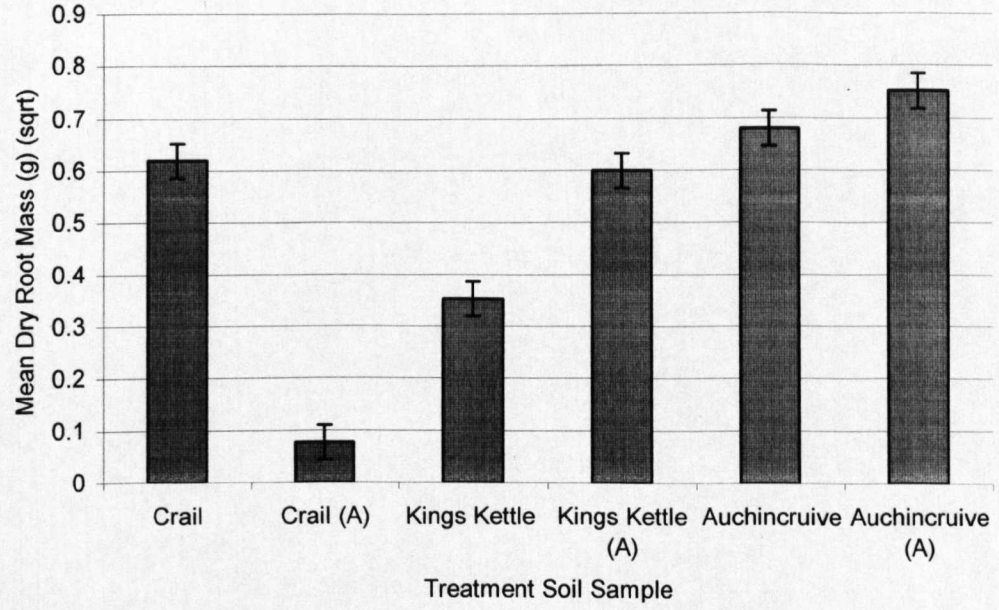
Autoclaving the Crail soil sample again resulted in phyto-toxicity, there was a significant decrease in the dry root (Graph 5.8, Table 5.5) and shoot mass (Graph 5.9, Table 5.5). As a result the disease

Graph 5.7. . Mean disease indices of plants grown in three autoclaved and non-autoclaved soil samples.



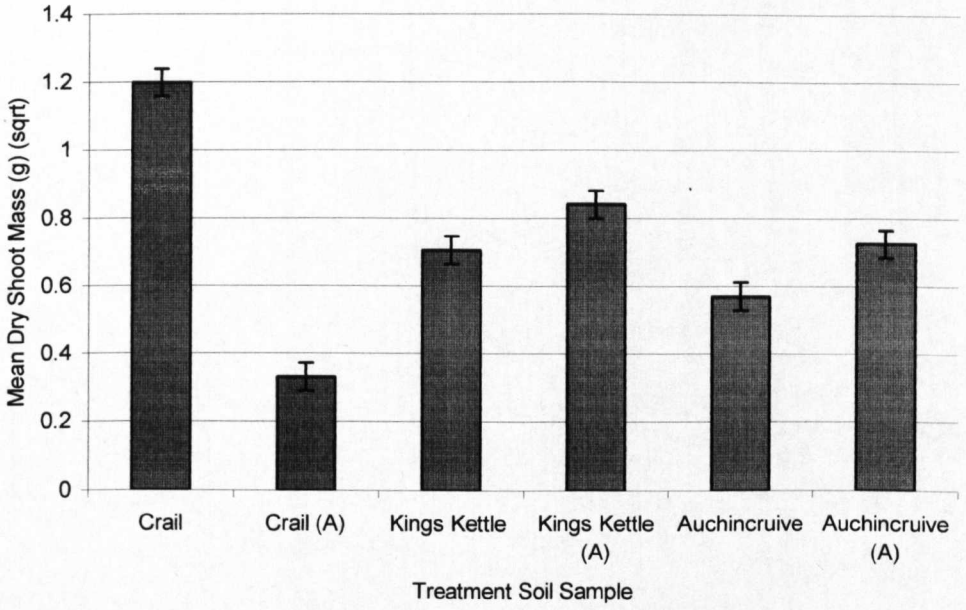
(A) indicates a soil which was autoclaved and then inoculated with resting spores.
s.e.d = 6.36

Graph 5.8. . Mean dry root masses of plants grown in three autoclaved and non-autoclaved soil samples.



(A) indicates a soil which was autoclaved and then inoculated with resting spores.
s.e.d = 0.0664

Graph 5.9. Mean dry shoot masses of plants grown in three autoclaved and non-autoclaved soil samples.



(A) indicates a soil which was autoclaved and then inoculated with resting spores.
s.e.d = 0.0812

index was also significantly decreased (Graph 5.7, Table 5.5), the host requires a good supply of carbohydrates in order *for P. brassicae* to stimulate gall formation (Evans and Scholes, 1995).

There were no significant differences in the disease indices of the two Kings Kettle soil samples when compared using the L.S.D value. The disease index of plants grown in Kings Kettle soil was lower than that of plants grown in autoclaved Kings Kettle soil, however and the difference was significant when the S.E.D value was used to compare the two treatments (Graph 5.7, Table 5.5). The same pattern is seen in the values obtained for dry shoot mass (Graph 5.9, Table 5.5).

The root mass was significantly lower in the non-autoclaved sample of Kings Kettle soil (Graph 5.8, Table 5.5) which corresponds to the lower level of disease in this sample. The plants in the autoclaved sample did however, have a high level of disease.

The mean disease index of the Auchincruive soil sample decreased upon autoclaving (Graph 5.7, Table 5.5) but there was no significant difference in the dry root (Graph 5.8, Table 5.5) or shoot masses (Graph 5.9, Table 5.5).

Conclusions

The results from this experiment involving the Crail soil sample are comparable to those obtained in experiment 5.2 in that autoclaving resulted in phyto-toxicity. As has been suggested it is considered that this was due to the autoclaving processes affecting pesticide residues in the soil.

The Auchincruive soil sample was included in experiments 5.2 and 5.3 as a control as it is known to be infected with *P. brassicae* and has not exhibited any signs of a suppressive nature. The results from experiment 5.2 support this as no differences were observed between autoclaved and non-autoclaved soil samples. In experiment 5.3 the level of disease decreased upon autoclaving of the soil but not significantly, further supporting the suggestion that soil from the Auchincruive site is not suppressive.

Although the level of *P. brassicae* infection in autoclaved Kings Kettle soil did not increase significantly in this experiment the level of infection did rise. In combination with the results from experiment 5.2, where the level of clubroot increased significantly in the autoclaved sample, the results suggest the suppressive nature of Kings Kettle soil is due to biotic rather than abiotic factors.

5.4. Determination of the micro-floral content of Crail, Kings Kettle and Auchincruive soil samples.

Experiments 5.2 and 5.3 established that the soil micro-flora has a role in the suppressive abilities of soil collected from Kings Kettle. This experiment was carried out to determine whether Kings Kettle and Crail soils had a higher level of micro-flora than soils collected from Auchincruive. The autoclaving of Crail soil samples resulted in phyto-toxicity and so this would allow comparison of the levels of micro flora to Kings Kettle soil which is thought to have a biotic suppressive character and Auchincruive soil which does not. This would allow further characterisation of the properties of the Crail soil sample.

Method and Materials.

The method and materials used are described under the heading soil dilution plates in Chapter 2. Malt extract (fungal colonies) and tryptic soya agar (bacteria) were chosen as Dhingra and Sinclair (1985) recommended them as good general agars for soil counts.

Results.

The number of colonies formed on each plate were counted after two and seven days. The results are given in Tables 5.6-5.9.

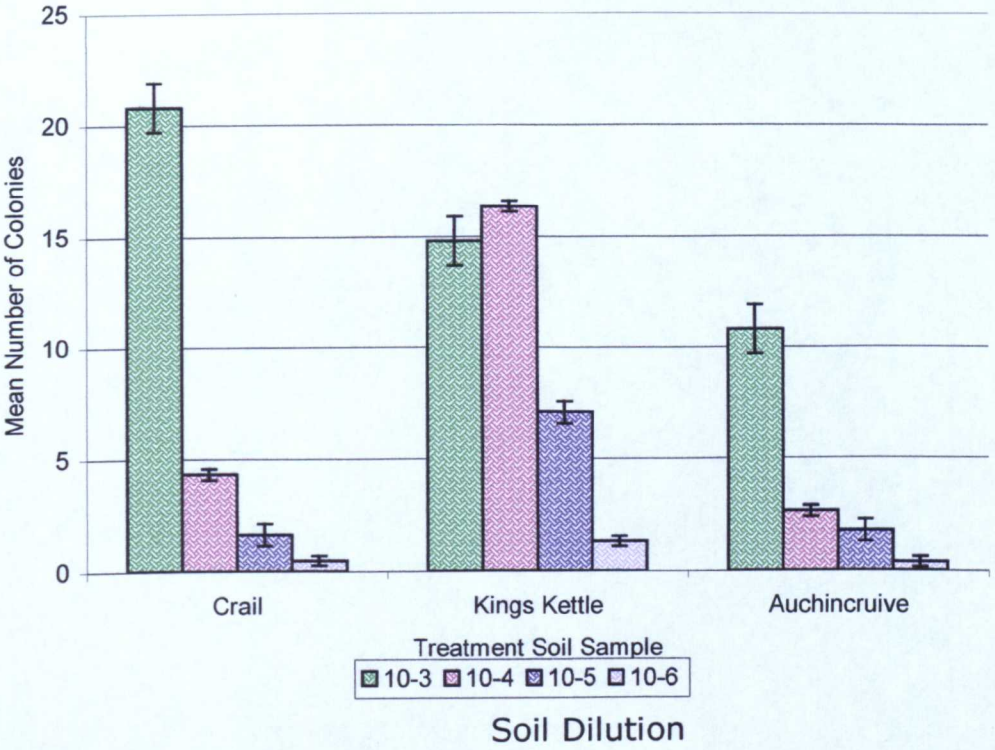
Table 5.6. Mean number of bacterial colonies recorded on tryptic soya broth agar following two days incubation.

	Soil Dilution			
Soil Sample	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Crail	20.81a	4.33b	1.65b	0.50a
Kings Kettle	14.79b	16.33a	7.06a	1.33a
Auchincruive	10.8b	2.63c	1.78b	0.37a
S.E.D	2.208	0.504	0.991	0.457
L.S.D	4.92	1.124	2.209	1.018

Numbers with the same letters are not significantly different using the L.S.D value.

The mean number of bacterial colonies after two days incubation on tryptic soya broth agar are illustrated in Graph 5.10.

Graph 5.10. Mean Number of bacterial colonies per tryptic soya broth plate following two days incubation.



s.e.d of 10^{-3} soil dilution = 2.208
s.e.d of 10^{-4} soil dilution = 0.504
s.e.d of 10^{-5} soil dilution = 0.991
s.e.d of 10^{-6} soil dilution = 0.457

The number of colonies observed on Crail plates was significantly higher than in other treatments at a soil dilution of 10^{-3}. At a soil dilution of 10^{-4} the number of colonies on Crail plates was significantly lower than that on Kings Kettle plates. The number of colonies on Crail plates was however, still significantly higher than the number forming on Auchincruive plates. The number of colonies on Kings Kettle plates at a dilution of 10^{-5} was significantly higher than those on Crail and Auchincruive plates. At 10^{-6} there was no significant difference in the number of colonies between treatments.

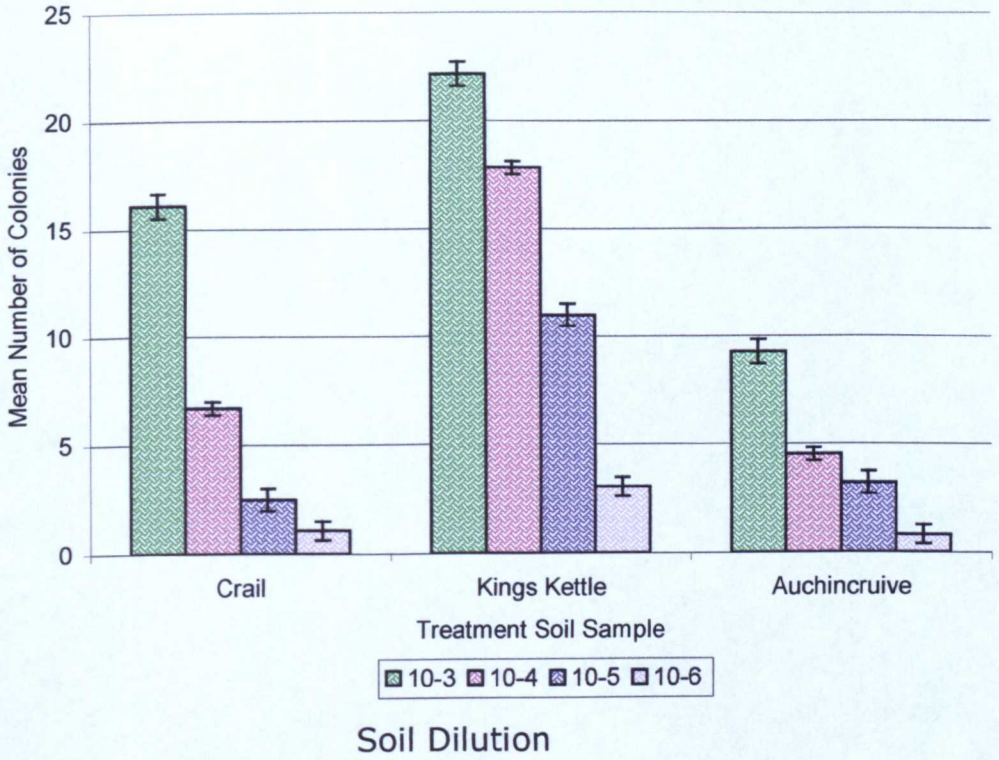
Table 5.7. Mean number of bacterial colonies recorded on tryptic soya broth agar following seven days incubation.

	Soil Dilution			
Soil Sample	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Crail	16.07b	6.68b	2.49b	1.06b
Kings Kettle	22.15a	17.81a	10.98a	3.0a
Auchincruive	9.25c	4.50c	3.21b	0.83b
S.E.D	1.116	0.598	1.026	0.893
L.S.D	2.486	1.332	2.286	1.990

Numbers with the same letters are not significantly different using the L.S.D value.

The mean number of colonies counted on tryptic soya broth agar after seven days incubation are illustrated in Graph 5.11.

Graph 5.11. Mean number of bacterial colonies per tryptic soya broth plate following seven days incubation.



s.e.d of 10^{-3} soil dilution = 1.116
s.e.d of 10^{-4} soil dilution = 0.598
s.e.d of 10^{-5} soil dilution = 1.026
s.e.d of 10^{-6} soil dilution = 0.893

A similar pattern of colony growth was observed after seven days incubation with the exception that there was a significantly higher number of colonies on Kings Kettle plates.

Table 5.8. Mean number of fungal colonies recorded on malt extract agar following two days incubation.

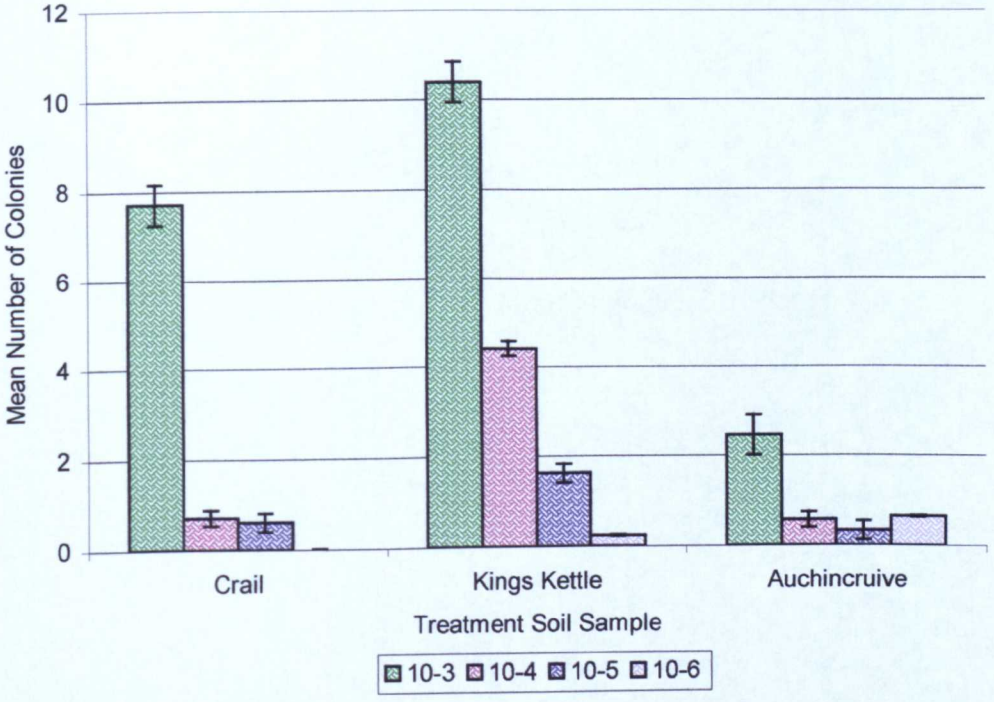
	Soil Dilution			
Soil Sample	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Crail	7.69b	0.71b	0.61b	0a
Kings Kettle	10.39a	4.41a	1.62a	0.24a
Auchincruive	2.45c	0.57b	0.33b	0.64a
S.E.D	0.0847	0.338	0.413	0.338
L.S.D	1.887	0.753	0.920	0.752

Numbers with the same letters are not significantly different using the L.S.D value.

The mean number of fungal colonies counted on malt extract agar after two days incubation are illustrated in Graph 5.12.

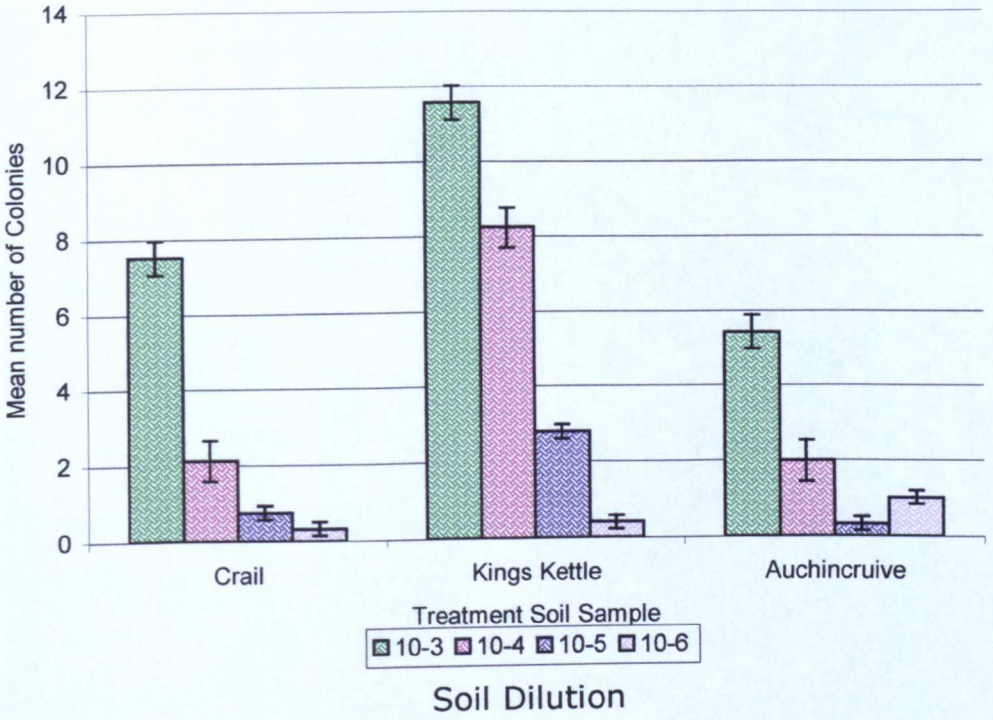
The number of colonies counted on malt extract agar was significantly higher for Kings Kettle soil dilutions than other treatments at all dilutions except at 10^{-6}.

Graph 5.12. Mean number of fungal colonies per malt extract agar plate following two days incubation.



s.e.d of 10^{-3} soil dilution = 0.847
s.e.d of 10^{-4} soil dilution = 0.338
s.e.d of 10^{-5} soil dilution = 0.413
s.e.d of 10^{-6} soil dilution = 0.338

Graph 5.13. Mean number of fungal colonies per malt extract agar plate following seven days incubation.



s.e.d of 10^{-3} soil dilution = 0.914
s.e.d of 10^{-4} soil dilution = 1.072
s.e.d of 10^{-5} soil dilution = 0.363
s.e.d of 10^{-6} soil dilution = 0.363

Table 5.9. Mean number of fungal colonies recorded on malt extract agar following seven days incubation.

	Soil Dilution			
Soil Sample	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Crail	7.52b	2.12b	0.75b	0.33a
Kings Kettle	11.56a	8.24a	2.78a	0.4a
Auchincruive	5.41c	1.98b	0.33b	1.02a
S.E.D	0.914	1.072	0.363	0.363
L.S.D	2.107	2.389	0.821	0.809

Numbers with the same letters are not significantly different using the L.S.D value.

The mean number of fungal colonies after seven days incubation on malt extract agar are illustrated in Graph 5.13. Again it can be seen that the number of colonies formed on agar inoculated with Kings Kettle dilutions was higher at all treatments when compared to plates inoculated with Auchincruive and Crail soil samples except at a dilution of 10^{-6}.

Conclusions.

The results indicate that Kings Kettle soil dilution plates had higher numbers of both bacterial and fungal colonies. This supports the results from experiments 5.2 and 5.3, which suggested that the suppressive nature of this soil sample was due to biotic factors. The number of colonies recorded growing on both malt extract agar (fungi) and tryptic soya broth agar (bacterial growth) was higher

for Kings Kettle samples indicating that there is a higher overall micro-flora within this soil rather than one specific area

Plates which were inoculated with the Crail soil sample gave mixed results. At dilutions of 10^{-3} and 10^{-4} on tryptic soya broth agar and malt extract agar the number of colonies were high but at lower dilutions the number of colonies decreased.

The soil dilution plates inoculated with Auchincruive soil samples generally had a lower number of colonies when compared with other treatments, although the number of colonies was not always significantly lower than Crail soil plates. The differences between Auchincruive plates and Crail plates were significantly less at higher soil dilutions indicating that the soil micro-flora may play at least a part in the suppressive character of Crail soil.

This experiment did not clarify the suppressive nature of Crail soils. The relevance of calcium, boron, and nitrogen levels in stimulating the soil micro-flora has not been established. An experiment was therefore carried out which examined whether or not raising the level of calcium in the soil influenced the soil microflora.

5.5. Investigation in to whether the addition of calcium to a soil influences the soil micro-flora population.

Experiment 5.4 determined that Kings Kettle soil may generally have a higher microflora content than Crail or Auchincruive samples. Crail soil samples also had a greater micro-floral content when compared with Auchincruive samples. Both Kings Kettle and Crail samples have high calcium levels, which were greater than those suggested by Hseih and Wang (1986) Wang and Hseih (1986) as required for suppressive soils. It is possible therefore that the level of calcium may play a role in disease suppression possibly stimulating the micro-flora. This experiment therefore aims to determine whether increasing the calcium content of soil encourages micro-flora growth. The effects of calcium are of particular interest as increased levels of calcium have already been associated with regression of *P. brassicae* by Webster (1985).

Method and Materials

One gram samples of air dried Auchincruive soil were placed into sterile 25ml conical flasks. Four ml of the appropriate treatment solution were added to each flask, sufficient to moisten the soil sample, the treatment solutions were :-

1. Control – Sterile de-ionised water
2. 0.082g $10ml^{-1}$ of calcium nitrate
3. 0.149 g $10ml^{-1}$ of calcium nitrate

The calcium nitrate concentrations were calculated to increase the calcium content of the Auchincruive soil sample to levels similar to those contained in Crail and Kings Kettle samples. An example of

the calculation used to determine the required levels of calcium nitrate is given below.

Example calculation :

The highest calcium level recorded was in the autoclaved Kings Kettle sample = 3680 mg l^{-1} Auchincruive soil contained 1160 mg l^{-1} and thus:-

3670 – 1160 = 2520 mg l^{-1} was required to raise the calcium level of the Auchincruive soil sample to the same level as Kings Kettle soil

To calculate the level of calcium nitrate required to obtain this level the formula weight was divided by the mass of calcium in calcium nitrate and multiplied by the required level of calcium.

$$= \frac{236.15}{40} \times 2520 \text{ mg } l^{-1} = 1489 \text{ mg } l^{-1} = 14.89 \text{g } l^{-1}$$

Where 236.15 = the formula weight of calcium nitrate and 40 = the atomic weight of calcium.

Hence, in order to make sufficient solution to treat both samples 0.1489 g of calcium nitrate was required in 10ml of de-ionised water.

The flasks were plugged with autoclaved non-absorbent cotton wool and incubated in the dark at 25^{0}C for two or seven days giving a total of six treatment flasks (three treatments, two

incubation periods). The flasks were then used to prepare soil dilution plates as described in Chapter 2.

Results

The number of colonies formed were counted on each replicate dilution plate at two and seven days incubation after plating. The square root of the mean colony counts are presented in Tables 5.10-5.17. The results were square rooted due to the data being skewed.

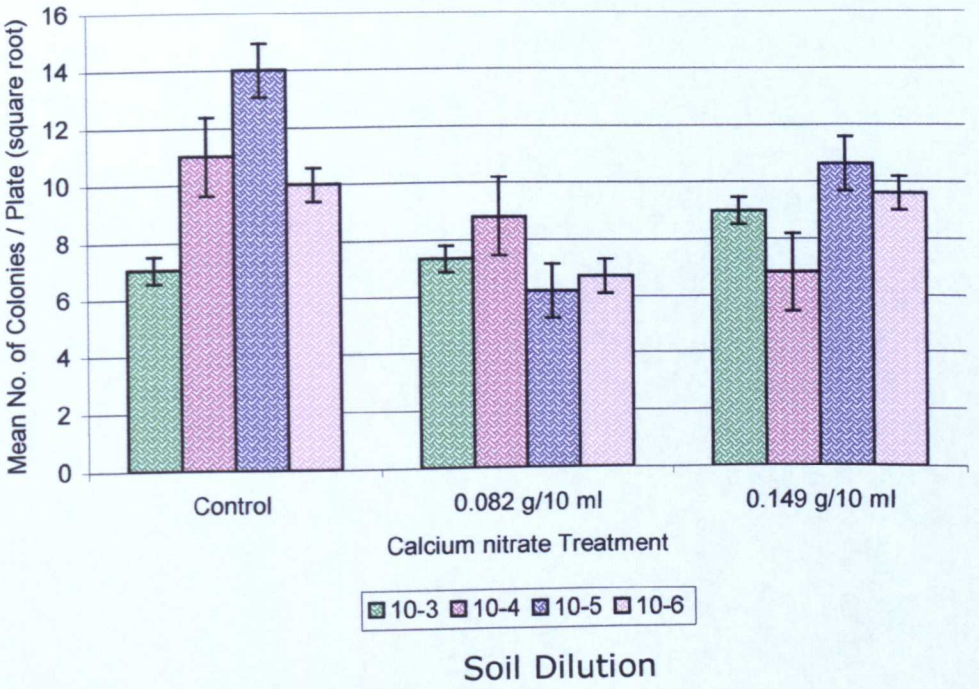
Table 5.10. Mean number of bacterial colonies recorded on tryptic soya broth agar following two days incubation with treatment solution and two days incubation following plating.

	Soil Dilution			
Treatment	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Control	7.03a	11a	14a	10a
0.082 g $CaNO_3$ 10 ml^{-1}	7.32a	8.8a	6.17b	6.69b
0.149 g $CaNO_3$ 10ml^{-1}	8.97a	6.8a	10.61a	9.58a
S.E.D	0.941	2.75	1.911	1.188
L.S.D	2.097	6.12	4.259	2.648

Numbers with the same letters are not significantly different using the L.S.D value.

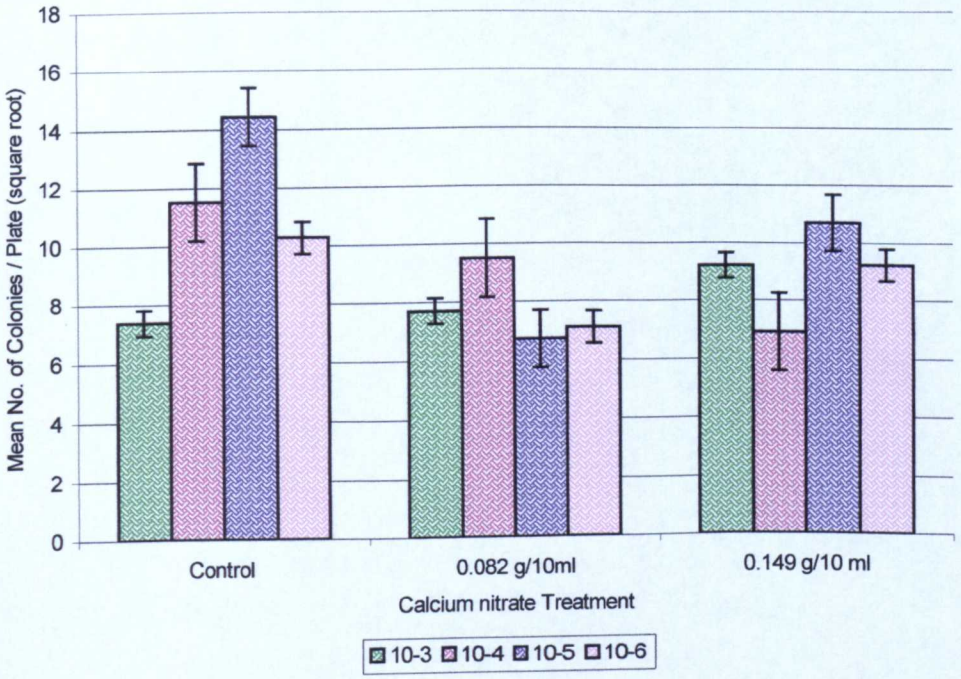
The number of bacterial colonies recorded on tryptic soya broth agar plates incubated for two days following inoculation with soil samples which had been incubated for two days prior to use, were

Graph 5.14. Mean number of bacterial colonies per tryptic soya broth agar plate following two days treatment and two days incubation.



s.e.d of 10^{-3} soil dilution = 0.914
s.e.d of 10^{-4} soil dilution = 2.75
s.e.d of 10^{-5} soil dilution = 1.911
s.e.d of 10^{-6} soil dilution = 01.188

Graph 5.15. Mean number of fungal colonies per tryptic soya broth agar plate following two days treatment and seven days incubation.



s.e.d of 10^{-3} soil dilution = 0.867
s.e.d of 10^{-4} soil dilution = 2.68
s.e.d of 10^{-5} soil dilution = 1.927
s.e.d of 10^{-6} soil dilution = 1.108

not significantly different between the treatments at dilutions of 10^{-3} and 10^{-4}. At soil dilutions of 10^{-5} and 10^{-6} soil treated with 0.082g calcium nitrate 10 ml^{-1} had a significantly lower number of colonies (Graph 5.14, Table 5.10).

A similar pattern was observed following seven days incubation with the exception that at soil dilutions of 10^{-5} and 10^{-6} the colony counts of plates inoculated with soil incubated in 0.082g calcium nitrate 10 ml^{-1} had a lower number of colonies than other treatments. The number of bacterial colonies was only significantly lower than the control treatment (Graph 5.15, Table 5.11).

After both incubation periods the number of bacterial colonies was higher on control plates although not significantly so.

Table 5.11. Mean number of bacterial colonies recorded on tryptic soya broth agar following two days incubation with treatment solution and seven days incubation following plating.

	Soil Dilution			
Treatment	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Control	7.37a	11.5a	14.44a	10.27a
0.082 g $CaNO_3$ 10 ml^{-1}	7.7a	9.5a	6.72b	7.09b
0.149 g $CaNO_3$ 10 ml^{-1}	9.21a	6.9a	10.70ab	9.23ab
S.E.D	0.867	2.68	1.927	1.108
L.S.D	1.932	5.97	4.293	2.469

Numbers with the same letters are not significantly different using the L.S.D value.

Table 5.12. Mean number of bacterial colonies recorded on tryptic soya broth agar following seven days incubation with treatment solution and two days incubation following plating.

	Soil Dilution			
Treatment	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Control	8.91b	9b	9.06b	7.89b
0.082 g $CaNO_3$ 10 ml^{-1}	11.58a	7.65b	10.21a	14.94a
0.149 g $CaNO_3$ 10 ml^{-1}	8.77b	12.85a	11.45a	7.54b
S.E.D	1.158	1.663	0.996	1.384
L.S.D	2.58	3.705	2.219	3.084

Numbers with the same letters are not significantly different using the L.S.D value

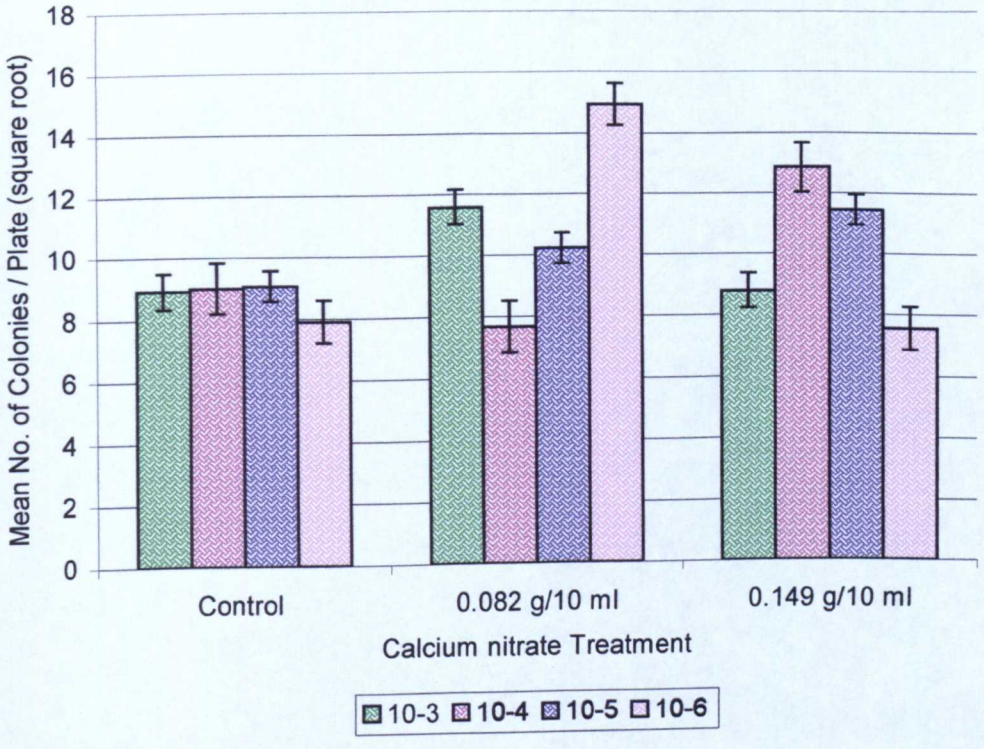
The number of bacterial colonies counted on tryptic soya broth agar plates incubated for two days following inoculation with soil treated for seven days, showed that at dilutions of 10^{-3} and 10^{-6} soil treated with 0.082 g $CaNO_3$ ml^{-1} calcium nitrate had a significantly higher number of colonies than other treatments (Graph 5.16, Table 5.12).

By contrast at soil dilutions of 10^{-4} this treatment produced a significantly lower number of colonies. The number of bacterial colonies on plates inoculated with soil treated with de-ionised water with a dilution of 10^{-5} was significantly lower than other treatments. A similar pattern of results was seen after seven days incubation (Graph 5.17, Table 5.13).

Table 5.13. The mean number of bacterial colonies recorded on tryptic soya broth agar following seven days incubation with treatment solution and seven days incubation following plating.

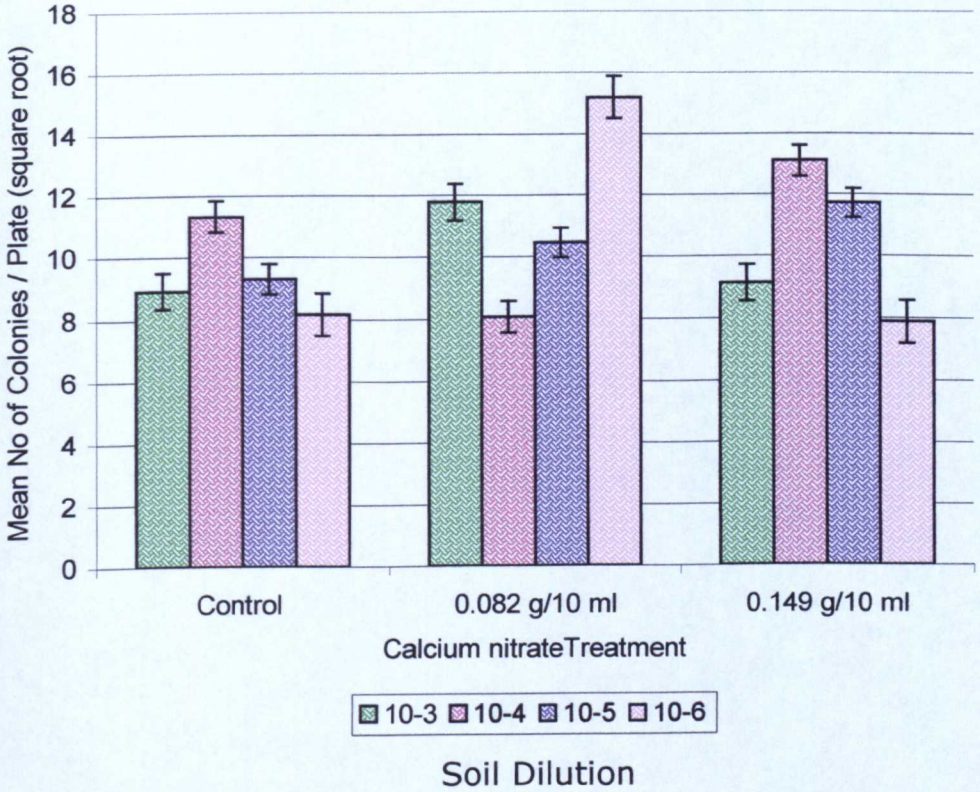
	Soil Dilution			
Treatment	10^{-3}	10-^{4}	10^{-5}	10^{-6}
Control	8.93b	11.33a	9.31b	8.16b
0.082 g $CaNO_3$ /10 ml^{-1}	11.77a	8.06b	10.44ab	15.2a
0.149 g $CaNO_3$ /10 ml^{-1}	9.12b	13.10a	11.71a	7.87b
S.E.D	1.181	1.016	0.965	1.372
L.S.D	2.631	2.265	2.151	3.057

Graph 5.16. Mean number of bacterial colonies per tryptic soya broth agar plate following seven days treatment and two days incubation.



s.e.d of 10^{-3} soil dilution = 1.158
s.e.d of 10^{-4} soil dilution = 1.663
s.e.d of 10^{-5} soil dilution = 0.996
s.e.d of 10^{-6} soil dilution = 1.384

Graph 5.17. Mean number of bacterial colonies per tryptic soya broth agar plate following seven days treatment and seven days incubation.



s.e.d of 10^{-3} soil dilution = 1.181
s.e.d of 10^{-4} soil dilution = 1.016
s.e.d of 10^{-5} soil dilution = 0.965
s.e.d of 10^{-6} soil dilution = 1.372

Numbers with the same letters are not significantly different using the L.S.D value

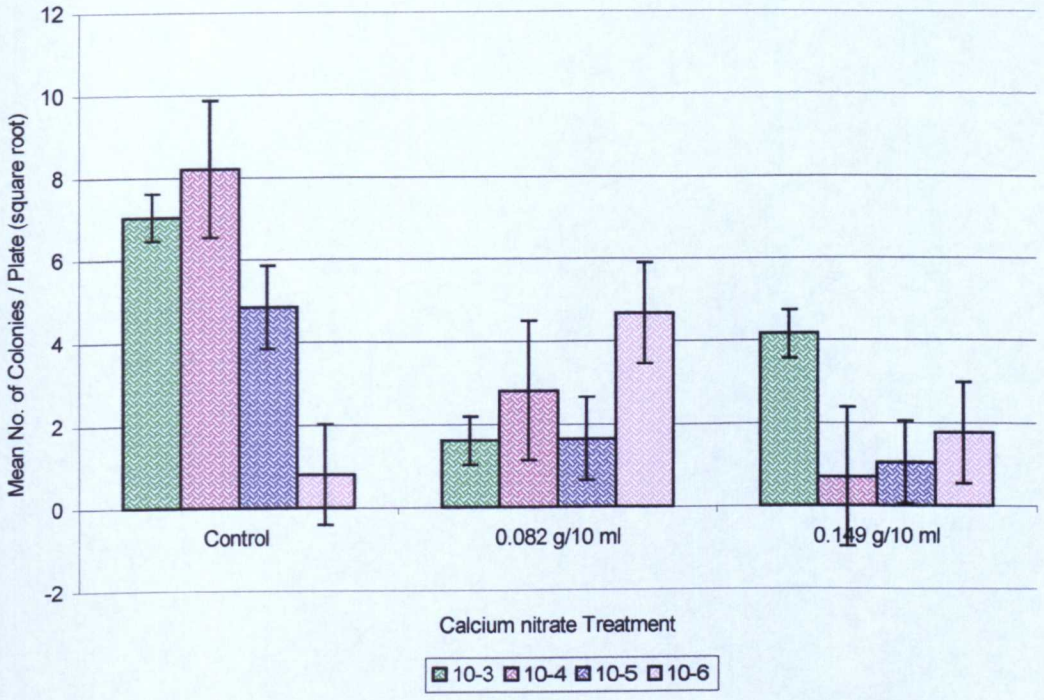
Table 5.14. Mean number of fungal colonies recorded on malt extract agar following two days incubation with treatment solution and two days incubation following plating.

	Soil Dilution			
Treatment	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Control	7.03a	8.2a	4.84a.	0.8a
0.082 g $CaNO_3$ / 10 ml^{-1}	1.61c	2.8a	1.65a	4.67a
0.149 g $CaNO_3$ /10 ml^{-1}	4.15b	0.7b	1.04a	1.76a
S.E.D	1.166	3.35	2.02	2.445
L.S.D	2.598	7.46	4.562	5.448

Numbers with the same letters are not significantly different using the L.S.D value

There were no significant differences in the mean number of colonies counted on malt extract agar incubated for two days following inoculation with soil treated for two days at soil dilutions of 10^{-5} and 10^{-6} (Graph 5.18, Table 5.14). At a dilution of 10^{-4} the number of fungal colonies was significantly lower on plates inoculated with soil treated with 0.149 g $CaNO_3$ 10 ml^{-1}. By contrast at a dilution of 10^{-3} the highest number of colonies was recorded on plates inoculated with soil treated with de-ionised water.

Graph 5.18. Mean number of fungal colonies per malt extract agar plate following two days treatment and two days incubation.



s.e.d of 10^{-3} soil dilution = 1.166
s.e.d of 10^{-4} soil dilution = 3.35
s.e.d of 10^{-5} soil dilution = 2.020
s.e.d of 10^{-6} soil dilution = 2.445

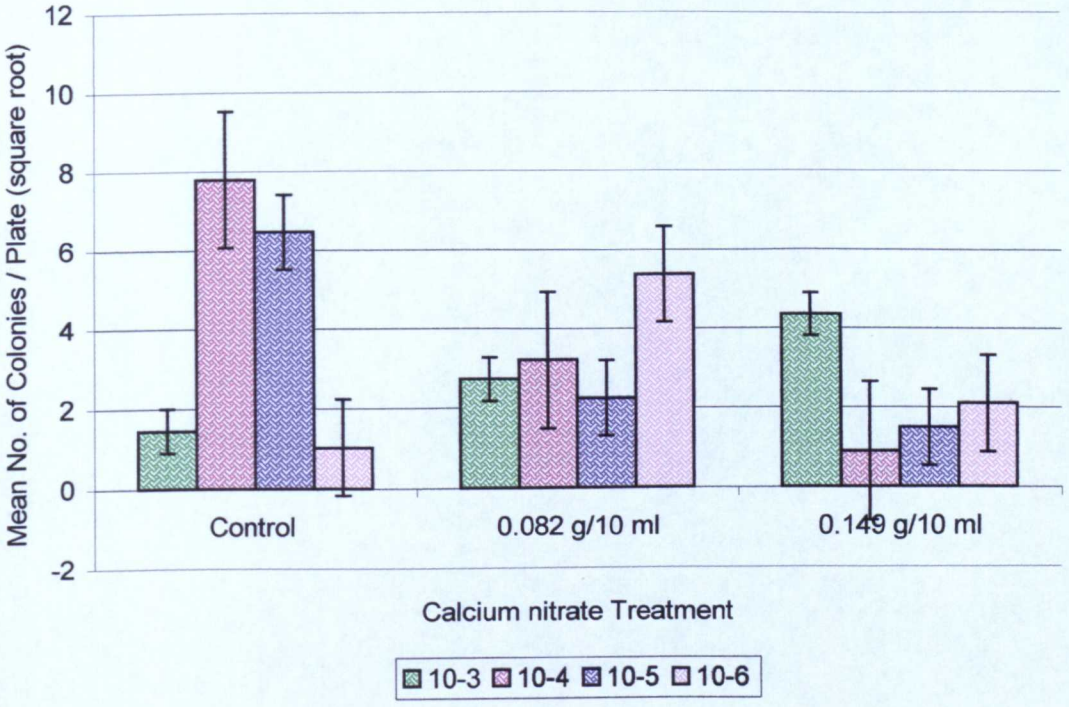
After seven days incubation there was no significant difference between the number of fungal colonies on plates inoculated with a soil dilution of 10^{-6} (Graph 5.19, Table 5.15). There were also no differences at a dilution of 10^{-4}. At a dilution of 10^{-5} the number of colonies was significantly higher on plates inoculated with de-ionised water. In contrast at 10^{-3} the lowest number of colonies was observed on plates inoculated with de-ionised water.

Table 5.15. Mean number of fungal colonies recorded on malt extract agar following two days incubation with treatment solution and seven days incubation following plating.

	Soil Dilution			
Treatment	10^{-3}	10-^{4}	10^{-5}	10^{-6}
Control	1.45b	7.8a	6.47a	1.03a
0.082 g $CaNO_3$ /10 ml^{-1}	2.72a	3.2a	2.0b	5.37a
0.149 g $CaNO_3$ /10 ml^{-1}	4.33a	0.9a	1.48b	2.09a
S.E.D	1.099	3.47	1.92	2.418
L.S.D	2.448	7.74	4.278	5.387

Numbers with the same letters are not significantly different using the L.S.D value

Graph 5.19. Mean number of fungal colonies per malt extract agar plate following two days treatment and seven days incubation.



s.e.d of 10^{-3} soil dilution = 1.099
s.e.d of 10^{-4} soil dilution = 3.47
s.e.d of 10^{-5} soil dilution = 1.920
s.e.d of 10^{-6} soil dilution = 2.418

Table 5.16. Mean number of fungal colonies recorded on malt extract agar following seven days incubation with treatment solution and two days incubation following plating.

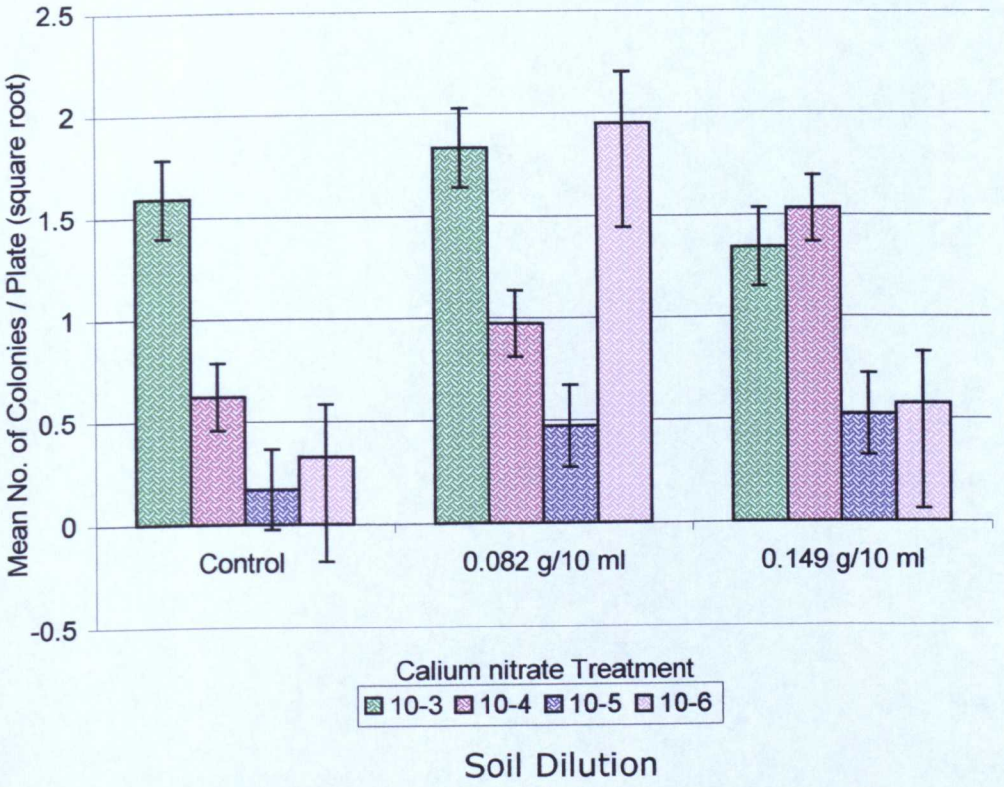
	Soil Dilution			
Treatment	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Control	1.59a	0.62b	0.17a	0.33b
0.082 g $CaNO_3$ /10 ml^{-1}	1.83a	0.97ab	0.47a	1.95a
0.149 g $CaNO_3$ /10ml^{-1}	1.34a	1.53a	0.52a	0.57b
S.E.D	0.386	0.328	0.391	0.502
L.S.D	0.861	0.731	0.871	1.115

Numbers with the same letters are not significantly different using the L.S.D value

Following seven days incubation with the calcium nitrate treatment and two days plate incubation no significant difference was observed on malt extract plates at dilutions of 10^{-3} and 10^{-5} (Graph 5.20, Table 5.16). The number of fungal colonies was significantly higher at dilutions of 10^{-6} for soil treated with 0.082 g calcium nitrate /10ml^{-1}. At a dilution of 10^{-4} the highest number of colonies was recorded for soil treated with 0.149 g calcium nitrate /10ml^{-1} but this was only significant when compared with plates treated with de-ionised water.

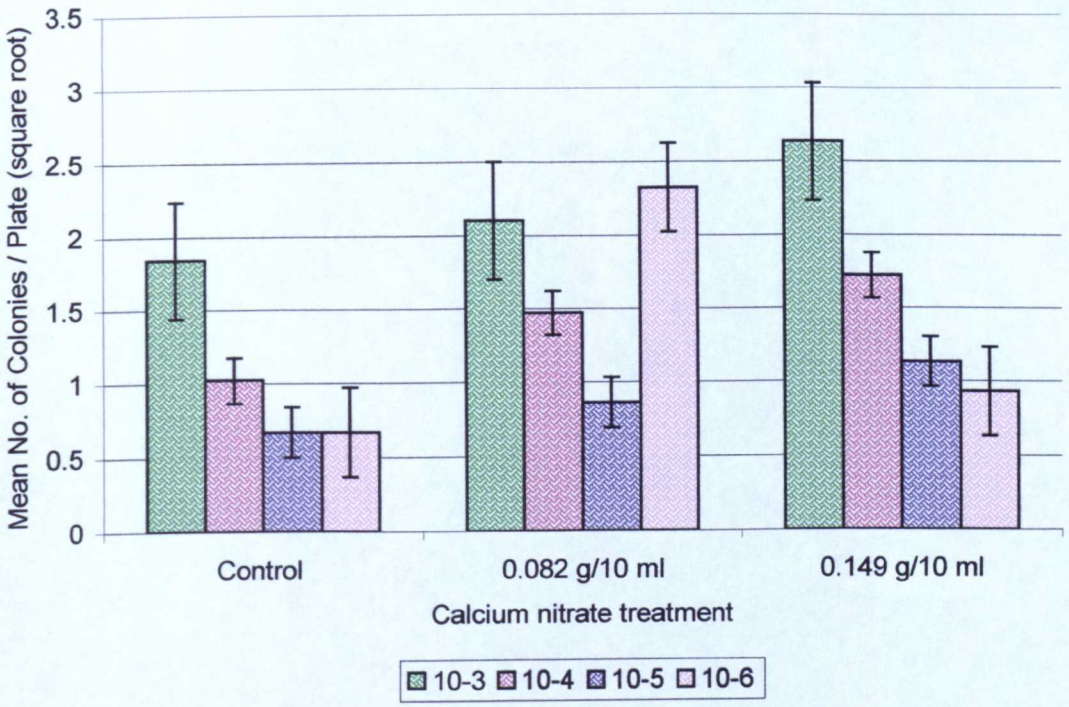
Following seven days incubation of malt extract plates a similar pattern of fungal colony formation was observed with the exception that there were no significant differences in the numbers of fungal

Graph 5.20. Mean number of colonies per malt extract agar plate following seven days treatment and two days incubation.



s.e.d of 10^{-3} soil dilution = 0.386
s.e.d of 10^{-4} soil dilution = 0.328
s.e.d of 10^{-5} soil dilution = 0.391
s.e.d of 10^{-6} soil dilution = 0.502

Graph 5.21. Mean number of colonies per malt extract agar plate following seven days treatment and seven days incubation.



s.e.d of 10^{-3} soil dilution = 0.8
s.e.d of 10^{-4} soil dilution = 0.361
s.e.d of 10^{-5} soil dilution = 0.348
s.e.d of 10^{-6} soil dilution = 0.662

colonies formed on plates inoculated with a soil dilution of 10^{-4} (Graph 5.21, Table 5.17).

Table 5.17. Mean number of fungal colonies recorded on malt extract agar following seven days incubation with treatment solution and seven days incubation following plating.

	Soil Dilution			
Treatment	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Control	1.84a	1.02a	0.67a	0.67b
0.082 g $CaNO_3$ /10 ml^{-1}	2.15a	1.47a	0.86a	2.32a
0.149 g $CaNO_3$ /10 ml^{-1}	2.63a	1.72a	1.13a	0.93ab
S.E.D	0.8	0.361	0.348	0.662
L.S.D	1.782	0.805	0.776	1.476

Numbers with the same letters are not significantly different using the L.S.D value

Conclusions

No increase in the number of colonies was observed when the soil sample was incubated in solutions containing calcium nitrate. In certain cases the number of colonies are increased and in others decreased, but the results from this experiment are inadequate to determine a consistent effect of treating soil with calcium nitrate upon the micro flora.

5.6. Does the addition of suppressive soil extracts to compost decreases the level of *P. brassicae* infection?

This investigation aimed to determine whether adding extracts of suppressive soils to Arthur Bowers' Seed and Potting Compost decreases the level of clubroot upon inoculation of the compost with *P. brassicae* resting spores.

Method and Materials

Chinese cabbage seed (*Brassica rapa* ssp. *pekinensis* cv Mariko) was sown into 7cm^2 pots filled with Arthur Bowers' seed and potting Compost. Each pot was subsequently treated with 30ml of a soil extract or de-ionised water.

Three soil extracts were used as treatments. These were prepared from Crail, Kings Kettle and Auchincruive soils by adding 1g of air dried soil to 10ml of sterile de-ionised water in a sterile universal bottle. The bottles were transferred to a Fisher Scientific Flask shaker and shaken vigorously for half an hour. The suspension in each of the universal bottles was subsequently transferred to a separate 1l plastic bottle containing 990 ml of de-ionised water, which had been autoclaved. This gave a 10^{-2} soil dilution extract.

The experiment consisted of six replicates with four pots in each replicate. Pots of the same treatment and in the same replicate were placed in small gravel tray. One week after treatment the pots were inoculated with 30ml of a 10^{-6} resting spores ml^{-1} solution. The experiment was carried out in a glasshouse at the University of Strathclyde (Chapter 2).

Results

The plants were harvested 50 days after inoculation of the pots with *P. brassicae* resting spores. Mean dry root and shoot masses are presented in Table 5.18.

All plants in the experiment were scored with a disease index of 100%.

Table 5.18: Mean Dry Root and Shoot masses of plants raised in compost inoculated with soil extracts.

Soil Extract	Mean Dry Root Mass (g)	Mean Dry Shoot Mass (g)
Control	0.882a	1.015a
Crail	0.913a	1.060a
Kings Kettle	0.983a	1.205a
Auchincruive	0.889a	1.100a
S.E.D	0.1030	0.1256
L.S.D	0.2048	0.2497

Numbers with the same letters are not significantly different using the L.S.D value

There was no difference in the level of *P. brassicae* infection and no significant difference between the dry root and shoot masses.

Conclusions

Treating the growing media with extracts of suppressive soils had no effect on the subsequent level of *P. brassicae* infection. Although the results suggest that adding suppressive soil extracts to non-suppressive compost does not decrease infection only one soil dilution was used which was only applied once. It is therefore possible that a more concentrated soil extract or multiple applications may encourage *P. brassicae* suppression.

The ability to promote suppressiveness in conducive soils by inoculating them with suppressive soil samples is however a characteristic of a soil which has a biotic suppressive character (Alabouvette, 1986). This experiment therefore questions whether the nature of the suppressiveness of Kings Kettle soil is solely due to the micro-flora. It is clear, however, that this experiment did not cover a wide range of treatment combinations and further investigations would be required in order to confirm that the suppressive soils fail to transfer their repressive nature to conducive soils.

Discussion

Experiments 5.1 and 5.3 illustrated the ability of soil samples collected from Crail and Kings Kettle to reduce the extent of clubbing in *P. brassicae* infected plants. Autoclaving Kings Kettle soil increased the level of clubbing as might be expected of a soil which is deemed to be suppressive due to the presence of antagonistic organisms. The level of infection however did not increase to a level comparable to that seen in non-autoclaved Auchincruive sample suggesting that the micro-flora was not the only suppressive factor.

Colony counts established that Kings Kettle soil had a higher micro-flora content than the other soils. Crail soil similarly had high levels of micro-flora but the number of colonies was not in general significantly higher than that of Auchincruive soil. The micro-flora of Auchincruive soil was not increased by incubating with solutions containing calcium. This suggests that the high levels of calcium present in Crail and Kings Kettle soils were not solely responsible for promoting suppressiveness through stimulation of the general micro-flora.

Applying extracts of suppressive soils to *P. brassicae* infected compost did not have an effect upon the level of clubroot.

From the results of experiments 5.1 – 5.5 it can be concluded that :-

- Kings Kettle and Crail soils are suppressive to clubroot.

- It was not possible to establish whether the suppressive nature of Crail soil was due solely or partly due to the microflora because of the phyto-toxicity induced upon autoclaving of the soil samples.

- In experiment 5.3 the level of micro-flora counted on plates was in general not significantly different to the level in Auchincruive soil. Crail soil has a high pH and calcium content and it is therefore likely that this is a major factor in reducing the level of clubroot. This would explain why levels of clubbing were generally higher in plants grown in Crail soil than those raised in Kings Kettle soil.

- The micro-flora level in Auchincruive soil is not increased by treatment with calcium nitrate.

- Kings Kettle soil is suppressive through a combination of a high calcium content, more alkaline pH and due at least in part to the soil microflora. This supports the hypothesis of this thesis that control may be obtained through multiple means.

Chapter Six

Interaction of *Plasmodiophora brassicae* With Growth Substrates.

6. The interaction of *Plasmodiophora brassicae* with growth substrates.

Vegetable brassica crops are frequently raised under protection in peat compost filled modules before being transplanted to the field. It may be possible for transplants to become infected prior to transplantation but plants most often become infected in the field. The hypotheses of "root camouflage" suggested by Gilbert *et al* (1984) has already been discussed in Chapter 1. The definition of root camouflage is

"roots with microbial communities more similar to the microbial community in the surrounding soil may be less attractive to pathogens."

This theory could be applied to plants grown in modules. When plants are transplanted into the field the roots move from the growing media into natural soil. As these composts differ greatly from the field soil composition they could alert the soil microflora to the presence of a plant, the micro-flora would be attracted to the compost and hence the plant, and with them plant pathogens. Support to this argument is given by Huang and Hseih (1987), who noted that when plants were transplanted into calcium carbonate amended soils, the bare rooted plants were more susceptible to disease than those transplanted with soil attached to the roots. All plants had been raised in soil collected from a non-*P. brassicae* infected field which was similar to the infected field.

The following experiments were aimed at determining whether raising brassica seedlings in composts other than those which are

peat based decreased the incidence of clubroot following transplantation to *P. brassicae* infested soil.

6.1. An investigation into the effect of growing cabbages in different media on subsequent *Plasmodiophora brassicae* infection.

Method and Materials.

Three composts and one soil sample were used in this experiment this experiment, they were :-

1. Levington's modular compost. A peat based compost formulated for raising plants in modules.

2. John Innes Seedling Mixture. This compost is soil based and might contain a micro-flora more similar to the field environment.

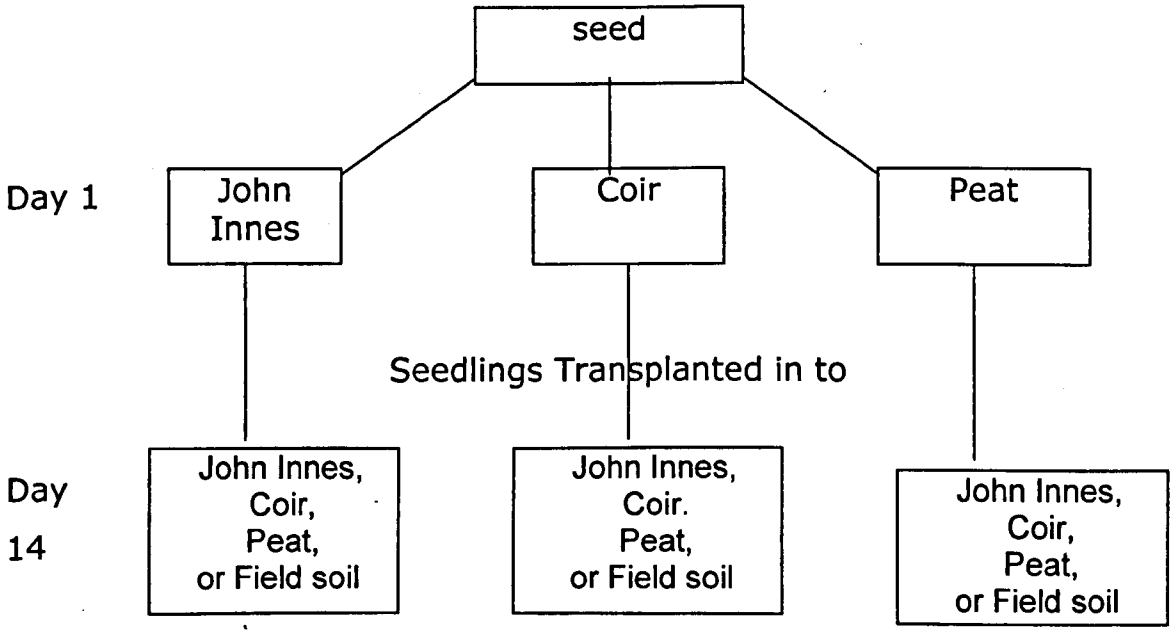
3. Sainsbury's Coir compost. This is sold as an "environmentally friendly" compost and does not contain peat. It is made from coconut husks (coir).

4. Field soil collected from the Auchincruive site (Grid Ref. 238415,623365). This soil was mixed with coarse gravel (0.5 cm) in a 4 soil : 1 grit ratio in order to aid drainage.

Experimental design.

Summer cabbage seed (*B. oleracea var capitata* cv Castello) was sown into p450 plug trays containing one of the compost / soil treatments. After 14 days seedlings raised in each compost were transplanted into $4cm^2$ pots containing either field soil, John Innes, coir or peat compost, giving a total of twelve treatments. Four plants were transplanted into each pot and there were three replicates of each treatment.

Figure 6.1. Experimental layout.



All growing media (except the field soil which was naturally infested), were inoculated with 10 ml of 10^6 resting spores ml^{-1} suspension using a glass pipette. This ensured that the growth media was highly infested with *P. brassicae* resting spores.

Four plants from each treatment replicate were sampled at one and two weeks after transplantation to examine root hair infection using the method described in Chapter 2. Following the second sample the number of plants per pot was reduced to 1.

The experiment was carried out in a glasshouse at Auchincruive (described in Chapter Two).

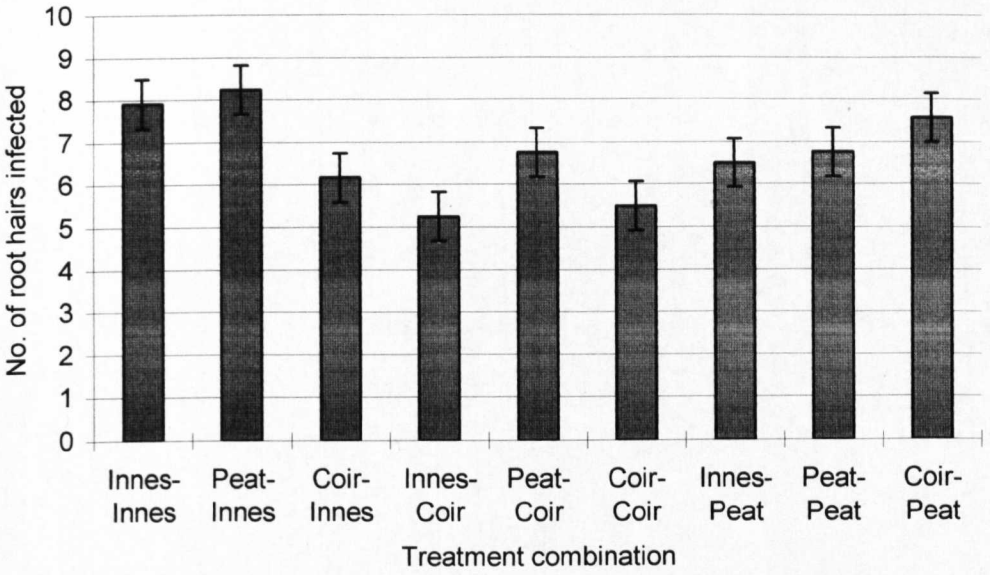
Results

The roots of plants growing in field soil were difficult to harvest due to the clay content of the soil. This resulted in damage to the roots and the root hairs making assessment un-reliable. Field soil results are therefore not presented.

The results of the first root hair assessment are given in Table 6.1 and Graph 6.1. The number of root hairs infected was counted but it was not possible to determine the stage of infection due to compost particles which remained on the roots making microscopic viewing unclear.

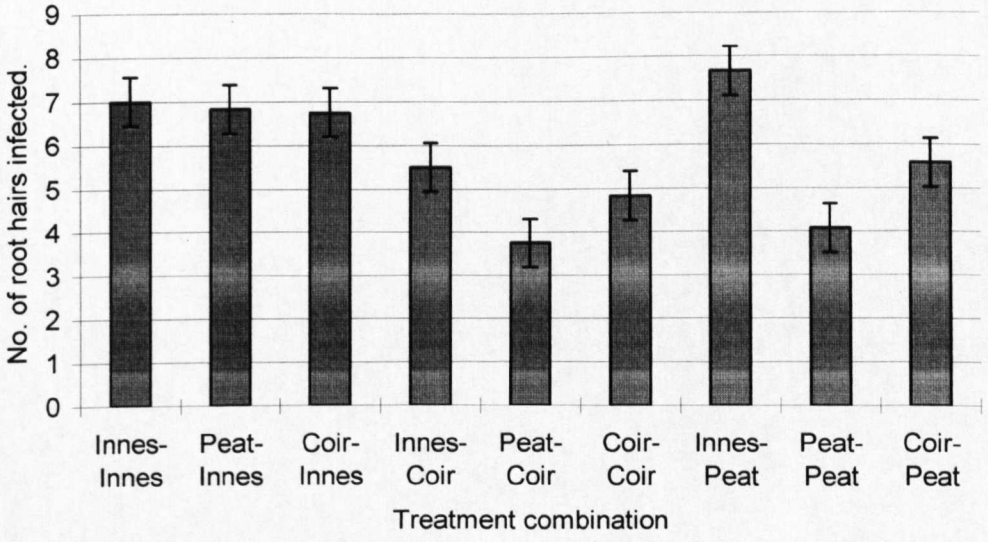
After one week the number of root hairs infected was low. The highest level of infection was obtained in roots raised in peat based compost and transplanted to John Innes but this was only significant when compared with plants raised in John Innes and coir and transplanted to coir which had the lowest level of infection.

Graph 6.1. Mean number of root hairs infected of 30 examined for each treatment. (Week 1).



s.e.d 1.157.

Graph 6.2. Mean number of root hairs infected of 30 examined for each treatment. (Week 2).



s.e.d = 1.107

Table 6.1. Mean number of *P. brassicae* infected root hairs examined for each treatment combination. week 1.

Treatment combination	Innes-Innes	Peat-Innes	Coir-Innes	Innes-Coir	Peat-Coir	Coir-Coir	Innes-Peat	Peat-Peat	Coir-Peat
No. of root hairs infected / 30	7.92a	8.25a	6.17ab	5.25b	6.75ab	5.5b	6.5ab	6.75ab	7.60a
S.E.D.	1.157								
L.S.D	2.297								

Numbers with the same letters are not significantly different using the L.S.D value.

The second sample taken two weeks after transplantation provided a different pattern of infection. After two weeks the pathogen could have been expected to move into the root cortex. This is shown by the lower level of root hair infection observed in the samples. The results for this sample are given in Table 6.2 and Graph 6.2.

Table 6.2. Mean number of *P. brassicae* infected root hairs for each treatment combination. Week 2.

Treatment combination	Innes-Innes	Peat-Innes	Coir-Innes	Innes-Coir	Peat-Coir	Coir-Coir	Innes-Peat	Peat-Peat	Coir-Peat
No. of root hairs infected / 30	7.0a	6.83a	6.75a	5.5ab	3.75b	4.83b	7.67a	4.08b	5.58ab
S.E.D.	1.07								
L.S.D	2.198								

Numbers with the same letters are not significantly different using the L.S.D value.

The highest level of infection was found in plants raised in John Innes and transplanted to peat based compost. The level of infection in this treatment was significantly higher than plants raised in a peat based compost and transplanted to coir and plants raised in coir and transplanted to coir.

The results from the root hair samples show no definite trend but the general trend in both samples was that the lowest levels of infection were observed in plants raised in John Innes and Coir and transplanted into Coir.

The trial was harvested 8 weeks after transplanting dry root and shoot mass, leaf area and disease category were recorded. The values for plants grown in field soil are not presented because the plants grew poorly. This was due to the difficulty in maintaining the soil moisture at an appropriate level.

The mean results are given in Table 6.3. The dry root mass was transformed by square rooting as the data was skewed. The disease index was also transformed to allow analysis of the values as they are calculated as percentages.

Table 6.3. Mean dry root and shoot masses and disease indices of plants raised in three different media prior to transplanting into infected composts.

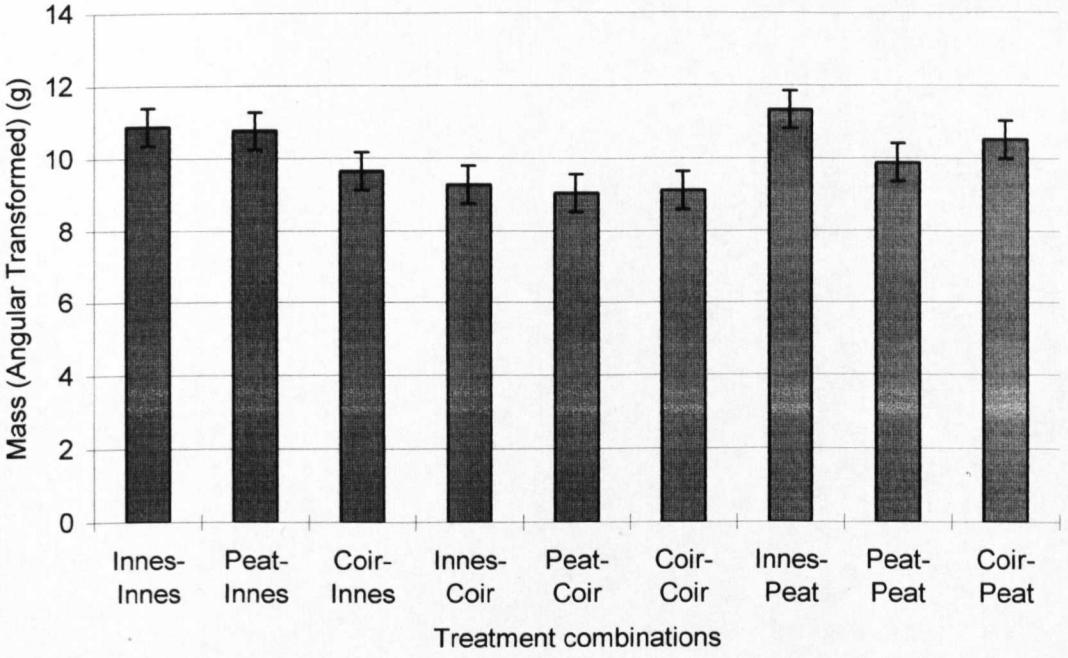
Treatment	Dry Root Mass (Angular) (g)	Dry Shoot Mass (g)	Leaf Area cm^2	Disease index (Angular)
Innes-Innes	10.88ab	5.50ab	515a	28ab
Peat-Innes	10.77ab	4.97a	638a	41.2abd
Coir-Innes	9.65ab	4.86a	549a	19.8a
Innes-Coir	9.27b	7.18b	961b	44.6abd
Peat-Coir	9.04b	8.98bc	1047bc	57.8bcd
Coir-Coir	9.11b	10.13c	1258c	32.3ab
Innes-Peat	11.33a	7.96bc	1007bc	56.8bcd
Peat-Peat	9.88ab	9.65c	1245bc	80c
Coir-Peat	10.51ab	5.83ab	682a	66.4cd
S.E.D	1.032	1.046	135.5	12.02
L.S.D	2.048	2.075	268.8	25.48

Numbers with the same letters are not significantly different using the L.S.D value.

The dry root mass is illustrated in Graph 6.3. It can be seen from this that the dry root mass was generally similar. The highest level of infection was observed in plants raised in John Innes and transplanted to peat based composts and was significantly higher than plants transplanted to coir.

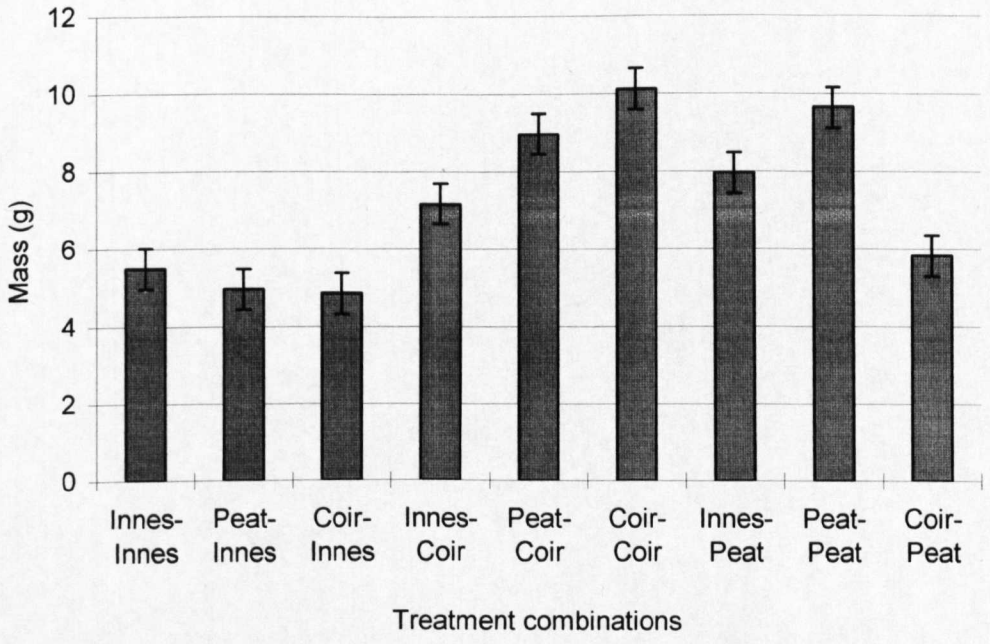
The leaf area of the plants corresponded well to the shoot masses (Table 6.3, Graph 6.5).

Graph 6.3. Root dry masses of plants raised in several composts before being transplanted to *P. brassicae* infected media.



s.e.d = 1.032

Graph 6.4. Shoot dry masses of plants raised in several composts before being transplanted to *P. brassicae* infected media.



S.E.D = 1.046.

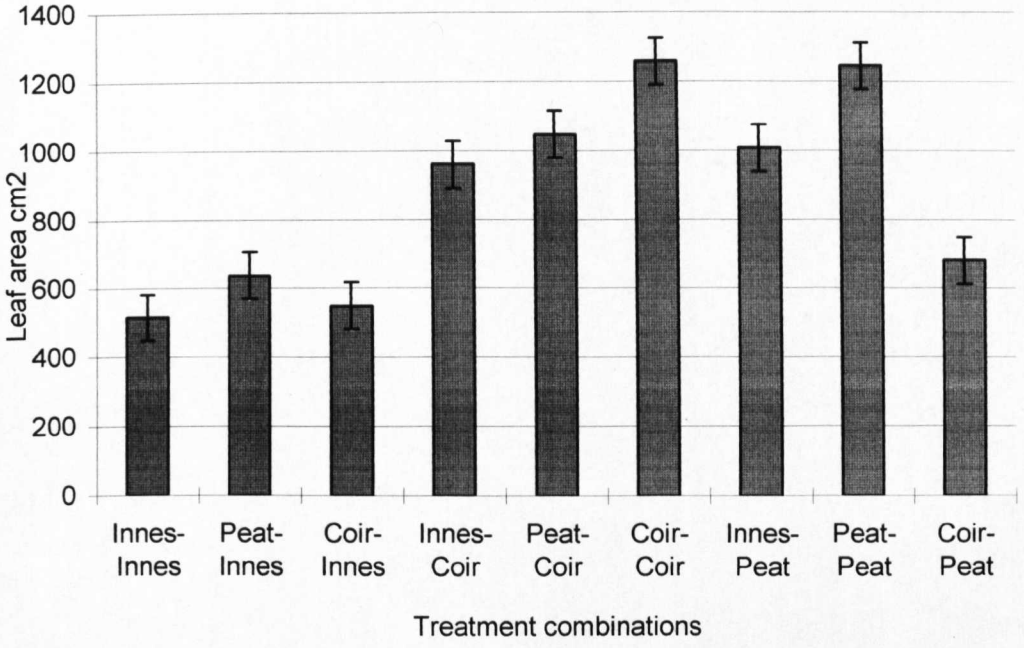
The dry shoot mass (Graph 6.4) was highest in plants raised in coir and transplanted to coir this is reflected in the leaf area (Graph 6.5). These plants also had low disease indices. Plants transplanted to John Innes had lower shoot masses and leaf areas despite low levels of clubbing. This indicates that the poor growth of the upper parts of the plant was not due to *P. brassicae* infection. The root mass was also high in this treatment.

The highest level of infection was observed in plants raised in peat and transplanted to peat based compost. The lowest disease level was observed in plants raised in coir and transplanted to John Innes. Plants transplanted to peat based compost had higher levels of disease irrespective of the media they were raised in before transplanting (Table 6.3, Graph 6.6). Plants transplanted to peat based compost had the highest level of disease irrespective of the compost they were raised in.

Low levels of infection were also observed in plants raised in Coir then transplanted to Coir and plants raised in John Innes then transplanted to John Innes, although these treatments did not produce significantly lower values than other treatments using the L.S.D value, the results are in line with the soil camouflage theory presented by Gilbert *et al* (1984) in that plants transplanted into the same composts that they were raised in produced low levels of disease (Table 6.3, Graph 6.6).

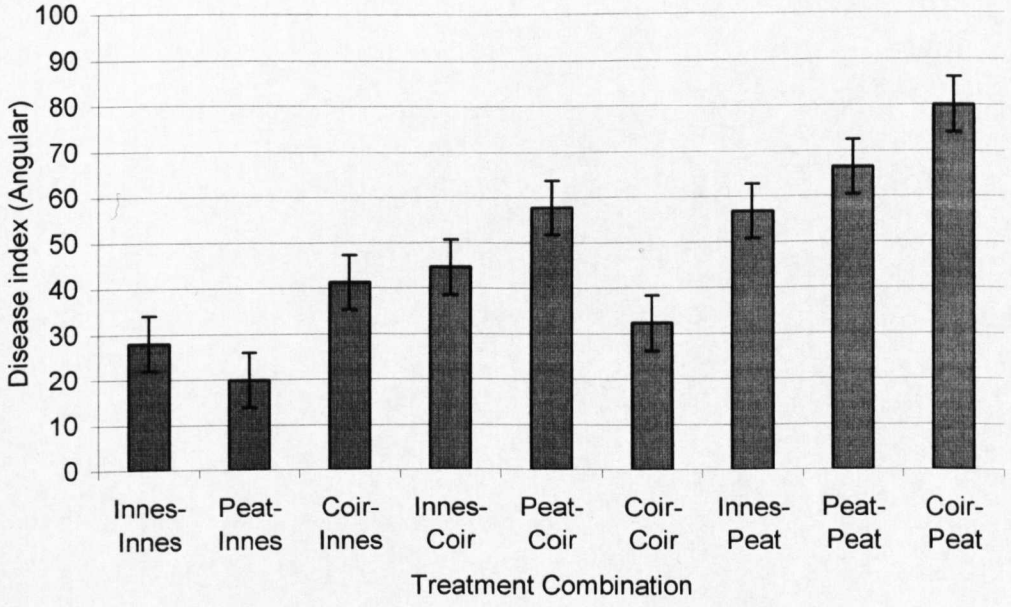
Results from plants transplanted to John Innes indicate that the compost did not provide adequate nutrition for the plant. The root mass was high which could be due to the roots searching for nutrients at the expense of shoot growth. The low level of infection by *P. brassicae* also indicated poor plant growth since high levels of

Graph 6.5. Mean leaf area of plans raised in several composts before being transplanted to *P. brassicae* infected media.



s.e.d = 135.5

Graph 6.6. Mean disease indices of plants raised in several composts before being transplanted to *P. brassicae* infected media.



s.e.d = 12.02

infection require carbohydrates from the host (Evans and Scholes, 1995).

Conclusions.

This experiment did not provide any clear evidence that raising plants in a media different from the eventual transplant medium had an effect on the level of clubroot infection. The highest level of infection was observed in plants raised and transplanted into Levington's modular peat compost. In contrast low levels of disease were seen in plants raised in Coir and transplanted to Coir and plants raised in John Innes and transplanted to John Innes.

It is not possible to say whether root camouflage was definitely responsible for the reduction in disease especially as the results were not significantly lower and because plants grown in John Innes did exhibit poor growth. The results do not however disprove that it is possible to reduce disease levels by camouflaging the plant roots.

The poor growth of plants transplanted into John Innes compost and Field soil made the results of this experiment difficult to relate solely to root camouflage. The use of these media in a field trial with less factors may make it clearer as to whether the media used to raise modules in can influence the level of *P. brassicae* infection.

6.2. The effects of raising cabbage seedlings in three media prior to transplanting into a *P. brassicae* infected field.

This experiment continued the research undertaken in experiment 6.1 to determine whether the substrate in which brassica seedlings were raised in influenced the level of *P. brassicae* infection following transplantation *to P. brassicae* infested soil infested field soil

Method and Materials

Summer cabbage seedlings (*B. oleracea* var. *capitata* cv Castello) were raised in p450 plug trays containing either John Innes seedling, Levington's modular or coir compost. After five weeks the plants were transplanted into the field area at Auchincruive (Chapter 2). The experiment consisted of six replicates per treatment and was prepared as described in Chapter 2.

Vigour assessments and chlorophyll fluorescence measurements were taken as described in Chapter 2. Both assessments indicated that the plants were not under stress.

The plants were harvested 13 weeks after planting out and the disease categories and plot masses were recorded as described in Chapter 2.

Results.

The mean disease indices and mean plot masses of plants harvested in the field experiment are given in Table 6.4 and Graphs 6.7 and 6.8.

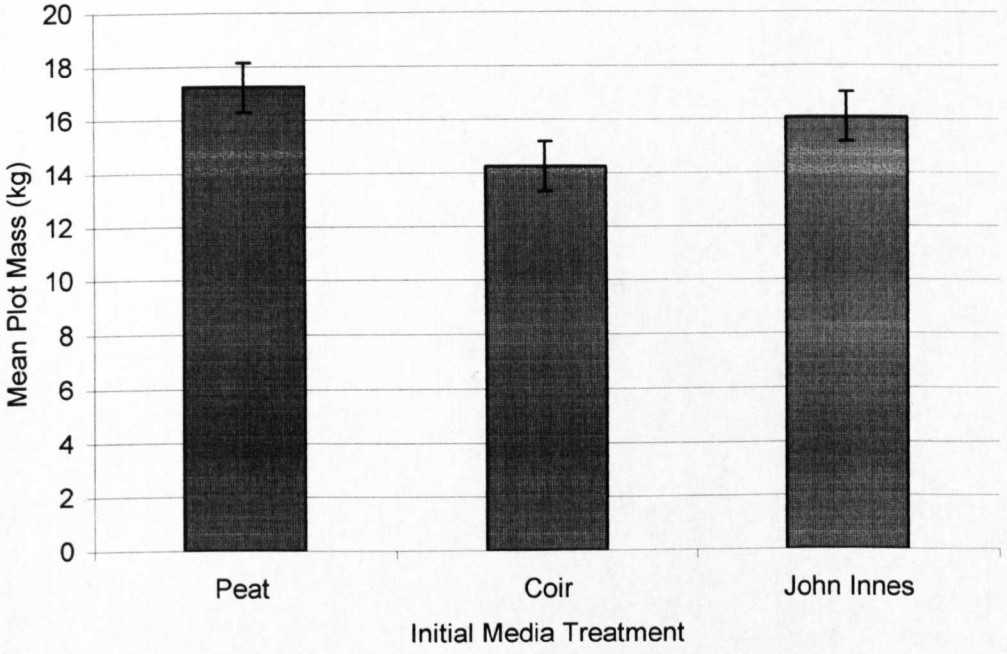
Table 6.4. Mean plot masses and disease indices of plants raised in three different media prior to transplantation into a infected field.

Treatment Compost	Mean Plot Mass (kg)	Mean Disease Index (Angular)
Modular Peat	17.23a	64.66a
Coir	14.27a	66.98a
John Innes	17.23a	64.99a
S.E.D	1.847	1.627
L.S.D	4.114	3.626

Numbers with the same letters are not significantly different using the L.S.D value.

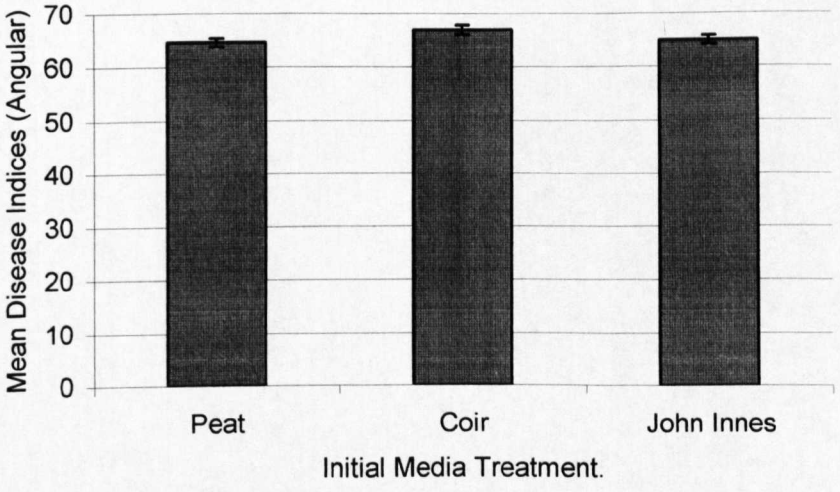
The results did not show any significant differences in the level of disease or the plot mass, although the mass of plants raised in coir prior to transplantation was lower than the other treatments and did have the highest disease index although these differences were not significant.

Graph 6.7. Mean plot masses of plants raised in three media prior to transplantation into a *P. brassicae* infected field.



s.e.d = 1.847

Graph 6.8. Mean disease indices of plants raised in three media prior to transplantation into a *P. brassicae* infected field.



s.e.d = 1.627

Conclusions

The results of this experiment are in line with those of experiment 6.2, in that the media used to raise the plants in prior to transplantation has no influence upon the subsequent level of *P. brassicae* infection.

From these results it would seem that the use of different media to raise transplants does not reduce subsequent levels of disease and it can be concluded that using different composts to raise modules in has no effect on the subsequent level of disease.

It would therefore appear that it is not possible to camouflage roots by using the three manufactured composts tested. The use of green composts however, may decrease the level of *P. brassicae* infection as they are thought to be more disease suppressive. The higher microflora content of these composts may also prevent pathogens present in the soil being able to move into the module area either by out competing them or by the production of chemicals which are detrimental to the pathogens.

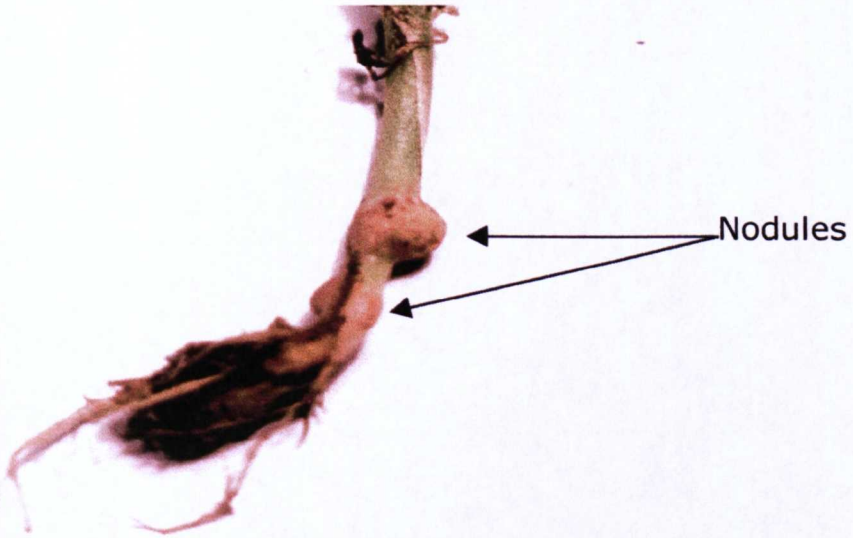
6.3. The effects of three commercial composts on infection by *P. brassicae*.

During the European Clubroot Differential (ECD) experiments defining the physiological race of *P. brassicae* present in field galls, nodules developed on some of the hosts (Plate 6.1). The nodules were small hard growths on the base of some plant stems. All of the ECD hosts were grown in Levington's multi-purpose compost and inoculated with a resting spore suspension prepared from galls collected at the Auchincruive field site.

Levington's compost contains the biological agent 'Stimulex'. Stimulex is claimed to reduce plant disease and promote host growth. It is possible that this biological agent could encourage the nodule formation and these may be an indication of a host hypersensitivity response.

This experiment therefore aimed to determine whether the compost used to grow *P. brassicae* infected brassicas affects the level of infection and possibly the formation of nodules.

Plate 6.1. Nodules formed on ECD host *B. oleracea var. capitata cv. Septa* grown in Levington's multi-purpose compost.



Method and Materials.

Three commercially available composts were used in this experiment SHL potting compost (Sinclair), Arthur Bower's seed and potting and Levington's multi – purpose. Two test host lines were chosen following the results from the ECD tests (Chapter 4) these were *B. oleracea* cv Verheul, which had the highest number of nodules and a low level of galling, and *B. rapa* ssp. *pekinensis* cv Granaat, which had a high level of galling and a low level of nodule formation. Granaat is thought to be universally susceptible to *P. brassicae* infection.

Twenty seeds of Verheul or Granaat were sown into small seed trays containing one of the three compost treatments. Three replicates of each compost gave a total of 18 trays. Trays were arranged in a random block design on the glasshouse bench. Each

tray was inoculated with 100ml of a 10^6 resting spore suspension three days after sowing, this results in a final concentration of 10^5 spores per cm^3 of compost. One week after inoculation the seedlings were reduced to 10 per tray.

Results

The plants were harvested 50 days after inoculation. The gall category was recorded and the dry root and shoot mass.

Table 6.5. Dry root and shoot masses and disease indices *for B. oleracea* cv Verheul plants raised in three compost treatments infected with *P. brassicae.*

Treatment	Disease Index (Angular)	Dry shoot Mass (g)	Dry root Mass (g)
SHL	22.7a	0.89a	0.312a
Arthur Bowers	36.5b	1.25a	0.224a
Levington's	10.5c	1.08a	0.163a
S.E.D	3.06	0.404	0.0329
L.S.D	8.5	1.121	0.0914

Numbers with the same letters are not significantly different using the L.S.D value.

Table 6.6. Dry root and shoot masses and disease indices *for B. rapa* ssp. *Pekinensis* cv Granaat plants raised in three composts infected with *P. brassicae.*

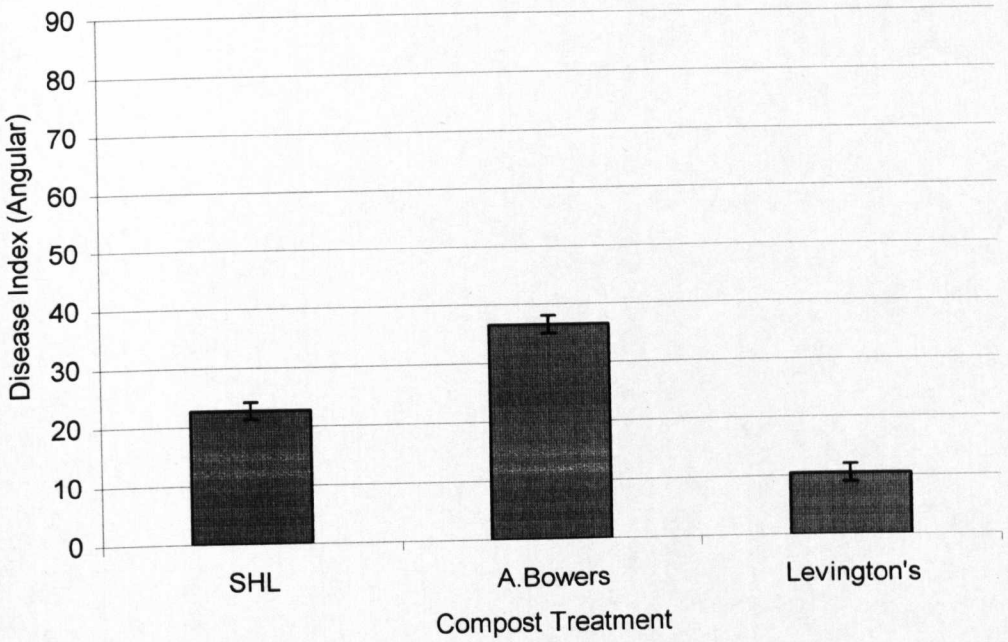
Treatment	Disease Index (Angular)	Dry shoot Mass (g)	Dry root Mass (g)
SHL	71.1a	0.225a	0.262a
Arthur Bowers	80.1a	0.448b	0.43a
Levington's	43.1b	0.843c	0.282a
S.E.D	7.81	0.0498	0.0644
L.S.D	24.86	0.1584	0.2049

Numbers with the same letters are not significantly different using the L.S.D value

Disease in Verheul plants was significantly different in each compost with the highest expression in Arthur Bowers' seed and potting compost and the lowest in Levington's multi-purpose compost. (Table 6.5, Graph 6.9). The disease index of Granaat grown in Levington's multi-purpose compost was also significantly lower than plants grown in SHL and Arthur Bowers' seed and potting compost. (Table 6.6, Graph 6.10) The dry root and shoot (Table 6.5, Graphs 6.11 and 6.13) masses of Verheul plants were not significantly different between treatments.

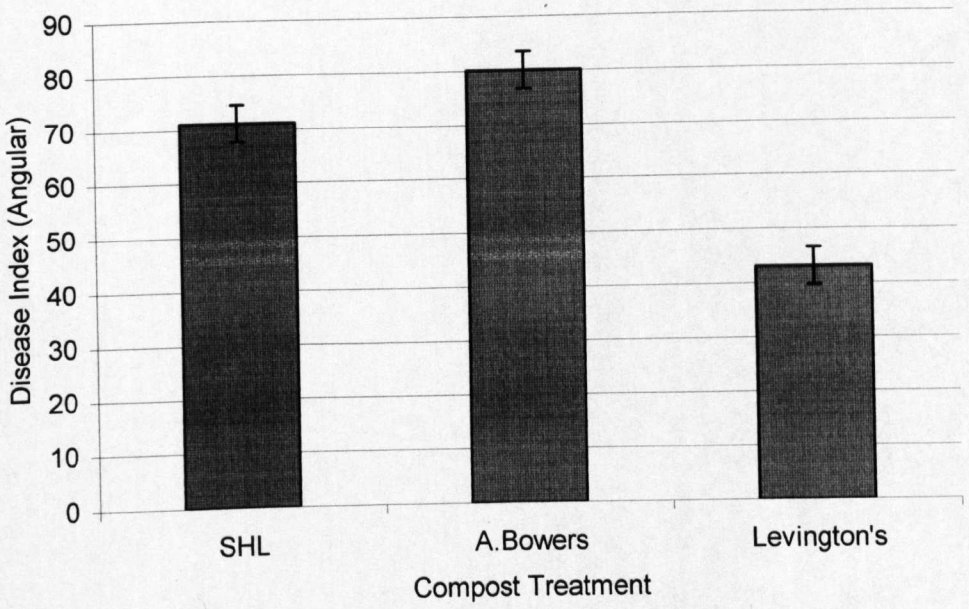
The dry root masses of Granaat plants (Table 6.6, Graph 6.14) were not significantly different between treatments. The dry shoot masses of Granaat plants (Table 6.6, Graph 6.12) were however significantly different from each other with the highest shoot mass occurring in plants grown in Levington's multi-purpose compost and the lowest in SHL multi-purpose compost.

Graph 6.9. Mean Disease Indices of cv Verheul grown in three composts.



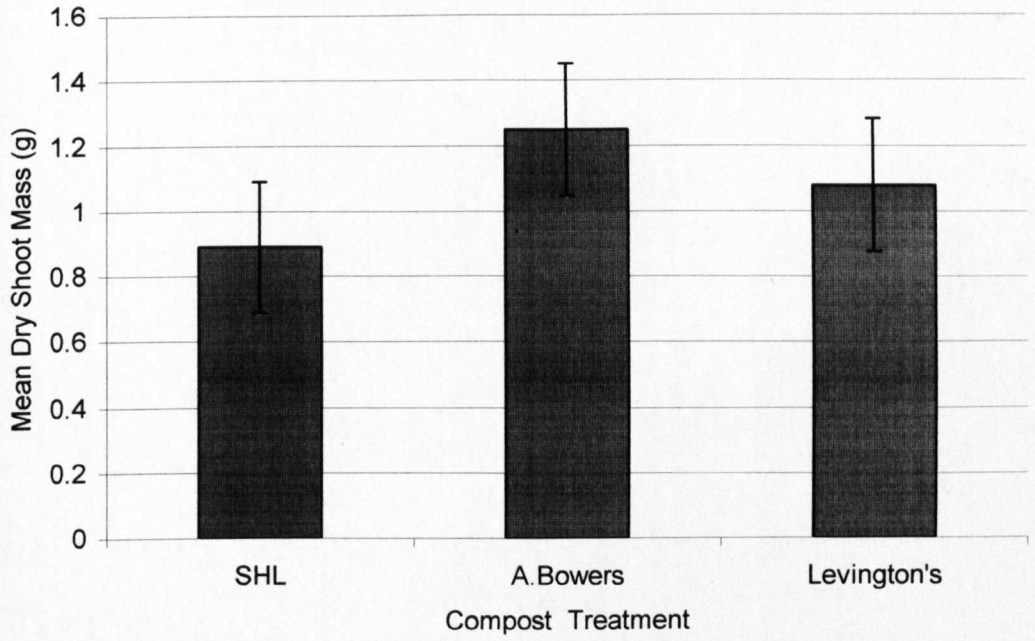
s.e.d = 3.06

Graph 6.10. Mean Disease Indices of cv Granaat grown in three composts.



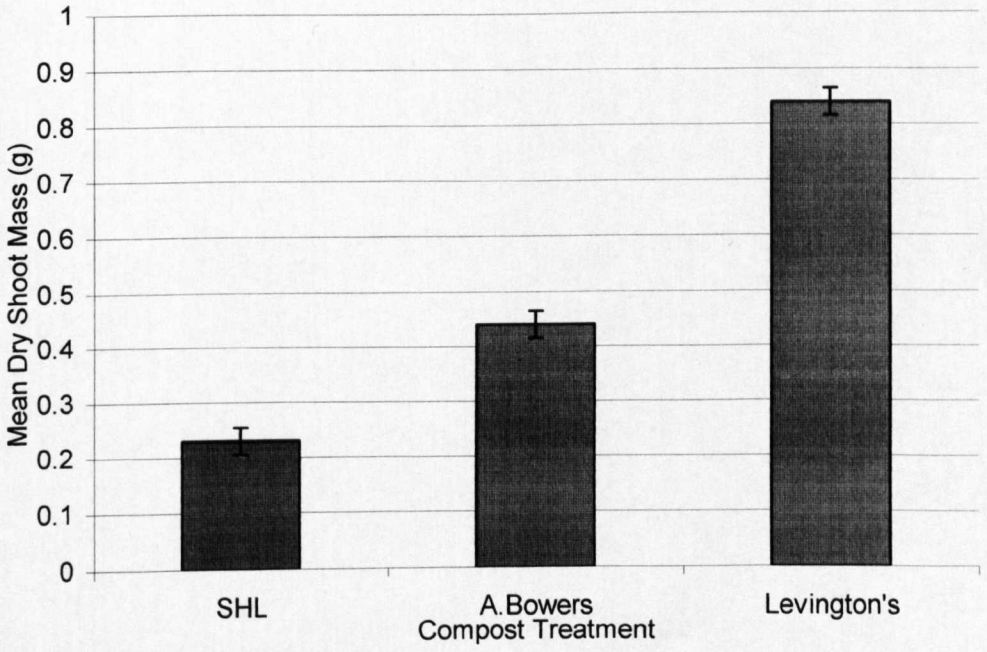
s.e.d = 7.81

Graph 6.11. Mean dry shoot mass of cv Verheul grown in three composts.



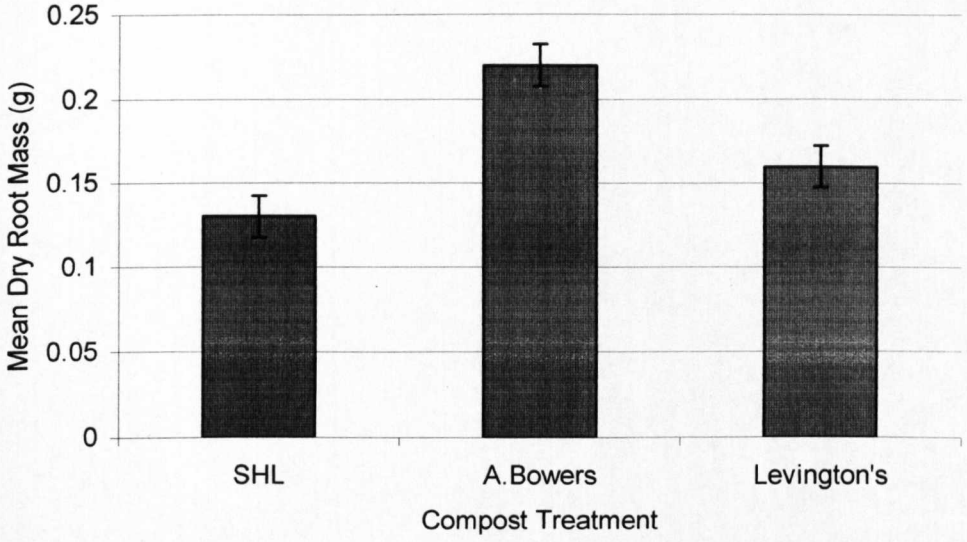
s.e.d = 0.404

Graph 6.12. Mean dry shoot masses of cv Granaat grown in three composts.



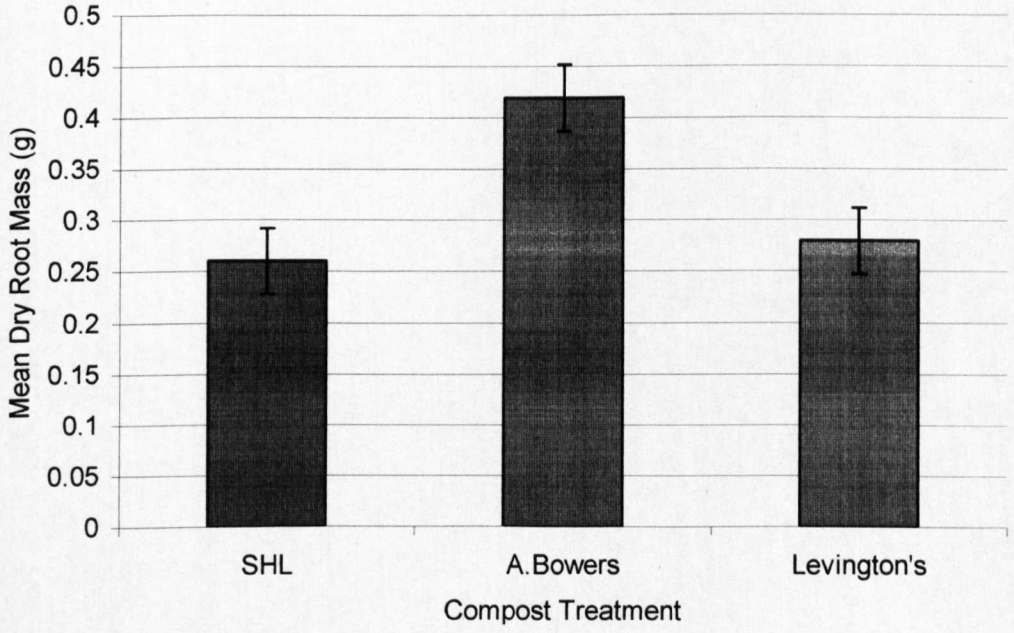
s.e.d = 0.0498

Graph 6.13. Mean Dry Root Masses of cv Verheul grown in three composts.



s.e.d = 0.329

Graph 6.14. Mean Dry Root Masses of cv Granaat grown in three composts.



s.e.d = 0.0644

Conclusions.

The results show that the commercial compost formulation used for an experiment will affect gall development. The lower level of disease in both Verheul and Granaat plants grown in Levington's multi-purpose compost indicated that this treatment, whether due to the presence of "Stimulex" or it's nutrient composition decreased *P. brassicae* infection. This may explain why different pathogen races were identified in each compost treatment.

There was however no formation of nodules in any of the treatments. It is therefore not possible to determine whether or not the formation of nodules is a hypersensitive response initiated solely by the Levington's multi-purpose compost.

A further experiment was therefore designed to determine whether nodules form in the absence of *P. brassicae*.

6.4. Investigation of whether nodule formation only occurs where there is *P. brassicae* infection.

Experiment 6.3 established that a lower level of *P. brassicae* infection developed when plants were grown in Levington's multi-purpose compost. It was not possible however to determine whether nodule formation was related to this reduction in disease intensity.

This experiment aimed to establish whether nodules form in the absence of *P. brassicae* and therefore whether they are related to disease infection.

Method and Materials.

Twenty seeds of the ECD host Verheul were sown into small seed trays containing either SHL, Arthur Bowers or Levington's compost. There were six replicates of each compost treatment and arranged in a random block design on a glass house bench. One week after sowing the number of seedlings was reduced to ten per tray.

The trial was harvested 50 days after sowing and the dry root and shoot masses determined.

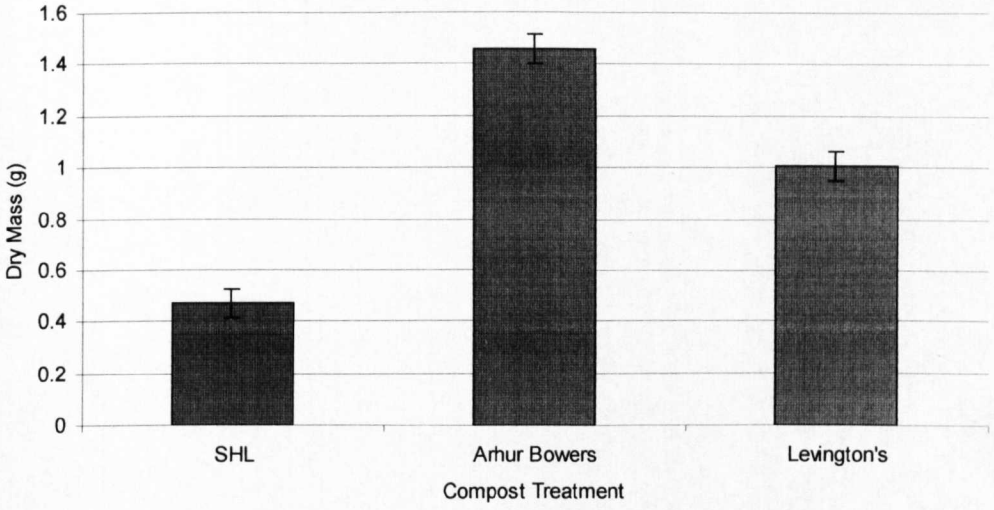
Results

Upon harvesting there were no signs of nodule formation on any of the plants. It is therefore unlikely that nodule formation is instigated in the absence of disease. It is possible that the nodules are formed when resting spores are splashed from the soil surface onto the plant stems during watering. It is unknown however whether the nodules are in effect galls or a host resistance response.

Table 6.7: Dry root and shoot masses *for B. oleracea* cv Verheul plants raised in three composts infected with *P. brassicae.*

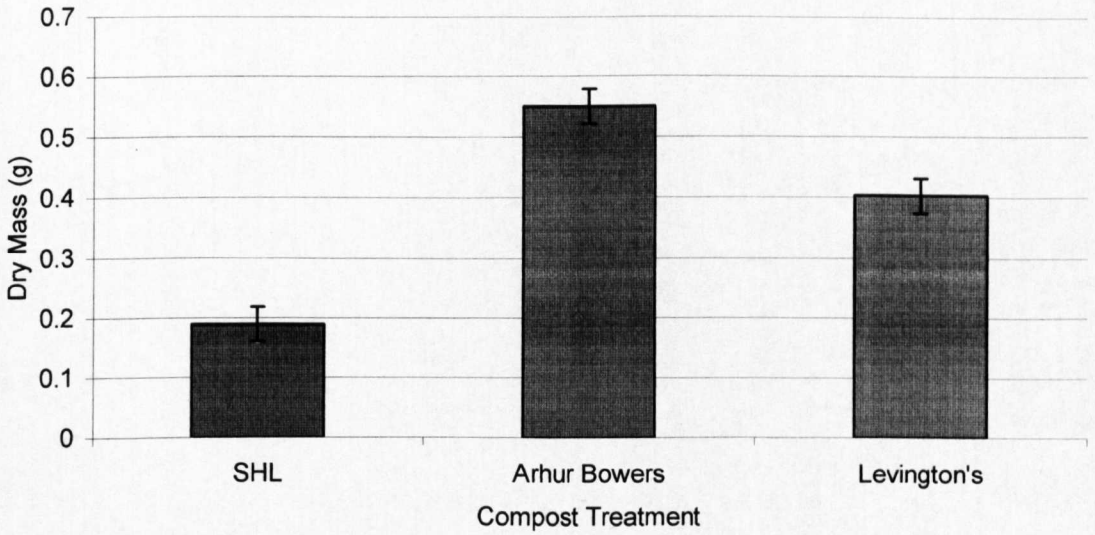
Treatment	Dry shoot Mass (g)	Dry root Mass (g)
SHL	0.472a	0.189a
Arthur Bowers	1.456b	0.551b
Levington's	0.472a	0.404c
S.E.D	0.1098	0.0572
L.S.D	0.2174	0.113

Graph 6.15. Dry shoot masses of cv Verheul plants raised in three composts un-infected with *P. brassicae*.



s.e.d = 0.1098

Graph 6.16. Dry root masses of cv Verheul plants raised in three composts un-infected with *P. brassicae*.



s.e.d = 0.0572

Numbers with the same letters are not significantly different using the L.S.D value

The dry root and shoot mass of plants grown in Arthur Bower's seed and potting compost had significantly higher shoot and root masses (Table 6.7, Graph 6.15 and 6.16) than plants grown in other compost treatments.

Conclusions.

Plants grown in Levington's multipurpose compost had significantly lower shoot and root dry masses than plants grown in Arthur Bower's compost. These results could provide the reason why plants grown in Levington's multi-purpose compost had lower levels of disease in experiment 6.3. It is not clear whether the reduced level of disease resulted from the biological agent "Stimulex" or due to the plants poorer growth in this compost resulting in smaller galls. In experiment 6.3, however, there were no significant differences in the root and shoot growth of Verheul in the three composts used.

Firm conclusions of the ability of Levington's multi-purpose compost to reduced *P. brassicae* infection can't be drawn. One plausible explanation would be that the high level of disease in plants grown in Arthur Bower's seed and potting compost led to a decrease in shoot growth and that the level of disease in Levington's multi-purpose compost grown plants was lower due to poorer plant growth rather than due to the action of the biological agent "Stimulex".

Nodule formation would also appear to be a result of *P. brassicae* infection as they did not form in the absence of disease. In experiment 6.3 however nodules failed to form in the presence of disease indicating that nodule formation is not always induced by infection. More research needs to be carried out into nodule formation as this phenomenon was not seen in any other experiments and may therefore be an aberration bought about by the growth environment and the types of compost used.

Discussion.

The results from experiment 6.1 suggested that Gilbert *et al's* (1984) theory of root camouflage could be used to decrease *P. brassicae* infection as the level of disease was lower in plants raised in Coir and transplanted to Coir and plants raised in John Innes and transplanted to John Innes. The highest level of infection was however observed in plants raised and transplanted into Levington's modular peat compost. In a field environment (experiment 6.2) however, there was no indication that the type of compost used to raise transplants in reduced the subsequent level of disease upon transplantation to a *P. brassicae* infested field soil.

The results of experiment 6.3 did show that other composts did influence the level of disease. It is therefore possible that using different composts to raise transplants in, prior to transplantation to the field could reduce the level of disease if the correct type of compost could be identified. Root camouflage may be more obtainable by the use of more 'green composts', such as those made from organic landfill waste, rather than commercial composts.

The formation of nodules on the host plant stems is most likely a result of *P. brassicae* infection as no infection occurred in the absence of disease (6.4). The nodules may be forming as a result of resting spores being splashed onto the stems during watering. Preliminary investigations to establish whether this was what was causing the nodules were unsuccessful. In these investigations it was attempted to inoculate the stems by attaching pieces of absorbent paper to the plant stems. It however proved difficult to attach the paper and prevent resting spores from dripping onto the compost surface, which would of resulted in the study being inconclusive as he inoculation would not of only occurred on plant stem.

In conclusion these investigations have shown how composts can reduce infection whilst other composts have no effect or may even increase infection. More research is therefore required to screen the many different kinds of composts available in order to identify a compost which is both suitable for raising brassica transplants in and decreases the level of *P. brassicae* infection. Green composts, which have already been shown by Pitt (Hedges, 1996) to decrease the level of *P. brassicae* infection, should be included in such an investigation despite the problems with their uniformity.

Chapter Seven.

Means of Increasing Soil Suppressiveness to *P. brassicae*

7. Experiments to determine whether alternative controls are effective in decreasing *Plasmodiophora brassicae* infection.

There is an increased need to establish the best basic control methods, for example are calcium carbonate applications to the soil more effective than using calcified seaweed. In consequence, this series of experiments aims to investigate the potential of four non-chemical control methods for reducing the extent of galling and subsequent yield reduction due to *P. brassicae* infection.

This type of protection is particularly important for *P. brassicae* has managed to evade many control measures. Using these broad based control measures the attack on the pathogen is less specific. It would therefore be more difficult for resistance to evolve in the pathogen.

7.1. A comparison of the effectiveness of calcium carbonate and calcified seaweed applications in reducing *P. brassicae* infections.

Calcified seaweed is a form of coral. It mainly consists of *Lithothamnion coralloides* and *Phymatolithon calcareum.* Only dead coral is harvested and processed limiting the environmental impact (www.seaweed.ucg.ie). Calcified seaweed has been used by farmers for many years as a fertiliser and has been associated with disease reduction. A chemical analysis of calcified seaweed is given in Appendix 2, from which it can be seen that there is a higher level of calcium present than in calcium carbonate.

Calcium carbonate has been used for many years to increase the soil alkalinity and decrease disease incidence (Campbell, 1989). The aim of the following experiments was therefore to establish whether calcified seaweed is more effective at controlling clubroot than calcium carbonate and hence whether there is any interaction between these two products and *P. brassicae*.

Method and Materials .

Pots (7 cm^2, FP8 Plantpak) were filled with Arthur Bower's seed and potting compost. The compost was then mixed with the appropriate treatment using the method described in Chapter 2.

Treatments.

1. Control
2. 1.146 g of calcium carbonate / pot
3. 0.573 g of calcium carbonate / pot
4. 1.146 g of calcified seaweed / pot
5. 0.573 g of calcified seaweed / pot

The experiment consisted of four pots per replicate and four replicates. Pots of one treatment in the same replicate were placed in full size seed trays to prevent cross contamination. Four Chinese cabbage seeds cv Mariko were sown into each pot and the trays placed in a glasshouse. Seven days after sowing the number of plants per pot was reduced to one and each plant inoculated by pouring 30 ml of a 10^6 *P. brassicae* resting spores ml^{-1} suspension over the surface of each pot.

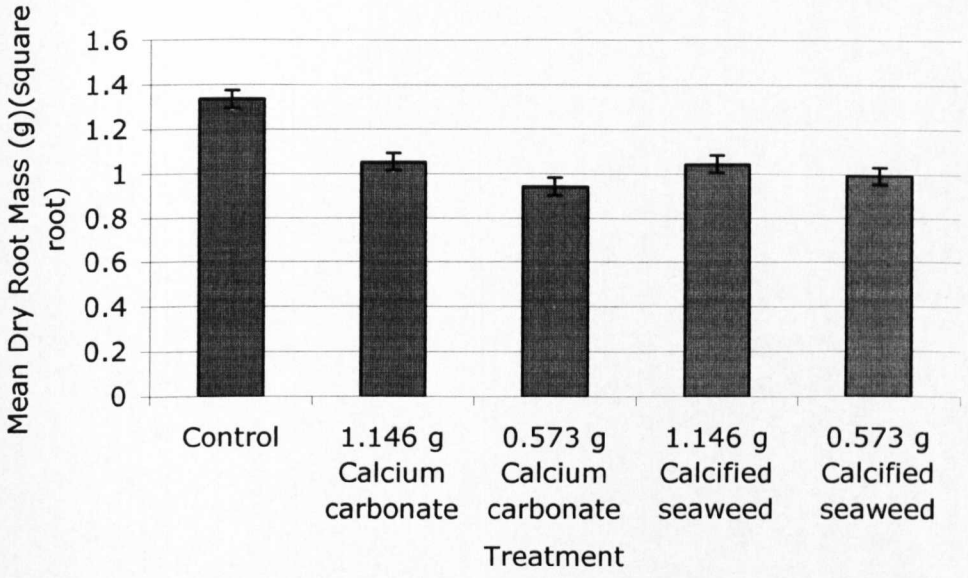
Results.

The plants were harvested six weeks after inoculation. The dry root and shoot masses and the gall categories of each plant were determined.

Graph 7.1 illustrates the mean dry root masses and the dry shoot masses are given in Graph 7.2. The figures have been transformed using the square root to enable statistical analysis due to the data being skewed.

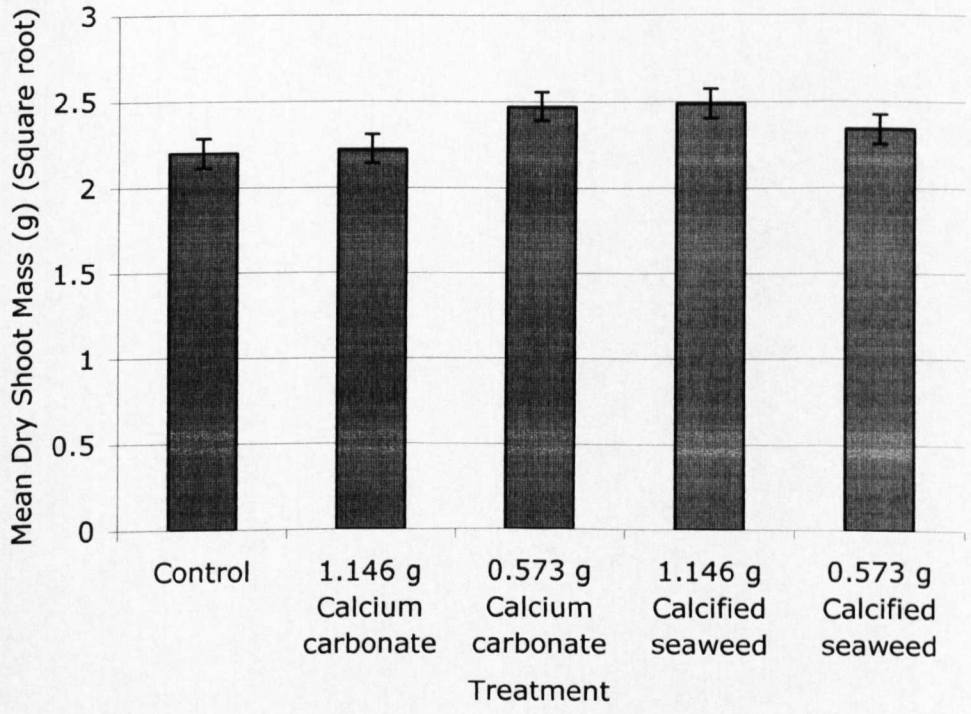
The mean disease indices were calculated from the gall categories using the formula in chapter 2. The disease indices were then transformed using the angular value to allow statistical analysis as these values are percentages. The transformed values are shown in Graph 7.3.

Graph 7.1. Mean dry root masses of plants treated with calcium carbonate and calcified seaweed at the recommended rates of application.



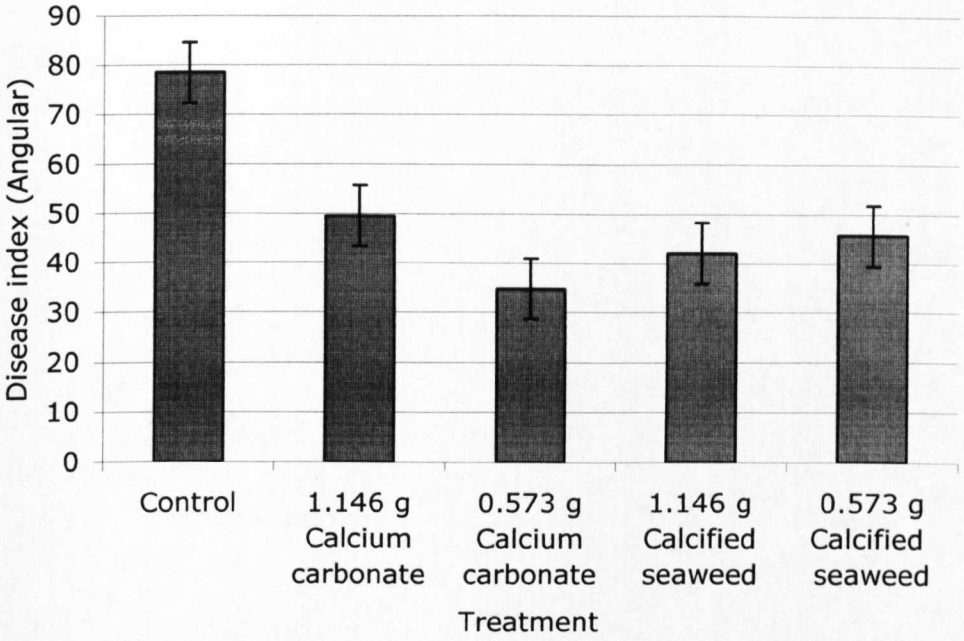
s.e.d = 0.0242

Graph 7.2. Mean dry shoot masses of plants treated with calcium carbonate and calcified seaweed at the recommended rates of application.



s.e.d = 0.047

Graph 7.3 Mean disease indices of plants treated with lime and calcified seaweed at the recommended rates of application.



s.e.d. = 4.7

Table 7.1. Mean dry root and shoot masses and disease indices (transformed) of plants treated with calcium carbonate and calcified seaweed.

Treatment / Pot	Dry Root Mass (g) (square root)	Dry Shoot Mass (g) (square root)	Disease Index (Angular)
Control	1.336a	2.2a	78.5a
1.146 g Calcium Carbonate	1.054b	2.223a	49.5b
0.573 g Calcium Carbonte	0.943b	2.473a	34.8b
1.146 g Calcified Seaweed	1.046b	2.49a	42.1b
0.573 g Calcified Seaweed	0.992b	2.341a	45.7b
S.E.D	0.0781	0.1688	12.24
L.S.D	0.1547	0.335	25.53

Values with the same letter are not significantly different using the L.S.D value.

Conclusions

There was no significant difference in dry shoot mass between treated plants or between treated and untreated plants.

Treated plants had significantly lower disease indices than untreated control plants and correspondingly lower root masses, however no significant differences were found between treatments.

At this rate of application therefore applications of calcified seaweed are no more effective than corresponding applications of calcium carbonate.

7.2. A further comparison of the effectiveness of calcium carbonate and calcified seaweed applications in reducing *P. brassicae* infections.

Higher application rates were used in this experiment in order to determine whether either of the treatments would become more effective in decreasing *P. brassicae* infection.

Method and Materials .

Round pots of 5.08 cm diameter were half filled with Arthur Bowers Seed and Potting compost, control pots were filled to within 0.5 mm of the top). The compost was then mixed with the required treatment using the method given in chapter 2.

Treatments.

1. 63.33 g / pot calcified seaweed
2. 31.51 g /pot calcified seaweed
3. 63.33 g / pot calcium carbonate
4. 31.51 g / pot calcium carbonate

Twelve pots of each treatment constituted a replicate and there were four replicates / treatment. The pots which formed each treatment replicate were placed together in one large gravel tray. Three seeds of Chinese cabbage cv Mariko were sown into each pot and placed into a glasshouse. Seven days later the number of plants per pot was reduced to one and then inoculated with *P. brassicae* by pouring 20 ml of a 10^6 resting spores ml^{-1} suspension over the surface of each pot. The plants were maintained for six weeks before harvest.

Results.

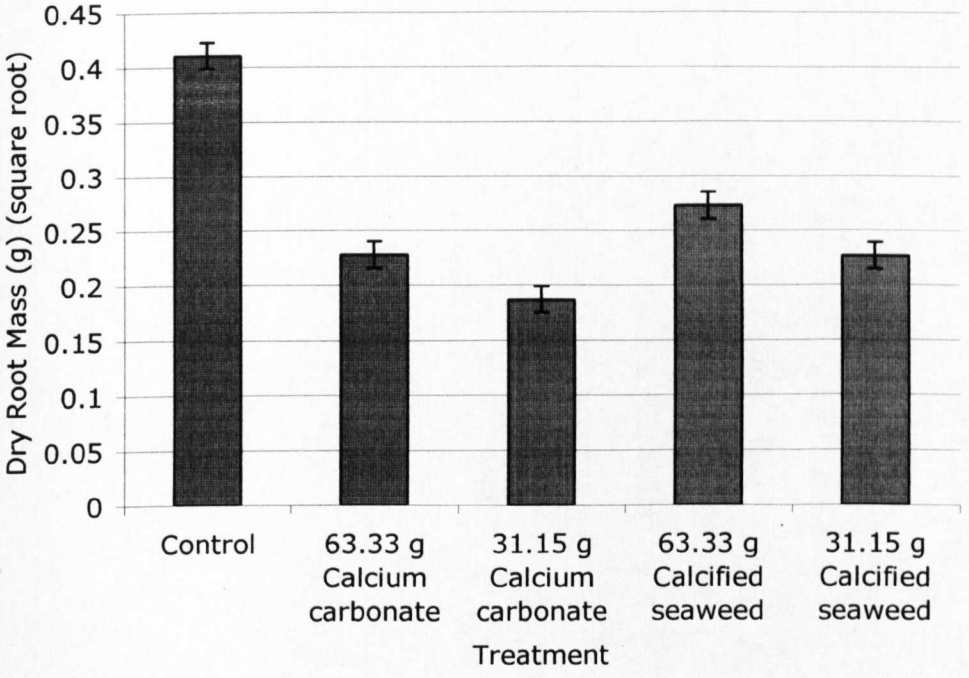
The dry root and shoot masses and gall categories of each plant were determined. The data of the dry root and shoot mass was skewed and was transformed using the square root to allow more accurate statistical analysis.

The transformed data for the dry root mass is given in Graph 7.4 and the transformed data for the dry shoot mass is given in Graph 7.5.

From the gall category a disease index was calculated using the formula given in chapter 2. These values are illustrated in Graph 7.6. The disease values were transformed to angles allowing an analysis of variance to be performed.

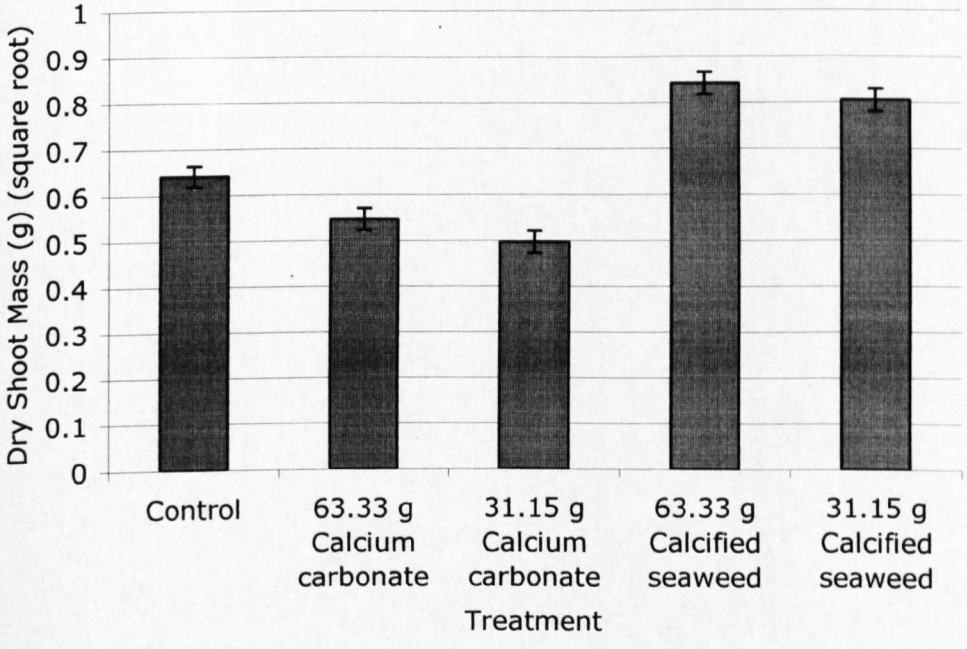
The transformed mean dry root and shoot masses and disease indices with the S.E.D and L.S.D values are given in Table 7.2.

Graph 7.4. Mean dry root masses of plants following treatment with calcium carbonate and calcified seaweed.



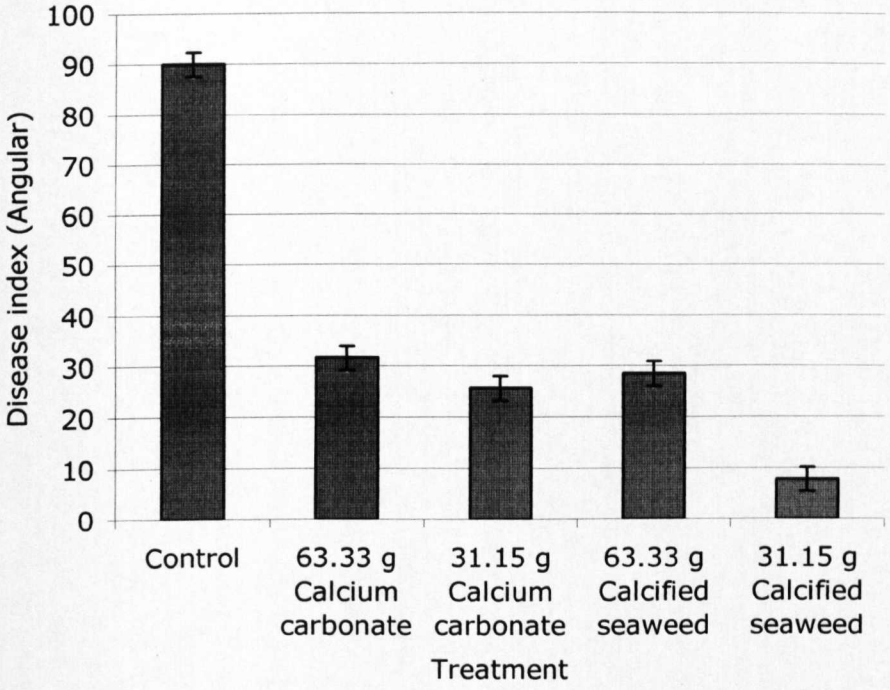
s.e.d = 0.0781

Graph 7.5. Mean dry shoot masses of plants plants following treatment with calcium carbonate and calcified seaweed.



s.e.d = 0.1688

Graph 7.6. Mean disease indices (angular) of plants plants following treatment with calcium carbonate and calcified seaweed.



s.e.d = 12.24

Table 7.2. Mean dry root and shoot masses and disease indices of plants treated with calcium carbonate and calcified seaweed.

Treatment / Pot	Dry Root Mass (g) (square root)	Dry Shoot Mass (g) (square root)	Disease Index (Angular)
Control	0.4106a	0.641a	90a
63.33 g Calcium Carbonate	0.228bc	0.547b	31.6b
31.15 g Calcium Carbonate	0.1867c	0.497b	25.7b
63.33 g Calcified Seaweed	0.2734b	0.842c	28.5b
31.15 g Calcified Seaweed	0.227bc	0.805c	7.8c
S.E.D	0.0242	0.047	4.7
L.S.D	0.04768	0.0926	10.23

Values with the same letter are not significantly different using the L.S.D value.

The mean disease index was significantly lower in plants treated with 31.15g of calcified seaweed than plants in any other treatments. Plants treated with calcium carbonate and calcified seaweed at a rate of 63.33g / pot had disease indices which were significantly lower than the untreated control plants but were not significantly different from other treated plants.

The mean dry shoot masses of plants treated with calcified seaweed were significantly higher than those of plants in other treatments. Plants treated with calcium carbonate had significantly higher shoot masses than control plants.

Plants treated with calcified seaweed and calcium carbonate had mean dry root masses which were significantly lower than untreated control plants. Plants treated with 63.33g of calcified seaweed had root masses which were significantly higher than plants treated with calcium carbonate.

Conclusions

The results from this experiment therefore indicate that applications of calcified seaweed at a rate of 31.15g / pot is the most effective *P. brassicae* control treatment.

In areas affected with *P. brassicae* applications of calcium carbonate are generally higher than is recommended in order to sufficiently raise the soil pH to suppress disease infection. The results of these experiments suggest that the use of calcified seaweed would be more effective in decreasing disease infection than calcium carbonate.

Calcified seaweed may be more effective at decreasing *P. brassicae* infection at higher concentrations due to its higher calcium content. Calcium carbonate and calcified seaweed both raise the soil alkalinity. At high levels however the soil alkalinity cannot be increased further, at this point the higher calcium content of calcified seaweed may serve to decrease *P. brassicae* infection further. It has already been established that high levels of calcium decrease disease infection and hence it is probable that the high level of calcium contained within calcified seaweed acts as a further environmental constraint in addition to a raised soil pH upon infection by *P. brassicae*.

7.3. Experiments to examine the impact of chitin on *P. brassicae* and clubroot disease.

Chitin is a natural by-product of the seafood industry. The shells of shell fish such as shrimp and crabs are made of chitin and it is the processed shells which are used in chitin based fertilisers.

Mitchell and Alexander (1962) found that soil additions of chitin decrease some plant diseases and increases the presence of chitinase, a chitin degrading enzyme in the soil environment. The increase in chitinase production corresponds to an increase in the population of actinomycetes. In one experiment they found that a significant decrease in infection by *Fusarium solani f. phaseoli* when ground chitin was applied to infected soil two weeks prior to seed sowing.

It has been reported that *Sclerotinia sclerotiorum* on vegetables was decreased by actinomycetes grown on oyster shell powder (Lin et al, 1990) and Ellis *et al.* (1995) noted that *Rhizoctinia solani* infection was decreased when a chitin based fertiliser was applied to the soil.

It has therefore been established that the application of chitin can decrease infection by soil borne pathogens. The following experiments were aimed at determining the effectiveness of chitin in decreasing brassica infection by *Plasmodiophora brassicae*.

7.3.1. The effectiveness of two forms of chitin in decreasing *P. brassicae* infection.

Method and Materials .

The chitin was supplied by Primex (Norway) who manufacture chitin under the product name of Chitosan. Chitosan is processed shrimp shells and is available in two forms ground and flaked.

Plant pots (7cm square pots, FP8 (Plantpak)) were filled with Arthur Bower's Seed and Potting Compost. The appropriate treatment was then mixed with the compost using the method described in Chapter 2. The experiment consisted of six replicates each with four plants. Replicate treatment pots were placed in small gravel trays to prevent cross contamination between treatments due to leaching and preventing contamination from the bench.

Treatments

An application rate equivalent to that found to be effective against *Fusarium solani f. phaseoli* by Mitchell and Alexander (1962) was used. The rate of application of chitin was therefore 3.15 g / pot the equivalent of 560g of chitin m^{-2}. This rate of application also falls between the rates of application used by Ellis *et al.* (1995) (270g m^{-2}–810 g m^{-2}).

Both forms of chitin (powdered and flaked) were used and were applied either two weeks prior to sowing or at the time of sowing to establish whether chitin application prior to sowing was more

effective in reducing disease infection than those made at the time of sowing.
The treatments were therefore :-

1. Untreated Control
2. Flaked chitin applied pre-sowing
3. Ground chitin applied pre-sowing
4. Flaked chitin applied at sowing
5. Ground chitin applied at sowing.

Pots treated with chitin pre sowing were irrigated and maintained in a glasshouse prior to sowing.

After the treatments had been applied had been applied to the pots they were sown with Chinese cabbage cv. Mariko. Four seeds were sown into each pot. One week after sowing the number of seedlings was reduced to one per pot and each pot inoculated with *P. brassicae* by pouring 30 ml of a 10^6 resting spores/ ml suspension over the surface.

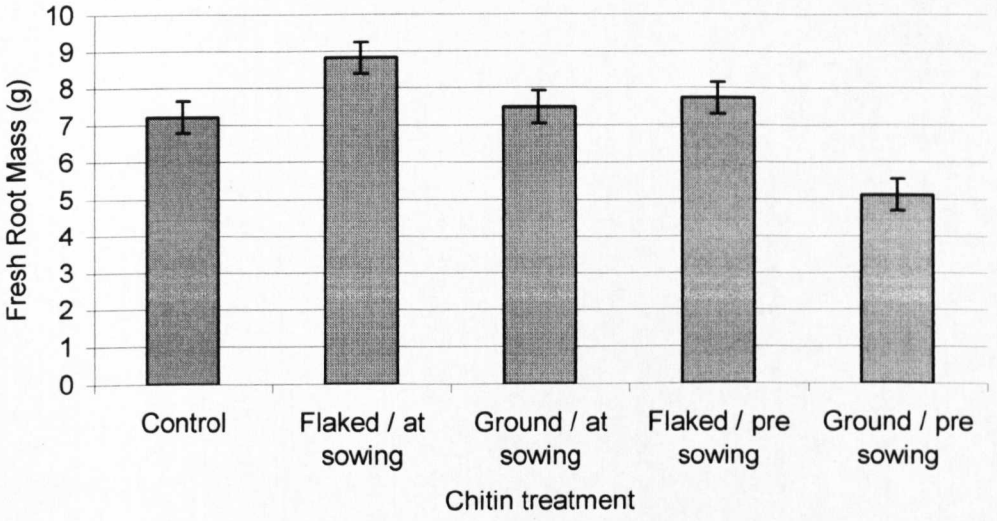
Results

The plants were harvested six weeks after it was inoculated.

The fresh root and shoot mass and gall category of each plant was determined and are illustrated in Graphs 7.7 and 7.8. The values are also given in Table 7.3.

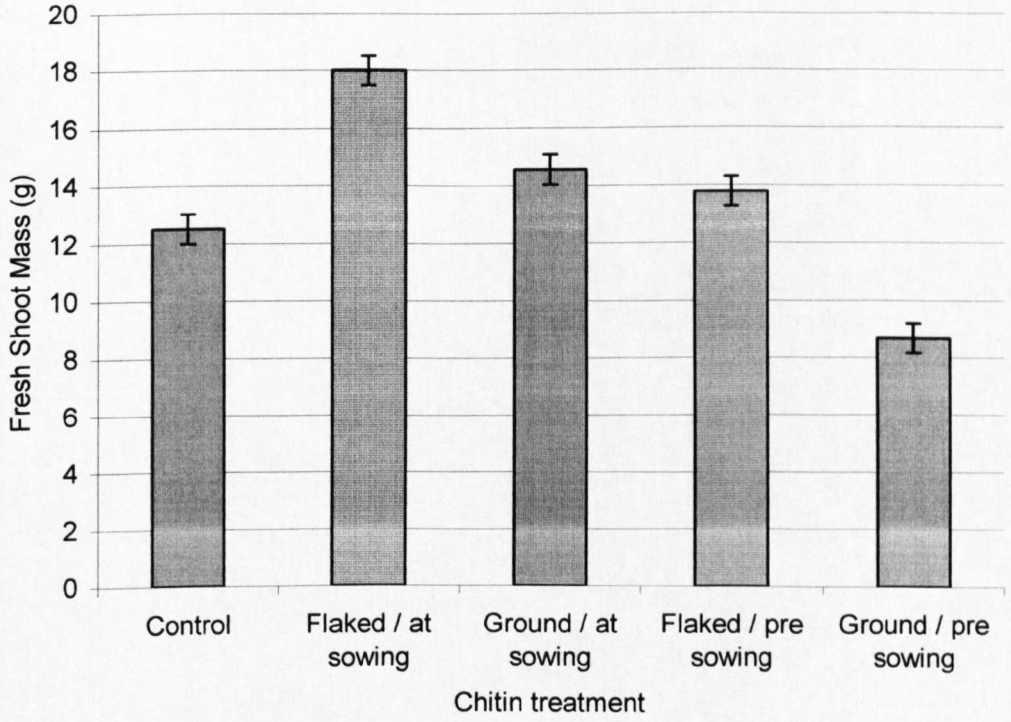
The gall category of each plant was categorised as 3 and therefore there was no differences in infection between treatments.

Graph 7.7. Mean fresh root masses of plants treated with two forms of chitin prior to sowing and at sowing.



s.e.d. = 0.85

Graph 7.8. Mean fresh shoot masses of plants treated with two forms of chitin prior to sowing and at sowing.



s.e.d. = 1.289

Table 7.3. Fresh root and shoot masses and disease indices of plants treated with chitin.

Chitin Treatment / pot	Mean Fresh Root Mass (g)	Mean Fresh Shoot Mass (g)	Disease Index %
Untreated Control	7.23a	12.51a	100
Flaked applied at sowing.	8.83a	17.99b	100
Ground applied at sowing.	7.49a	14.57a	100
Flaked applied pre-sowing.	7.73a	13.78a	100
Ground applied pre-sowing.	5.12b	8.68c	100
S.E.D	0.85	1.289	
L.S.D	1.685	2.555	

Values with the same letter are not significantly different from each other using the L.S.D values

Conclusions

The mean root mass of plants treated with ground chitin applied pre-sowing was significantly less than plants in other treatments. This would suggest that the galls on these plants were smaller than those on other plants, however, the shoot masses of these plants were also significantly lower than other plants. These results would suggest that applying ground chitin to plants pre-sowing is detrimental to plant growth.

This is in contrast to the findings of Mitchell and Alexander (1969) who found that ground chitin applied to two weeks prior to sowing decreased infection by *F. solani f sp. phaseoli*. The chitin in the

experiment carried out by Mitchell and Alexander (1969) was applied to soil already infected with the pathogen. In contrast in this experiment the chitin was applied three weeks before the plants were inoculated. Ellis *et al.* (1995) applied a chitin based fertiliser to compost 2 and 3 weeks before inoculation with *Rhizoctonia solani* and found that there was no further decrease in infection if the compost was treated with chitin at the same time as inoculation when plants were sown seven days later.

Although there was no decrease in disease infection, root and shoot masses of plants treated with flaked chitin at the time of sowing increased when compared to the infected control plants, significantly so for the shoot mass. A further experiment was therefore carried out to establish whether flaked chitin applied at higher rates would decrease *P. brassicae* infection.

7.3.2 Investigation in to whether chitin affects *P. brassicae* at higher concentrations.

Experiment 7.3.1 established that flaked chitin applied at the time of sowing increased subsequent shoot growth despite the plants being heavily infected with *P. brassicae*. The aim of this experiment was therefore to determine whether increasing the application rate of flaked chitin would decrease the level of disease infection.

Method and Materials

Plant pots (7 cm square pots, FP8 (Plantpak)) were filled with Arthur Bower's Seed and Potting Compost and mixed with the appropriate amount of flaked chitin. The pots were then each sown with four Chinese cabbage seeds cv Mariko and moved to the glasshouse.

Treatments

1. Untreated control
2. 1.575 g of flaked chitin / pot
3. 3.15 g of flaked chitin / pot
4. 7.875 g of flaked chitin / pot

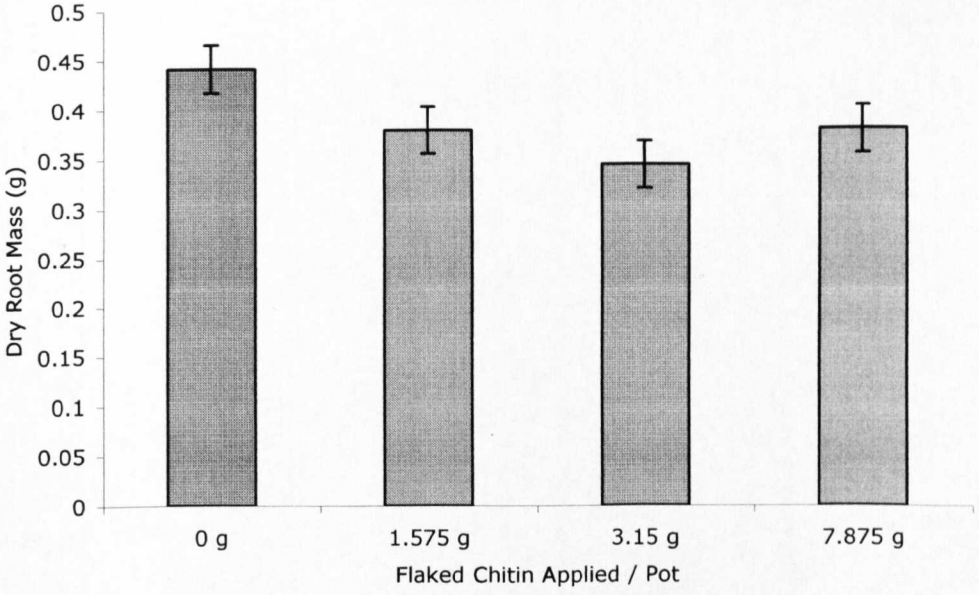
The experiment consisted of four treatments and five replicates. Each replicate consisted of four pots which were put in to the same small gravel tray to prevent cross contamination between treatments and contamination from the glass house bench.

One week after sowing the number of seedlings per pot was reduced to one and each pot was inoculated with *P. brassicae* by pouring 30 ml of a 10^6 resting spores / ml spore suspension over the surface of each pot.

Results

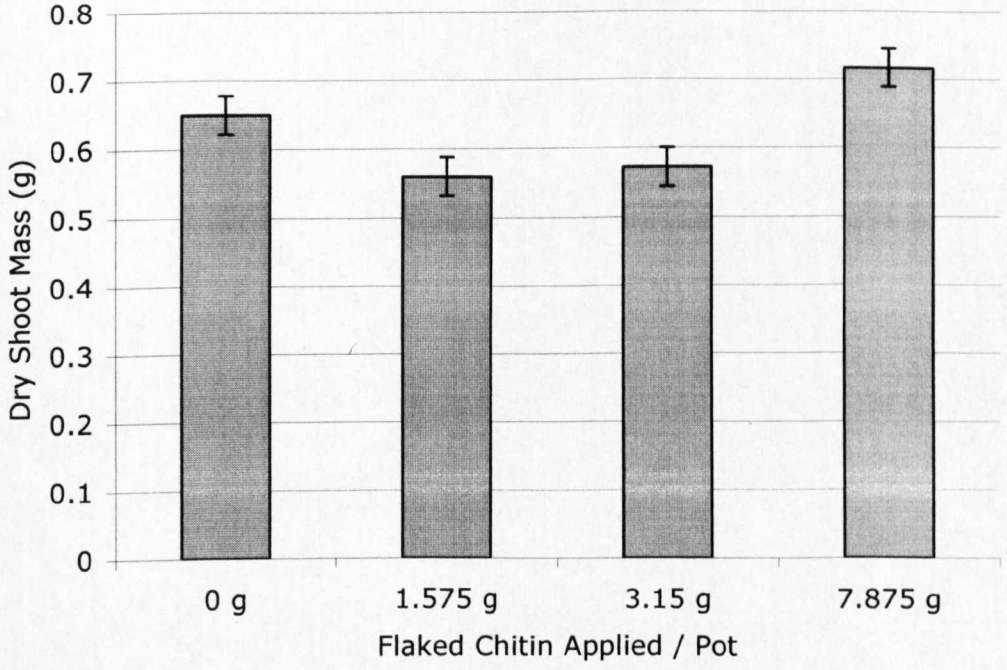
The plants were harvested six weeks after inoculation. The dry root and shoot masses of each plant were recorded along with the gall category. The mean dry root and shoot masses are illustrated in Graphs 7.9 and 7.10 and are given in Table 7.4.

Graph 7.9. Mean dry root masses of plants treated with chitin.



s.e.d. = 0.0489

Graph 7.10. Mean dry shoot Masses of plants treated with chitin.



s.e.d. = 0.0567

The gall categories of all the plants were recorded as three and therefore there was no difference in the level of infection between treated or untreated plants (Table 7.4).

Table 7.4. Table illustrating the dry root and shoot masses of plants treated with flaked chitin.

Treatment / pot	Mean Dry Root Mass (g)	Mean Dry Shoot Mass (g)	Disease Index %
Untreated control	0.441a	0.65a	100
1.575 g	0.379a	0.56b	100
3.15 g	0.345a	0.572b	100
7.875 g	0.382a	0.717a	100
S.E.D	0.0489	0.0567	
L.S.D	0.0974	0.1130	

Values with the same letters are not significantly different from each other using the L.S.D.

There was no significant difference between the mean dry root masses of treated plants or between treated and untreated plants further indicating that there was no difference in the level of infection.

None of the shoot masses were significantly higher than the untreated control when using the least significant difference value. If the standard error of difference value is used then plants treated with 7.875 g of chitin / pot then the shoot mass is significantly greater than the control plants.

Conclusions

The results of experiments 7.3.1 and 7.3.2 are not conclusive in determining whether chitin is a suitable control measure for *P. brassicae*. In experiment 7.3.1 flaked chitin applied at the time of sowing significantly increased the shoot mass of treated plants, however in experiment 7.3.2 higher application rates of flaked chitin did not have the same effect. It may be that higher rates of chitin are less effective in improving plant growth.

The effectiveness of chitin may be more effectively assessed in field trials. The results in these experiments may be variable due to the microbial environment within the compost being variable. A plant pot only holds a limited micro-flora environment whereas in a field environment the variation is much greater. As the ability of chitin to decrease infection is dependent on the micro-flora the field may be a more suitable environment for the testing of chitin.

It has also been suggested (Ellis, 1999) that chitin may be more effective as a seed coating or in combination with another control method.

7.4 Determination of the effectiveness of a seaweed extract in decreasing *P. brassicae* infection.

The use of a seaweed extract (Maxicrop, Triple) has been found to decrease the level of *Pythium* disease in Brassicas and conifers (Walsh, 1998). This experiment was therefore aimed at determining the effectiveness of decreasing *P. brassicae* infection.

Method and Materials.

Plant pots (7 cm^2 square pots, FP8 (Plantpak)) were filled with Arthur Bowers Seed and Potting Compost. The pots were treated with the seaweed extract at the following rates

Treatments.

1. Sterile de-ionised water control
2. 1:3000 dilution of Maxicrop Triple
3. 1:1000 dilution of Maxicrop Triple

The treatments were applied by pouring 10 ml of the appropriate treatment over the surface of each pot once a week. The experiment consisted of five replicates of each treatment with four plants in each replicate. The pots were then placed into a glass house and maintained for two weeks before being sown with four Chinese cabbage seeds cv Mariko. One week later the number of seedlings per pot was reduced to one and each pot was inoculated with 30 ml of a 10^6 resting spores ml^{-1} suspension.

Results

The plants were harvested six weeks after inoculation. The mean dry root and shoot masses are illustrated in Graphs 7.11 and 7.12 and (Table 7.5). The gall category of each plant was also recorded and each plant had a category of 3. There was therefore no difference in the level of infection between treatments (Table 7.5).

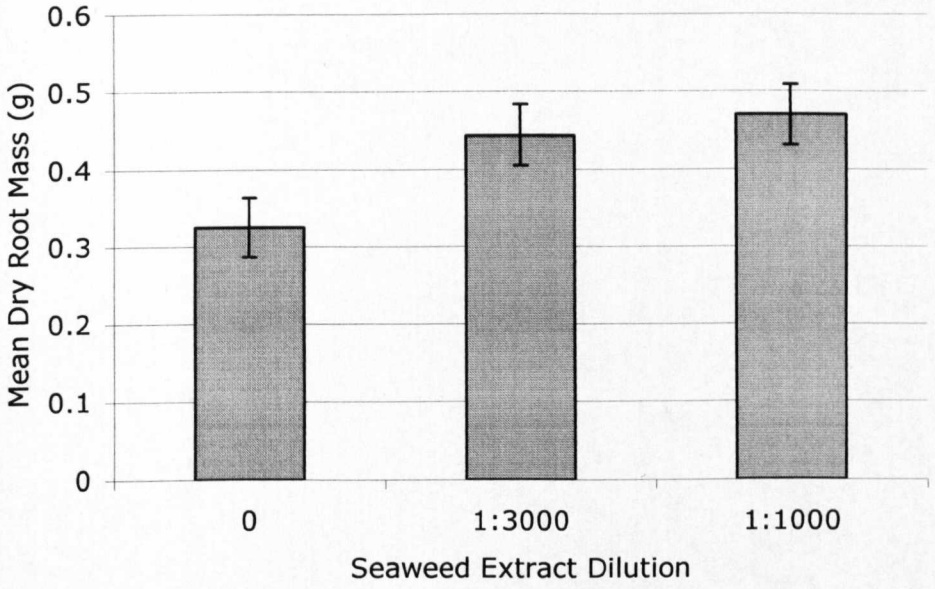
Table 7.5. Mean dry root and shoot masses and disease indices of plants treated with a seaweed extract.

Seaweed Extract Treatment	Mean Dry Root Mass (g)	Mean Dry Shoot Mass (g)	Disease Index %
Untreated Control	0.325a	0.518a	100
1:3000 Dilution	0.444a	0.513a	100
1:1000 Dilution	0.470a	0.599a	100
S.E.D	0.0776	0.0851	
L.S.D	0.1557	0.1708	

Values with the same letter are not significantly different using the L.S.D values

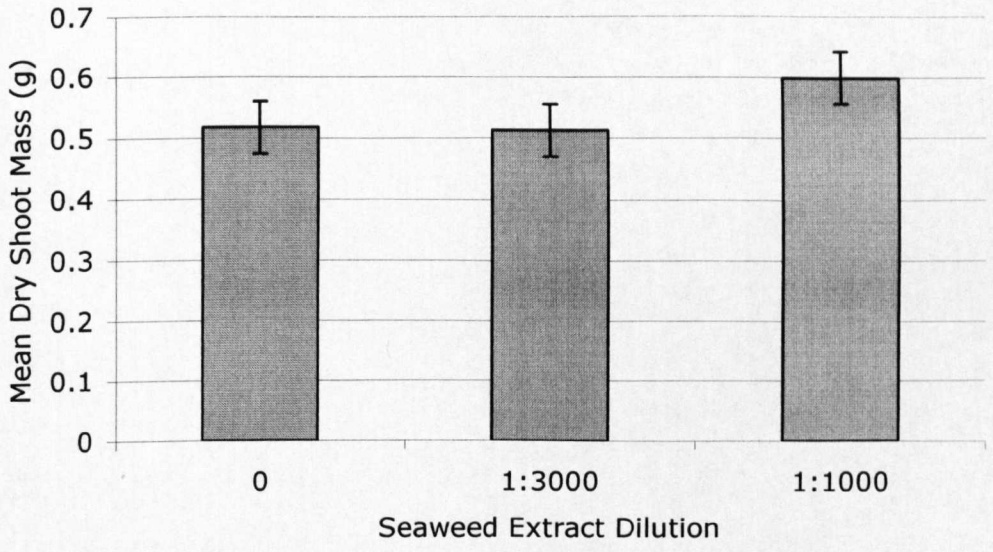
There was no significant difference between dry root and shoot masses of treated and untreated plants.

Graph 7.11. Mean dry root masses of plants treated with seaweed extract.



s.e.d. = 0.0776

Graph 7.12. Mean dry shoot masses of plants treated with seaweed extract.



s.e.d. = 0.0851

Conclusions

The results indicate that the regular application of seaweed extract to *P. brassicae* infected plants did not decrease the level of infection.

Discussion

Calcium carbonate proved to be an effective control measure, confirming the findings of Colhoun (1953) Campbell *et al.*, (1985) Fletcher *et al.* (1982) and Hamilton and Crête (1978). Calcified seaweed also reduced the level of *P. brassicae* infection. It was not possible however to determine from these results whether calcified seaweed was a more effective control measure than calcified seaweed. From the results in experiments 7.1. and 7.2 it would appear that there is no difference in their ability to control *P. brassicae*. Where high levels of these products are to be used to substantially increase the alkalinity of the soil calcified seaweed may offer more control than calcium carbonate.

The other control measures tested, chitin and seaweed extract, proved to be less effective in controlling *P. brassicae*. In experiment 7.3.1 the results indicate that higher application rates of flaked chitin applied at the time of sowing would result in better shoot growth and possibly a decrease in *P. brassicae* infection. This however, was not the case. Ellis *et al.* (1995) found chitin applications to decrease *Rhizoctinia solani* infection but also found that increasing the application rate of chitin did not result in increased control. In this experiment the rates of chitin applied were much higher than those made by Ellis et al (1995) but the results agree in that increasing the application rates of chitin does

not improve the disease control. In addition further increases in chitin application results in the loss of the increase in shoot growth.

Chitin may be ineffective against *P. brassicae* due to the structure of the resting spore wall. A resting spore has a coat which consists of alternating lipid and chitin layers. An increase in chitinase production may result in the first chitin layer being digested but unless there is also an increase in lipase production the resting spore is still protected. Chitin may therefore be more effective when applied in combination with other treatments. Chitin may prove to be effective in a field situation or when applied as a seed treatment but from the results in experiments 7.3.1 and 7.3.2 chitin provides no effective control against *P. brassicae,* but at a rate of application of 560 g m^{-2} an increase in shoot growth is observed.

The application of seaweed extract at weekly intervals also failed to provide any control against *P. brassicae* and no compensatory increase in shoot growth was observed. This again may be due to the structure of the resting spore wall. Walsh (1998) observed an increase in some enzymes including chitinases, which led to a subsequent decrease in *Pythium* spp. infection. If these enzymes are unable to attack the resting spore or zoospore wall then this may be the reason for it's apparent ineffectiveness. At different application rates however, applications of seaweed extract may promote plant growth as it has already been shown to be a plant stimulant (Dixon, Pers. com)

The research in this chapter has established that the use of calcium carbonate and calcified seaweed still remains the best non-chemical control method for protection against *P. brassicae*.

Although chitin and seaweed extracts were unable to decrease the level of infection, when applied in combination with other treatments they may stimulate plant growth producing higher shoot masses where there is a low level of infection.

Chapter Eight

General Discussion

Discussion

Treatment of *Plasmodiophora brassicae* with calcium nitrate decreased the extent of gall formation. The application of calcium nitrate prior to planting of the crop was the most effective mode of application. Further investigations revealed that applying calcium nitrate pre-planting not only slows development of the pathogen within the host plants root hairs (Webster, 1985) but also decreases germination of resting spores and the invasive success of primary zoospores when incubated in a calcium nitrate solution. The mode of action of calcium would therefore appear to be two fold, first the resting spore / zoospore viability is reduced and secondly the rate of pathogen maturation is reduced.

The discovery that incubation of *P. brassicae* resting spores with calcium nitrate reduces spore viability is in contrast to the observations of Myers and Campbell (1985) who considered that both calcium and hydrogen ions had a low toxicity to resting spores. In their experiments incubation of resting spores in 1.0 M calcium chloride solution at a pH of 6.2-7.2 for 1 week reduced infectivity only slightly when compared to resting spore suspensions incubated in sodium chloride or control buffer solutions. However, as the optimum germination time for resting spores is 36 hours (Aist and Williams (1971) it is possible that one week was too long a period for the spores to have been left to allow an accurate comparison of the effects of incubating resting spores in a calcium environment. Alternatively, the source of calcium may have been ineffective against the resting spore viability.

Suzuki *et al.* (1992) suggested that calcium could stimulate the germination of resting spores and this may be part of the reason why a decrease in infection was seen. In incubating the resting

spores in a calcium environment they may have been stimulated to germinate prematurely and so by the time the plants were inoculated a higher percentage of spores may have already germinated. This may also explain why calcium nitrate applications were found to be more effective when applied one week in advance of planting. This in combination with the effect of calcium nitrate upon primary resting spores makes calcium nitrate a very potent control measure against *P. brassicae*.

The application of calcium nitrate to seedlings prior to transplantation to a *P. brassicae* environment did not decrease the extent of galling upon host infection. Treatment of seeds with calcium nitrate prior to sowing into *P. brassicae* infected soil also failed to provide control. Applications of calcium nitrate post infection also failed to decrease infection. The most efficient use of calcium nitrate as a control measure would therefore appear to be to apply the treatment approximately one week prior to seedling transplantation.

Investigations using the European Clubroot Differential Series further revealed the influence of calcium nitrate upon *P. brassicae* resting spores. The pathogen race differed in galls collected from plants treated with calcium nitrate from that found in wild type galls. It is therefore, hypothesised that the presence of calcium nitrate in the root environment acts as a selection pressure on the *P. brassicae* population. Further investigations using the ECD series and applications of combinations of resting spore suspensions from wild type and calcium nitrate treated galls, revealed that the pathogenic race contained in calcium nitrate galls was more virulent than the one found in wild type galls.

The result of applying the more virulent resting spores from calcium nitrate treated galls was that the resistance of some of the ECD hosts was compromised. The resistance of some but not all plants of an ECD host sample being overcome highlighted the instability of the resistance and the variability of the ECD hosts. Jones (1981 and *et al.* 1982b) also expressed concern over the reliability of ECD series host resistance but in the absence of a more reliable system the ECD series remains in use. It is therefore vital that a more efficient system for the identification of *P. brassicae* pathogen races is developed. This improved identification system almost certainly lies in the development of molecular methods such as those of Ito *et al* (1999), however much more work needs to be done before a reliable and fast technique can be released for use in the identification of *P. brassicae* pathogen races.

The availability of calcium in the root environment is only one of many factors which influence the infective ability of *P. brassicae*. Indeed this research demonstrated that variances in the type of compost used can decrease or increase the extent of gall formation. Use of the ECD series demonstrated that the type of compost used to grow hosts in influenced the predominant pathogen race that was identified.

The result of applying a wild type resting spore suspension to these ECD hosts resulted in the determination of a different predominate host in each compost. It may therefore be possible to use the compost a seedling is raised in to manipulate the extent of infection or even to select for less virulent pathogen races.

The finding that even the compost type can affect pathogen variability highlights the need to find control measures which are less specific than chemical control and hence more difficult for the

pathogen to circumvent. This thesis has potentially identified several such methods of control.

The identification of two brassica growing areas, Kings Kettle and Crail, which have soils suppressive to *P. brassicae* is an advance towards finding an alternative control measure. It must be remembered however, that the soils suppressed disease, but did not eradicate it. The suppressive nature of the soils can be attributed to both biotic and abiotic factors. Soils which are suppressive to *P. brassicae* have been previously identified by Rouxel (1984) as well as soils which are suppressive to other pathogens (Rouxel, 1991). However, there has been little development from their discovery in identifying biological control agents. It is highly unlikely therefore that a single biological control agent for the control of *P. brassicae* could be identified from these soil samples but, with further research, it may be possible to determine which microbial populations need to be promoted in a soil to obtain suppressiveness, and how this could be done.

The investigations into these soil samples established that although these soils did contain a high level of calcium, applying calcium to non-suppressive soils did not promote an increase in micro-flora or suppressiveness. These studies were however limited and further investigations using a wider range of microbial growth substrates and techniques would be recommended to establish this.

The pathogen races present in the suppressive soils were not determined but it may be of greater advantage to do this when more reliable techniques are available. In identifying the race it would be interesting to see how it varies from other pathogen populations in the same area contained in conducive soils.

Identification of the soils has been a necessary step in the development of possible control measures but they now need to be further examined in a manner which will result in their application to a brassicae growing environment. Transferring bio-control populations from these suppressive soils would probably not be possible but if further research could determine how to manipulate the soil environment to promote suppressiveness, even to a small extent, in combination with other controls it may provide a necessary weapon to reduce the levels of *P. brassicae* infection.

The use of different composts to manipulate the rhizosphere may be one measure that could be used in combination with suppressive soils or other control methods.

The research using the E.C.D series illustrated how changing the compost in which a plant is grown is sufficient to affect the *P. brassicae* population. Investigations into how composts could be used to protect plants against *P. brassicae* produced variable results.

Although in a glasshouse environment raising module plants in different composts did have an effect on subsequent infections, the composts used did not influence the level of infection in the field. In other glasshouse experiments where plants were grown in a different range of composts the level of infection was affected. These conflicting results highlight one of the main difficulties in working *with P. brassicae*, in that the pathogen is so variable that where one compost may have no effect another may reduce disease. The research has also highlighted a fact that should be born in mind in future work, which is that although the pathogen race may be influenced, the infection level may not necessarily be reduced.

Work by Dr Pitt of Exeter University (pers. comm and Hedges (1996)) has established that some green waste composts which are claimed to virtually eliminate *P. brassicae* infection. Raviv *et al.* (1998) have established that raising cabbage transplants in a compost based on cattle manure reduces the level of *Pythium aphanidermatum* upon transplantation. Research into the use of composted onion waste has also been shown to decrease the incidence of onion white rot (*Sclerotinia cepivorum*) (www.Hortips.co.uk). It may therefore, be more sensible to investigate the properties of these types of more organic composts rather than those which are currently sold on a large scale commercially.

Due to the variability of the pathogen, which has been demonstrated again in this thesis, and the failure to obtain a chemical control it is necessary to identify other control measure for *P. brassicae*. It was recognised that more 'holistic' control measures which affect the whole system and not just the pathogen may be effective and prevent the evolution of pathogen resistance.

The investigations into the effects of suppressive soils and composts have shown that chemical controls are not always necessary in order to achieve a reduction in disease. The remaining research also identified some further non-chemical control methods. These investigations were aimed at identifying control measures which could be used in organic as well as conventional cultivation.

During this research the possibility of using calcified seaweed was explored and this proved to be at least as effective as applications of calcium carbonate. The use of chitin and seaweed extract were also examined but they were found, in this instance, to have little

effect on the level of *P. brassicae* infection. Further research needs to be carried out though as in the time period of this project it was not possible to test a wide range of concentrations or to test the products in a field environment. The testing of possible control measures in a field environment is particularly important in the development of *P. brassicae* control methods as, has already been illustrated what succeeds in a glasshouse may have a lesser effect in the field.

This thesis has, however, identified several possible control measures; calcium nitrate, suppressive soils, composts and calcified seaweed, all of which require further investigation. The work on calcium nitrate has answered several questions and an application rate of between 300 and 400 kg ha^{-1} of nitrogen applied one week before planting is suggested.

Further investigation is needed into the remaining control methods identified before any recommendations on their use can be made.

The investigations using the E.C.D. series have proved to be particularly useful in highlighting the high degree of pathogen variability and how *P. brassicae* interacts with even the smallest changes in the soil environment. This should be taken into account in future research and control methods should ideally be tested on as wide a range of populations and over as many generations as possible.

One of the most important messages contained within this thesis is that whilst new avenues for control can be identified, complete protection may not be possible with a single control measure. During further research into control measures it is important to consider that a holistic combination of less effective controls may

prove to be more successful than a single control measure and as many combinations as possible should be examined. If *P. brassicae* is attacked on more than one front there is a reduced chance of the pathogen overcoming the control measure. A combined control system could provide protection to the plants through many stages of infection and therefore even if the pathogen overcomes the first control measure it may not be able to overcome the second.

For example, even the most effective control measure identified in this thesis does not provide total control of *P. brassicae*. A better system of control may therefore be to apply calcium nitrate which attacks the pathogen at three stages of infection, resting spore germination, primary zoospore infection and disease maturation, to a soil which has been encouraged to develop suppressiveness. In this manner the disease would not only be prevented from infecting the host in the first place but it may also be protected through later stages of development. The further application of plant stimulants such as seaweed extracts may help to promote plant disease resistance to over come any infections which do then occur, resulting in a marketable crop.

This thesis has demonstrated that reductions in *P. brassicae* are possible with the use of calcium nitrate and suppressive soils, but much more research is needed into the pathogen itself. Unless the pathogen is more fully understood e.g. how does the pathogen population alter so rapidly to a change in the soil environment, does the DNA of the pathogen change, are there further parts of the lifecycle to be discovered, finding a control measure may remain a hit and miss affair. There is much more research to be carried out but this thesis may at least have suggested a few answers and identified many more questions.

Summary of main findings

The main findings of this research, in relation to the original aims were :-

1. Calcium nitrate is effective in decreasing *P. brassicae* infection.

2. Calcium nitrate decreases galling after *P. brassicae* infection has occurred.

3. The viability of resting spores and the ability of primary zoospores to infect the host are reduced in the presence of calcium nitrate.

4. The predominant pathogen race present at the Auchincruive field site was 16/6/14

5. The presence of calcium nitrate in the rhizosphere caused a change in the predominant pathogen race. The race present in galls from plants treated with calcium nitrate was 16/12/14

6. The growth substrate in which the E.C.D. series is raised can affect the predominant pathogen race.

7. It was not possible to initiate "root camouflage" using commercially available composts.

8. Soil samples from Kings Kettle and Crail were identified as suppressive to *P. brassicae*. The nature of the suppression was investigated and found to be due to both biotic and abiotic factors.

9. Both calcium carbonate and calcified seaweed were found to reduce the level of *P. brassicae* infection It was not possible, however to determine whether calcified seaweed was a more effective control.

10. Applications of liquid seaweed extract did not decrease *P. brassicae* infection.

11. Applications of chitin to the growth medium did not decrease *P. brassicae* infection.

Posters presented at conferences and Publication list

PAGE NUMBERING AS IN THE ORIGINAL THESIS

A5.1. Poster presented at ISHS Symposium on Brassicas, Rennes, France

PROTECTING BRASSICA TRANSPLANTS FROM *PLASMODIOPHORA BRASSICAE*.

L. PAGE & G.R. DIXON

Deapartment of Horticulture, SAC / University of Strathclyde, SAC Auchincruive, Ayr, KA6 5HW, Scotland UK

Introduction.

Brassica seedlings raised in peat appear more susceptible Plasmodiophora brassicae (clubroot) than those grown in other media. Hypotheses regarding root camouflage (Gilbert et al., 1993) suggest that rhizosphere communities differ between growing media, some of which expose the host to increased disease risk. While soil-borne pathogens such as Pythium spp and Colletotrichum spp were controlled by organic composts added to the root zone (Zhang et al., 1996).

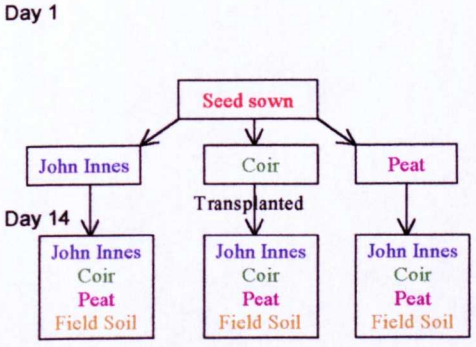
The aim of this project is to understand the possible suppression of *P. Brassicae* in organic media. Work has commenced by surveying the interaction of several growing media as substrates in which clubroot is either repressed or enhanced. Preliminary results support the view that peat based composts induce greater disease development.

Materials and Method.

Equal amounts of Summer cabbage seed, cv Castello were sown into plug trays containing either John Innes, Coir based or Peat based composts.

After 14 days seedlings raised in each compost were transplanted into pots containing either field soil, John Innes, Coir or Peat compost giving a total of twelve treatment combinations.

Fig.1: Experimental method.



The field soil was naturally infested with *P. brassicae* The pots containing compost were sampled at weekly intervals for three weeks to examine root hair infection. A *P.brassicae* infected root hair is illustrated in plate 1, demonstrating zoosporangia.

Results.

The experiment was harvested eight weeks after inoculation. The fresh weight and dry weight of both the roots and shoots were recorded as were disease category and leaf area.

Fig. 2. Disease category.

The disease category is a measure of the extent of root galling. Higher values correlate with increases severity of infection. In summary, greater galling developed in plants transplanted into peat based compost.

Fig:1. Disease values obtained from plants raised in several media, eight weeks after

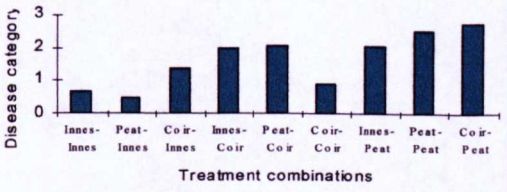


Fig. 3 & 4: Root and Shoot Dry Mass.

The dry masses were obtained by drying plants at 80ºc for 48 hours.

Fig:3. Root dry mass of plants raised in several media eight weeks after transplanting to clubroot infested media

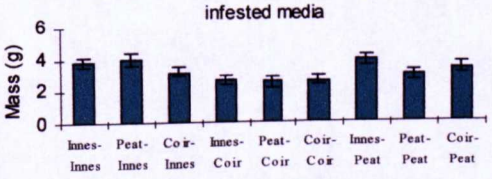


Fig:4. Shoot dry mass of plants raised in several media, eight weeks after transplanting to clubroot infested media.

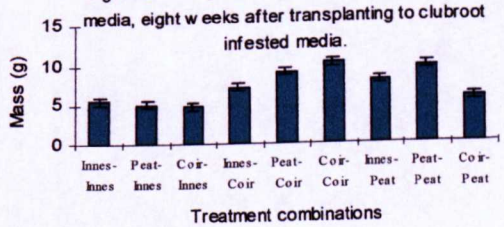


Plate 1: A root hair infected with primary plasmodia.

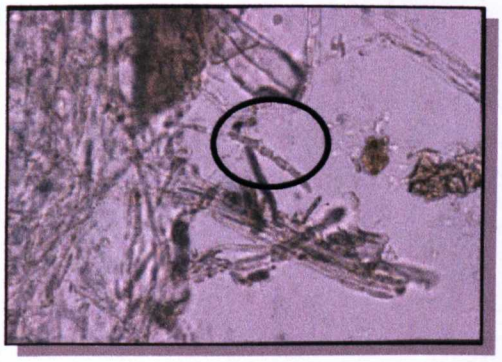
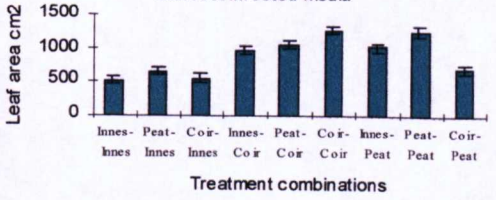


Fig.6: Leaf area

The leaf area of each leaf was recorded using a Delta T area meter.



Conclusions.

- The highest disease categories tended to be associated with plants inoculated and transplanted into Peat and Coir.
- Root dry masses did not correlate with galling
- Plants transplanted into John Innes had low disease categories, but also poor shoot growth associated with poorer plant establishment.
- Plants transplanted to peat gave higher disease values and with the exception of those plants also raised in peat modules these treatments also produced poor shoot growth.
- Field soil tended to be associated with poor plant establishment and led to poor results.
- In this experiment it is clear that plants grown in peat are more susceptible to clubroot than those grown in other media. However what is not clear from these results is whether plants raised inn peat before transplanting are more susceptible. Field trials are therefore currently comparing the amount of clubroot infection between plants raised in John Innes, Coir and Peat composts.

References.

Gilbert, Handelsman & Parke, 1994, Phytopathology, 84, 222-225.

Zhang, Dick and Hoitink, 1996, Phytopathology, 86, 1066-1070

A5.2. Poster presented at the International Congress of Plant Pathology 98.

ABSTRACT 951: THE REGRESSION OF *PLASMODIOPHORA BRASSICAE* (CLUBROOT) BY CALCIUM.

L.V. PAGE, G.R. DIXON, M.N. BURGE.

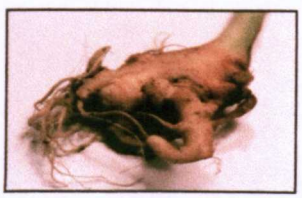
Dept. Bioscience & Biotechnology, University of Strathclyde, Glasgow.

Introduction.

Infection of *Brassica spp* by *Plasmodiophora brassicae* (clubroot), a plasmodial fungus which parasitises the root hair and cortical cells, leads to massive morphological, biochemical and molecular changes which decrease crop yields.

One traditional treatment for *P. brassicae* is the application of lime, which may operate through changes to soil pH. However, research has shown that calcium itself has a role in controlling the growth and reproduction of *P. brassicae*. Field experiments demonstrate that the use of calcium nitrate leads to a reduction in *P. brassicae* infection. Calcium itself has a wide range of effects on plants, from strengthening cell walls to involvement in signaling [1]. Research is therefore attempting to establish how calcium affects this fungus and subsequent pathogenesis [2].

Plate 1: Chinese cabbage root affected by clubroot.



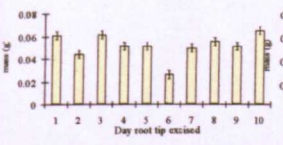
Method and Materials : Split root technique.

Originally the root tips of seedlings were removed after ten days. However the root length before the point of bifurcation was too long. To determine the optimum time for root tip removal Chinese cabbage seed cv Mariko, was germinated on moist filter paper for 24 hours. Seedlings were then transferred to petri dishes containing sand and irrigated with modified Hoaglands solution. The root tips of groups of ten seedlings were removed at 24 hour intervals for a period of ten days. The seedlings then grew for a further ten days permitting development of the root system.

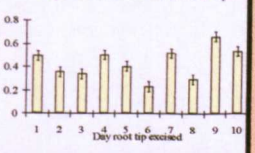
Results.

Plants were harvested seven days after root tip removal and the dry root and shoot masses recorded.

Dry root mass of seedlings 10 days after removal of root tip.



Dry shoot mass of seedlings 10 days after removal of root tip.



With the exception of day six, the time of tip removal had little effect on the root mass. Day six also had a low shoot mass. It was decided that removing root tips two days after germination produced the most useable root system.

Discussion.

Now the optimum time for root tip removal has been determined it is possible to divide the root system between two bijoux bottles each containing nutrient solutions differing in calcium content. Both sides of the root system are inoculated with *P. brassicae* and the infection process monitored to determine if the difference in calcium levels causes a difference in the infection process. Preliminary results suggest that the effect of calcium on *P. brassicae* is not translocated.

Plate 2: A cabbage seedling growing with its root system divided between two bijoux bottles.

To gain a thorough understanding of the effects of calcium on *P. brassicae* will require the following questions to be answered:

1. Are the effects of calcium, due to an increase in pH immediately in the root zone, leading to a variation in the uptake of selected minerals?
2. Is calcium an inhibitor of the disease process?
3. If the effects of calcium are not translocated is the effect limited to the rhizosphere?
4. Does the presence of calcium affect the resting spores, primary stages or secondary stages of infection?
5. Is calcium strengthening the plant cell walls against invasion by *P. brassicae?*
6. Is calcium, or other minerals whose uptake is increased / decreased by the presence of calcium, invoking a hypersensitive response?
7. Is the presence of calcium selecting for the presence of antagonistic microorganisms in the rhizosphere?

References.

1. **Roux SJ, Slocum RD. 1982.** Calcium and cell function **3**, 409-453
2. **Dixon GR, Page LV. 1998.** Acta horticulturae **459**, 343-350

A5.3. The Impact of Calcium on Resistance in Brassica Genotypes to *Plasmodiophora brassicae* Wor. (Clubroot).

The Impact of Calcium on Resistance in Brassica Genotypes to *Plasmodiophora brassicae* Wor. (Clubroot).

G.R. DIXON and L.V. PAGE

Dept. Bioscience & Biotechnology, University of Strathclyde, Glasgow G1 1XQ. United Kingdom.

E-Mail:- geoffrey.dixon@strath.ac.uk

•Introduction

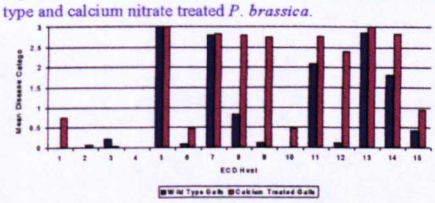
The European Clubroot Differential Series (ECD) is the standard means for classifying physiological races of *Plasmodiophora brassicae* Wor., the causal agent of clubroot disease in the Brassicaceae. Substantial microgeographical variations between populations of *P. brassicae* are reported. These may stem from interactions between the hosts, pathogen and their environments. This project examined the impact of calcium on variation.

•Materials & Methods

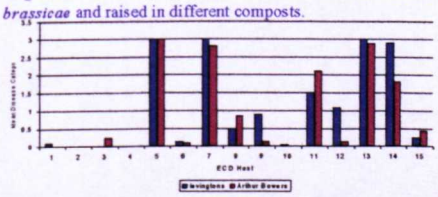
Seedlings of the ECD Series were grown in composts artificially infested with *P. brassicae* at 10^6 resting spores gm^{-1} and maintained in a glasshouse at 20 °C approx. for 42 days. Clubbing symptoms were assessed on a rising scale of 0 - 3 symptom expression.

•Results

Figure 1. Mean disease categories of ECD hosts inoculated with wild type and calcium nitrate treated *P. brassica*.



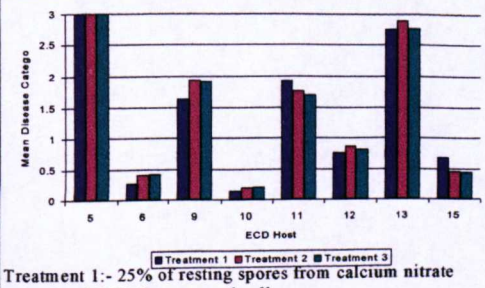
•Figure 2. Mean disease categories of ECD hosts inoculated with *P. brassicae* and raised in different composts.



•Conclusions

1. The addition of calcium to the compost environment altered symptom expression in seedlings of ECD 1 (*B. rapa* var. *rapifera* line AABBCC), ECD 6, 8, 9, 10 (*B. napus* lines DC 101,128, 129 & 130), ECD 12 & 14 (*B.oleracea* var. *capitata* cv. Bindsachsener & Septa) and ECD 15 (*B. oleracea* var. *fimbriata* cv. Verheul).
2. Variation in symptom expression by ECD hosts resulted from using two differing compost mixtures.
3. Mixing differing ratios of wild type resting spores of *P. brassicae* with those derived from hosts grown with high calcium nutrition produced only limited variation in symptom expression.

Figure 3. Mean Disease Categories of ECD hosts inoculated with mixtures *P. brassicae* spores from wild type and calcium nitrate treated galls.



Treatment 1:- 25% of resting spores from calcium nitrate treated and 75% from normal galls.
Treatment 2:-.50% of resting spores from calcium nitrate treated and 50% from normal galls.
Treatment 3:- 75% of resting spores from calcium nitrate treated and 25% from normal galls.

Acknowledgements Funding for this Project was received from the Ministry of Agriculture, Fisheries & Food, London and from Hydro - Agri ASA, Norway.

A5.4. Publications

- **Dixon, G.R & Page, L.V. 1997.** Calcium and Nitrogen Eliciting Alterations to Growth and Reproduction of *Plasmodiophora brassicae* (clubroot). *Acta Horticulturae*, **459:** 343-349.

- **Page, L.V & Dixon G.R. 1997.** Protecting Brassica Transplants from *Plasmodiophora brassicae. Poster presented at ISHS Symposium on Brassicas, Rennes, France.*

- **Page, L.V. 1998.** Report on Brassica 97, Rennes, France. *BSPP Newsletter,* **32:** 26-28.

- **Page, L.V. 1998, Dixon G.R. Burge, M.N & Harriman, M. 1998.** The regression of *Plasmodiophora brassicae* by Calcium Nitrate. *Poster presented at the International Congress of Plant Pathology 98.*

- **Dixon, G.R and Page, L.V.** 2001 The Impact of Calcium on Resistance in Brassica Genotypes to *Plasmodiophora brassicae* Wor. (Clubroot). *Poster presented at ISHS Symposium on Brassicas, Wellesbourne.*

References.

PAGE NUMBERING AS IN THE ORIGINAL THESIS

References

Agrios G. 1988. Plant Pathology 3rd Edn. Academic Press Inc. London pp 288-291.

Aist J.R, Williams P.H. 1971. The cytology and kinetics of cabbage root hair penetration by *Plasmodiophora brassicae*. *Canadian Journal of Botany* **49**:2023-2034.

Alabouvette C.1986. *Fusarium* wilt-suppressive soils from the Chateaurenard region: review of a 10-year study. Agronomie 6: 273-284.

Anderson M.D. 1853. Proceedings in the Laboratory: Report on the disease of finger and toe in turnips. *Transactions of the Highland Agricultural Society, Scotland*. Series 3, 6: 118-140.

Anon. 1982. How to control clubroot in agricultural brassicas. Scottish Agricultural College, Publication No. 96.

Anon. 1984. A.D.A.S Clubroot Leaflet No. 276. Agricultural Development and Advisory Service. London.

Antonova G.G, Shestiperove Z.I and Shuvalova G.V. 1974. Effect of root feeding with boron and manganese in reducing damage by clubroot. *Zap. Leningr. Del.'-Koz. Inst , 239 :81-86. Seen in Review of Plant Pathology 1975, 54: Abstract 5611.*

Arie T, Kobayashi Y, Okada G, Kono Y, Yamaguchi I. 1998. Control of soilborne clubroot disease of cruciferous plants by Epoxydon from *Phoma glomerata*. *Plant Pathology* **47 (6):** 743-748.

Ayers G.W. 1944. Studies on the history of the clubroot organism, *Plasmodiophora brassicae*. *Canadian Journal of Research Series B* **22**:143-149.

Baker R. 1986. Biological Control – An overview. *Canadian Journal of Plant Pathology 8: 218-*

Bangerth F. 1979. Calcium related physiological disorders of plants. *Annual Review of Phytopathology* **17**:97-122.

Barber S.A and Ozanne P.G. 1970. Autoradiographic evidence for the differential effect of four plant species in altering the

calcium content of the rhizosphere soil. *Soil Sci. Am. Proc.* **34**: 635 - 637.

Baydoun E. A. H and Northcote D.H. 1981. The extraction from maize (*Zea mays*) root cells of membrane bound protein with Ca^{2+} dependent ATPase activity and its possible role in membrane fusion in vitro. *Biochem. J.* **193**: 781-792.

Benhamou N, Lafontaine P.J, Nicole M. 1994. Induction of systemic resistance to fusarium crown and root rot in tomato plants by a seed treatment with chitosan. *Phytopathology* **84 (12)**: 1432-1444.

Bjorkman O and Demming B. 1987. Photon yield of O2 eveolution and chlorophyll fluorescence characteristics at 77k among vascular plants of diverse origin. *Planta* **170**: 489-504

Bolhar-Nordenkampf H.R, Long S.P, Baker N.R, Ouquist G, Schreiber U and Lechner E.G. 1989. Chlorophyll fluorescence as a probe of the photosynthetic competence of leaves in the field: a review of current instrumentation. *Functional ecology* **3**:497-514.

Blunden G. 1991. Agricultural uses of seaweeds and seaweed extracts. In *Seaweed Resources in Europe: Uses and Potential* (Edited by M.D Guiry and G. Blunden). John Wiley and sons Ltd. pp 65-81.

Blunden G and Wildgoose P.B. 1977. The effects of aqueos seaweed extract and kinetin on potato yields. *Journal of Science, Food and Agriculture* **28**: 121-125.

Boehm M.J and Hoitink H.A. 1992. Sustenance of microbial activity in potting mixes and its impact on the severity of Pythium root rot of Poinsettia. *Phytopathology* **82** : 259-264.

Braselton J. P. 1995. Current status of the Plasmodiophorids. *Critical reviews in microbiology* **21**: 263-275.

Bremer H. 1924. Untersuchungen uuber Biologie und Bekämpfung des Erregers der Kohl hernie *Plasmodiophora brassicae* woronin. 2 Mitt. Kohlhernie und Bodenaziitae. *Landw. Jb* **59**: 673-685.

Broadbent D. and Baker K.F. 1974. Behaviour of Phytophthora cinnamomi in soils suppressive and conducive to root rot. Australian journal of agricultural Research **25**: 121-127.

Brokenshire T, Channon A.G, Wale S. 1984. Recognising oilseed rape diseases. *Publication No. 185. Scottish Agricultural College.*

Buczaki S.T. 1988. *Plasmodiophora brassicae* Woronin. In *The European Handbook of Plant Diseases*. Eds. Smith I, Archer S.A, Dunez J, Lelliot R & Phillips D.H. Blackwell scientific Publications Ltd, Oxford. pp 243-245.

Buczaki S.T. 1983a. Plasmodiophora : An inter-relationship between biological and practical problems. *In: Zoosporic Plant Pathogens – A Modern Perspective*. Ed. Buczaki S.T. Academic press pp161-192.

Buczaki S T 1983b. *Tests of Agrochemicals and Cultivars No.4 (Annals of Applied Biology 102 Supplement)* pp 48-49.

Buczaki S T. 1977. Root infections from single resting spores of Plasmodiophora brassicae. *Transactions of the British Mycological Society* **69**: 328.

Buczaki S.T, Clay C.M. 1984. Some observations on secondary zoospore development in *Plasmodiophora brassicae*. *Transactions of the British Mycological Society* **82**:339-342.

Buczaki S T, Stevenson K. 1981. Clubroot Research report pp75 In report of the National Vegetable Research Station for 1980.

Buczaki S.T, Moxham S.E, Turner R.H. 1979. Some morphological features of the resting spore of *Plasmodiophora brassicae*. *Transactions of the British Mycological Society* **73**: 343-376.

Buczaki S.T, Moxham S.E. 1980. Karyogamy in Plasmodiophora brassicae. *Transactions of the British Mycological Society, 75:439-444*

Buczaki S.T, White , Moxham S.E. 1977. Fungicidal control measures. Nat*ional Vegetable Research Station Report* **pp 99-100**

Buczaki S.T, Toxopeus H, Mattusch P, Johnston T.D, Dixon, G.R, Holbooth L.A. 1975. Study of the physiological specialisation in Plasmodiophora brassicae: Proposals for attempted rationalisation through an international approach. *Transactions of the British Mycological Society* **65**: 295-303.

Burge M.N, Isaac I. 1977. Predisposition of Aster to *Phialophora* wilt. *Annals of Applied Biology* **86**: 353-358.

Butcher, D.N, El-Tigani S, Ingram D.S. 1974. The role of indole glucosinolates in the clubroot disease of the cruciferae. *Physiological Plant Pathology* **4**:127-141.

Carlile W.R. 1988. *Control of Crop Diseases*. Edward Arnold, London. pp 18-19

Castlebury L.A, Maddox J.V, Glawe D.A. 1994. A technique for the extraction and purification of viable Plasmodiophora brassicae resting spores from host root tissue. *Mycologia* **86**: 458-460.

Cameron R.F.1993. Low temperature stress in woody perennials with special reference to Rhododendron. PhD Thesis, University of Strathclyde.

Campbell R. 1989. Biological control of microbial plant pathogens. Cambridge University Press, UK.

Campbell R.1994. Biological control of soil-borne diseases: some present problems and different approaches. *Crop protection* **13:** 4-13.

Campbell A.G, Greathead A.S, Myers D.F, de Boer G.J. 1985. Factors related to control of clubroot of crucifers in the Salinas Valley of California. *Phytopathology* **75**: 665-670.

Cannon A.G. 1959. Clubroot of brassicas. *Annual Report of the National Vegetable Research Station for 1958*, pp 37

Chellemi D.O, Rhodas F.M, Olson S.M and Rich J.R. 1998. Tomato production using minimum tillage in Bahiagrass pasture as an alternative to fumigation with methyl bromidefor soil disinfestation. *International Congress of Plant Pathology 98, Abstracts*. **3:5.1.9.**

Cogram K.J. 1994. The effects of seaweed extracts on soilborne diseases, soil microbiology and growth of wheat. PhD Thesis, *University of Bristol.*

Colhoun J. 1958. Clubroot disease of crucifers caused by *Plasmodiophora brassicae* Woron: A monograph. The Commonwealth Mycological Institute, Kew Surrey.

Colhoun J. 1953b. observations on the incidence of clubroot disease in limed soils in relation to temperature . *Annals of Applied Biology* **40:** 639-644.

Colhoun J. 1953b. A study of the epidemiology of clubroot disease of brassicae. *Annals of Applied Biology* **40**: 262-282.

Conway W.S and Sams C.E. 1983. Calcium infiltration of Golden Delicious apples and its effect on decay. *Phytopathology* **73**: 1068-1071.

Cook R.J and Baker K.F. 1983. The Nature and Practice of Biological Control of Plant Pathogens, St Paul Minnesota: American Phytopathological Society pp 539.

Cook R.J and Reis E. 1981. Control of soil-borne pathogens of wheat in the Pacific North-West of the U.S.A. In : *Strategies for the control of cereal disease, Eds Jenkyn J.F and Plumb R.T. Blackwell Scientific Publications. Oxford.* pp 170-173.

Cook W.R and Schwartz M.A. 1929. The life history, cytology and method of infection of *Plasmodiophora brassicae* Woron, the cause of Finger and Toe disease of cabbages and other crucifers. *Philosophical Transactions of the Royal Society of London Series B Biological sciences* **218**: 283-314

Craig M.A, Dixon G.R. 1993. The influence of boron on the growth of *Plasmodiophora brassicae* (Clubroot) in summer cabbage. *Proceedings of the Crop Protection in Northern Britain*, Dundee. pp 277-282.

Crouch I.J and van Staden J. 1991. Evidence for rooting factors in a seaweed concentrate prepared from *Ecklonia maxima. Journal of Plant Physiology* **137**: 319-322.

Crute I.R, Gray A.R, Crisp P, Buczacki S.T. 1980. Variation in *Plasmodiophora Brassicae* and resistance to clubroot disease in *brassicas* and allied crops-a critical review. *Plant Breeding Abstracts* **50**: 91-103.

Deidrechessen E, Sacristan M.D. 1996. Disease response of resynthesized *Brassica napus L.* lines carrying different combinations of resistance to *Plasmodiophora brassicae* Wor. *Plant Breeding* **115**: 5-10.

Dekhuijzen H.M. 1979. Electron microscope studies on the root hairs and cortex of a susceptible and resistant variety of *Brassica campestris* infected with *Plasmodiophora brassicae. Netherlands journal of Plant Pathology* **85**:1-17.

Dekhuijzen H.M and Overeem J.C. 1971. The role of cytokinins in clubroot formation. *Physiological Plant Pathology* **1**: 151-62.

Delacruz J, Rey M, Lora J.M, Hidalgogallego A, Dominguez F, Pintotoro J.A, Llobell, Benitez T. 1993. Carbon source control of Beta Glucanases, Chitobiase and Chitinase from *Trichoderma harzianum. Archives of Microbiology* **159 (4):** 316-322.

Delp C.J, Klopping H.L. 1968. Performance attributes of a new fungicide and mite ovicide candidate. *Plant Disease Reporter* **52**:95-99.

Demarty M, Morvan C and Thellier M. 1984. Calcium and the cell wall. *Plant Cell and the Environment* **7**: 441-448.

Dhingra O.D, Sinclair J.B. 1985. Basic Plant Pathology Methods. CRC Press.

Dixon G.R. 1991. Repression of the morphogenesis of Plasmodiophora brassicae Wor. by Boron – a review. *Acta Horticulturae* **407** : 393-401.

Dixon G.R. 1984. Galls caused by fungi and bacteria. In *Plant Diseases, Infection, Damage and Loss,* Eds. R Wood and G J Ellis . Blackwell Scientific Publications, Oxford pp 189-197

Dixon G.R. 1981. Vegetable crop diseases. Macmillan Publishers Ltd pp 137-143

Dixon G.R, Thomas J.E, Kenyon D.M, Davies J and Oxley S.J.P (1998). Flusulfamide: New oppurtunities for the control of clubroot (Plasmodiophora brassicae). *7th International Congress of Plant Pathology, Edinburgh* Vol 3. 5.6.6.

Dixon G.R, Webster M.A. 1988. Antagonistic effects of boron, Calcium and pH on pathogenesis caused by *Plasmodiophora brassicae* Woronin (Clubroot) – A review of recent work. *Crop Protection in Northern Britain Conference*, Dundee. pp 399-404.

Dixon G.R, Williamson C.J. 1984. Factors affecting the use of calcium cyanamide for control *of Plasmodiophora brassicae.*

Proceedings of better Brassicas 1984 conference, St Andrews, September 1984, pp 238-244.

Dixon G.R, Wilson F. 1985. Fungicides applied to propagation blocks and the field to control *Plasmodiophora brassicae*, clubroot, on cabbage. *Tests of Agrochemicals and Cultivars No. 6: (Ann. Appli. Biol. 102, suppl), pp 44-45*

Dixon G.R, Wilson F. 1984a. Field evaluation of WL105 305 (NK483) for control of clubroot (*Plasmodiophora brassicae*). *Tests of agrochemicals and cultivars No.5, (Ann. appli. Biol. 104, suppl.), pp 34-35.*

Dixon G.R, Wilson F. 1984b. Field evaluation of chemicals for control of clubroot of clubroot *Plasmodiophora brassicae. Proceedings of Crop Protection in Northern Britain Conference, Dundee,* pp 400-405.

Dixon G.R, Wilson F. 1983. Evaluation of calcium cyanamide for control of *Plasmodiophora brassicae* (clubroot). *Tests of agrochemicals an cultivars No.4, (Ann. appli. Biol. 102, suppl.), pp 50-51.*

Djatnika I. 1991. Prospects of biological control of clubroot *cruciferae newsletter* 14/15 eucarpia.

Dobson G.R, Gabrielson R.L, Baker A.S. 1983. Effects of lime particle size and distribution and fertiliser formulation on clubroot disease caused by *Plasmodiophora brassicae. Plant Disease* **67**: 50-52.

Donaldson S.P and Deacon J.W. 1993. Changes in motility of *Pythium* zoospores induced by calcium and calcium-modulating drugs. *Mycological Research* **97 (7)**: 877-883.

Duffy B.K and Weller D.M. 1995. Use of Gaeumannomyces gramminis var graminis alolne and in combination with Fluorescent Pseudomonads spp. To suppress Take All of Wheat. *Plant Disease* **79** (9): 907-911.

Einhorn G, Bochow H, Huber J, Krebs B. 1991. Methodological studies to detect antagonists of the clubroot pathogen, *Plasmodiophora brassicae* Wor. Archiv fur *Phytopathologie und Pflanzenschutz – Archives of Phytopathology and Plant Protection* **27** (3): 205-208.

Ekeberg E, Riley H.C.F. 1997. Tillage intensity effects on soil properties and crop yields in a long term trial on moranic loam soil in SE Norway. *Soil and Tillage Research* **42**:277-293.

Elad Y. 1988. Ultrastructural scanning electron microscopy study of parasitism of *Botrytis cinnerea* Pers. On flowers and fruits of cucumber. *Transactions of the British Mycological Society* **91**: 185 - 190.

Elad Y, Yunis H, Volpin H. 1993. Effect of nutrition on susceptibility of cucumber, eggplant and pepper crops *to Botrytis cinnerea. Canadian Journal of Botany* **71**: 602-608.

Ellis S. 1999. The Grower. 19th August pg 22.

Ellis S.A, Hallam M.L, Ottway C.J and Winters D.1995. An evaluation of a chitin based fertiliser against potato cyst nematode, cabbage root fly and *Rhizoctonia solani. BCPC : symposium Proceedings* **63:** 203-208.

Elsherif M and Grossman F. 1991. Investigations on biological control of some plant pathogenic fungi by fluorescent Psuedomonads using different methods of application. *Zeitschrift fur Pflanzenkrankheiten und Pflanzenschutz - Journal of Plant Diseases and Protection* **98**(3): 236-249.

Engelhard A.W. 1996. Soilborne Plant Pathogens: Management of Diseases with Macro - and Micro - elements. APS Press. The American Phytopathological Society. Minnesota.

Evans J.L and Scholes J.D. 1995. How does clubroot alter the regulation of carbon metabolism in its host? *Aspects of Applied Biology* **42**: 125-132.

Finnie J.F and vanStaden J. 1985. The effect of seaweed concentrate and applied hormones on in vitro cultured tomato roots. *Journal of Plant Physiology* **120**: 215-222.

Fletcher J.T, Hims M.J, Archer F.C and Brown A. 1982. Effects of adding calcium and sodium salts to field soils on the incidence of clubroot. *Annals of Applied Biology* **100**: 245-251.

Gabrielson R.L. 1979. Clubroot control in crucifers with hydrated lime and PCNB. . *Proceedings of Woronin +100 Conference 1977.* University of Wisconsin-Madison College of Agriculture and Life sciences pp 145.

Garrett S.D. 1965. Towards biological control of soil borne pathogens. (Editors K.F. Baker & W.C Snyder) pp4-15, John Murray, London.

Gilbert G.S, Handelsman J and Parke J.L, 1994, Root Camouflage and Disease Control. *Phytopathology*, **84:** 222-225.

Graham R.D. 1983. Effects of nutrient stress on susceptibility of plants to disease with particular reference to the trace elements. *Advances in Botanical Research* **10:** 221-276.

Gorbatenko I.Y, Onishchuk I.A, Krivtsov G.G, Vanyushin B.F. 1996. Eliciting and growth regulating effects of chitosan on plants. *Izvestiya Akademii Na auk Seriya Biologicheskaya* No.4 pp 402-405 (Bids Abstract).

Gorodecki B and Hadar Y. 1990. Suppression *of Rhizoctonia solani* and *sclerotium rolfsii* diseases in container media containing composted grape marc. *Crop Protection* **9:**271-274.

Gustaffson M, Liljeroth E, Gunarsson M, Lundborg T. 1986. Effects of infection by *Plasmodiophora brassicae* on root anatomy of rape. *Journal of Phytopathology* **117**: 144-151.

Hadar Y and Mandlebaum R. 1992. Suppressive compost for biocontrol of soilborne plant pathogens. *Phytoparasitica* **20 (Suppl):**113-115.

Hadar Y and Mandlebaum R. 1986. Suppression of Pythium aphanidermatum damping off in container media containing composted liqourice roots. *Crop Protection* **5**: 88-92.

Hamilton H A and Crête R. 1978. Influence of soil moisture, soil pH and liming sources on the incidence of clubroot, the germination & growth of cabbage produced in mineral and organic soils under controlled conditions. *Canadian Journal of Plant Science* **58**: 45-53.

Hanseler C.M. 1937. Control of clubroot of crucifers. *Phytopathology* **27**:130.

Hedges S. 1996. Compost is a natural-born killer. New Scientist 21st September pp25.

Hepler P.K and Wayne R.O. 1985. Calcium and Plant Development. *Annual Review of Plant Physiology* **36**: 397-439.

Hildebrand P.D and McRae K.D. 1998. Control of clubroot caused by *Plasmodiophora brassicae* with non-ionic surfactants. *Canadian Journal of Plant Pathology - Revue Canadienne De Phytopathologie* **20 (1)**:1-11.

Horiuchi S, Hori M, Takashi S, Shimizu K. 1982. Factors responsible for the development of clubroot suppressing effect of soil solarization. *Bulletin of the Chigoku National Agricultural Experiment Station.* No. 20 pp 48

Hornby D. 1979. Take all decline: a theorists paradise. In: Soil-borne plant pathogens, B. Schippers &M.W. Gams (Eds), London Academic Press pp 133-156.

Hseih W.H, Wang J.1986. Investigation on suppressive soils of clubroot crucifers in Taiwan. Plant protection Bulletin (Taiwan ROC) **28:**353-362.

Huber D.M. 1996. The role of nutrition in the Take-All disease of wheat and other small grains. *Soilborne Plant Pathogens: In Management of Diseases with Macro and microelements.* Ed Engelhard A.W. pp 46-74. APS press The American Phytopathological society. Minnesota.

Huber D. M. 1980. The role of Mineral Nutrition in Defense In: *Plant Disease Vol. 5.* Eds. Horsfall J.G, and Cowling E.B. Academic Press N.Y. pp 381-406.

Humpherson –Jones F.M. 1993. Effect of surfactants and fungicides on clubroot *(Plasmodiophora brassicae)* of brassicas. *Annals of Applied Biology* **122**: 457-465.

Humpherson-Jones F.M, Dixon G.R, Craig M.A and Ann D.M. 1992. Control of clubroot using calcium cyanamide – A review. *Brighton Crop Protection Conference – Pests and Diseases* pp1147-1154.

Il'ina M.N and Shekunova. 1981. The effect of chemical substances on cabbage yield and on the limitation of the development of clubroot (trudy Vsesoyuznogo Nauchno-Isseledovatel'skogo). Instituta Zashchity Rastenii Leningrad. Lenin *Acad. Sci 74-79. Res. Inst. Pl. Prot* seen in Review of Plant Pathology 62: 1253 (1983).

Inbar J and Chet I. 1991. Evidence that chitinase produced by *Aeromonas caviae* is involved in the biological control of soil borne plant pathogens by this bacterium. *Soil Biology and Biochemistry* **23** (10) : 973-978.

Ingram D.S. 1969. Growth of *Plasmodiophora brassicae* in host callus. *Journal of General Microbiology* **56**: 55-67.

Ingram D.S and Tommerup I.C. 1972. The life history of *Plasmodiophora brassicae* Woron. Proceedings of the Royal Society of London series B **180**: 103-112.

Irving H.R, Grifith J.M, Grant B.R. 1984. Calcium efflux associated with encystment of *Phytophthora palmivora* zoospores. *Cell Calcium* **5**: 487-500.

Iser J.R, Griffith J.M, Balson R, Grant B.R. 1989. Accelerated ion fluxes during differentiation in zoospores of *Phytophthora palmivora*. *Cell Development and Differentiation* **26**: 29-38.

Jacobsen B.J and Williams P.H. 1969. Control of cabbage clubroot using benlate. *Phyopathology* **59**:1033 (Abstract).

Jaw-Fen W, Wen-Hsui. 1986. Studies on the suppressive factors and characteristics of suppressive soils of clubroot in crucifers. *Plant Protection Bulletin (Taiwan ROC)* **28**:363-370.

Jones D.R. 1981. Differential pathogenicity pf *Plasmodiophora brassicae* Wor. *PhD Thesis, University of Cambridge*.

Jones D.R, Ingram D.S and Dixon G.R. 1982a. Characterization of isolates derived from single resting spores of *Plasmodiophora brassicae* and studies of their interactions. *Plant Pathology* **31:**239-245

Jones D.R, Ingram D.S and Dixon G.R. 1982b. Factors affecting tests for differentials pathogenicity in populations of *Plasmodiophora brassicae*. *Plant Pathology* **31:** 229-238.

Jones D.G and Clifford C. 1978. Cereal diseases their pathology and control BASF. Hadleigh. pp 132-135.

Jones J.P, Engelheard A.W, Woltz S.S. 1996. Management of Fusarium wilt of vegetables and ornamentals by macro and microelement nutrition. *Soilborne Plant Pathogens: In Management of Diseases with Macro and microelements.* Ed Engelhard A.W. pp

18-32. APS press The American Phytopathological society. Minnesota.

Kai H, Veda T, Sakaguchi M.1990, Antimicrobial activity of bark compost extracts. *Soil Biology and Biochemistry* **22:** 983-986.

Karling J.S. 1968. The *Plasmodiophorales* 2nd edition. Halfner Publishing Group. New York, London.

Katznelson H, and Rouatt J.W (1957). Manometric studies with the rhizosphere and non-rhizosphere soil. *Canadian Journal of Microbiology* **3**: 104-118.

Kelman A, Mc Guire R.G, Tzeng K. 1996. Reducing the severity of bacterial soft rot by increasing the concentration of calcium in potato tubers. *In soil borne plant pathogens: Management of Diseases with Macro and microelements.* Ed Engelhard A.W. pp 102-123. APS Press The American Phytopathological Society. Minnesota.

Keniath A.P, Loria R. 1996. Soilborne Plant Pathogens: Management of common scab of potato with plant nutrients. *In soil borne plant pathogens: Management of Diseases with Macro and microelements.* Ed Engelhard A.W. pp 152-166. APS Press The American Phytopathological Society. Minnesota.

Khalifa O. 1965. Biological control of *Fusarium* wilt of peas by organic soil amendments. *Annals of Applied Biology* **56**: 129-137.

Kindshoven J. 1928. Entsenchung des Bodens und Bekämpfung der Kohlhernie mit Kalkstickstoff. *Mitteilungen Deutschen LanndwirtschaftsGesellschaft* **43**: 522-523.

Klase H.J. 1996. Calcium cyanamide – An effective tool to control clubroot – A review. *Acta Hort.* **407**: 403-409.

Kunkel L.O. 1918. Tissue invasion by *Plasmodiophora brassicae. Journal of Agricultural Research* **14:** 543-572.

Kroll T.K, Lacey G.H Moore L.D. 1984. Root surface inhabiting bacteria and the expression of clubroot of radish in controlled environments. *Soil Biol. Biochem.* **16(3):** 265-267.

Lampkin N. 1990. The principles of organic farming. Farming press books. Ipswich. pp 221-227.

Larkin R.P, Hopkins D.L, Martin F.N.1996. Suppression of Fusarium Wilt of Watermelon by Non-pathogenic *Fusarium oxysporum* and other Microorganisms Recovered from a Disease suppressive soil. *Phytopathology* **86**:812-819.

Lennartsson M. 1988. Effects of organic soil ammendments and mixed species cropping on take all disease of wheat. In: Global Perspectiveson Agroecology and Sustainable Agricultural Systems Vol.2, P. Allen & D. van Drusen (Eds), Santa Cruz, California: University of California pp 575-580a.

Lewis D. H. 1980. Boron, lignification and the origin of vascular plants – a unified hypothesis. *New Phytologist* **84**: 209-229.

Liu D, Anderson N.A, Kinkel L.1995. Biological control of potato scab in the field with Antagonistic Streptomyces scabies. Phytopathology 85: 827-831.

Lorang J.M, Anderson N.A, Lauer F.I and Wildung D.K.1989. Disease decline in a minnesota potato scab plot. *American Potato Journal* **66**:531.

Lumsden R.D, Lewis J.A,.Papavizas G.C.1983. Effect of organic matter on soilborne plant diseases and pathogen antagonists. In: Environmentally Sound Agriculture, W. Lockeretz (Ed), New York; Praeger Press pp 51-70.

Macfarlane I. 1952. Factors affecting the survival of *Plasmodiophora brassicae* Wor. in the soil and its assessment by host test. *Annals of Applied Biology* **39**: 239-256.

Macfarlane I. 1955. Variation in *Plasmodiophora brassicae* Woron. *Annals of Applied Biology* **43**: 297-306.

Macfarlane I. 1970. Germination of resting spores of Plasmodiophora brassicae. *Transactions of the British Mycological Society* **55**:97-112.

Mattusch P. 1979. Chemical control of *Plasmodiophora brassicae* with a calcium cyanamide fertilizer under field conditions. *Proceedings of Woronin +100 Conference 1977.* University of Wisconsin-Madison College of Agriculture and Life sciences, pp139-141.

McQuilken M.P.1995. Promoting Natural Biological Control of Soil-borne Plant Pathogens. *BCPC Symposium Proceedings No. 63: Intergrated Crop Protection: Towards Sustainability ?* pp 59-66.

Mehorta R.S. 1980. Galls and abnormal growth. In: *Plant Pathology*. Tate McGraw-Hill Publishing Company Limited, New Delhi pp 565-567.

Menzies J.D.1959. Occurrence and transfer of a biological factor in soil that suppresses potato scab. *Phytopathology* **49**:648-653.

Millard A.W and Taylor C.B. 1927. Antagonism of microorganisms as the controlling factor in the inhibition of scab by green manuring. *Annals of Applied Biology* **14:**202-216.

Miller C. 1979. Incidence and control of *Plasmodiophora brassicae* in Malaysia. *Proceedings of Woronin +100 Conference 1977.* University of Wisconsin-Madison College of Agriculture and Life sciences pp142-143.

Mitani S, Araki S, Matsuo N. 1998. IKF- A novel systemic fungicide for the control of oomycete plant diseases. *The 1998 Brighton Conference- Pests and Diseases* pp 351-358.

Mithen R, Magrath R. 1992. A contribution to the life history of Plasmodiophora brassicae secondary plasmodia development in root galls of *Arabidopsis thaliana. Mycological Research* **96**: 877-885.

Monteith J. 1924. Relation of soil temperature and soil moisture to infection by *Plasmodiophora brassicae. Journal of Agricultural Research* **28**: 549-559.

Muchovej J.J, Muchovej R.M.C, Dhingra O.D and Maffia L.A. 1980. Supression of anthracnose of soybeans by calcium. *Plant Disease* **64** (12): 1088-1089.

Murakami H, Tsushima S, Akimoto T, Murakami K, Goto I, Shishido Y. 2000. Effects of growing leafy daikon (Raphanus sativus) on populations of *Plasmodiophora brassicae* (clubroot). *Plant Pathology* 49 (5): 584-589

Murakami H, Tsushima , Shishido Y. 1997. Factors associated with the suppressiveness of soil to clubroot disease of Chinese cabbage. Agronomy abstracts 1997 annual meetings pp218.

Myers D F, Campbell R N. 1985. Lime and the control of clubroot of crucifers: Effects of pH, calcium, magnesium and their interactions. *Phytopathology* **75**: 670-673

Naiki T, Dixon G.R. 1987. The effects of chemicals on developmental stages of *Plasmodiophora brassicae* (clubroot). *Plant Pathology* **36:** 316-327.

Narisawa K, Tokumasu S, Hashiba T. 1998. Suppression of clubroot formation in Chinese cabbage by the root endophytic gungus Heteroconium chaetospira. *Plant pathology* **47**:206-210.

Naumov N.A. 1925. Contribution to the knowledge of club root of cabbage (In Russian) *Morbi Plant* **14**: 49-73. Seen in Colhoun J. 1958. Clubroot disease of crucifers caused by *Plasmodiophora brassicae* Woron: A monograph. The Commonwealth Mycological Institute, Kew Surrey.

O' Brien D.G and Dennis R.W.G. 1936. Further information relating to control of raan in swedes. *Scottish Journal of Agriculture* **19**: 1-18.

Odunfa V.S.A and Oso B.A. 1979. Fungal populations in the rhizosphere and rhizoplane of cowpea. *Transactions of the British Mycological Society* **73**: 21-26.

Ohmori K, Nakagawa T. and Koike K. 1982. New salicylamide derivatives as soil fungicide. *Fifth International Congress of Pesticide Chemistry, Kyoto Japan*. Abstract No. IIb-13.

Ordentlich A, Migheli Q, Chet I. 1991. Biological control activity of 3 Trichoderma isolates against Fusarium wilt of cotton and muskmelon and lack of correlation with their lytic enzymes. *Journal of Phytopathology* **133 (3):** 177-186.

Palm E.T. 1963. Effect of mineral nutrition on the invasion and response of turnip tissue to *Plasmodiophora brassicae Wor. Contr. Boyce Thompson Inst* **22**: 91-112.

Parry D.W. 1990. Plant Pathology in Agriculture, Cambridge University Press. Cambridge. pp 92-96, 129 & 146.

Pidoplichko N.M, Moskovets V.S. Zhdanova N.M. 1965. Influence of some fungi fungi from the maize rhizosphere on the growth of its seedlings. In plant Microbes Relationships (ed. I.

Mancura and V. Mancura pp 220-227. Prague: Checoslovak Academy of Sciences Publishing house.

Porter I.J, Donald E.C, Cross S.J. 1998. Field evaluation of fluazinam against clubroot (*Plasmodiophora brassicae*) of cruciferous vegetable crops. Annals of Applied Biology **132:** 12-13, (Suppl. S Jun).

Porter I.J, Merriman P.R and Keane P.J. 1991 Soil solarisation combined with low rates of soil fumigants controls clubroot of cauliflowers caused by Plasmodiophora brassicae. *Australian Journal of Experimental Agriculture*. 31 (6): 843-851 1991

Porter I.J and Merriman P.R. 1985. Evaluation of soil solarisation for control of clubroot of crucifers and white rot of onions in south eastern Australia. In: *Ecology and management of soilborne plant pathogens.* Edited by Parker C A, Rovira A D, Moore K J and Wong P T W. The American Phytopathological Society pp 282-284.

Punja Z.K. 1996. Influence of nitrogen and calcium compounds on development of disease of disease due to *Sclerotium rolfsii. Soilborne Plant Pathogens: In Management of Diseases with Macro and microelements.* Ed Engelhard A.W. pp 75-89. APS press The American Phytopathological society. Minnesota.

Punja Z.K and Zhang Y.Y. 1993. Plant chitinases and their roles in resistance to fungal diseases. *Journal of nematology* **25(4):** 526-540.

Raaijmakers J.M, Leeman M, van Oorschot M.M.P, van der Sluis I, Schippers B, Bakker P.A.H.M. 1995. Dose-response Relationships in Biological Control of Fusarium Wilt of Radish by *Pseudomonas* spp. *Phytopathology* 85:1075-1081.

Raviv, M, Reuveni, R, Zaidman, BZ. 1998. Improved medium for organic transplants. Biological Agriculture and Horticulture, **16**: 12

Renard P. 1935. Une maladie du chou: la hernieou gros pied. *Vie Agricole Rurale, Paris* **24**:167-169.

Renwick A, Campbell R and Coe S. 1991. Assessment of in vivo screening for potential biocontrol agents of *Gaeumannomyces gramminis. Plant Pathology* **40**: 524-532.

Ressignol M, Lamant D, Salsac L and Heller R. 1977. Calcium fixation by the roots of calcicole and calcifuge plants: The importance of membrane systems and their lipid composition. *In transmembrane ionic exchange in plants. Eds Thellier M, monnier, A, Demarty M and Dainty J. Éditions du CNRS. Paris, et Éditions de l' Universitè, Rouen.* pp 483-490.

Rhode G. 1952. Kohlhernie und Bor. D'tsch. *Landw* 3: 642-646. Seen In Dixon (1991).

Rhodes D.J, Logan C, Gross D.C. 1986. Selectionof Pseudomonas spp. inhibitory to potato seed tuber decay. *Phytopathology* **10:** 1078-1078.

Robak J. 1979. Fungicidal control of clubroot on muck soil in Poland. *Proceedings of Woronin +100 Conference 1977.* University of Wisconsin-Madison College of Agriculture and Life sciences pp 144.

Roux S.J and Slocum R.D. 1982. Role of calcium in mediating cellular functions important for growth and development in higher plants. In: *Calcium and cell function vol.3* Ed. Cheung W.Y, New York Academic Press. **13**: 409-451.

Rouxel F. 1984. Studies on soil receptivity to clubroot. In: Methods for studying the biological control of diseases caused by soilborne pathogens. British Society for Plant Pathology Anglo-Franch workshop, Rothamstead Experimental Station 12-13 September 1984.

Rouxel F. 1991. Natural suppressiveness of soils to plant diseases. In Biotic Interactions and Soil-borne diseases, A.B.R Beemster, G.L Bollen, M Gerlag, M.A Ruissen, B. Schipperfs and A. Tempel (Eds), Amsterdam: elselvier pp 287-296.

Rouxel F, Briard M, Lejeune B. (1988) Studies of soil receptiveness to clubroot caused by *Plasmodiophora brassicae*: Experiments on reponses of a series of vegetable soils in Brittany. *Progress on Pest Management in Field Vegetables*: Proceedings CEC-IOBC Expert Group Meeting, Rennes 20-22 Nov 1985. pp 145-152.

Samuel G, Garrett S.D. 1945. The infected root hair count for estimating the activity of *Plasmodiophora brassicae* Woron. in the soil. *Annals of Applied Biology* **32**: 96-101.

Sanders D.H. 1990. Statistics a fresh approach. Chapter 3 pp 98.McGraw-Hill book company. Singapore.

Sarathchandra S.U, Watson R.N, Cox N.R, diMenna M.E, Brown J.A, Burch G, Neville F.J. 1996. Effects of chitin amendment of soil on micro-organisms, nematodes, and growth of white clover (Treifolium repens) and perennial ryegrass (Lolium perenne). *Biology and Fertility of Soils* **122 (3):** 221-226.

Schüler C, Biala J, bruns C, Gottschall R, Ahlers S, Vogtmann H.1989. Suppression of root rot on peas, beans and beetroots caused by *Pythium ultimum* and *Rhizoctonia solani* through amendment of growing media with composted household waste. *Journal of Phytopathology* **127**: 227-238.

Seaman W.L, Larson R.H. 1963. A new race of *Plasmodiophora brassicae* affecting Badger Shipper Cabbage. *Phytopathology* **53**: 1426-1429.

Senn T.L and Skelton J. 1969. The effect of Norwegian seaweedon metabolic activity of certain plants. *Proceedings of the 6th International Seaweed Symposium* pp 724-730.

Shear C.B. 1975. Calcium related disorders of fruits and vegetables. *Horticultural Science* **10**: 361-65

Shimotori H, Yanagida H, Enomoto Y, Igarashi K,Yuoshinari M, Umemoto M. 1996. Evaluation of Benzenesulfonanilide derivatives for the control of crucifers clubroot. *Journal of Pesticide Science* **21** (1) :31-35.

Shirama K. 1955. Control of clubroot disease of cruciferous plants. *Agriculture and Horticulture* **30**:197-201.

Shirashi T. 1999. Supressor as a factor determining plant pathogen specificity. *In Plant Microbe Interactions vol 4. Chapter 6* pp 121-162. Eds Stacey G and Keen N T. American Phytopathological Society, St Paul, Minnesota, USA.

Simon E.W.1978. The symptoms of calcium deficiency in plants. *New Phytologist* **80:** 1-15.

Smeiton M.J. 1939. On the use of chlorinated nitrobenzenes for the control of clubroot disease of brassicae. *Journal of Pomology and Horticultural science* **17**:195-217.

Smiley R.W and Cook R.J. 1973. Relationship between take all of wheat and rhizosphere pH in soils fertilised with ammonium vs. nitrate nitrogen. *Phytopathology* **63:** 882-890.

Sneh B, Katan J, Henis Y. (1971). Mode of inhibition of rhizoctonia solani in chitin amended soil. *Phytopathology* **61 :** 1113-1117.

Slepyan E.I and Minina E.V. 1978. The structure of galls produced by plasmodiophora brassicae Wor. On the roots of head cabbage plants during the formation of resting spores at the end of the growing period. *Mikologiya I Fitopatologiya* **12:**512-516. Seen in *Review of Plant Pathology* 1979, **58:** abstract 5072

Stephens C.T and Stebbins T.C.1985. Control of damping off pathogens in soiless container media. *Plant Disease* **69:** 494-496.

Stephenson W.M. 1976. The effects of a seaweed extract on the yield of a variety of field and glasshouse crops. *Proceedings of the 8th International Seaweed Symposium* pp.740-744.

Stephenson W.M. 1968. Seaweed in Agriculture and Horticulture, Faber and Faber, London.

Stephenson W.M. 1965. The effect of hydrolysed seaweed on certain plant pests and diseases. *Proceedings of the 6th International Seaweed Symposium* pp. 405-415.

Subba Roa N.S, Bidwell R.G, Bailey D.L.1961. Effect of fungi on the uptake and metabolism of nutrients by tomato plants. *Canadian Journal of Botany* **39:**1759-1764.

Suzuki K, Sugimoto K, Hayashi H, Komyoij T. 1995. Biological mode of action of fluazinam, a new fungicide, for Chinese cabbage clubroot. *Ann. Phytopathol. Soc. Japan* **61:**395-409.

Suzuki K, Matsumiya E, Ueno Y, Mizutani J. 1992. Some properties of germination-stimulating factor from plants for resting spores of *Plasmodiophora brassicae*. Annals of the Phytopathological Society of Japan **58**: 699-705.

Takahashi K.1994. Biological Agents Affecting the viability of resting Spores of Plasmodiophora brassicae Wor. In soil without Host roots. *Annals of the Phytopathological Society of Japan* **60**: 667-674.

Takahashi K, Yamaguchi T. 1988. A method for assessing the pathogenic activity of resting spores of Plasmodiophora brassicae by fluorescence microscopy. Ann. Phytopath. Soc. Japan **54**: 466-475.

Tanaka S, Kochi S, Kunita H, Ito S Kameyaiwaki M. 1999. Biological mode of action of the fungicide, flusulfamide, against Plasmodiophora brassicae (clubroot). *European Journal of Plant Pathology* **105 (6):** 577-584.

Thulsen J and Lisbjerg B. 1992. Report on trials with calcium nitrate against clubroot. *Internal Hydro Agri Report. Norsk Hydro, Oslo, Norway.*

Thuma B.A, Rowe R.C, Madden L.V. 1983. Relationships of soil temperature and moisture to clubroot (*Plasmodiophora brassicae*) severity on Radish in organic soil. *Plant Disease* **67**:758-762.

Timonin M.I. 1940. The interaction of higher plants and sol micro-organisms II. Study of the microbial population of the of the rhizosphere in relation to resistance of plants to soilborne diseases. *Canadian journal of Research* **18:** 444-456.

Tommerup I.C, Ingram D.S. 1971. The life cycle of *Plasmodiophora brassicae* Woron. in brassica tissue cultures and intact roots. *New Phytologist* **70**:327-332.

Tsushima S, Murakami H, Kanno T. 1996. Soil suppressive to clubroot disease of chinese cabbage caused by *Plasmodiophora brassicae.* ***Phytopathology* S66:** abstract 582A.

Tsushima S, Murakami H, Shishido Y, Yoshida N, Nomura Y, Narisawa K. 1997. Biocontrol of Clubroot Disease of Chinese Cabbage by Endophytic fungi. Agronomy abstracts 1997 annual meetings pp218.

Utkina N.I, Nazarova L.P, Danilova T.A and Bazherova I.N. 1980. Effectiveness of chemical measures against clubroot and cabbage flies on swedes sowings. *Khimiya v. Sel'skom Khozaistve* 18:13-15. Seen in Review of Plant Pathology 60: 5163 (1981)

Venter, F. 1979. Modellversuche zur Bekämpfung der Kohlhernie. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* **86**(11): 667-669.

Volpin H and Elad Y. 1991. Influence of calcium nutrition on susceptibility of rose flowers to Botrytis blight. *Phytopathology* 81: 1390-1394.

Von Broembsen S.L and Deacon J.W. 1997. Calcium interference with zoospore biology and infectivity of *Phytophthora parasitica* in nutrient irrigation solutions. *Phytopathology* **87**: 522-528.

Von Broembsen S.L and Deacon J.W. 1996. Effects of calcium on germination and further zoospore release from zoospore cysts of *Phytophthora parasitica. Mycologocal Research* **100** (12): 1498-1504.

Voorips R.E. 1995. *Plasmodiophora brassicae*: aspects of pathogenesis and resistance in *Brassica oleracea. Euphytica* **83**: 139-146.

Vytisii V.A. 1979. Control of clubroot of crucifers. *Zashchita Rastenii* 6 : 34. Seen in Review of Plant Pathology **60**:1119 (1981).

Wallenmammar A.C. 1999. Monitoring and control of Plasmodiophora brassicae in Spring Oilseed Brassica Crops. *Doctoral thesis, Swedish University of Agricultural Sciences.*

Wallenmammar A.C. 1998.Pers. Comm. Conversation during a meeting of the Clubroot Working Group at ICPP 1998.

Wallenmammar A.C. 1996. Prevalence of *Plasmodiophora brassicae* in a spring oilseed rape growing area in central Sweden and factors influencing so8il infestation levels. *Plant Pathology* **45**:710-719.

Walker J.C, Larson R.H. 1935. Calcium cyanamide in relation to control of clubroot of cabbage. *Journal of Agricultural Research* **51**: 183-189.

Walsh U.F. 1997. The effects of *Ascophyllum nodosum* liquid seaweed extract on microbial growth, activity and pathogenicity. PhD Thesis University of Strathclyde.

Wang J, Hseih W. 1986. Studies on the suppressive factors and characteristics of suppressive soils of clubroot in crucifers. *Plant Protection Bulletin (Taiwan R.O.C)* **28**: 363-370.

Watson A.G. 1967. The movement of *Plasmodiophora brassicae* in soil. *Phytopathology* **57:** 835

Webb P.C.R. 1949. Zoosporangia believed to be those of *Plasmodiophora brassicae* in root hairs of no-cruciferous plants. *Nature* **163:** 608

Webster M.A. 1986. pH and nutritional effects on infection by *Plasmodiophora brassicae* Wor. and on clubroot symptoms. *PhD Thesis Aberdeen University.*

Webster M.A and Dixon G. R. 1991a. Boron, pH and inoculum concentration influencing colonization by *Plasmodiophora brassicae. Mycological Research* **95**(1): 74-79.

Webster M.A and Dixon G.R. 1991b. Calcium, pH and inoculum concentration influencing colonisation by *Plasmodiophora brassicae. Mycological Research* **95**: 64-73.

Weller D.M. 1988. Biological control of soilborne pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology* 26 : 39-407.

West P.M and Lochead A.G. 1940. The nutritional requirements of soil bacteria – A basis for determining the bacterial equilibrium of soils. *Soil Science* **50**: 409-420.

Wilczek C.A and Ng T.J.1982. Promotion of seed germination in table beet by an aqueous seaweed extract. *Horticultural science* **17**:629-630.

Williams P.H. 1966. A cytochemical study of hypertrophy in clubroot of cabbage. *Phytopathology* **56**: 521-524.

Williams P.H, Aist S.J, Aist J.R. 1971. Response of cabbage root hairs to infection by *Plasmodiophora brassicae. Canadian Journal of Botany* **49**: 41-47.

Williamson C.J. 1987. Assessment of resistance to *Plasmodiophora brassicae* in swedes. *Plant Pathology* **36:** 264-275.

Williamson C.J, Dyce P.E. 1989. The effect of calcium cyanamide on the reaction of swede cultivars to poulations of *Plasmodiophora brassicae. Plant Pathology* **38**: 230-238.

Wisniewski M, Droby S, Chalutz E and Eilam Y. 1995. Effects of Ca^{2+} and Mg^{2+} on *Botrytis cinnerea* and *Penicillium expansum* in vitro and on the biocontrol activity of *Candida oleophila*. *Plant Pathology* **44**: 1016-1024.

Workhu Y, Gerhrdson B. 1996. Supressiveness to clubroot, pea root rot and fusarium wilt in Swedish soils. *Journal of Phytopathology* **144**:143-146.

Woronin M.S. 1878. *Plasmodiophora brassicae. Urheber der kohlpflanzen-Hernie. Jb. Wiss.Bot.* **11:** 548-574. Translation: Chupp C. 1934. Plasmodiophora brassicae the cause of cabbage hernia *Phytopathological classics no. 4. American Phytopathological Society.*

Yano S, Tanaka S, Kameya-Iwaki M, Katumoto. 1991. Relation of Ca^{2+} efflux to germination of resting spores of clubroot fungus. *Bulletin of the Faculty of Agriculture, Yamaguchi University* **39**: 105-112.

Yoshikawa H, Buczaki S.T. 1978. Clubroot in Japan: research and problems. *Review of Plant Pathology* **57**: 253-257.

Zadoks J C, Schein R. 1979. Epidemiology and Plant Disease Management. Oxford University press. Oxford pp 325.

Zvára J, Rod J. 1967. Results of a field experiment of chemical extermination of plasmodiophora brassicae Wor. in brassica crops. *Sbornik Provozne-Ekonomické Faculty Vysoké Skoly Zemedelske v Ceských Budejovicich* **167** (11): 11-19.

Appendix One.

Soil Analyses

A1. Soil Analyses

Soil samples collected from the Auchincruive, Crail and Kings Kettle sites were sent to the Scottish Agricultural College in Edinburgh for analysis.

Table A1.1. Analysis of Auchincruive Crail, Kings Kettle and soil samples.

Determination	Auchincruive	Crail	Kings Kettle
pH	5.4	7.5	7.7
Extractable Phosphorous	22.8 mg/l	43.9 mg/l	44.7 mg/l
Extractable potassium	420 mg/l	251 mg/l	193 mg/l
Extractable magnesium	140 mg/l	482 mg/l	325 mg/l
Extractable calcium	1440 mg/l	3130 mg/l	3520 mg/l
Extractable sodium	25.8 mg/l	37.3 mg/l	11.2 mg/l
Extractable boron	1.1 mg/l	2.2 mg/l	2.0 mg/l
Organic matter	6%	4%	4%
Conductivity	8.41 CF	1.62 CF	1.35 CF
Total nitrogen	2430 mg/kg	1500 mg/kg	1620 mg/kg

Table A1.2. Analysis of autoclaved Auchincruive Crail, Kings Kettle and soil samples.

Determination	Auchincruive	Crail	Kings Kettle
pH	5.8	7.5	7.7
Extractable Phosphorous	17.5 mg/l	53.1 mg/l	43.6 mg/l
Extractable potassium	237 mg/l	232 mg/l	164 mg/l
Extractable magnesium	92 mg/l	393 mg/l	276 mg/l
Extractable calcium	1160 mg/l	25550 mg/l	3680 mg/l
Extractable sodium	41.7 mg/l	51 mg/l	33.1 mg/l
Extractable boron	0.7 mg/l	2.2 mg/l	1.7 mg/l
Organic matter	5%	4%	4%
Conductivity	2 CF	2.77 CF	2.64 CF
Total nitrogen	3.87 mg/kg	4.54 mg/kg	9.42 mg/kg

Appendix Two

Product Formulations

A 2. Product Formulations.

Table A2.1 Formulation of the PBI product Baby Bio

Ingredient	% w/w
Caustic potash 100 TW 49/50%	3.64
Monammonium phosphate	5.37
Potassium nitrate	1.01
Phosphoric acid 130 TW	1.98
Urea prills	21.41
Marinure 30	0.30

Information supplied by PBI Home & Garden

Table A2.2. Chemical analysis of calcified seaweed.

Element	Concentration in calcified seaweed (%)
Phosphorous	0.04%
Potassium	0.04%
Calcium Oxide	46.3%
Magnesium Oxide	4.8%
Sodium	0.65%
Sulphur	0.26%
Chloride	0.48%
Fluoride	
Iodine	31ppm
Selenium	2.8ppm
Zinc	109ppm
Copper	46ppm
Molybdenum	
Manganese	297ppm
Iron	
Boron	4ppm
Cobalt	25ppm
Bromine	3ppm
Calcium carbonate	
Magnesium carbonate	
Fluorine	0.09%
Nickel	1ppm
Arsenic	18ppm
Aluminium	0.15%

Information about calcified seaweed was obtained from the Cornish Calcified Seaweed Company.

Appendix Three.

Addresses of suppliers

A3. Addresses of suppliers.

Table A3.1. Names and addresses of growth media manufactures and suppliers.

Growth Media	Address of manufacturer	Supplier
John Innes No. 2 compost.	Wessex Horticultural Products, Wessex House, Units 1-3 Hilltop Business Park, Devizes Road, Salisbury, Wiltshire, UK. SP3 4UF	Sainsbury's Homebase
Coir compost	Homebase Ltd. Stamford House, Stamford Street, London SE1 9LL	Sainsbury's Homebase
Multipurpose compost	Homebase Ltd. Stamford House, Stamford Street, London SE1 9LL.	Sainsbury's Homebase, Ayr
Arthur Bower's Seed and Potting compost	William Sinclair Horticulture Ltd, Firth Rd, Lincoln. LN6 7AH	Daggs and Son, Glasgow
Levington's Multi – Purpose Compost	Levington Horticulture Paper Mill Lane, Bramford, Ipswich, Suffolk, England, IP8 4BZ	Daggs and Son, Glasgow
SHL Potting Compost	William Sinclair Horticulture Ltd, Firth Rd, Lincoln. LN6 7AH	Daggs and Son, Glasgow
Silvaperl Perlite	William Sinclair Horticulture Ltd, Firth Rd, Lincoln. LN6 7AH	Daggs and Son, Glasgow
Silvaperl Vermiculite	William Sinclair Horticulture Ltd, Firth Rd, Lincoln. LN6 7AH	Daggs and Son, Glasgow
Silvaperl Sharp Sand (3mm down)	William Sinclair Horticulture Ltd, Firth Rd, Lincoln. LN6 7AH	Daggs and Son, Glasgow

Other Suppliers

BDH-Merck – Chemicals, Autoclave and general equipment
The General Scientific Company Limited
Unit 2, 72 - 86 Garlands Road,
Redhill,
Surrey,
RH1 6NT,
UK.

Beckman centrifuge
Global Medical Instrumentation, Inc.
3874 Bridgewater Drive,
St. Paul, Minnesota, 55123 USA

Bibby Sterilin Ltd and Stuart Scientific :- Sterile Bijoux Bottles
Tilling Drive Stone,
Staffordshire,
ST15 0SA

Daggs Garden Suppliers :- Seed Trays, Gravel Trays, Growth Media,
Insecticides, and general supplies
Bath Street
Glasgow
Lanarkshire

Decon Labs Ltd :- Decon 90
Conway Street
Hove
E. Sussex
BN3 2LY

DuPont :- Ludox

Distributer =

Marlow Chemical Company Ltd,

Osprey House

Black Eagle Square

Westerham

Kent

TN16 1PA

Gallenkamp :- Incubaters, Colony Counter

Belton Road

West Loughborough

Leicestershire

LE11 OTR

Jenway :- pH meter

Gransmore Green

Felstead

Dunmow

Essex

CM6 3LB

LMS :- Cooled Incubater

The Modern Forge

Riverhead

Seven oaks

Kent

TN13 2EL

Nickel Electro Ltd :- Vortex mixer

Oldmixon Crescent

Weston Super Mare

BS24 9BL, UK

Nickersons Seeds
Joseph Nickerson Research Centre
Rothwell
Lincoln. LN7 2DR

Plantpak
Synprodo Plantpak Ltd:
Burnham Road,
Mundon,
Maldon,
Essex CM9 6NT

Primex Ingredients ASA
Karmsund Fiskerihavn
PO Box 114
N-4299 Avaldsnes
Norway

Scottish Agricultural College
Central Analytical Laboratory
West Mains Road
Edinburgh

Sigma-Aldrich :- Chemicals and General Supplies

Fancy Road
Poole
Dorset BH12 4QH

Stuart Scientific see Bibby Sterelin

Water bath, Flask Shaker.

USF Ltd :- Water De-Ioniser
Harford court
Foxholes Buisness Park
John Tate Road
Hertford
SC13 7NW

Ward

Wednesbury

West Midlands

BLANK IN ORIGINAL

Appendix 4

Experimental Methods Which Proved Unsuitable For Use With *P. brassicae* In This Research.

A.4. Experimental Methods Which Proved Unsuitable For Use With *P. brassicae* In This Research.

This appendix provides information on experimental methods that were attempted during the research programme but which proved to be either unsuitable for use in a particular experiment or failed to work in the way intended.

A4.1 Experiment to investigate the possibility of growing plants in agar inoculated with *P. brassicae* resting spores.

It would be a great advantage to the study of the early stages of *P. brassicae* infection if the host plants could be grown in agar. The agar could easily be removed from the roots and any residue would not obscure the field of vision in the same way as other growing media. A small scale experiment was set up to investigate whether it would be possible to grow plants in *P. brassicae* inoculated agar. An earlier experiment had been carried out where the spore suspension was mixed directly with the agar but this resulted in a high level of contamination by other fungal and bacterial pathogens.

In this experiment the *P. brassicae* resting spore suspension was prepared from galls which had been harvested before rotting of the galls had begun. The spore suspension from these galls was prepared using method described in A4.3.

The appropriate amount of each spore suspension was added to agar prepared with Hoaglands' solution to give final spore concentrations of 10^7 ml^{-1}. The agar was poured into sterile Bijoux bottles to which one surface sterilised summer cabbage seed, cv Castello was added. The tubes were then placed into an incubator at 20^0C.

Root hair examinations of samples taken from the experiment three weeks after it was begun confirmed that the plants were infected with the clubroot pathogen. After eight weeks however, there was no gall formation present on the plants.

One of the largest problems with this experiment was that once the tops were removed from the bijoux bottles, in order to achieve good plant growth, the agar lost moisture which could not be reabsorbed by the agar.

In order to obtain gall formation the plants need to be actively growing. The plants growing in the agar did not grow very well probably due to the problems of the agar loosing moisture. This could be one factor in the lack of gall formation. The viability of resting spores may also be influenced by the agar.

A4.2. Fluorescence microscopy.

The application of fluorescence microscopy to assess the viability of *P. brassicae* resting spores is based on using two fluorescent stains calcofluor white M2R and ethidium bromide. Calcofluor white binds to the chitin contained within the resting spore wall and fluoresces in the blue spectrum making the spores visible and simple to count. Ethidium bromide penetrates slowly into the cytoplasm of intact viable spores but quickly into non-viable spores causing them to fluoresce in the red part of the spectrum (Takahashi & Yamaguchi 1988).

By staining for less than four hours, ethidium bromide will distinguish non-viable spores since during this time only non-viable spores are affected. A combination of calcofluor white and ethidium bromide allows determination of the total numbers of viable and no-viable spores present and the number of non-viable spores (red fluorescence).

Protocol for assessing the viability of resting spores (after Takahashi & Yamaguchi, 1988)

1. Extract the spore suspension from galls in the way described in chapter 2.
2. Clean the spore suspension further using Ludox protocol
3. Prepare fluorescent stains Ethidium bromide (EB) 50μg/ml and calcofluor white M2R (CFW) 100 μg/ml.
4. Mix equal volumes of EB and CFW solutions to prepare the fluorescent stain.
5. Mix an equal volume of spore suspension with the fluorescent stain solution and leave to stand for no more than 4 hours

6. Place a drop of sample solution onto a microscope slide and cover with a cover glass. Place one drop of non-fluorescent immersion oil onto the cover glass.
7. Observe the sample using a high pressure mercury lamp fluorescent microscope with an UG1 exciter filter and a L420 barrier filter.
8. Assess 350 spores in the field of view for viability, blue = viable, red = non-viable.

Unfortunately despite taking samples from experiment 3.5 and preparing individual samples specifically for observation, in which the resting spores had been left for sufficient time viability to be affected, it was not possible to see any red fluorescing spores. It was therefore not possible to use this method for further experiments.

A4.3. Preparation of purified resting spore suspensions.

For experiment A4. 1, where the growing of *P. brassicae* plants in agar was attempted and experiments using fluorescence microscopy (A4.2) it was essential to remove all debris from the spore suspensions. The following methods were used to try and obtain cleaner spore suspensions.

Method and Materials 1: Use of sodium hypochlorite, ethanol and Decon 90 to improve the quality of spore suspensions.

1. Five defrosted galls were washed in 5% sodium hypochlorite (Sigma) for 30 mins.

2. Galls were cut into small pieces and placed in a Warring blender.

3. Three hundred ml of sterile de-ionised water was added to the blender and the material macerated on full power for 30 seconds. The suspension was filtered through two layers of cotton cloth.

4. The filtrate was divided equally into four tubes and centrifuged for 10 minutes at 2000g. The pellets were re-suspended in sterile de-ionised water. Centrifugation was repeated a further a further three times.

5. Each pellet was re-suspended in 40 ml of one of the following treatments :- 5% Decon 90, 5% sodium hypochlorite, 5% ethanol or sterile de-ionised water. The tubes were centrifuged twice for 10 mins at 2000g. Between centrifugation's the pellets were re-suspended in the appropriate treatment solution.

Spore suspensions were then prepared from each of the four treatments using sterile de-ionised water.

Method 2: The use of Ludox to improve the quality of resting spore suspensions.

Ludox (DuPont) is a silica (SiO_2) suspension in sodium hydroxide. This method to improve *P. brassicae* spore suspensions was adapted from that used for purifying malaria parasites (*Plasmodium falciparum*) extracted from mosquito salivary glands (Castlebury *et al*, 1994).

1. A suspension of *P. brassicae* resting spores was prepared as described in chapter 2.

2. The resulting pellet was re-suspended in 5ml of a 50% sucrose solution and centrifuged for 10 min at 2000g.

3. The supernatant was transferred to new centrifuge tubes and diluted with 30 ml of sterile de-ionised water and centrifuged for 10 min at 2000g.

4. The supernatant was discarded and the pellet re-suspended in 5 ml of sterile de-ionised water.

5. 1ml of the resulting spore suspension was layered onto a 40ml continuous gradient of Ludox. This was prepared using a density gradient mixer (Sigma). Twenty ml of sterile de-ionised water was placed into one chamber and 20 ml of Ludox into the remaining chamber. Releasing the valve between the two chambers allowed a gradient to form in a centrifuge tube.

6. The gradient and spore suspension were centrifuged for 30 minutes at 1000g.

7. A band of resting spores formed one third of the way down the tube, and was removed with a Gilson pipette (1ml).

8. The spores were transferred to another centrifuge tube and 30 ml of sterile de-ionised water added. The spore suspension was centrifuged at 1000g for 5 minutes.

9. The supernatant was discarded and the pellet re-suspended in 30ml of sterile water, and re-centrifuged.

10. The pellet was re-suspended in 5 ml of sterile de-ionised water and the concentration of the resulting resting spore suspension was determined by haemocytometery.

Plates A4.1 & A4.2 compare spore suspensions before the Ludox protocol and afterwards. The spore suspensions were stained with methyl blue improving the visibility of resting spores and other fungal material within the suspension.

Plate. A4.1. Spore suspension after cleaning with Ludox gradients. mag. x400

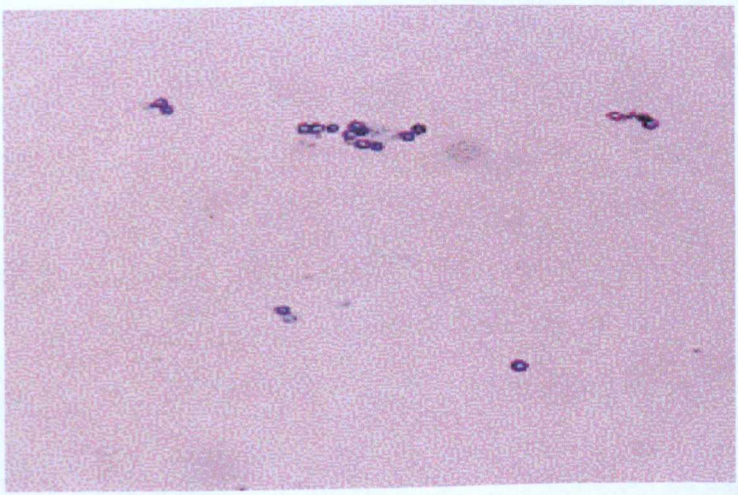
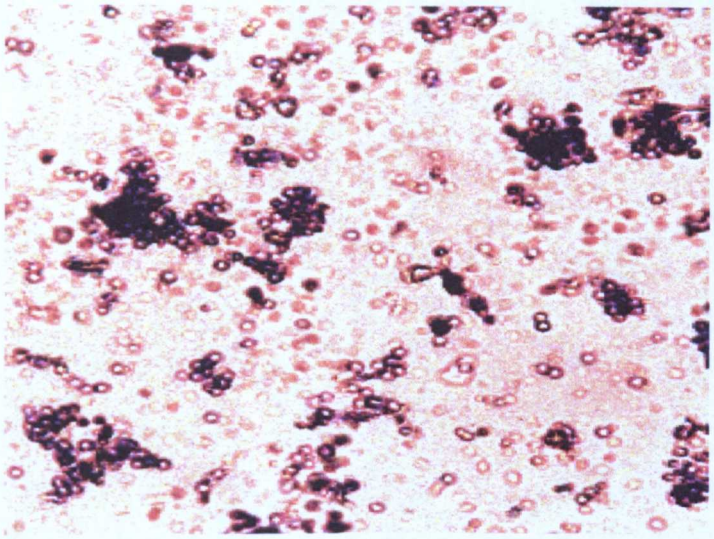


Plate A4.2. Uncleaned spore suspension. mag. x400



Conclusions

Suspensions produced by this method were much purer but only small amounts of low spore concentrations resulted making this method unsuitable for large scale experiments.

The use of solely a sucrose gradient (steps 2 & 3 in the Ludox protocol) to improve the quality of spore suspensions (Fraser & Buckzacki, 1983.) proved unsatisfactory resulting in a large loss of spores without the suspension becoming noticeably cleaner

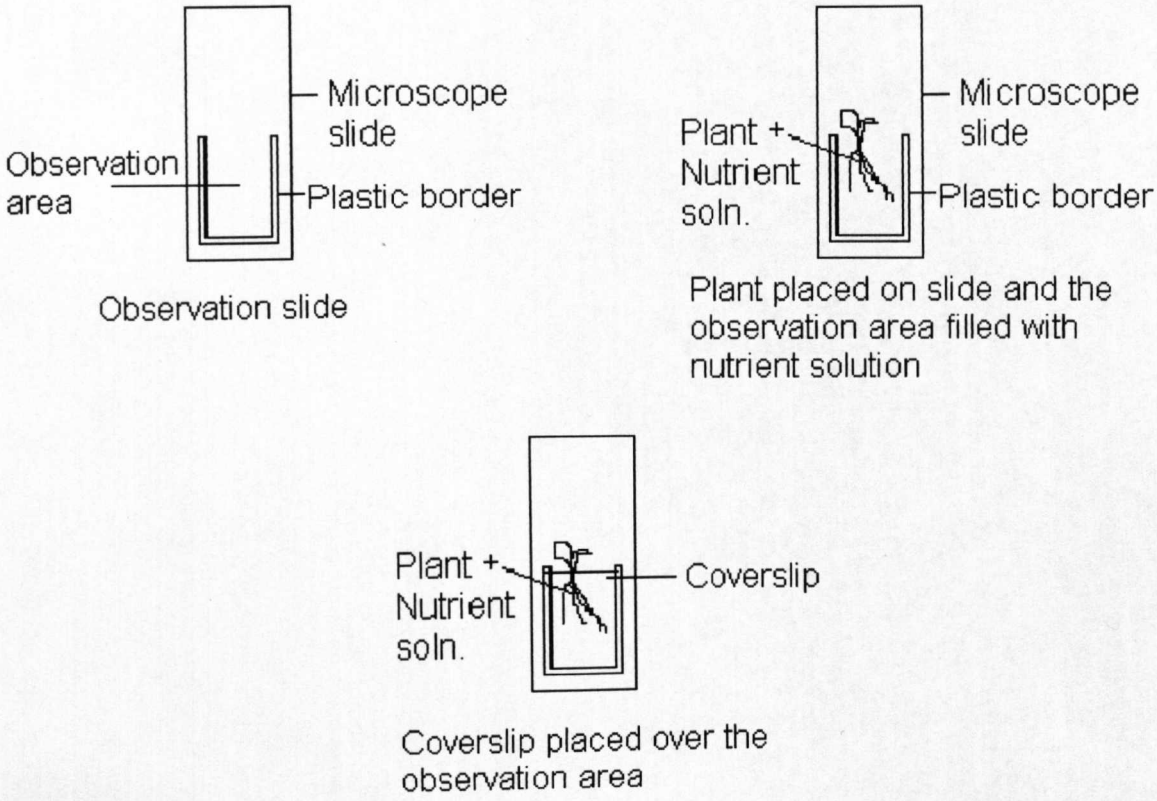
A4.4. Observation slide design.

Slides were designed to allow the continuous observation of a seedling throughout the infection process by *P. brassicae*. A piece of weighing boat (BDH) plastic was cut into a rectangular shape and glued to a microscope slide forming an observation area (Fig A4.1).

Seedlings of cv Castello were raised in a method E nutrient culture (Chapter 2). The seedlings were removed from the culture system once the first true leaf emerged.

One seedling was placed into each of the observation slides (Fig.A4.1). The plastic around the observation area was coated with petroleum jelly. The seedling was placed onto the slide within the cavity area. Nutrient solution (Kristalon) was added to the cavity area using a glass pipette until the area was filled (3-5 drops). A coverslip (22mm x 50 mm) was placed over the observation area so that the root system was enclosed within the observation area and of the seedling rested on the remaining portion of the slide (Fig A4.1).

Figure A4.1. Microscope slides made for the observation of the P. brassicae infection process.



Slides were placed inside a plastic box lined with absorbent paper soaked in distilled water forming a humid environment. A transparent lid was placed over the box. The box was placed in a growth chamber (LMS) set to 24^0C and a 16 hour photoperiod. Slides remained in the incubator for 48 hours allowing the seedlings to acclimatise.

After 48 hours 2 drops of a 10^{-6} resting spores ml^{-1} *P. brassicae* suspension were added to the observation area of each slide. The slides were incubated for a further 48 hours. Seedlings were

observed using an Olympus microscope every 24 hours monitoring the infection process by *P. brassicae*. The nutrient solution level within the observation area was maintained throughout the experiment.

Conclusions

Although this method provided good views of the plant roots, the plants were not able to survive the experimental period and seedlings were often pulled from the slide on turning the microscope objective. This method was therefore unusable for experiments.

A4.5 Systemic Acquired Resistance.

This experiment aimed to stimulate the plants own defence responses in order to provide the plants with protection against *P. brassicae* infection.

Method and Materials.

Cabbage seed cv Castello was surface sterilised. One seed was sown into each sterile Bijoux pot containing autoclaved vermiculite. Once the plants had produced three true leaves, after approximately four weeks, either the 1st leaf was inoculated with a clubroot spore suspension at a resting spore concentration of 10^1 or the roots were inoculated by adding 1ml of a 10^1 resting spore suspension. The 1st leaf on each plant was inoculated by gently

resting the leaf on a piece of absorbent tissue and covering the leaf in the spore suspension using a soft bristled brush.

The leaves or roots of control plants were treated in the same way but with sterile de-ionised water instead of a *P. brassicae* resting spore suspension.

All plants were challenged at 7 and 14 days after the first inoculation with 1ml of a 10^6 resting spore suspension.

Samples for root hair analysis were taken at 7, 14 and 21 days following challenge and a final harvest was made eight weeks after each of the challenge times. At the final harvest the number of galls per treatment were recorded along with the shoot and root dry masses.

Results

The plants grew poorly in this experiment and although some root galling was seen the galls were very small and it was not possible to categorise them. The number of galls observed on plants which had, had their leaves inoculated was not lower than that of non-inoculated controls.

Conclusions

During the set up of this experiment several problems with the method of inoculation were noted which would of led to any results which had been obtained being questionable. It was very difficult to inoculate the leaves without the spore suspension dripping into the growth tubs. The addition of a surfactant to the spore suspension may help to improve the inoculation technique.

The growth media and nutrient solution were also not suited to plant growth or gall formation and as has been discussed in Chapter 2 (Nutrient culture systems, pp 75)

This experiment was later repeated using a modified Hoaglands' solution but plant growth was again poor. Plant leaves were inoculated by wiping them with absorbent paper, which had been soaked in a *P. brassicae* resting spore suspension. This did not however, did not prevent the resting spore suspension form rolling off the leaf and into the growth media. Gall formation was very poor even in inoculated plants suggesting that this plant growth method did not promote gall formation.

A4.6 Split root experiments.

These experiment aimed to develop a method to produce Chinese cabbage plants with bifurcated root systems. It was hoped that this method could be used in experiments to investigate :-

1. Whether applying calcium nitrate to one side of the root system would decrease galling on both sides.

2. How mobile *P. brassicae* is in the plant. This experiment would investigate whether applying resting spores to one side of the root system would lead to infection on the un-inoculated side of the plant.

3. Are some calcium salts more effective than others. Experiments whereby each of side of the root system would be supplied with a different calcium salt to determine the most effective calcium salt.

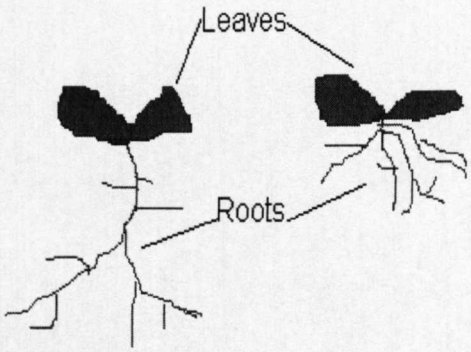
Method and materials.

Initial methods had been used by other researchers working on clubroot (Khatun, *pers. comm*). Surface sterilised Chinese cabbage seed cv. Mariko were germinated in petri dishes. Two filter papers were placed into the base of each petri dish base and were moistened with 3.5 ml of sterile de-ionised water and one filter paper was placed in the lid of each dish and moistened with 1.5 ml of sterile de-ionised water (Webster, 1985). Following germination, the seed was transplanted into autoclaved 'Magenta' tubs containing vermiculite and Hoaglands' solution.

In the first trial of this experiment the root tips were removed after seven days, however it was determined that this bifurcated the root system to far down the root making it difficult to place the two root systems into separate bijoux bottles (BDH).

In further trials the seeds were germinated in a similar manner but the root tips were removed after 24 and 48 hours. The seedlings were then placed in to petri dishes containing sand and Hoaglands' solution. A petri dish base was placed over the seedlings allowing room for growth but preventing moisture loss. Within three days the root systems were splitting satisfactory manner. It was determined that removing the root tips after 48hours produced a better root system than removing them after 24 hours. The poster in Appendix 5 entitled the regression of *P. brassicae* (clubroot) by calcium nitrate describes this experiment in more detail. Figure A4.2 demonstrates how the root systems of the seedlings differed when the root tips were removed after 48 hours and seven days.

Figure A4.2. Illustration of the differences in the root systems where the root tips were removed at varying times.



Left = root tip removed after 7 days

Right = root tip removed after 48 hrs

Conclusions

Although several experiments were attempted using the split root system the results were always poor. Plant growth was poor in all the nutrient culture systems (Chapter 2) and gall formation did not occur. Use of the system therefore ceased, however further work may establish a more suitable system.