

**Development of a systems approach for the control of diseases in
organic greenhouse crops**

By

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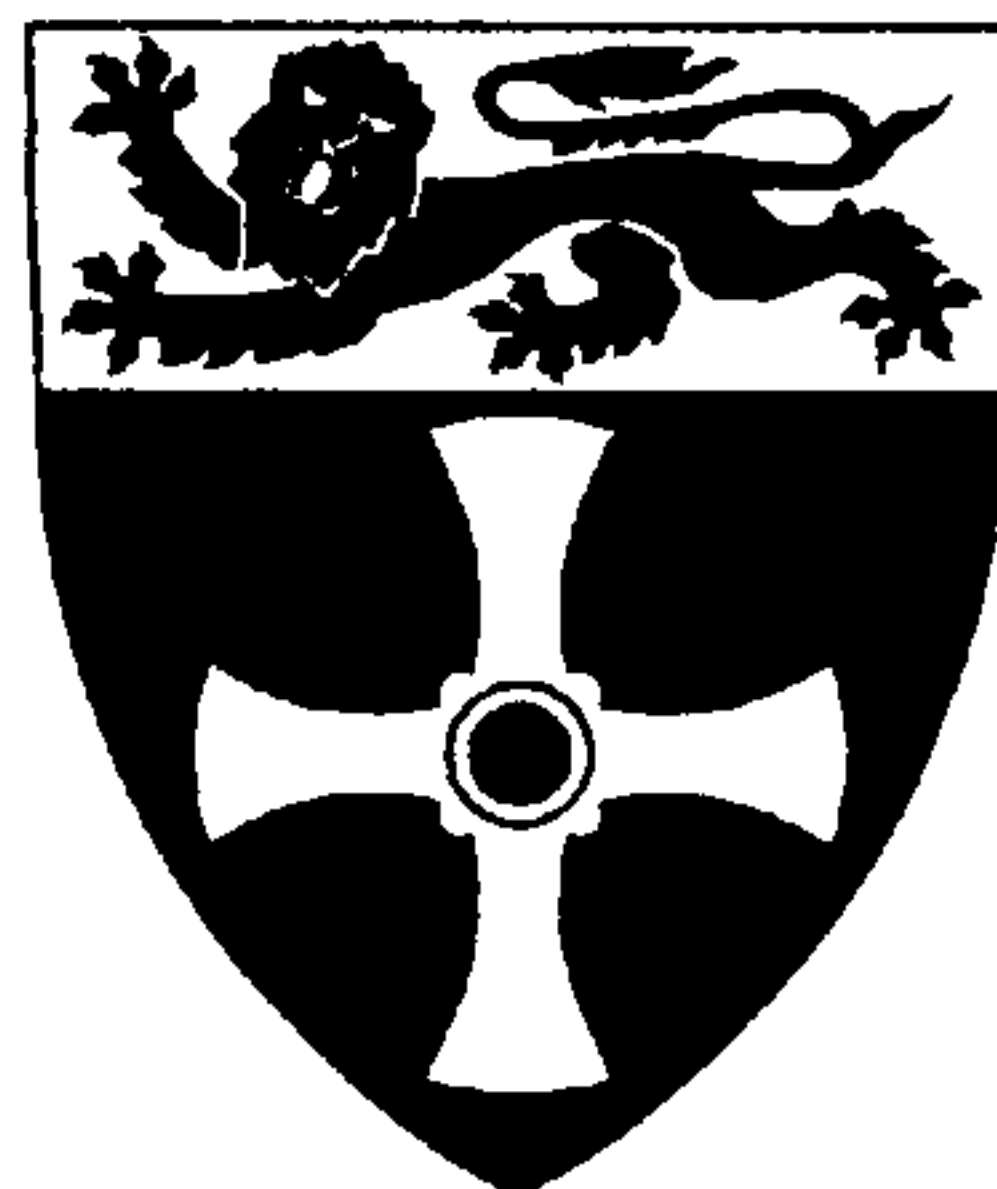
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A thesis submitted for the degree of Doctor of Philosophy (PhD) at the University of

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Newcastle upon Tyne, UK

April, 2006

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Abstract

The study aimed to develop integrated crop management (ICM) systems in organic greenhouse production for improved control of tomato corky root rot (*Pyrenochaeta lycopersici*), tomato verticillium wilt (*Verticillium albo-atrum*), and cucumber powdery mildew (*Podosphaera xanthii*), compatible with and/or utilizing organic fertility management practices, while minimizing or eliminating permitted practices such as soil disinfection by steaming and foliar application of sulphur fungicide.

Different concentrations of chitin (or chitosan – a water soluble form), fresh *Brassica* tissues, and cow manure based compost applied to soils infected with *P. lycopersici* alone and *P. lycopersici* plus *V. albo-atrum* reduced disease incidence and/or increased yield. However, efficacy differed between treatment types and concentrations. Compost or fresh *Brassica* tissue significantly increased soil microbial activity which may have increased competition and/or antagonism against soil pathogens, lowering disease incidence. Combining different treatments gave no synergistic or additive effects on incidence of both diseases, but the *Brassica* tissues plus chitosan combination improved yield. In tests of cultivar resistance/tolerance to the two pathogens, two “standard” cultivars were susceptible to both pathogens whilst the cherry type was tolerant to corky root rot, but not *Verticillium*. In soils infected with corky root rot only, grafting the cultivars onto a resistant root-stock effectively reduced disease incidence, but when infected with both pathogens, only one “standard” cultivar showed reduced disease incidence.

In cucumbers, foliar application of chitosan reduced powdery mildew but Milsana® VP 2002 (a plant extract) was significantly better. *Ampelomyces quisqualis* and *Pythium oligandrum* (Biological Control Agents) were ineffective. There was no synergistic or additive effect detected with any combinations. Resistance/tolerance of cultivars significantly affected disease incidence. However, the more susceptible cultivar outyielded the tolerant one despite greater infection.

Several components of an integrated strategy for control of diseases were identified with significant potential for development of more sustainable, organic protected-cropping systems.

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CHAPTER 1. GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1. Organic greenhouse production systems

The estimated world area covered by greenhouses is about 300,000 ha, 50,000 ha. of which are covered with glass and 250,000 ha with plastic (Albajes *et al.*, 1999).

Greenhouses allow a significant extension of the growing season for crops and high quality products to be produced in large quantities on a relatively small surface area. For example, in the Netherlands, only 0.5% of the agricultural area is covered with glasshouses, but produces approximately 20% of the total value of agricultural production (van Lenteren, 2000).

Organic (also described as biological and/or ecological in some parts of Europe) greenhouse production is a rapidly expanding sector and currently accounts for between 0.2 and 5% of total greenhouse area in different Western European countries (Lampkin, 2002).

Although greenhouse growers are usually well trained, there is still a relative hesitation in conversion from conventional to organic greenhouse production and growers often identify the higher risk associated with disease control, lower yields and increased production costs (thus making profitable productions reliant on a significant price premium being achieved by organic crops) as the main barriers for conversion (Greer and Diver, 2000; Lampkin, 2002; Food and Agriculture Organization of the United Nations, 2001).

1.2 Main challenges/problems in organic greenhouse production systems

For some aspects of greenhouse technology and practices, conventional and organic greenhouse production is very similar. For example, the (a) same type of greenhouses and environmental control methods are used and (b) pest control is now mainly based on the release of biological control products (based on parasites and parasitoids) rather than chemosynthetic pesticides (especially in the northern EU countries).

However, in other areas there are significant differences between organic and conventional systems. Most importantly organic standards prescribe: **(i)** soil based growing system (in particular in Northern Europe conventional systems are now mainly based on rockwool or other out-of soil, hydroponic growing systems), **(ii)** that no chemosynthetic N (e.g. NH_4 , NO_3) and P (e.g. super-phosphate) and, KCl based fertilizers are used (compounds which constitute the main NPK inputs in conventional systems) and **(iii)** no chemosynthetic fungicides, nematocides and soil sterilants are used (crop protection chemicals which are widely used in conventional production).

These restrictions and the requirement for a 2-year “conversion period” pose the main challenges/problems in organic greenhouse production (Dodson *et al.*, 2002) and are described in detail below.

1.2.1 Conversion period

Greenhouse production systems require substantially higher levels of capital and variable cost investments (which are often financed via loans) than any other crop production systems (van Lenteren *et al.*, 1992). Conversion to organic production usually results in additional pressure on both gross and profit margins, because during the (usually 2 year) conversion period **(a)** marketable yields decrease, **(b)** additional capital costs are often incurred and **(c)** products cannot yet be sold as “organic” and thereby achieve the price premiums required to compensate for lower yields and additional capital costs. Also additional labour costs are usually associated with establishing new markets or marketing systems for organic products (Lampkin, 2002). Therefore the decision to convert carries with it a high element of risk and uncertainty.

These risks are compounded in many countries by the lack of detailed information and advice on organic growing methods, but this situation is likely to improve as the industry expands and due to the fact that many countries have increased their investment in research and advisory systems that support organic greenhouse production (Lampkin, 2002).

1.2.2 Fertility management in organic greenhouse systems

The main crops grown in greenhouse production systems in Western Europe (e.g. tomato, peppers, cucumbers) produce large amounts of biomass and are relatively long season crops. They therefore have a high demand for fertility inputs (e.g. an organic tomato crop is estimated to require fertility inputs providing between 500 to 600 kg N per ha) (Sampson *et al.*, 2002). In conventional greenhouse farming there are many types and mixtures of chemo-synthetic mineral N, P and K fertilizers that can be used to satisfy the nutritional needs of plants.

In organic greenhouse production, inputs used include mainly organic matter based fertility inputs, with either a high (e.g. chicken manure pellets, manure slurry, fermented fish waste slurries, spent microbial fermentation media) or low (manure, compost, plant residues) level of water soluble, readily plant available nitrogen sources (NH_4 and NO_3). These amendments also provide significant inputs of P, K, Ca, Mg, S and micronutrients, but may be supplemented with permitted mineral P (e.g. finely ground rock phosphate) or K (potassium sulphate, rock-dust) inputs. Most mineral micronutrient fertilizers are also permitted for use if they are shown to be necessary as supplements to the main organic matter inputs via either soil or plant analysis.

Different to outdoor organic production systems, nitrogen supply via inclusions of legume crops into the rotation or legume intercrops is either absent or plays a minor role in the supply of nitrogen to organic greenhouse crops. Providing a balanced fertility supply to organic crops is therefore more complex and requires significant experience/knowledge of farm specific soil and environmental conditions and often on-farm experimentation. However, in the absence (or without effective control) of major disease and pest problems, yield levels with organic fertility management practices may be similar to those obtained in conventional production systems (Sampson *et al.*, 2002; Sullivan, 2004).

1.2.3 Crop protection in organic greenhouse systems – overall trends

The establishment of greenhouse crops is expensive (both in terms of capital and variable cost) and losses in marketable yield, caused by pests and diseases have to be minimized to maintain satisfactory gross and profit margins (van Lenteren, 2000). While greenhouses optimize growing conditions for crops, (i) the higher temperatures (and often also higher humidity levels), (ii) monoculture and/or short rotations used (apart from perennial crops, greenhouse systems are the only crop production systems in which organic standards permit monocultures to be used, although there are increasing efforts to prohibit this practice), at the same time offer excellent conditions for the fast reproduction of pests and diseases (van Lenteren and Woets, 1988). For this reason the intensive use of soil sterilants (to control soil-borne diseases and nematodes), pesticides (to control both foliar and root damage by invertebrate pests) and fungicides (to control foliar diseases such as powdery mildew, downy mildew/*Phytophthora* and grey mould) was widespread in the 1960s, 70s, 80s and 90s. The variable costs of chemical crop protection are minimal representing less than the 2% of the total overall cost of production (van Lenteren, 1995).

1.2.4 Crop protection in organic greenhouse systems - PEST CONTROL

Since the 1980s and 90s the use of biological control products based on parasites and predators of invertebrate pests, has increasingly replaced the use of pesticides in greenhouse systems. This is mainly because the level of environmental control that can be provided under protected conditions, and the more or less continuous presence of crops (and associated pests), makes the management of pests via natural enemies more effective in protected rather than outdoor production systems. The adoption of biological control agent (BCAs) and integrated pest management **IPM** over the last 20 years in protected conventional production, first in North-Western Europe and later in other greenhouse areas, has been remarkable (van Lenteren and Woets, 1988; van Lenteren, 2000; Parrella *et al.*, 1999). As a result even many conventional greenhouse production systems BCAs have completely replaced the use of chemosynthetic pesticides, and BCAs

also provide excellent control against invertebrate pests in protected organic production (van Lenteren, 2000).

1.2.5 Crop protection in organic greenhouse systems - DISEASE CONTROL

Different to the situation with respect to crop pests, disease control is still heavily reliant on highly toxic soil sterilants (e.g. methyl-bromide) and chemosynthetic fungicides in conventional and IPM systems: strategies that are not permitted in organic production.

The approaches and methods for disease control in organic farming are less effective and/or have significant negative side effects for some diseases (e.g. *Phytophthora infestans*, *Botrytis cinerea* and some soil-borne disease). The currently available disease control methodologies are described in separate sections below.

1.2.5.1 The use of sulphur and copper fungicides

Sulphur and copper based fungicides are permitted in organic farming against a range of diseases but **can have significant negative side effects**. Most importantly

- **The use of sulphur reduces the populations of many natural enemies (including those of predators and parasites released as BCA-products)**
- **Sulphur damages the plastic cladding material and**
- **Cu-fungicides are likely to be banned in organic farming due to their negative effects on soil biological activity and biodiversity and potential toxic effects on people handling Cu-fungicides**

1.2.5.2 Soil steaming/solarization

Major crop protection strategies used in organic farming to control soil-borne pathogens and nematodes include soil steam pasteurization and/or solarization (Runia, 2000).

Soil solarization is the process of covering moist soils with clear polyethylene to trap solar radiation and raise soil temperatures to levels that kill fungal pathogens and weed seeds (Katan *et al.*, 1976).

However, heat treatments can have significant negative side effects and limitations. Most importantly

- **Currently used high temperature steaming of soil eliminates not only the soil pathogens, but also much of the beneficial soil microflora and fauna (Bennett *et al.* 2003). The application of lower temperatures for longer periods could overcome this disadvantage (van Loenen *et al.*, 2003) but commercial systems for the use of low temperature steam are not yet available.**
- **Soil solarization is not effective against all soil pathogens and nematodes and can be used only during summer in South EU countries (Runia, 2000)**

1.2.5.3 Resistant varieties/cultivars and rootstocks

The use of resistant varieties/cultivars and rootstocks has developed into one of the most important strategies for the control of soil-borne pathogens and some foliar diseases, but resistant varieties/cultivars are not available for all greenhouse crops and against all pathogens (Greer and Diver, 2001). Most commercial breeding for resistance has focused on resistance against major soil-borne diseases in greenhouse crops (e.g. *Verticillium*, *Fusarium*, corky root rot) (Vakalounakis and Fragiadakis, 2003).

However, there are also tomato cultivars with resistance to foliar and/or vascular fungal and bacterial pathogens such as *Cladosporium fulvum*, *Alternaria alternata*, *Stemphyllium spp.* and *Pseudomonas syringae* and viruses such as tomato mosaic tobamovirus ToMV (Vakalounakis and Fragiadakis, 2003).

Most of the resistances currently available are known to rely on single genes. **This results in a significant risk for such resistances to be overcome by the pathogen especially if resistant crop varieties are regularly used in the same greenhouse (Pegg and Brady, 2002).**

1.2.5.4. Environmental Control

Highly effective control of some foliar diseases (e.g. grey mould, late blight etc.) may also be obtained by appropriate environmental control (e.g. computer regulated temperature, humidity, light, ventilation and CO₂ supplementation) that minimizes periods in which the glasshouse climatic conditions are favorable for infection and spread

of diseases (van Lenteren, 2000). **However, such systems require very high capital and energy inputs** (one of the main mechanisms that minimizes disease development is the installation of ground heating systems which create an upwards movement of warm air and thereby remove the humidity in the crop). **They are therefore mainly used in heated glasshouse systems used in Northern Europe** (where such systems are economically viable), but are rarely used in plastic greenhouses systems in Southern parts of Europe (Albajes *et al.*, 1999; van Lenteren, 2000).

1.2.5.5 Biological control agents (BCAs)

Some commercial products are available for foliar plant diseases in greenhouse crops (see Tables 1&2), but are not widely used in both organic and conventional glasshouse crops (van Lenteren, 2000). **This is mainly because they have often not shown adequate control under high disease pressure** (Cook and Baker, 1983; Paulitz and Belanger, 2001). The activity of the biological control agents currently commercially available against diseases, such as grey mould and/or powdery mildews, **is also dependent of specific environmental conditions and their use may therefore also be limited to greenhouse systems with sophisticated environmental control** (Elad *et al.*, 1996). For example, in order to obtain the maximum colonization by hyperparasites (such as *Trichoderma spp.*) temperature and vapour pressure deficit on the plant surface should be kept close to the optimum for the hyperparasites for considerable periods of time (Elad *et al.*, 1996).

Table 1.1. Commercially available Biological Control Agents (BCAs) for soil-borne diseases of greenhouse crops (van Lenteren, 2000)			
Microorganism	Trade name	Target pathogen	Registration Countries
<i>Coniothyrium minitans</i>	Contans® Koni®	<i>Sclerotinia sclerotium</i> , <i>S. minor</i>	Germany Switzerland Hungary
<i>Gliocladium virens</i> (= <i>Trichoderma virens</i>)	SoilGard®	<i>Pythium ultimum</i> <i>Rhizoctonia solani</i>	USA
<i>Trichoderma harzianum</i> (Different strains)	RootShield® PlantShield® Trichodex® Binab T® Supresivit®	<i>Fusarium sp.</i> <i>Rhizoctonia solani</i> <i>Pythium sp.</i>	USA Israel Sweden Czech Republic
<i>Streptomyces griseoviridis</i> (Strain K61)	Mycostop®	<i>Fusarium sp.</i> <i>Pythium spp.</i> , <i>Phytophthora spp</i>	USA, Finland
<i>Gliocladium catenulatum</i> (Strain J1446)	Primastop®	<i>Pythium sp.</i> <i>Rhizoctonia sp.</i> <i>Fusarium sp</i>	USA
Nonpathogenic <i>Fusarium oxysporum</i> (Strain Fo47)	Biofox® Fusaclean®	<i>Fusarium sp.</i>	Italy France

Table 1.2. Commercially available Biological Control Agents (BCAs) for foliar diseases of greenhouse crops (van Lenteren, 2000)			
Microorganism	Trade name	Target pathogen	Registration Countries
<i>Ampelomyces quisqualis</i>	AQ10®	Powdery mildews	USA
<i>Sporothrix flocculosa</i>	Sporodex®	Powdery mildews	USA
<i>Trichoderma harzianum</i>	Trichodex®	<i>Botrytis cinerea</i> <i>Fulvia fulva</i> , <i>Pseudoperonospora cubensis</i>	Israel
<i>Pythium oligandrum</i>	Polyversum®	<i>Botrytis cinerea</i> <i>Phytophthora infestans</i>	Czech Republic
<i>Verticillium lecanii</i>	Vertalec®	Whitefly (Also active against powdery mildews)	USA Europe
<i>Ulocladium atrum</i>	Under registration	<i>Powdery mildews</i>	USA

Certain formulations can increase the environmental “window of activity” (in particular the requirement for high relative humidity/free water availability during infection) for BCAs. For example, formulation with certain oils, was shown to improve the effectiveness of the hyperparasites *Ampelomyces quisqualis*, and *Verticillium lecanii* under dry conditions (Verhaar *et al.*, 1999; Elad *et al.*, 1998).

1.2.5.6 Suppressive composts

Some soils and compost preparations have been shown to be “suppressive” to certain soil-borne diseases. This suppressiveness was often linked to high levels of antagonistic and antibiotic producing organisms or high levels of anti-microbial compounds in these composts that are thought to reduce the populations of disease causing organisms (Schneider, 1982). Disease-suppressive composts are now commercially available and used widely in pot plant or transplant production media (both peat and soil-based media) (Kuepper, 2004; Hoitkin *et al.*, 1991). The use of suppressive composts in the greenhouse could provide an additional method for the control of soil-borne diseases in organic production systems. **However, the potential of using suppressive composts has not so far been tested in detail and suppressive composts are not widely used in organic greenhouse production** (Hoitink *et al.*, 1997).

More recently, the potential of composts for the control of soil-borne plant pathogens has received increased interest in both organic and conventional farming (Abbasi *et al.*, 2002; Chellemi *et al.*, 1992; Hardy and Sivasithamparam, 1991; Workneh and van Bruggen, 1994). In addition, microorganisms that are present in suppressive soils can be isolated and formulated in commercial products as for example the bacteria *Streptomyces spp.* (Mycostop®) against soil-borne pathogens (Lahdenpera *et al.*, 1990).

1.2.5.7 Cultural practices (crop rotation, cover crops, green manures)

Cultural practices that promote soil health include crop rotation and the incorporation of cover crops and/or green manures that have “suppressive” effects on soil-borne diseases (Sampson *et al.*, 2002).

The use of rotations or break crops in greenhouse cropping system is limited, due to the small range of crops that can be grown profitably under protected conditions. For example in Northern Europe only cucurbits (e.g. cucumbers, melons and courgettes) and solanaceous (tomato, peppers and aubergine) and, to some extent winter lettuce crops are grown in greenhouses, with more than 60% of greenhouse being used for solanaceous crops (Sullivan, 2004). This lack of botanical diversity, the fact that some soil-borne

pathogens are shared by both solanaceous and cucurbit crops (e.g. *Verticillium spp*) and economic pressures to focus production on specific crops (supermarkets often only give contracts for individual crops to growers) make it virtually impossible to establish rotations that make significant contributions to disease control (Lampkin, 2002).

However, some benefits of rotational cropping may be introduced into glasshouses via different mechanisms. For example, applying fresh crop residues/wastes from out-door crops (e.g. *Brassica spp.* that were shown to reduce soil-borne disease incidence when grown as breakcrops in outdoor rotational cropping systems), into greenhouse soils, may reduce soil-borne disease incidence (Sullivan, 2004). For example amendment of high levels (>40 t/ha) of plant residues/processing wastes of Brassica and crucifer plants to outdoor soils was shown to result in reductions in soil-borne disease due to the release of isothiocyanate and other antimicrobial compounds (Xiao *et al.*, 1998; Mojtahedi *et al.*, 1991; Bailey and Lazarovits, 2003).

However, the effect of such soil amendments on soil-borne diseases in organic greenhouse systems has not, so far, been investigated in detail.

1.3 Objectives of the present research programme

Due to the limitations in the current disease management practices used in organic greenhouse systems –**highlighted in section 1.2.5 above**- it is essential to develop improved disease control methods and strategies to reduce yield and quality losses in existing organic production systems and to increase confidence among conventional greenhouse producers that would like to convert to organic production.

More specifically the objectives of this research programme were to:

- I. compare the effects of different alternative, non-chemical strategies on the control of root, vascular and foliar diseases of the two more important greenhouse crops, tomato and cucumber**

- II. quantify the effect of **combinations** of different alternative, non-chemical soil and foliar treatments on disease development and fruit yield of tomato and cucumber and to identify potential **antagonistic, additive or synergistic interactions** between the different combinations
- III. quantify the **level of resistance** of different tomato and cucumber cultivars against root, vascular and foliar diseases
- IV. identify interactions between **cultivar resistance** and **foliar treatments**
(= compare the relative effect of alternative treatments in different cultivars)
- V. to identify **potential mechanisms of action** for disease suppression and yield increases associated with different treatments

1.3.1 Crop Diseases targeted

Under section 1.3 the soil-borne and foliar diseases targeted by experimental work presented in this thesis are described in more detail in separate sections below.

1.3.2. *Tomato Corky root rot (Pyrenochaeta lycopersici)*

Corky root is a serious soil-borne disease of tomato that causes a slow progressive rot of the root system of infected plants (Termohlen, 1962; Last *et al.*, 1966; Gerlach and Schneider, 1964). Corky root rot can cause significant crop losses and in commercial tomato production systems, yield losses of up to 75% have been reported (Campbell *et al.*, 1982). In addition to tomato, the pathogen infects a range of other field and glasshouse crops including spinach, safflower, pepper, aubergine, cucumber, squash, and melon (Grove and Campbell, 1987; Risser and Laugie, 1968; Termohlen, 1962). Its wide host range and long persistence in soil makes it extremely difficult to control the disease through rotational and break crop approaches, especially in greenhouse production systems (Ebben *et al.*, 1978; Lemaire *et al.*, 1998).

The symptoms of infection are initially localized tan lesions that appear on the main and secondary roots, later, the lesions become dark brown, furrowed and develop a corky texture (Last *et al.*, 1969). Finer lateral roots may also show local lesions of brown discoloration (Last *et al.*, 1968). Severely infected roots are frequently girdled and the development of furrowed fractures provides entry of secondary microorganisms. A severe attack of *P. lycopersici* can lead to root pruning, which leads to an overall reduced root system. Also since new, small roots around the tap roots develop, the root system becomes “bushy” in appearance (Forsberg *et al.*, 1999).

Above ground infected plants typically display stunting and excessive wilting when water stressed (Forsberg *et al.*, 1999). In young seedlings the growing point may die back and profuse adventitious shoots may subsequently develop (Jones *et al.*, 1987).



Figure 1.1. Left; symptoms of tomato roots infected by *P. lycopersici*. Right; healthy tomato root.

The causal agent of corky root was known as “grey sterile fungus (GSF)” until a method to induce sporulation was developed and it could be identified as *P. lycopersici* (Schneider and Gerlach, 1966).

The fungus is a poor saprophytic competitor compared to soil-borne pathogens such as *Fusarium* spp., *Colletotrichum coccodes* and *Rhizoctonia solani* (Davet, a,b, 1976). Microsclerotia of *P. lycopersici* are formed within infected cortical cells and in culture (Ball, a, b, 1979; Preece, 1964; White and Scott, 1973). Microsclerotia can survive in soils for up to 5 years (White and Scott 1973; Forsberg *et al.*, 1999).

1.3.2.1 Effect of environmental conditions on disease development

Soil temperature appears to play an important role in the development of corky root rot, with disease being most severe at constant temperatures between 15- 20° C (Termohlen, 1962).

Early planted tomatoes in California suffered more from the disease than later planted tomatoes, most likely because of cooler soil temperature during the early stages of tomato growth favored disease development (Campbell *et al.*, 1982). Disease in tomato plants in Crete was found to be most severe in winter until the middle of March. After March the less severely infected plants recovered progressively probably because

soil temperatures increased to levels sub optimal for fungal attack and disease development (Malathrakis *et al.*, 1983).

Greenhouse and field production in temperate regions, where tomato crops are grown continuously and exposed to low soil temperatures over longer periods of time, appear particularly prone to disease development (Shishkoff and Campbell, 1990).

Soil moisture also seems to affect disease development and severity. It has been suggested that high levels of soil moisture favour disease development and the appearance of symptoms due to *P. lycopersici* (Ciccarese and Ciruli, 1983). It is supported by studies into the effect of different soil irrigation levels on disease development, where increasing water supply resulted in higher disease index (D' Amato *et al.*, 1993).

1.3.2.2 Control of corky root rot

Soil disinfection Under the intensive production systems in glasshouses in the United Kingdom and other Northern European countries, corky root rot is ubiquitous and unless soils are at least partially sterilized, the severity of root symptoms increase over the years, while growth rates and yields decrease, especially in monoculture systems of tomatoes (Ebben, 1974).

Soil fumigation with methyl bromide, chloropicrin, isothiocyanide and other chemicals (Clerjeau *et al.*, 1973; Campbell *et al.*, 1982;) can be effective, but compared to methyl bromide most other chemical soil disinfectants are either less effective or currently uneconomic and are therefore used for only a small proportion of glasshouse production (Jones *et al.*, 1987).

Application of chemicals such as dazomet, fenamiphos, calcium cyanamide, methyl isothiocyanate 20%+dichloropropane mixture 80% (Di-Trapex), Vapam [metham-sodium]+ethoprophos 10%, alone or with soil solarisation, have been reported to give high levels of control against *P. lycopersici* (Malathrakis *et al.*, 1989; Fiume and

Parisi, 1995). The extensive use of fungicides may lead to the development of pathogen strains resistant to chemicals.

Health and environmental concerns associated with the use of soil fumigants (especially methyl-bromide, which is known to be chemical contributing to the depletion of the atmospheric ozone layer), chemo-synthetic pesticides, but also soil steaming (which significantly increases the energy/fuel use in glasshouse production) have resulted in the need to develop alternative, environmental friendly approaches for the control of soil-borne diseases such as corky root rot (Workneh and van Bruggen, 1994).

Prior to the widespread availability and use of methyl bromide, steam disinfection was a common method used for the treatment of greenhouse soils contaminated with corky root and other soil-borne pathogens (Last *et al.*, 1966; Haine *et al.*, 1968; Granges *et al.* 1998). More recently the use of steam sterilisation has increased again, due to the increasing restrictions on methyl bromide use and to the expansion in organic glasshouse production (C. Leifert, personal communication).

Soil solarization is a method of hydrothermal disinfection, which does not increase energy/fuel use. It is accomplished by covering a moistened soil with a transparent polyethylene film for several weeks, usually during the hottest period of the year. This disinfection method can achieve commercially acceptable control of pathogenic fungi, nematodes, mites, insects and weeds (Katan *et al.*, 1976).

Prolonged exposure periods to high temperatures are also thought to weaken the pathogens and make them more vulnerable to antagonistic microorganisms and reduce their infectivity and longevity (Moura and Palminha, 1994). Control of soil-borne pathogens by soil solarization usually leads to improved plant growth and yield (Tjamos and Skretis, 1990).

Soil solarization has been used against *P. lycopersici* with good results in Southern Europe and the Middle East but is thought to be less or not effective in cooler climatic regions of Northern and Eastern Europe (Bourbos and Skoudridakis, 1991; Cartia, 1989; Cocksull *et al.*, 1994; Fiume, 1995; Fiume and Parisi, 1995; Moura and Palminha, 1994).

Resistant rootstocks and cultivars Plant breeders have successfully transferred genes providing tolerance to *P. lycopersici* from wild *Lycopersicon* species to *L. esculentum* cultivars (Hoggenboom, 1970). The roots of tolerant plants are infected by *P. lycopersici*, but lesions are restricted and root loss is lower than that observed on root systems of susceptible plants (Ebben, 1974).

Grafting of susceptible cultivars onto resistant rootstocks has also been developed as an effective alternative to steam and chemical soil disinfection and sterilisation (Smith and Proctor, 1965; Haine *et al.*, 1968; Upstone, 1968; Ginoux and Dauple, 1985; Palminha, 1987; Morra *et al.*, 1997; Mazollier, 1999). Grafting onto resistant rootstocks has been repeatedly reported as a method that gives “lasting” and consistently high levels of control of corky root rot, but has initially not been widely adopted, because it is labor intensive, and expensive compared to methyl bromide soil treatment (Ebben *et al.*, 1978). However, since it was announced that methyl bromide has to be withdrawn from use (due to its negative environmental impacts), the use of grafted plants has increased substantially.

The development of grafting protocols in which two stems are left to be developed after grafting onto one resistant root system (and thereby allows planting at half the standard density), has further improved the commercial viability of production systems based on grafted plants (Granges and Leger, 1996).

It has been suggested that genetic resistance offers a satisfactory method of long-term control of *P. lycopersici* (Jones *et al.*, 1987). However, the relatively short period of time that resistant rootstocks have been used in commercial practice (5-6 years) makes it difficult to assess the risk of resistance development in the pathogen population (C. Leifert personal communication)

Biological control Only few studies report potential biological control approaches for *P. lycopersici*. For example, certain *Trichoderma* strains were shown to show antagonistic activity against *P. lycopersici* (Vanachter *et al.*, 1998; Whipps, 1987). Non-volatile antagonistic substances and/or mycoparasitism were the suggested modes of action of *Trichoderma spp* against *P. lycopersici* (Vanachter *et al.*, 1998; Whipps, 1987).

In another study, significant cross protection was reported, when pre-inoculation of roots with non-aggressive strains of *P. lycopersici* or *Rhizoctonia solani* resulted in significant reductions in corky root rot disease development in young tomato plants. (Lemaire *et al.*, 1998; Ciccicarese *et al.*, 1994).

Pre-planting applications to roots of antagonistic strains of *Bacillus subtilis* and *Streptomyces graminofaciens* was also reported to reduce disease symptom development in infested soils, enhanced crop growth rates and higher yields (Bochow, 1989). Also a combination of soil treatment with *Bacillus subtilis* and VA-mycorrhizal fungal (*Glomus caledonium*) spores resulted in a lower disease index, increased crop growth rates and yield (Boscow and Abou-Shaar, 1990).

Organic management practices and organic matter soil inputs In studies in which corky root rot disease development was compared between soils managed to organic and conventional standards, corky root of tomatoes was found to be less severe in soils from organic farms compared to soil from conventional farms (Workneh and Van Bruggen, 1994). This could have been due to (a) lower levels of pathogen soil inocula being present in organically managed soils, (b) higher levels of competition for the pathogen in organic soil (organic soils were shown to be more biologically active) and/or (c) a more balanced supply of mineral nutrients in the soils from organic system resulting in a more resistant plant.

These results are supported by another comparative study in which 27 organic and conventional on-farm systems were assessed, which also showed that the severity of corky root caused by *P. lycopersici* was lower in the organic farms (Workneh and Van

Bruggen, 1993). Discriminated analysis of 11 soil and plant variables indicated that tissue nitrogen and soil nitrate were positively correlated with corky root rot severity, whereas microbial activity was negatively associated with the disease (Workneh and Van Bruggen, 1994). These results suggest that high nitrogen concentrations in conventional farms might have rendered tomato plants more susceptible to corky root rot than those grown in organically managed soils. This confirms previous reports, which suggested that increased susceptibility to root rots is caused by high nitrogen concentrations in plant tissues (Huber and Watson, 1974).

1.3.3 Tomato *Verticillium* wilt (*Verticillium albo-atrum*)

Verticillium wilts occur worldwide, but are most important in temperate regions. They attack more than 200 mainly dicotyledonous species of plants, including most vegetable and many flower crops, fruit trees, strawberries, field crops and shade and forest trees (Pegg and Brady, 2002).

There are six species of *Verticillium* causing *Verticillium* wilt diseases according to a recent comprehensive monograph (Pegg and Brady, 2002). Of these, the hemi-biotrophic species (*V. albo-atrum* and *V. dahliae*) are more widespread. *Verticillium albo-atrum* produces only dark-resting mycelium and *V. dahliae* produces only microsclerotia as survival/resting structures.

Three other closely related species are weaker plant pathogens and/or soil saprophytes. These are *Verticillium tricorpus* which produces dark-resting mycelium, microsclerotia and chlamydospores and *V. nigrescens* and *V. nubilum* which produce only chlamydospores. The sixth species, *V. theobromae* can produce a pale brown resting-mycelium and it is mainly associated with fruit rots (cigar end) of bananas (Barbara and Clewes, 2003).

Three *Verticillium* species have been found to cause *Verticillium*-wilt in tomato, *Verticillium dahliae* and *V. albo-atrum* (that are most widespread) and *V. tricorpus* (Agrios, 1997).

The temperature requirements for growth and survival for the microsclerotial and dark resting mycelial types of *Verticillium* spp. are the most important characteristic used for the separation of *V. albo-atrum* and *V. dahliae* as taxonomically distinct species (Pegg and Braddy 2002). *V. albo-atrum* grows best between 20 and 25° C whereas *V. dahliae* shows maximum growth at slightly higher temperatures 25-28° C (Agrios, 1997).

Fungal mycelium invades the root tissues through wounds, but undamaged roots may also be infected. For example, *V. albo-atrum* has been observed to penetrate undamaged roots through root hairs or epidermis and cortex cells and invade intact tomato seedling

roots in sterile culture (van den Ende, 1958). Selman and Buckley (1959) reported that the infection of tomato roots by *V. albo-atrum* increases by 4 to 10 fold when tomato roots are injured or cut during transplantation.

For successful infection, the infection hyphae have to be relatively close to the roots, since the fungus is a poor saprophyte in field soils (Schnathorst, 1981). It was reported by Sewell (1959) that *V. albo-atrum* hyphae could not be detected more than 2mm from germinating propagules.

After invasion of the root epidermis, the fungus enters the xylem vessels where the mycelium proliferates and produces conidiospores on conidiophores (Beckman, 1987; Buckley *et al.*, 1969; Tolmsoff, 1973) and/or by autoconidiation (Schnathorst, 1981).

In some hosts, *Verticillium* wilt develops in seedlings, which usually die rapidly (seedling damping-off). However, infections of later development stages of plants are more common. Typical symptoms of *Verticillium* infection are the dropping of younger upper leaves and the development of irregular chlorotic patches that later become necrotic on older leaves. Older plants are usually stunted and their vascular tissues show characteristic discolorations (Agrios, 1997).

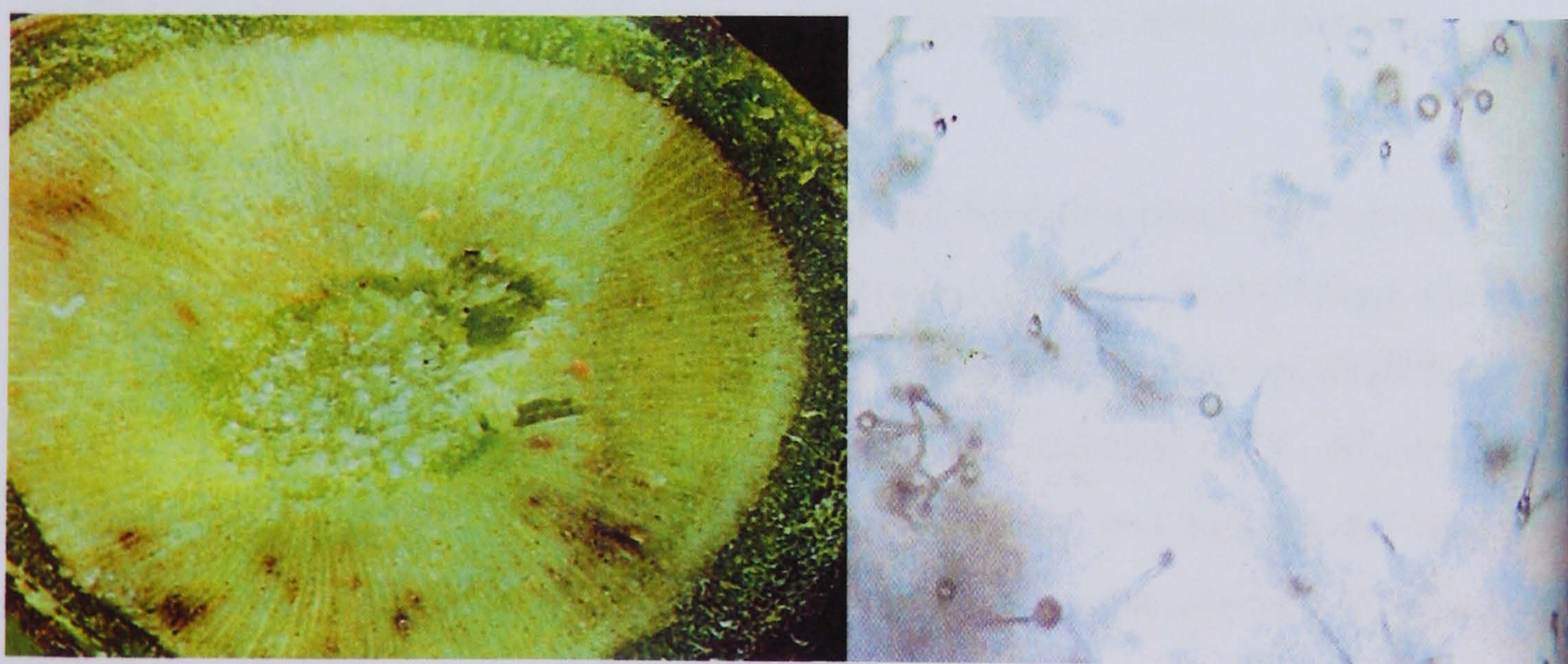


Figure 1.2. Vascular discoloration (left) of tomato vessels infected by *V.albo-atrum*. Conidiophores and conidiospores of *V. albo-atrum* (right). (Pronti and Laffi, 1990).

The pathogen inoculum for initial infection usually comes from infected soil and infected plant material (contaminated seeds, vegetative cuttings and tubers) and can be spread by the wind, the surface water and machinery (Pegg and Brady, 2002; Bourbos & Scoudridakis, 1987).

When *Verticillium* first appears in a field, symptoms on plants are usually mild and limited to specific areas in the field. However, as the inoculum builds up and/or when more aggressive strains appear, the disease symptoms become more severe and widespread and as a consequence the use of the susceptible crop species in the rotation has to be discontinued or resistant varieties have to be introduced (Beckman, 1987; Agrios, 1997).

1.3.3.1 Effect of environmental conditions on disease development

Temperature is the most important environmental factor affecting (a) the germination of propagules and infection process of *Verticillium*, (b) the disease progress and severity and (c) the geographical distribution of the disease (Pegg and Brady, 2002).

In a recent survey in Crete (Greece), 92 *Verticillium* isolates were taken from different crops including vegetables, olive trees and weeds and were all found to be *Verticillium dahliae* (Ligoxilakis and Vakalounakis, 1994).

According to Isaac (1949) the optimum temperature for growth of *V. albo-atrum* was 22.5° C, whilst at 30° C, *V. albo-atrum* was reduced to a yeast-like budding.

pH Conidial germination of *Verticillium* species has been described to be optimal within a pH range of 5.0-7.0, while growth of mycelium stops below pH 3.0 (Pegg and Brady 2002; Puhalla and Bell, 1981). Different *Verticillium* species appear to react differently to soil pH, for example, Isaac (1949) reported that the pH optimum for *V. albo-atrum* was between 5.3 and 7.2, while the pH optimum for *V. dahliae* was between 8.0 and 8.6. It has been reported though that *V. albo-atrum* and *V. dahliae* can tolerate pH values up to 10 (Isaac, 1949; Malca *et al.*, 1968).

Nitrogen supply Though the literature is conflicting about the role of nitrogen in disease development, high nitrogen supply is generally thought to increase wilt severity and occurrence (Pegg and Brady, 2002). For example, Roberts (1943) and Selvaraj (1975) reported that high levels of nitrogen increased tomato's susceptibility to *V. albo-atrum*.

Supply of other mineral nutrients According to Dutta and Bremner (1981) increasing the supply of copper sulphate *in vitro* reduced spore germination and germ tube length and inhibited growth of *V. albo-atrum*. On the other hand, addition of manganese and molybdenum stimulated fungal growth.

In an *in vivo* study by Baruah and Dutta (1979) into the effect of foliar application of organic and inorganic chemicals on tomatoes infected with *V. albo-atrum*, increasing the supply of the trace elements B and Mo was observed to reduce disease severity.

Lighting regime Jones *et al.* (1975), demonstrated that symptom development in *V. albo-atrum* infected tomato plants was affected by the length of the daily light period (in experiments a root-dip method of inoculation was used and plants incubated in growth rooms at a constant temperature of 22⁰ C and a light intensity of 8.608 lux). At a 4-hour daily light period, symptoms developed most rapidly and were most severe, and decreased with increasing daily light period (8, 12 and 16 hours).

1.3.3.2 Control methods of *Verticillium albo-atrum*

Soil disinfection and fungicide soil treatments For more than 20 years control of *Verticillium* spp. in many conventional crops was based on methyl bromide soil disinfection. However, Munnecke *et al.* (1978) categorised *V. albo-atrum* as the pathogen least sensitive to methyl bromide when compared to several other soil-borne plant pathogenic fungi. Moreover, Bourbos (1986) demonstrated that 12 days after treatment with methyl bromide in unheated tomato glasshouses *V. albo-atrum* started to re-appear, and that the treatment did not affect fungi at the depth of 30-40cm, and that *Verticillium*

inocula present at such depths can re-colonise upper layers of soil within one growing period. This is thought to be the main reason for the very frequent need to re-apply methyl bromide treatments in *Verticillium* infected soils.

Other soil fumigants such as methyl isothiocyanate, chloropicrin, 1,3-dichloropropan have been used and reported to control the pathogen effectively (Overman and Jones, 1986), but are all highly toxic and require (like methyl-bromide) specialist equipment and trained operators for application.

Sodium azide, used as soil fumigant, also controlled the pathogen well, compared with dazomet which did not give satisfactory levels of control in tomatoes (Wambeke *et al.*, 1984).

A range of systemic fungicides is also currently available for the control of *V. albo-atrum* including benomyl, benzimidazol-2-yl carbamate (carbendazim, MBC) and several other compounds with similar mode of action (Pegg and Brady, 2002).

Soil solarization is a soil disinfection technique pioneered by Katan *et al.*, (1976), which is based on the use of polyethylene or PVC to cover the soil, enclosing the solar radiation and delaying the loss of heating. The increase in temperature in the upper layers of the soil to above the thermal death point of pathogens, results in a reduction of soil inocula of fungal pathogens, including *Verticillium* (Bourbos and Skoudridakis, 1996), nematodes, weed seeds and other organisms (e.g. non-spore forming bacteria).

Soil solarization has been also reported to increase the competitiveness of antagonistic fungi such as *Trichoderma flavus* and *Aspergillus terreus* (Tjamos and Skretis, 1990), *Trichoderma*, *Talaromyces flavus* and certain lytic bacteria (Elad *et al.*, 1980; Tjamos and Paplomatas, 1986, 1987 a,b, 1988; Greenberger *et al.*, 1987) and results in an increase in population density of thermophilic and thermotolerant fungi and bacteria (Stapleton and DeVay, 1982).

Control of soil-borne pathogens by soil solarization usually leads to improved plant growth and yield (Katan 1996). Tjamos and Faridis (1981) using solarization

reported an increase of 100% on yield of tomatoes grown in soils infected by *V. albo-atrum*.

Biological disinfection is a non-chemical control method developed by Blok *et al.* (2000). This method is aimed at imposing a general soil anaerobiosis by incorporating green plant material into moist soil and covering the soil by thick ensilage plastic for at least six weeks. Inoculum density of soil-borne pathogens such as *F. oxysporum* f.sp *asparagi*, *Verticillium dahliae*, *Pratylenchous penetrans*, *Meloidogyne chitwoodi* and *Globodera pallida* was reduced by at least 95%.

Biological control During the 1970s numerous attempts were made to develop methods which allow the suppression of soil-borne plant pathogen based on application of antagonistic microorganisms, but overall these attempts were judged to be “not that successful” (Cook and Baker, 1983). However, from the 1980s and throughout the 1990s, consumer concern about the use of soil disinfectants and pesticides (in particular methyl bromide, which was shown to be a major ozone depleting chemical) increased rapidly, resulting in an renewed effort to identify biological control strategies for soil-borne pathogens such as *Verticillium* spp. (Pegg and Brady, 2002).

Various fungi have been tested as biological control agents but the genera of *Trichoderma*, *Gliocladium*, *Trichoderma* and *Penicillium* have dominated the more recent literature (Pegg and Brady, 2002), though most of the research has been focused on *V. dahliae*.

T. viride has been reported to be antagonistic to *V. albo-atrum* (Aube, 1967) and more recently Dutta (1981) reported that *Trichoderma viride*, *Gliocladium* spp., *Fusarium culmorum*, *Penicillium chrysogenum*, *P. vermiculatum* and *P. chrysogenum* effectively suppressed *V. albo-atrum* in tomato crops and resulted in fruit yields similar to un-infected, healthy tomato plants. *Penicillium* spp. and *Trichoderma* spp. are thought to be among the most effective antagonists, and their mode of action was shown to

include the production of strong broad range antibiotics and direct competition (Pegg and Brady, 2002).

Kurzawinska and Pacyna (2000) demonstrated that the growth of *V. albo-atrum* was inhibited in tomato cultivation by the saprobial community of, mainly *Trichoderma* spp., *Penicillium* spp. and Kowalic (1994), reported that *Fusarium* spp. *Trichoderma aureoviride*, *T. harzianum*, *T. piluliferum*, *T. pseudokoningii* and *T. viride*, controlled *V. albo-atrum* when introduced in hydroponic tomato cultures based on peat and mineral/rock wool substrate.

Wilderspin and Heale (1984) in laboratory experiments found out that the use of an isolate of *Talaromyces flavous*, Tf1, resulted in a 50% reduction in *albo-atrum* infection in *Antirrhinum*.

Other fungi reported as antagonists for *V. albo-atrum* include *Zygorrhynchus moelleri* (Brown, 1986), which produces lytic enzymes, *Tuber melanosporum* (Bonfante *et al.*, 1972), *Blastomyces luteus* (Isaak, 1954), and *Phaeotheca dimorphospora* (Yang *et al.*, 1993).

Another antagonist that was described to have potential as a biological control agent for *Verticillium* is the oomycete *Pythium oligandrum* (Berry *et al.*, 1993; Deacon, 1976; Martin and Hancock, 1987). Benhamou *et al.*, (1999) demonstrated the suppression of *V. albo-atrum* by *P. oligandrum* is associated with the rapid lysis of the pathogen's cytoplasm, resulting in cell wall disorder and eventually to an entire collapse of the host cell.

El-Abyad *et al.* (1996) tested three *Streptomyces* species (*S. pulcher*, *S. canescens* and *S. citreofluorescens*) for their activity against *V. albo-atrum* in tomato. He used three different *in vivo* treatments: soaking tomato seeds in filtrate of the antagonists before sowing, inoculating the soil with the antagonist seven days before sowing and coating

tomato seeds with spores of the antagonist before sowing. The seed-coating treatment was the most effective in controlling the pathogen at 42 and 63 days after sowing.

Streptomyces plicatus was tested for chitinase production and found to be the best producer among three hundred and seventy-two isolates belonging to the genus *Streptomyces* (Abd-Allah, 2001). *S. plicatus* chitinase was shown to significantly inhibit germ tube elongation, spore germination and radial growth of *V. albo-atrum* *in vitro*.

These *in vitro* study results were verified through greenhouse trials, where the application of *S. plicatus* spores to the root system of tomato plants significantly reduced disease development by *V. albo-atrum*.

The potential of *Bacillus* spp. as biocontrol agents for fungal diseases has also been repetitively reported. With regard to *V. albo-atrum* there are numerous studies showing effective control when *B. subtilis* was applied (Podile and Dube, 1985; Podile *et al.* 1985).

There is also a report of a nematode (*Aphelenchus avenae*) having potential as a biological agent; this nematode is known to feed on the mycelium of fungi such as *V. albo-atrum* (Mankau and Mankau, 1962).

Resistant varieties and rootstocks A single gene *Ve* from the Peruvian wild species *Lycopersicon pimpinellifolium* was incorporated into commercial cultivars of *L. esculentum* by Blood in 1925 (Pegg and Brady, 2002) and confers resistance to race 1 of *V. dahliae* and to strains of *V. albo-atrum* (Pegg and Dixon, 1969).

In Greece recently, in a survey of different soils, only the *V. dahliae* race 1 was found and for this reason Verticillium wilt of tomato crops is not concerned as a major problem as there are many cultivars with *Verticillium* (race 1) resistance (Vakalounakis and Fragiadakis, 2003). Though the successive use of resistant varieties could lead to the development of *Verticillium* strains capable to overcome this resistance (Beckman and Talboys, 1981).

For example Patternotte and Van Kestern (1993) described a new aggressive strain of *V. albo-atrum* in Verticillium resistant cultivars of tomato in The Netherlands.

Race 2 pathogenic to race 1 resistance appeared in 1962 (Alexander, 1962) and it was suggested by Grogan et al. (1979) that the race 2 was already in existence with race 1 but it increased under the selection pressure from an increased planting of *Ve* lines.

Recently a partial resistance to race 2 was described in c.v. IRAT (Gold and Robb, 1995).

Cultivars IRAT L3, Morden Lac, Okitsu Sozai and UC82 have showed a tolerance to race 2 quite different from the single *Ve* gene resistance to race 1 and at present what appears to be polygenic partial resistance (tolerance) is the best defence against *Verticillium* species (Pegg and Brady, 2002).

Crop Rotation For outdoor crops there are several reports indicating that crop rotation was successful in reducing *V. albo-atrum* population densities and disease incidence in infested soils. For example, a 3-4 year rotation with non-host crops effectively reduced the *Verticillium* wilt in potato crops and a 3-5 years rotation with non-hosts reduced the *Verticillium* wilt incidence and increased yield in mint crops (McKay, 1926; Green, 1967). Xiao *et al.*, (1998) evaluating broccoli residues or crops on *V. dahliae* infection in cauliflower and irrigation methods and regimes, showed a 94% reduction in propagules after two cauliflower crops in contrast to a fivefold increase after two cauliflower crops in the absence of broccoli residues. However, in glasshouse production systems rotation is less likely to be of significant importance as a wilt control method, due to the lack of suitable non-host crops (van Lenteren and Woets, 1988; Agrios, 1997).

Plant Extracts When a range of plant extracts were tested for their inhibitory effect against *V. albo-atrum* of tomato and other pathogens (Kuprashvili, 1996) extracts of garlic and celery had a disinfecting effect on tomato seed and increased yield of tomato crops grown from treated seed. Application of (-)-Usnic acid (extracted from the lichen *Alectoria ochroleuca*) at a concentration of 100 micro g/disc also controlled the pathogen in *in vitro* tests (Proksa *et al.*, 1996).

1.3.4. Cucumber Powdery mildew (*Podosphaera xanthii*)

Powdery mildews (*Erysiphales*) are one of the most commercially important groups of crop diseases and most of the vegetable and ornamental plants grown in greenhouses suffer from powdery mildews (Lucas, 1998).

“Powdery mildews are so common, widespread and ever present among crop plants and ornamentals that the total losses, in plant growth and yield, they cause every year on all crops probably surpass the losses caused by any other single type of plant disease” (Agrios, 1997).

Podosphaera xanthii is the most important species in greenhouse grown cucurbit crops (Molot and Lecoq, 1986) and is currently the only disease that prevents long “English”-type cucumbers from being produced without the use of synthetic pesticides, (Daayf *et al.*, 1995).

The most important species of powdery mildew in the greenhouses are *Podosphaera xanthii*, which attacks mainly cucumber, melon and courgette; *Erysiphe cichoracearum* which also attacks cucurbits; *Oidium spp.* which affects tomatoes; *Sphaerotheca panosa* var. *rosae* which damages roses; and *Leveillula taurica* which damages tomatoes, peppers, eggplants, artichokes and to a limited extent, cucumber (Blancard *et al.*, 1994).

Under greenhouse conditions with higher temperature and humidity levels and in outdoors field crops (especially during autumn) *P. xanthii* is predominant (Bourbos and Scoudridakis, 1993).

The number of asci (sexual ascospore containing sacs) contained in cleistothecia (fruiting bodies containing the asci with ascospores) can be used to discriminate *P. xanthii* from *E. cichoracearum*.

Cleistothecia of *P. xanthii* contain one ascus while cleistothecia of *E. cichoracearum* more than one ascus.

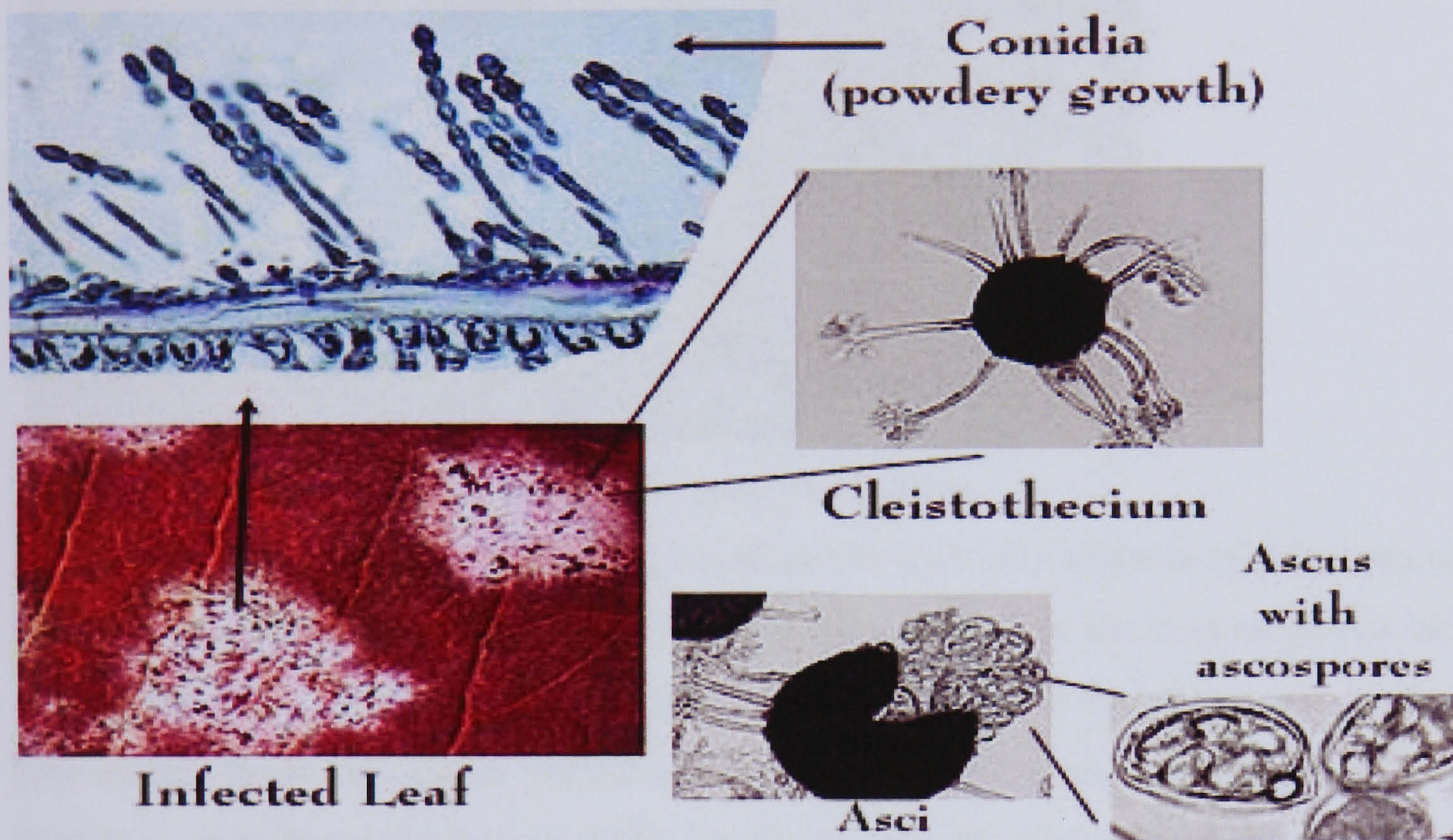


Figure 1.3. Conidia, cleistothecium, and asci of *P. xanthii*

(Source http://botit.botany.wisc.edu:16080/courses/botany_130/Div/Mildew.html)

The characteristic symptoms of powdery mildews (see Figure 4) are caused by grey to white sporulating mycelium covering areas of the upper leaf surface. However, infections and symptoms may also appear on the lower leaf surface (for example in peppers the fungus sporulates on the lower leaf surface). In severe epidemics sporulating lesions may even appear on stems and flowers (Zitter *et al.*, 1996).



Figure 1.4. Cucumber leaves infected by powdery mildew

The primary inoculum of powdery mildew infections is usually airborne conidia (asexual spores). Conidia are easily transferred from greenhouse crops to the field crops and vice versa (Elad *et al.*, 1996) and viable conidia of *P. xanthii* can be found on crop residues. Late season crops and weeds (which can act as alternative hosts of the pathogen) are thought to contribute significantly to the perpetuation of the disease from one year to the other (Bourbos and Skoudridakis, 1993), especially in Southern European countries. Spores produced as a result of sexual reproduction (ascospores) may also play a role in the persistence of the disease and cleistothecia of *P. xanthii* are easily found in greenhouses (Zitter *et al.*, 1994).

1.3.4.1 Effect of environmental conditions on disease development

Temperature Powdery mildews usually are more severe in summer crops when higher temperatures favor the infection process and disease development. Temperatures of between 22 and 30⁰ C are considered optimum for disease development, and while higher humidity favors the initial infection of leaves, dry conditions do not restrict the spread of disease after primary infection. Powdery mildews are therefore often the predominant diseases in geographic areas with dry warm/hot climates (Dixon, 1981).

The temperature range over which disease development by powdery mildews takes place, overlaps with the conditions generally prevailing in greenhouses and disease epidemics may occur up to maximum temperatures of about 30 –35⁰ C, (Blancard *et al.*, 1994).

Infection by powdery mildew conidia is favored by low vapor pressure deficit, but spores can be severely damaged when immersed in water on the plant surface, (Elad *et. al.*, 1996).

Humidity Experiments under controlled conditions demonstrated, that water-saturated air was needed for infection of the powdery mildew and, daily fluctuations in temperature increase the air humidity to a sufficiently high level for conidial germination and infection (Sundeim, 1982).

Powdery mildew fungi are obligate parasites. They grow on the surface of the host plant tissue (except *L. taurica*, which is entophytic) and extend haustoria into epidermal cells, through which nutrients are extracted from the host cell (Lucas, 1998). Once infection has occurred the spread of disease within the plant is much less dependent of humidity conditions (Sundeim 1982).

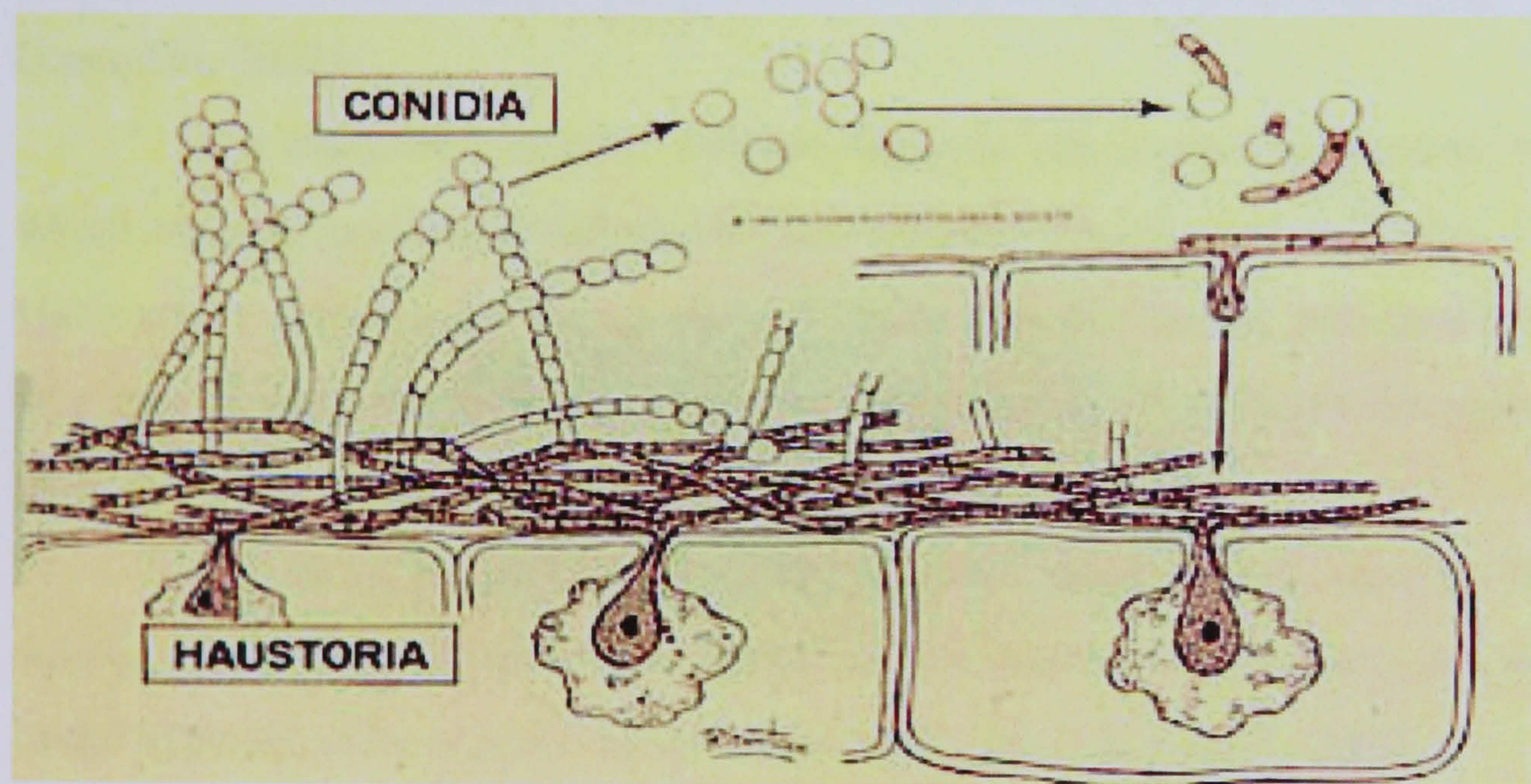


Figure 1.5 Haustoria of *P. xanthii* (Source, <http://www.apsnet.org/Education/k12plantpathways/TeachersGuide/Activities/PowderyMildew/text/FIG10.HTM>)

The host plant is rarely killed, but crops often suffer severe yield losses, through reduced levels of photosynthesis, changes of the physiology of plants and nutrients scavenging from the host plant by the pathogen (Agrios, 1997).

1.3.4.2 Control of powdery mildew diseases

The two main methods for the disease control in conventional crop production are repeated applications of fungicides and the use of cultivars that are resistant or tolerant to powdery mildew (Kiss, 2003), but both methods have limitations.

However, a range of alternative control methods based mainly on (a) improving environmental control in glasshouses, (b) biological control agents and (c) plant extracts have been described or are currently under development. These are described below.

Disease resistance/tolerance Cultivars resistant to powdery mildews have been developed in a number of cucurbit crops. For example, there are several cultivars of melon with vertical resistance against races 1, 2, and 3 of *P. xanthii* (Elad *et al.*, 1998).

Vertical resistance is where specific resistance against an individual race of pathogen is contained in a single gene. This can be overcome relatively easily by the pathogen (Lampkin, 2002).

For cucumber, several tolerant varieties (horizontal resistance) are available, which are only moderately infected by powdery mildew (Elad *et al.*, 1998).

Horizontal resistance -polygenic traits- is more general, aiming not for a total resistance to a specific race but a partial resistance (tolerance) to all races of the pathogen so it is more difficult to be overcome by the pathogen (Agrios, 1997).

Tolerance to powdery mildew involves mechanisms such as the host cell structure, inhibition of haustorium formation, the nutrition absorption through haustorium or the hypersensitive reaction of epidermal cells (Morishita *et al.*, 2002).

For example Awad (2000) tested different cultivars for their resistance levels against powdery mildew and found that there was a positive correlation between susceptibility to powdery mildew and leaf sugar content in most of the cultivars tested.

Schlosser, (1990) testing different cucumber cultivars found that the epidermic cells of the tolerant to powdery mildew cultivars became increasingly resistant with age,

due to progressive depletion of cytoplasm and concomitant vacuolization, and to increasing numbers of papillae which inhibited fungal penetration.

Fungicides In conventional greenhouse production, the use of chemosynthetic fungicides to minimise either **(a)** infection rates (in the early stages of the disease) or **(b)** spread of the disease following infection has remained a major approach for the control of powdery mildews. However, the development of pathogen strains resistant to the main mildew fungicides used such as EBIs and benzimidazoles has been reported and is now widespread (Dekker and Gielink, 1979; Schepers, 1983; Elad *et al.*, 1996).

Moreover, the public concerns about the environmental impact and potential consumer health risks associated with pesticide residues in foods have resulted in:

- (i) public pressure to reduced the use of chemosynthetic pesticides and
- (ii) the search therefore for alternative control methods for powdery mildew diseases in protected crops.

In both conventional and organic agriculture the use of Sulphur fungicides is permitted. They provide effective control against powdery mildew, but are toxic for plants at temperatures above 27⁰ C and can have negative side effects on beneficial invertebrates (see section 1.2.5.1, above). In Southern European countries this limits their use to the cooler seasons (Bourbos and Skoudridakis, 1993).

Biological control A wide range of microorganisms was reported to be effective as biological control agents (BCAs) against powdery mildew fungi (see recent review by Kiss, 2003). Table 1.3 lists the wide range of reports reviewed by Kiss (2003), in which specific fungal species were reported as natural antagonistics of powdery mildews and/or have been tested for their potential as BCAs in commercial greenhouse production. Table 3 also lists the mode of action ascribed to the different antagonists.

A range of modes of action can be responsible for the biological control of fungal pathogens by antagonists, most frequently described and studied modes of action being mycoparasitism, competition and antibiosis (Elad, 2000).

Biotrophic pathogens such as powdery mildews are not dependent on exogenous nutrients during the germination and host infection process. This ability is essential for infection to take place in a nutrient-depleted phyllosphere (Staples *et al.*, 1962).

It has been postulated that the ecological niche they occupy in the phyllosphere could only be colonized by other plant pathogens, but not by saprophytic biocontrol agents (Blakeman, 1993). Biological control approaches based on competition for nutrients/space by the antagonist are therefore not considered to be feasible with respect to powdery mildews (Elad *et al.*, 1996).

Kiss (2003) therefore suggested that for powdery mildew control, only biological control agents which act via antibiosis or mycoparasitism, as the principal modes of action, are likely to be successful.

The induction of plant defence has been proposed by some authors to be part of the antagonistic effect – mode of action observed against certain pathogens (Belanger and Labbe, 2002) and this mode of action may be responsible for the control of powdery mildew observed with some BCAs.

Table 1.3. Fungal species reported as naturally occurring antagonists or potential biological control agents against powdery mildew diseases (Kiss 2003)

<u>Antagonist</u>	<u>Powdery mildew genera/species</u>	<u>Host plant</u>	<u>Mode of action</u>
<i>Acremonium byssoides</i>	<i>Qidium heveae</i>	<i>Hevea brasiliensis</i>	?
<i>A. alternatum</i>	<i>Sphaerotheca fuliginea</i>	<i>Cucurbitaceae</i>	Mycoparasitism?
<i>A. strictum</i>	<i>S. fuliginea</i>	<i>Cucumis sativus</i>	?
<i>A. lanosoniveum</i>	<i>S. macu/aris</i>	Strawberry	?
<i>Ampelomyces spp.</i> (syn <i>Cicinnobolus spp</i>)	Many species	Many species	Mycoparasitism
<i>Aphanocladium album</i>	<i>Erysiphe cichoracearum</i>		?
	<i>S. fuliginea</i>	<i>C. sativus</i>	
	<i>S. pannosa var rosae</i>	<i>Rosa sp.</i>	
<i>Acrodontium crateriforme</i>	<i>E.pisi</i>	<i>Medicago lupulina</i>	Mycoparasitism?
	<i>S.fuliginea</i>	<i>C. sativus</i>	
<i>Aspergillus fumigatus</i>	<i>E.cichoracearum</i>	<i>Cucurbita maxima</i>	?
<i>Cladosporium oxysporum</i>	<i>Phyllactinia corylea</i>	<i>Morus alba</i>	Mycoparasitism?
<i>C. spongiosum</i>	<i>Ph. dalbergiae</i>	<i>Dalbergia sissoo</i>	?
	<i>Ph. corylea</i>	<i>M. alba</i>	?
<i>C. cladosporioides</i>	<i>E. cichoracearum</i>	<i>Xanthium strumarium</i>	?
<i>C/adosporium sp.</i>	<i>S. fulliginea</i>	<i>C. pepo</i>	?
<i>Cephalosporium Sp.</i>	<i>Leveillula taurica</i>	<i>Capsicum annuum</i>	?
	<i>S. fuliginea</i>	<i>Citrullus lanatus</i>	
<i>Calcarisporium arbuscula</i>	<i>S. fuliginea</i>	<i>C. sativus</i>	Antibiosis?
<i>Cladobotryum varium</i>	<i>S. fuliginea</i>	<i>C. sativus</i>	?
<i>Chaetomium spp.</i>	<i>Podosphaera leucotricha</i>	<i>Malus domestica</i>	?
<i>Drechslera spicifera</i>	<i>E. cichoracearum</i>	<i>C. maxima</i>	?
<i>Dissoconium aciculare</i>	<i>E. pisi</i>	<i>M. lupulina</i>	Antibiosis?
	<i>E. martii</i>	<i>Lupinus polyphyllus</i>	
	<i>E. betae</i>	<i>Beta vulgaris</i>	

Table 1.3 cont. Fungal species reported as naturally occurring antagonists or potential biological control agents against powdery mildew diseases (Kiss, 2003)

Antagonist	<u>Powdery mildew</u> <u>genera/species</u>	<u>Host plant</u>	<u>Mode of action</u>
<i>Fusarium oxysporum</i>	<i>E. cichoracearum</i>	<i>C. maxima</i>	?
<i>Paecilomyces farinosus</i>	<i>E. martii</i>	<i>L. polyphyllus</i>	Antibiosis?
	<i>S. fuliginea</i>	<i>C. sativus</i>	
		<i>C. fanatus</i>	
	<i>L. taurica</i>	<i>C. annuum</i>	
<i>Penicillium chrysogenum</i>	<i>S. fuliginea</i>	<i>C. sativus</i>	?
<i>P. fellutanum</i>	<i>E. cichoracearum</i>	<i>C. maxima</i>	?
<i>Peziza ostracoderma</i>	<i>S. fuliginea</i>	<i>C. sativus</i>	?
<i>Phoma glomerata</i>	<i>Microsphaera penicillata</i>	<i>Platanus occidentalis</i>	?
<i>Pseudozyma spp.</i>	<i>E. polygoni</i>	<i>Trifolium pratense</i>	Antibiosis
(syn. <i>Sporothrix spp.</i>	<i>S. fluliginea</i>	<i>C. sativus</i>	
<i>Stephanoascus spp)</i>	<i>S. pannosa</i> var <i>rosae</i>	<i>Rosa sp.</i>	
	<i>Blumeria graminis</i>	<i>Triticum aestivum</i>	
<i>Ramichloridium apiculatum</i>	<i>E.pisi</i>	<i>M. Lupulina</i>	?
	<i>S. fuliginea</i>	<i>C. sativus</i>	
<i>Scopulariopsis brevicaulis</i>	<i>S. fuliginea</i>	<i>C. sativus</i>	?
<i>Sesquicillium</i>	<i>S. fuliginea</i>	<i>C. sativus</i>	?

<i>candelabrum</i>			
<i>Sepedonium chrysospernum</i>	<i>S. fuliginea</i>	<i>C. sativus</i>	?
<i>Trichotecium roseum</i>	<i>E. martii</i>	<i>L. polyphyllus</i>	Antibiosis
	<i>S. fuliginea</i>	<i>C. sativus</i>	
<i>Tilletiopsis albescens</i>	<i>S. fuliginea</i>	<i>C. sativus</i>	Antibiosis
	<i>B. graminis</i>	<i>Hordeum vulgare</i>	
<i>T. minor</i>	<i>E. martii</i>	<i>L. polyphyllus</i>	Antibiosis
	<i>S. fuliginea</i>	<i>C. sativus</i>	
	<i>B. graminis</i>	<i>H. vulgare</i>	
<i>T. pallescens</i>	<i>B. graminis</i>	<i>H. vulgare</i>	Antibiosis
	<i>S. pannosa</i> var <i>rosae</i>	<i>Rosa</i> sp.	Antibiosis
	<i>S. fuliginea</i>	<i>C. sativus</i>	Antibiosis
<i>T. washingtonensis</i>	<i>S. fuliginea</i>	<i>C. sativus</i>	Antibiosis
<i>Tilletiopsis</i> sp.	<i>S. fuliginea</i>	<i>C. sativus</i>	Antibiosis
<i>Trichoderma harzianum</i>	<i>S. fusca</i>	<i>C. sativus</i>	Induced resistance?
<i>T. viride</i> D	<i>S. fuliginea</i>	<i>C. sativus</i>	?
<i>Verticillium lecanii</i>	<i>Oidium tingtanium</i>	<i>Citrus</i> spp.	Mycoparasitism?
	<i>S. fuliginea</i>	<i>C. sativus</i>	
	<i>U. necator</i>	<i>Vitis</i> sp.	
<i>V. fungicola</i>	<i>S. fuliginea</i>	<i>C. sativus</i>	?

While a wide range of fungal antagonists have been tested against powdery mildews in *in vitro* tests, bioassays and glasshouse or field trials, only a few antagonists have been developed into commercially available products (Paulitz and Belanger, 2001).

So far only two fungal antagonists have been registered and commercialised in some countries as biofungicides. These are AQ10 Biofungicide®, which contains conidia of a strain of the hyperparasite *Ampelomyces quisqualis*, and Sporodex®, which is based on conidia of a basidiomycete yeast, *Pseudozyma flocculosa*. These two antagonists and some of the other antagonists that are thought to have potential for development as commercial products, are described in more detail below. Reasons for the variable performance of antagonists in glasshouse trials are described.

Ampelomyces quisqualis The most commonly found hyperparasite on powdery mildew is the coelomycete *Ampelomyces quisqualis* (Elad *et al.*, 1996). It is a hyperparasite that was first described 130 years ago (Cesati, 1852). It is thought to occur naturally on many species of powdery mildews (Sundeim, 1982).

Hyphae of *Ampelomyces* penetrate the hyphae of powdery mildews, continue their growth internally, and produce their pycnidia in the cells of the hyphae, conidiophores and immature cleistothecia of their fungal hosts. As intracellular mycoparasites they suppress the sporulation of infected powdery mildew mycelium and eventually kill all parasitized mildew hyphael cells, which can be observed as a gradual degeneration of their cytoplasm. It seems that the early stage of mycoparasitism is a nearly biotrophic association, but one that gradually changes into necrotrophic interaction without production of any toxins (Kiss, 2003).

Several authors have demonstrated biocontrol activity of *A. quisqualis* against *P. xanthii* in cucumbers (Jarvis and Slingsby, 1977; Philipp and Cruger, 1979; Sundeim, 1982; Sundeim and Amundsen, 1982; Szejnberg *et al.*, 1989).

The commercial product AQ10 Biofungicide® is currently available in USA and a few European countries (i.e. Italy, Denmark) and has been included in the studies reported here.

AQ10 Biofungicide® contains not less than 5×10^9 spores/g and is being used against powdery mildew in different crops (i.e. vegetables, vine). Powdery mildew can cause severe problems under dry conditions but *A. quisquallis* requires high humidity conditions for its development. To overcome this incompatibility, the use of different oils is recommended by the producers.

Pseudozyma flocculosa is the most recently identified and probably the most efficient natural antagonist of powdery mildews (Paulitz and Belanger, 2001). It was discovered along with another closely related species *P. rugulosa*. Both species were initially described as yeast-like fungi belonging to the Endomycetaceae, *Stephanoascus flocculosus* (Traquair *et al.*, 1988). However, they were later redefined as basidiomycetous yeasts related to anamorphs of Ustilaginales belonging to the genus *Pseudozyma bandoni* emend. Boekhout (Paulitz and Belanger, 2001).

P. flocculosa and *P. rugulosa* were shown to effectively control powdery mildew in rose and cucumber greenhouse crops (Belanger *et al.*, 1994; Hajlaoui and Belanger, 1991; Jarvis *et al.*, 1989).

P. flocculosa and *P. rugulosa* have been reported to act against powdery mildews through antibiosis and the production of antifungal metabolites which cause a collapse of powdery mildew cells (Hajlaoui *et al.*, 1994; Choudbury *et al.*, 1994).

Tilletiopsis spp. Another biological control agent tested against powdery mildew of cucumber are *Tilletiopsis spp.* For example, strong antagonism between a *Tilletiopsis sp.* isolate and powdery mildew was shown in field experiments by Hoch and Providenti (1979).

The mode of action of *Tilletiopsis spp.* is thought to be antibiosis, since they were shown *in vitro* to cause plasmolysis of powdery mildew cells and to rapidly kill powdery mildew colonies (Kiss, 2003). Hijwegen (1986) demonstrated that *T. minor* was

very effective in controlling the disease on cucumber plants, under controlled conditions, when applied twice after artificial inoculation of cucumber plants with *P. xanthii*. However, in greenhouse trials the effect of the antagonist on the disease was disappointing, most likely because the vapour pressure deficit was too high (Hijwegen, 1992).

Recently, two other species of *Tilletiopsis*, *T. washingtonensis* and *T. pallescens*, were found both to reduce the density of powdery mildew spores on greenhouse-grown cucumbers (Urquhart *et.al.*, 1994).

Verticillium lecanii is an antagonist of *P. xanthii*, which significantly reduced powdery mildew of cucumber in greenhouse experiments (Spencer and Ebben, 1981; Verhaar *et al.*, 1996 and 1998; Askary *et.al.*, 1997;1998). The mode of action of *V. lecanii* was initially thought to be hyperparasitism, since *V. lecanii* hyphae were observed to penetrate powdery mildew cells. However, according to more recent studies antibiosis may also play an important role in this pathogen/antagonist interaction (Belanger and Labbe, 2002).

V. lecanii was initially recognised as a parasite of insect pests and certain strains have been registered as commercial products (e.g. Vertalec®), and are marketed specifically for the control of whiteflies in greenhouse systems (Paulitz and Belanger, 2001).

The humidity conditions at the plant surface were described as the most important factor influencing the germination, growth and survival of *V. lecanii* and its biocontrol activity against cucumber powdery mildew. For example, the efficacy of *Verticillium lecanii* against *P. xanthii* was significantly reduced at lower humidity levels (Verhaar *et al.*, 1998;1999b).

Other antagonists A range of other antagonists were shown to have significant activity in some glasshouse trials, but only under specific environmental conditions. These included *Acremonium alteratum* and *Cladosporium cladosporioides* which both parasitised and destroyed the thallus of *P. xanthii* under conditions of low vapour pressure deficit (Malathrakis, 1985; Malathrakis and Klironomou, 1992).

In addition to fungal antagonists, the bacterial species *Bacillus brevis* has been tested *in vitro* and *in vivo* on cucumber plants for its potential as a biocontrol agent against *P. xanthii*. The results indicated that *B. brevis* had the potential to be used as BCA to control powdery mildew of cucumber, since it reduced the number of lesions on cucumber leaves caused by *P. xanthii* (Schmitt *et al.*, 1999). *Bacillus* species are known to produce antifungal antibiotics, but induced resistance has also been hypothesised as a mode of action of *B. brevis* (Schmitt *et al.*, 1999). As with many fungal antagonists *Bacillus* spp. were shown to require relatively high temperatures and levels of relative humidity for significant growth on leaf surfaces.

For both fungal and bacterial BCAs, a mismatch between (a) the environmental conditions (especially temperature and humidity) required for germination, infection, growth and/or expression of antagonistic activity of antagonists and (b) the environmental conditions over which mildews can infect and cause disease in host plants, therefore appears to be the main reason for the poor biological control activity under glasshouse conditions (Elad *et al.*, 1996; Dik *et al.*, 1998)

Once infection has taken place, disease progress by powdery mildew fungi is not affected by the levels of relative humidity within the glasshouse and can proceed rapidly even under dry conditions. Most biocontrol agents for foliar diseases on the other hand require relative humidities above at least >70%, (Hajlaoui and Belanger, 1991; Philipp and Hellstern, 1986). One strategy to overcome this problem that has been proposed, is to develop formulations and/or additives for biological control agents that allow activity over a wider range of relative humidity conditions (Elad *et al.*, 1996). A large number of additives have been tested to overcome humidity requirements of different biocontrol agents (Kiss, 2003). For example, AQ10 Biofungicide® is recommended in conjunction with a wetting/dispersing agent, such as AddQ, to overcome humidity requirements of the fungus (Paulitz and Belanger, 2001). Also, formulation of *V. lecanii* spores in arachid oil was shown to significantly reduce the humidity dependence. Since it is considered safe

for human consumption, it can be used as an additive to increase the effectiveness of *V. lecanii* against *P. xanthii*, (Verhaar *et al.*, 1999b).

The activity of *P. flocculosa* and *T. pallescens* in the biocontrol of cucumber powdery mildew was also shown to improve when formulated in certain oils (Urquhart & Punja, 1997; Belanger *et al.*, 1994). In some cases the additives themselves provide control against powdery mildews (Verhaar *et al.*, 1996). A similar effect was found when rape oil formulations were used to reduce the high humidity requirement of the fungus *Ascochyta caulina*, which is currently under development as a mycoherbicide (Ghorbani *et al.* 1999, 2000 & 2002).

A range of studies has compared the activity of the most promising candidate antagonists described above.

In experiments under controlled conditions, *P. flocculosa* showed more rapid colonization of powdery mildew colonies than *P. rugulosa* or *Tilletiopsis washingtoniensis* and was less affected by unfavorable climatic conditions (Hajlaoui and Belanger, 1991).

Verhaar *et al.* (1996) compared the activity of *V. lecanii* and *Pseudozyma rugulosa* against powdery mildew on two cucumber cultivars with different levels of susceptibility to *P. xanthii*. They found out that *V. lecanii* applications resulted in significantly higher levels of control with both cucumber cultivars when compared with *P. rugulosa*.

More recently a comparison of bio-control activity of the antagonists *Ampelomyces quisqualis*, *Verticillium lecanii*, and *Pseudomyza flocculosa* against *P. xanthii* was carried out under semi-commercial glasshouse conditions. In this experiment *A. quisqualis* did not control the disease, *V. lecanii* had a small effect on the pathogen and *P. flocculosa* again gave significantly higher levels of control than the other two BCAs (Dick *et al.*, 1998).

In another comparative experiment, *Ampelomyces quisqualis* and *Trichoderma harzianum* were tested for their ability to control *P. xanthii* and *Botrytis cinerea* on

greenhouse cucumber (Elad *et al.*, 1998). In these trials *Trichoderma harzianum* T39 (TRICHODEX) reduced powdery mildew severity by up to 97% but its efficacy declined to 18-55% as the epidemic progressed. *Ampelomyces quisqualis* (AQ10®) was also very effective in these trials achieving up to 98% control.

In these trials, two aliphatic petroleum distillate oil formulations were compared to the use of spores without formulation. Formulations were found to improve the efficacy of both biocontrol agents.

When *T. harzianum* was applied to soil (instead of foliar sprays), it also resulted in 75-90% lower coverage of powdery mildew on cucumber leaves. This led to the conclusion that the mode of action of *T. harzianum* in powdery mildew is, at least, partially due to induced resistance and not solely mycoparasitism and/or antibiotic action.

Compost extracts An alternative approach for control powdery mildews, which was widely promoted among organic farmers for use in organic farming systems, is the use of aqueous extracts of composts that contain a mixture of microorganisms (Elad *et al.*, 1996).

Extracts obtained from composts made from cattle, sheep or chicken manures, chicken manure + seaweeds and straw, were tested for their effectiveness against *B. cinerea* and *P. xanthii* (Malathrakis *et al.*, 1995). Compost extracts resulted in a reduction of the infection by up to 60% in relatively high air humidity conditions. Bacterial isolates obtained from the extracts were found to be effective against *B. cinerea* and *P. xanthii* and it was suggested that the effectiveness of the extracts was related to the bacterial population developed during the fermentation process.

In another study, compost extracts effectively controlled powdery mildew in peas and beetroot (Thom and Moller, 1988).

There is a potential that compost extracts can be used as sources for biological control, but the inconsistency in the sources of composted materials and in process of composting, makes it difficult to be adopted as a disease control method (Elad *et al.*, 1996).

Plant Extracts Several plant extracts have been tested against powdery mildews. Konstantinidou-Doltsinis and Tzempelikou (2000) tested 69 ethanolic extracts of native and introduced plant species in Greece and found out that Cassia extracts were effective, if applied as protective sprays (prior to disease symptoms becoming visible), against cucumber powdery mildew.

Plant extracts of the perennial weed *Reynoutria sachalinensis* (currently commercially available under the trade name Milsana) have been extensively studied. It has been reported that aqueous and ethanolic plant extracts from *R. sachalinensis* protected greenhouse cucumber, tomato and begonia from powdery mildew when applied prophylactically (Herger and Klingauf, 1990; Petsikos-Panayotarou *et al.*, 2002).

It was shown that treatment of cucumber plants with this extract resulted in the build up of phytoalexins of phenolic nature and it was suggested that induced resistance is the mode of action of this extract against cucumber powdery mildew (Dayff *et al.*, 1997).

A liquid *R. sachalinensis* extract formulation, under the trademark Milsana®, is now commercially available and has been proved to be effective against powdery mildew in different cases (Dik and Van der Staay, 1995; Daayf *et al.*, 1995; Konstantinidou-Doltsinis and Schmitt, 1998).

Over the last 5 years, and due to the time-consuming preparations of fresh aqueous extracts, conservation problems and visible residues on plants (Dik and Van der Staay, 1995) several new liquid formulations of Milsana® have been developed, namely: Milsana® V 1999, Milsana® VP 220 and VP 2001 (Petsikos-Panayotarou *et al.*, 2002).

One of these new formulations has been lately produced under the commercial name Milsana® 2002 and this formulation will be tested in these trials.

Other natural compounds have been reported to control cucumber powdery mildew. These include phosphate and potassium salts (Reuveni *et al.*, 1995 and 1996; Dik *et al.*, 2003), salicylic acid (Conti *et al.*, 1996; Trdan *et al.*, 2004), micronutrient solutions (Reuveni *et al.*, 1997), silicon used in hydroponics nutrient solution and foliar treatments (Menzies *et al.*, 1990; Casulli *et al.*, 2002) and dried milk (Casulli *et al.*, 2002).

CHAPTER 2. Effect of different organic amendments, plant residues and biological control agents (BCAs) on tomato corky root rot (*Pyrenochaeta lycopersici*)

2.1 Introduction

Corky root rot is among the most serious soil-borne disease of tomatoes in soil based greenhouse production systems (Termohlen, 1962; Last *et al.*, 1966; Gerlach and Schneider, 1964). Losses of up to 75% have been reported for tomato (Hogeboom, 1970; Campell *et.al.*, 1982) and the pathogen also attacks and reduces yields in a range of other field and glasshouse crops including spinach, safflower, pepper, eggplant, cucumber, squash, and melon (Grove and Campbell, 1987; Risser and Laugie, 1968; Termolhen, 1962). Its wide host range and long persistence in soil makes it difficult to control via rotational and break crop approaches in greenhouse production systems (Ebben *et al.*, 1978; Lemaire *et al.*, 1998).

The disease is currently controlled mainly by chemical soil disinfection (e.g methyl bromide) in conventional, and soil steaming in organic, greenhouse production systems. However, significant environmental and/or potential human health problems have been linked to the use of both chemical and steam disinfection methods (see chapter 1). As a result, there is significant consumer, supermarket and legislative pressure to phase out methyl bromide or reduce soil steaming in the use of soil disinfection (see chapter 1).

There is a range of **important gaps in knowledge** about alternative strategies that could potentially replace the widespread use of soil disinfection in both (a) organic (where soil steaming is widely used) and (b) conventional (where both chemicals such as methyl bromide and steam based technologies are used) glasshouse production systems (see chapter 1 section 1.3.).

Most importantly, there is a relative lack of studies comparing (a) different alternative strategies (biological control agents, resistant rootstocks and organic matter soil amendments), (b) alternative strategies with steam treatments (the only non-chemical soil

treatment for corky root rot currently used in commercial practice) and (c) the relative activity of alternative treatments at different levels of soil disease pressure.

The **main objectives of work** under chapter 2 are therefore

- (i) to compare the effects of different alternative, non-chemical strategies for the control of corky root rot on root disease development, crop growth, fruit yield, and yield distribution pattern of tomato
- (ii) to compare the effects of alternative non-chemical strategies with those of soil steam treatment
- (iii) to study the effect of soil treatments at different soil pathogen inoculum levels

2.2 Materials and methods

2.2.1 Soils and pathogen inocula used

In 2001 a glasshouse pot trial was conducted at Newcastle University's Close House Experimental station, to test the efficacy of different soil treatments against *P. lycopersici* that causes the corky root rot in tomato.

Soil infected with corky root rot was collected from an organic glasshouse tomato-producing farm (Cantelo Nurseries Ltd.) in Somerset, Southwest England. The soil collected in different parts of the glasshouse was mixed and homogenised using a concrete mixer to minimise differences in substrate structure, texture and inoculum density and then placed into 15 litres volume pots.

Fresh tomato root pieces from the same farm (Cantelo nurseries) infected by the pathogen were chopped and homogenised using a food processor. The chopped up roots were added as "extra inoculum" to half of the pots in order to create a high (+) inoculum treatment.

2.2.2 Soil treatments used

For each treatment six pots containing naturally infected soil and six pots containing naturally infected pots plus 100 cm³ of finely chopped-up root inoculum were used. The concrete mixer used to mix soil with the different treatment preparations was cleaned with bleach solution (approx. 1% active chlorine final concentration; Domestos) after each soil/treatment combination had been prepared.

The following treatments were then applied to pots:

(i) Cow manure compost

Composted cow manure from an organic farm (Tio Ltd, Inverness, Scotland) was mixed with the soil. Compost was added at a volume of 50% (v/v), which represents the standard volume of compost added in commercial organic tomato glasshouse production systems immediately after conversion to organic management. Compost and soil were mixed using a concrete mixer.

(ii) House-waste compost

A house-waste compost product obtained from a local (NE England, Municipal Newcastle Recycle Company) company. Compost was added at a volume of 50% (v/v) and mixed with soil using a concrete mixer.

(iii) Chopped-up fresh *Brassica* tissue (Brussels sprouts, *Brassica oleracea* var *gemmifera*)

To mimic and evaluate the effect of *Brassica* wastes (which have previously been shown to suppress soil-borne disease when added to soil), we used chopped up (using a Food processor) Brussels sprouts, which were mixed with soil at a volume of 50% (v/v) and was mixed with soil using a concrete mixer.

(iv) Chitin

Chitin (made from crab shells, Sigma catalogue product) was added to infected soil at a level of 1% (v/v).

(v) Seaweed extracts

A commercial seaweed extract product (“Marinure”, Glenside Organics Ltd. Stirling, UK) was used by watering at the rate recommended by the manufacturers (1ml/lt/pot weekly).

(vi) Conifer extracts

A conifer extract product was applied by watering at the concentration recommended by the manufacturers (5ml/lt/pot weekly).

(vii) Fish emulsion

Fish emulsion, a product of a local company (Nugro, Hortifeed, Lincoln, UK), was applied by watering at the concentration recommended by the manufacturers (5ml/lt/pot weekly).

(viii) *Bacillus subtilis*

The *Bacillus subtilis* preparation used was a commercial product based on the *B. subtilis* strain MBI500, supplied by MicroBio Ltd. (St Albans, UK). Its mode of action was previously shown to be based, at least partially, on the production of anti-fungal volatiles. The manufacturer recommended concentration (2g/1000lt water) was used and 1lt of suspension added per pot at planting. A month later the same volume was applied again.

(ix) Polyversum (*Pythium oligandrum*)

Polyversum is a commercial product (Biopreparaty, Prague, CH) containing the BCA *Pythium oligandrum*. The manufacturers recommended application rate for *P.*

oligandrum was 1g/100 m². The tomato plants density in greenhouse crops varies from 200-300 plants per 100m². The lower density was used for the calculation and therefore a 0.005g/lt/pot was applied by watering. A month later the same volume was applied again.

2.2.3 Resistant rootstocks used

Tomato plants of the same fruiting cultivar “Star Fighter” were grafted onto rootstock “Beaufort” that is resistant to corky root rootstock.

2.2.4 Steam disinfection treatment used (positive control)

For the steam treatment a commercial soil-steaming machine used for horticultural substrates, (Camplex HD5116 Electric Soil Steriliser, Thermoforce Limited, Cumbria, U.K), was used. Soil was heat treated at 81° C for 30 minutes.

2.2.5 No soil treatment or resistant rootstock used (negative control)

Non-grafted plants planted in untreated soils were used as “negative” control treatments.

2.2.6 Experimental design

For each treatment six pots with naturally corky root rot infected soil and six pots with naturally infected soil, plus extra chopped-up root tissue inoculum were used. A completely randomised block was designed with six blocks (=rows in the glasshouse) and used (in each row there was one replicate pot for each treatment and the position of individual treatments within each row was randomised).

Six weeks old tomato plants of the commercial hybrid “Star Fighter” were transplanted into the pots on the 10th of March, 2001 and grown until the 10th of September, 2001.

2.2.7 Irrigation and temperature regimes used

Soil was kept always watered to field capacity every 1 to 2 days. Watering was slightly more frequently than in commercial glasshouse production to provide optimum conditions for disease development (the pathogen is favoured by moisture) and the air

temperature was kept between 18 and 22⁰ C, as the disease is more severe at relatively low temperature regimes.

2.2.8 Assessments

Assessments of fruit weight and size were made according to colour charts used in commercial glasshouse production (=when fruit were uniformly red).

After harvesting was finished in September 2001, root systems were removed from the soil and adhering soil removed by gentle washing in a bucket of tap water. Root fresh weight was assessed as an indicator of disease incidence: as infection and disease severity increased, root fresh weight decreased.

2.3 RESULTS

As there were no significant differences between the ‘extra’ and ‘standard’ inoculum treatments on any of the characteristics measured only the averages are considered in the results sections.

2.3.1 Effect of treatments on Fruit Yield

Six of the 10 soil treatments resulted in an increase in yield ($P<0.05$; see Fig. 2.1) compared to non-grafted plants grown in untreated soil (negative controls).

This included: (i) plants grafted onto resistant rootstocks, and (ii) ungrafted plants grown in soil treated with steam, and ungrafted plants grown in soil amended with one of the following organic matter inputs, (iii) cow manure based compost, (iv) household waste based compost, (v) fresh *Brassica* tissue or (vi) chitin. There were no significant differences in yield between these treatments.

The two BCA treatments (*Pythium oligandrum* and *Bacillus subtilis*), the two extract based organic matter inputs (seaweed extracts and conifer extracts), and the fish emulsion did not have any significant effect on yield when compared to the negative controls ($P<0.05$).

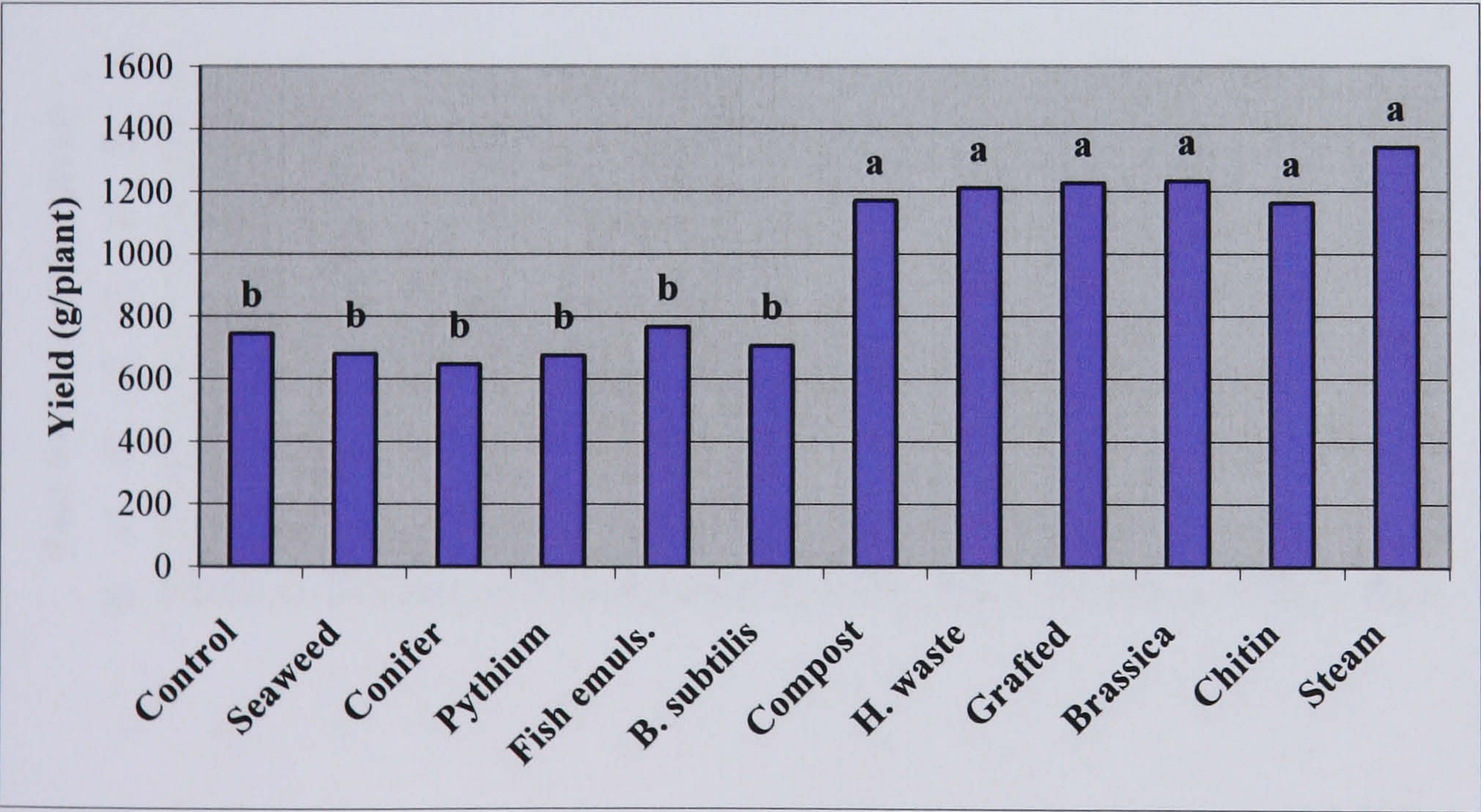


Figure 2.1. Effect of different treatments on tomato cumulative yield. Different letters indicate significant differences ($P<0.05$) according to Tukey’s Honestly Significant Difference (HSD) test.

2.3.2 Effect of treatments on Root Fresh Weight (RFW)

As with yield assessments, the root fresh weight of non-grafted plants grown in soils treated with either (a) one of the two BCAs (*Pythium oligandrum*, *Bacillus subtilis*), (b) one of the two extract (seaweed extracts, conifer extract) or (c) the fish emulsion, was not significantly different when compared to non-grafted plants grown in untreated soil (negative controls; see Fig. 2.2).

Cow manure compost also did not have a significant effect on RFW when compared to the negative controls ($P<0.05$; see Fig.2.2). This was different to the results of yield assessments, where the amendment of cow manure compost resulted in significant yield increases (see above).

However, (i) plants grafted onto resistant rootstocks and (ii) ungrafted plants grown in soils treated with steam and soil treated with one of the following organic matter inputs (iii) household waste based compost, (iv) chopped-up *Brassica* tissue or (v) chitin significantly increased RFW ($P<0.05$) compared to the negative controls, thus mimicking the effect observed for yield. There was no significant difference in RFW between these 5 treatments.

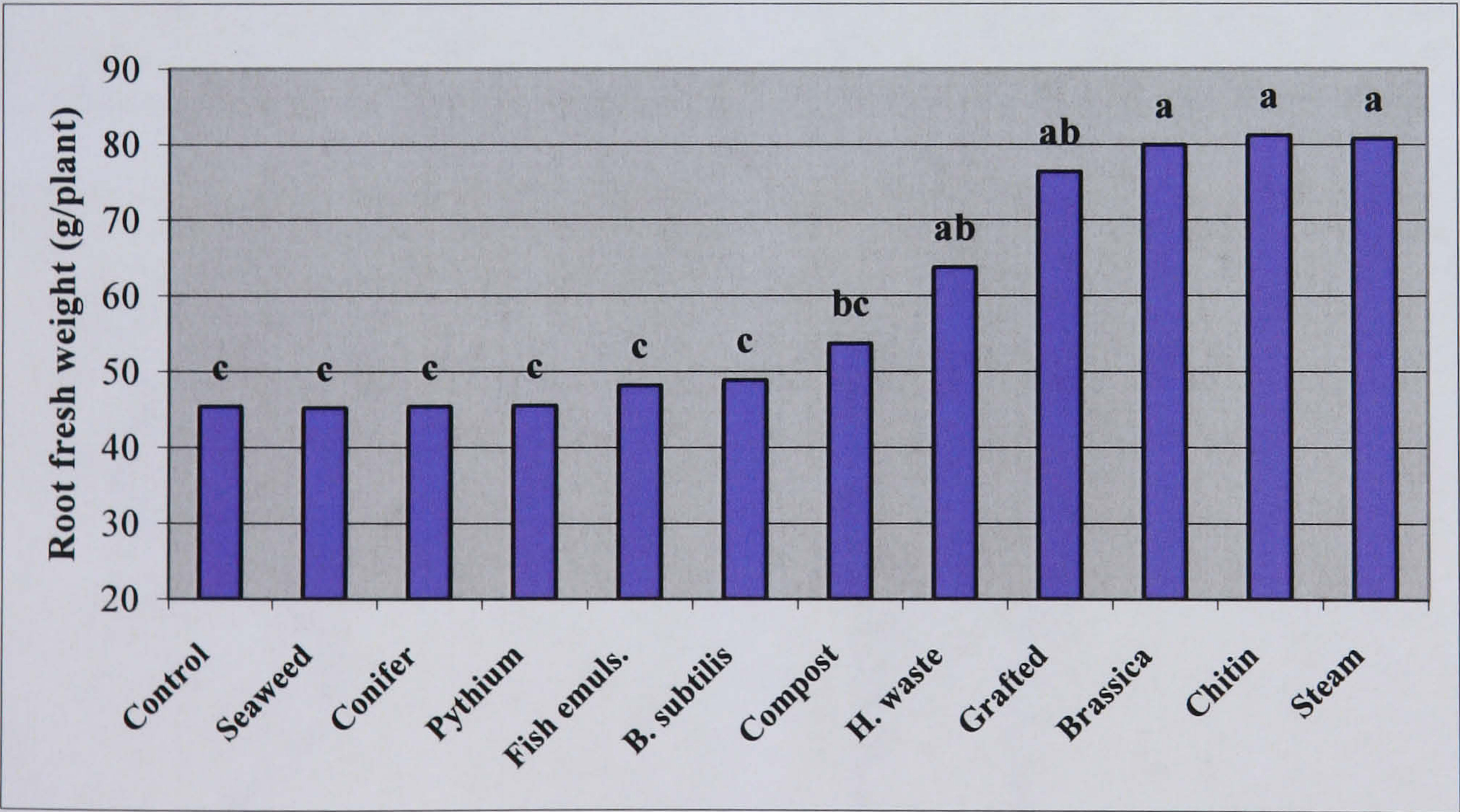


Figure 2.2. Effect of different treatments on root fresh weight. Different letters indicate significant differences ($P<0.05$) according to Tukey’s Honestly Significant Difference (HSD) test



A. Fish emulsion (mean weight 48g)



B. Conifer extracts (mean weight 45g)



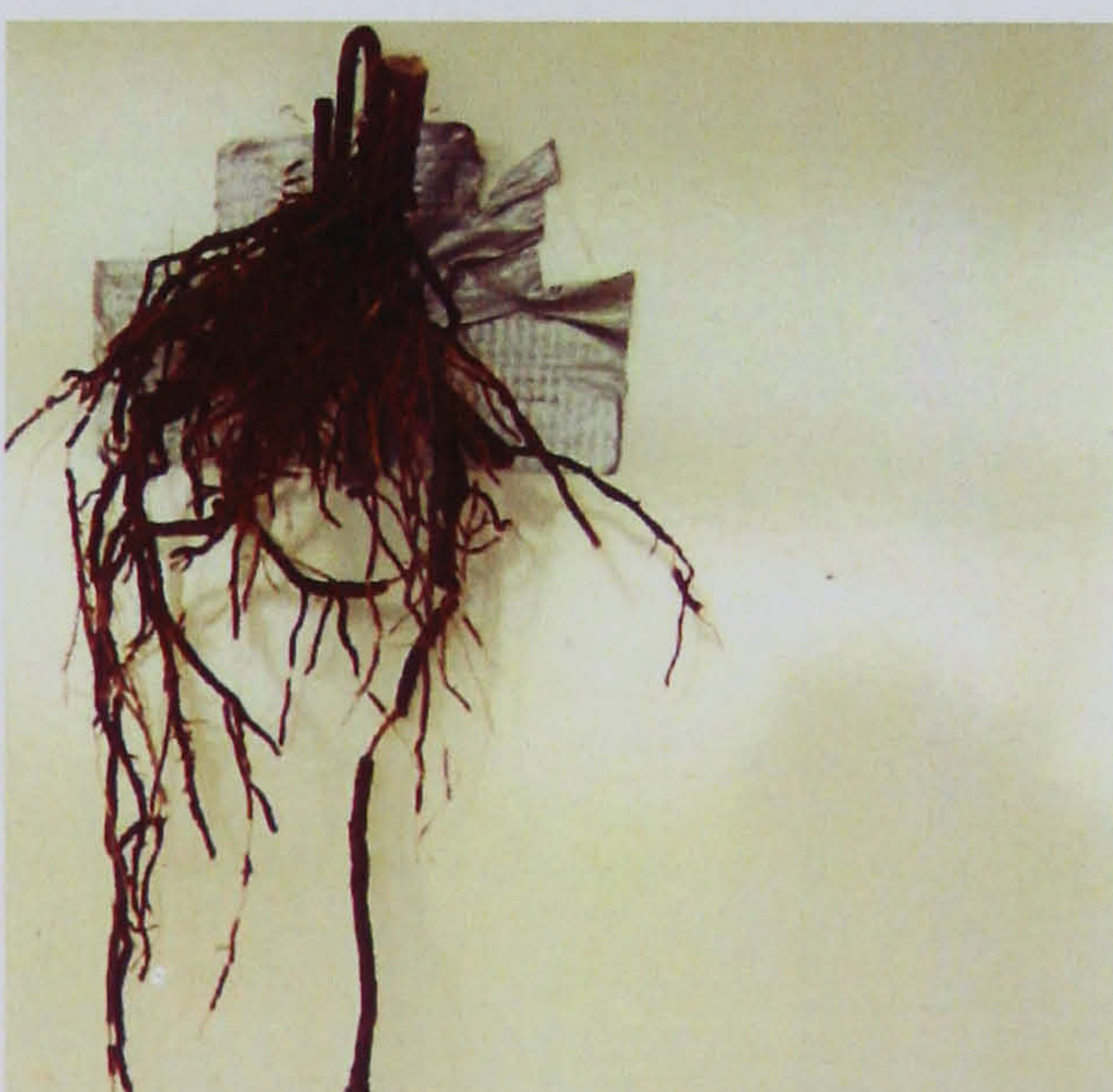
C. Seaweed extracts (mean weight 45g)



D. *Bacillus subtilis* (mean weight 49g)



E. *P. oligandrum* (mean weight 45g)



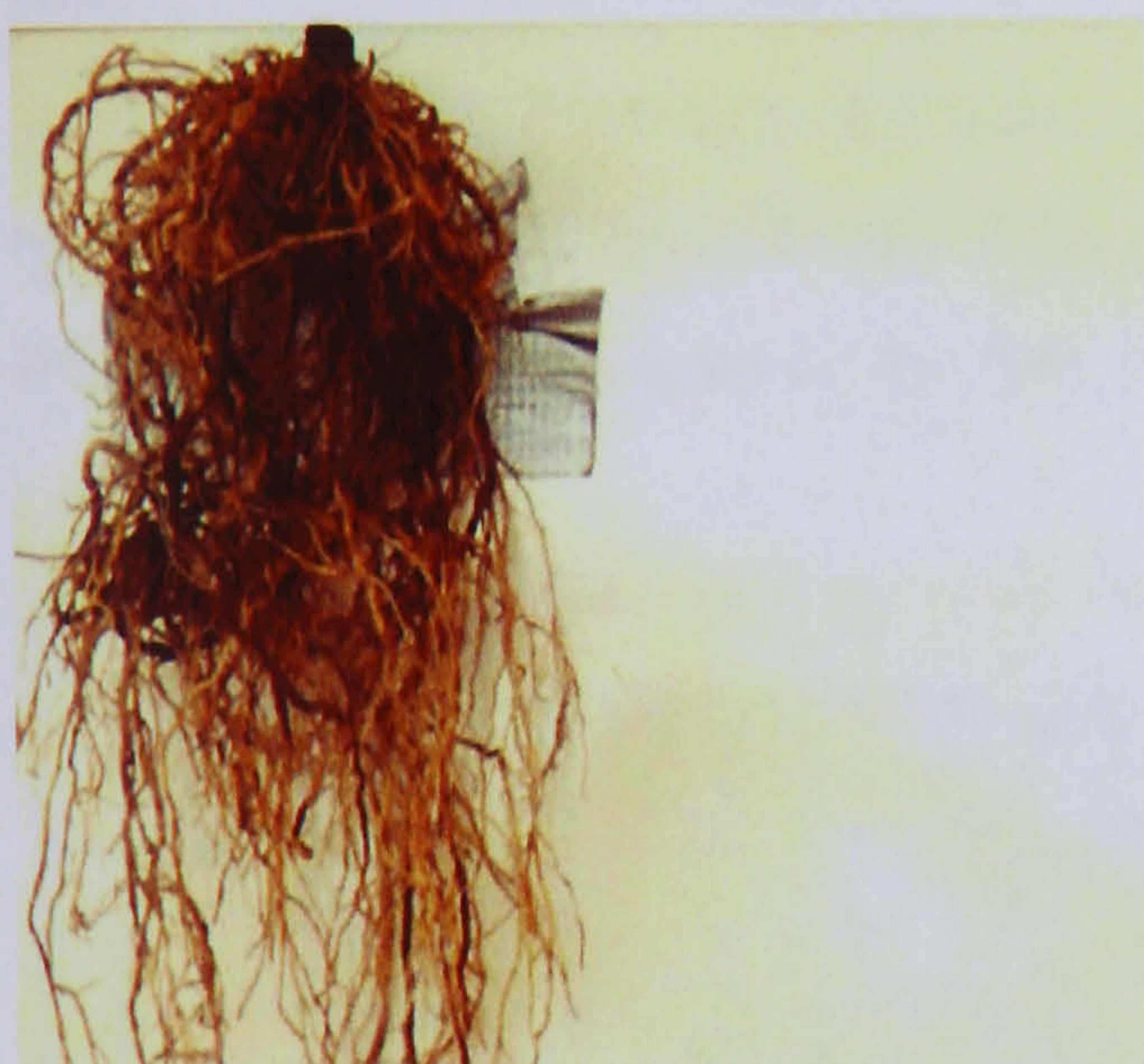
F. Untreated (control) (mean weight 45g)



G. Housewaste (mean weight 64g)



I. Grafted (mean weight 76g)



J. Steam (mean weight 81g)



K. Brassica (mean weight 80g)



L. C. man. Comp. (mean weight 54g)



M. Chitin (mean weight 81g)

Figure 2.3. Roots of tomato plants (different treatments) infected with *P. lycopersici* (plus extra inoculum)

(Photographs were taken from different distances and therefore are not to the same scale)

2.3.3. Effect of treatments on Fruit Size

None of the treatments had a significant ($P<0.05$) effect on fruit size when compared to non-grafted plants grown in untreated soil (negative controls; see Fig. 2.4).

However, the fruit size of tomato plants grafted onto resistant rootstocks was significantly ($P<0.05$) higher than that of plants grown in soils treated with the two BCAs, the two extracts and the fish emulsion (=the treatments which did not significantly increase root weight and fruit yield compared to ungrafted plants grown in untreated soil).

Also, the fruit size of tomato plants grown in soils amended with cow manure based compost was significantly higher than that of plants grown in soil treated with the BCA *Pythium oligandrum* or the fish emulsion.

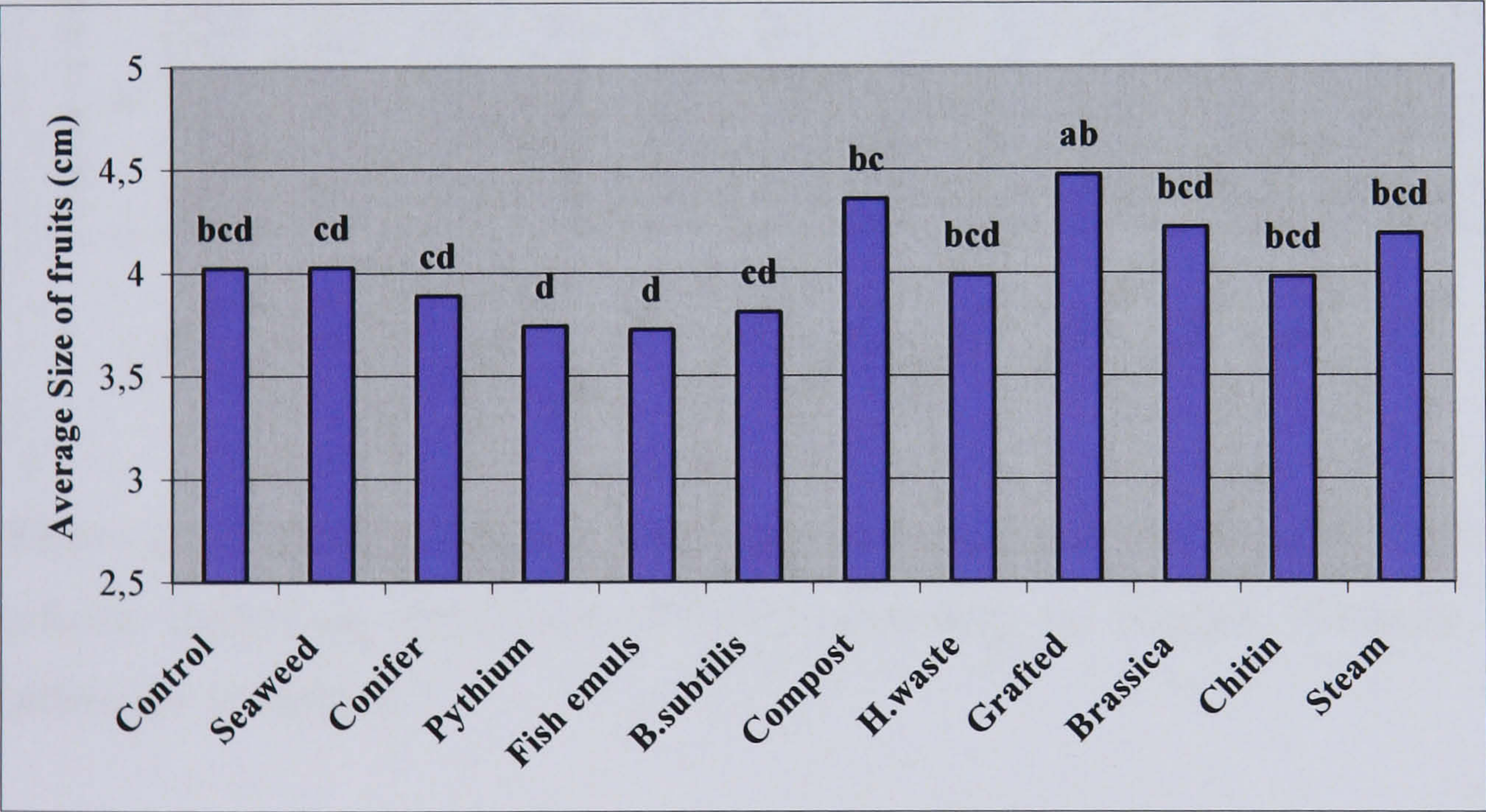


Figure 2.4. Effect of different treatments on average fruit size (diameter). Different letters indicate significant differences ($P<0.05$) according to Tukey's Honestly Significant Difference (HSD) test

2.3.4. Effect of treatments on Fruit Number

Chitin, *Brassica*, and house-waste amendments resulted in plants with number of fruits similar to the number of fruits of the plants grown in to steam sterilized soils ($P<0.05$).

All the other treatments did not have any significant effect on the number of fruits compared to the negative control (untreated plants) (see Fig. 2.5).

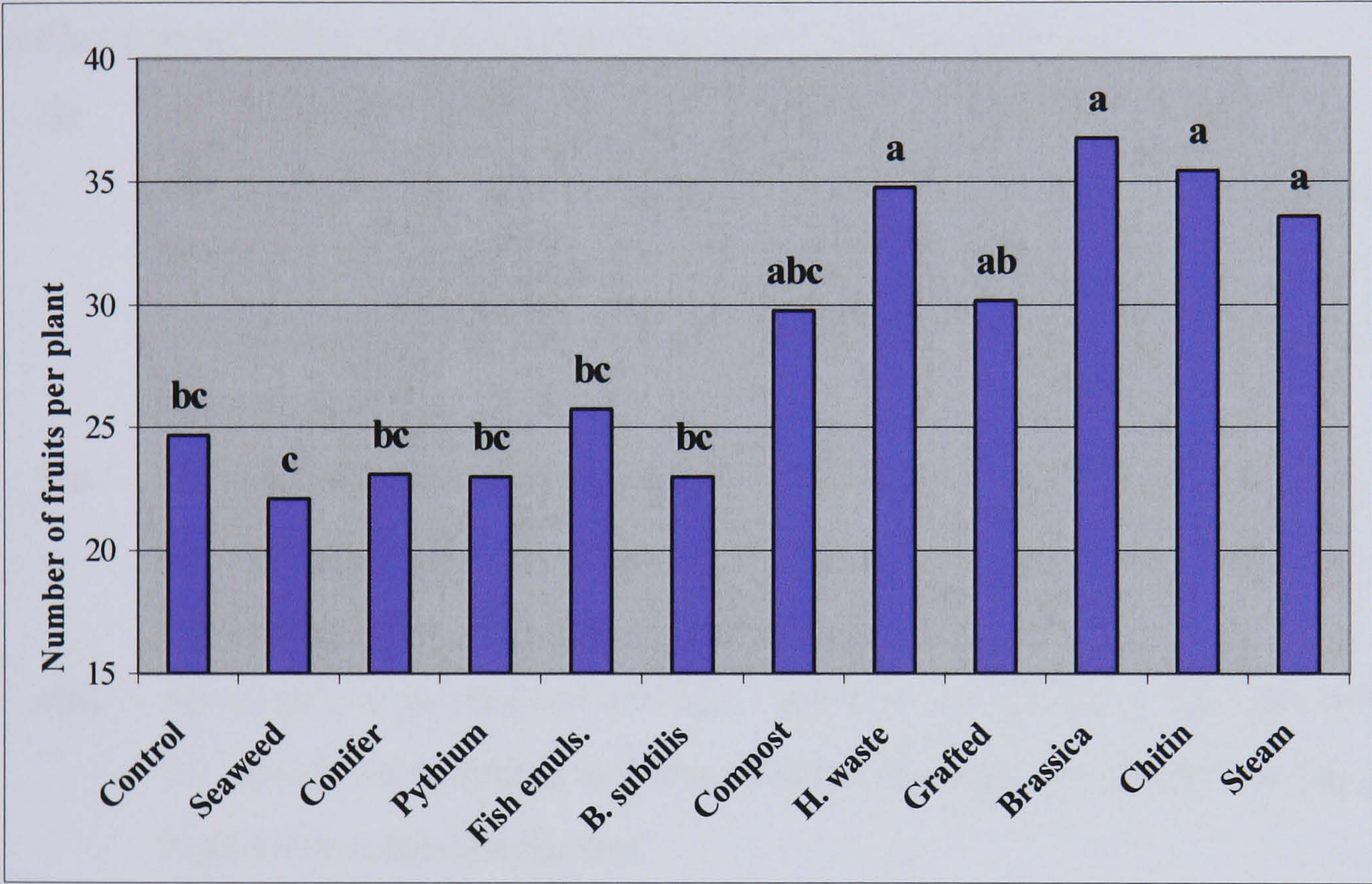


Figure 2.5. Effect of different treatments on cumulative fruit number. Different letters indicate significant differences ($P<0.05$) according to Tukey's Honestly Significant Difference (HSD) test

2.3.5 Effect of treatments on Fruiting Pattern

Fruits (at a specific ripening stage) were harvested at regular intervals until day 33 of the harvesting period. For the final yield assessment at day 35 all remaining fruit were harvested (including green or unripe fruits), which is the main explanation for the much higher yield at the last harvesting date compared to previous harvesting dates (see Fig. 2.6).

Fruiting pattern differed between harvesting dates most importantly:

- (i) all treatments which did not result in a significant difference in total cumulative yield (see section 2.3.1 above) and/or root fresh weight (see section 2.3.2 above) compared to un-grafted plants grown in un-treated soil (negative controls), also showed very similar fruiting pattern to the untreated controls.
- (ii) all soil treatments that increased total cumulative fruit yield (see section 2.3.1 above) except for plants grown in steamed soil, showed higher yields at all harvesting dates compared to negative controls.
- (iii) plants grown in steamed soil had relatively low yields at harvests before day 35, which were similar to those obtained for negative controls but had a very high yield at the last harvest.

Although fruiting pattern appeared to be different between the treatments that had resulted in significant increases in yields (see Fig. 2.5), these differences were not found to be significant.

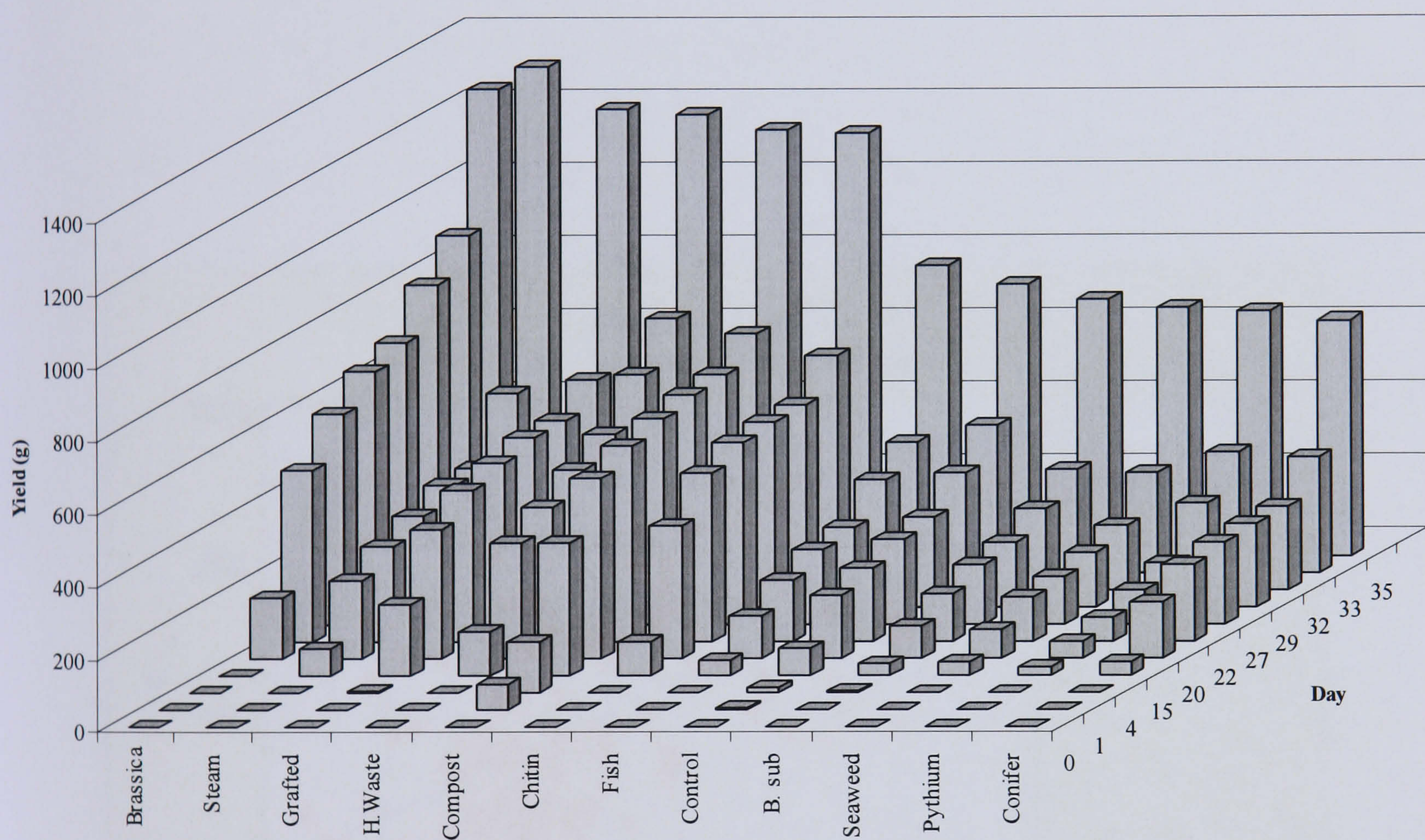


Figure 2.6. Effect of treatments on cumulative yield per plant

2.3.6 Correlation between fresh root weight and yield

There was a clear correlation between root fresh weight and yield as it is shown in figure 2.7.

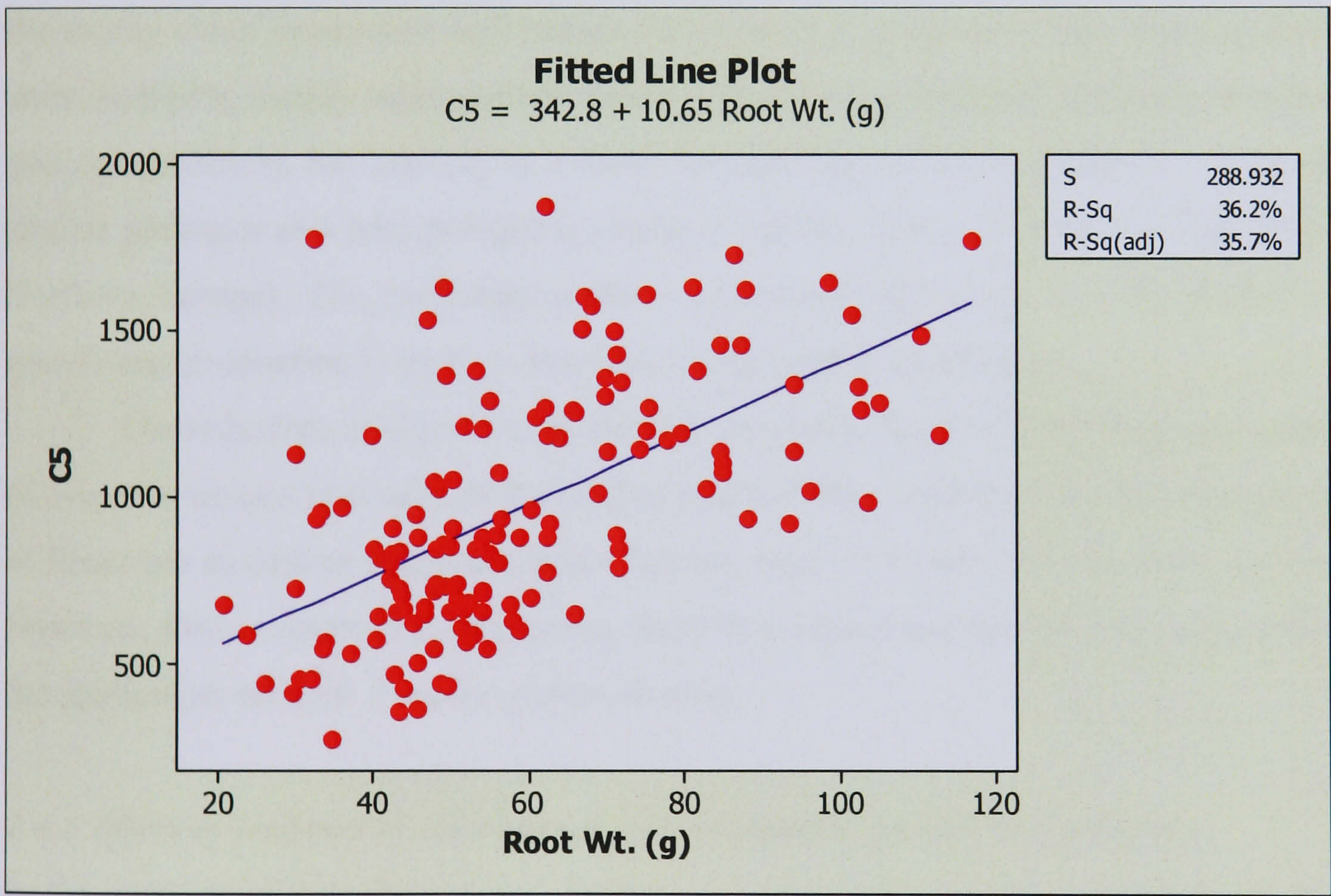


Figure 2.7 Correlation between fresh root weight and yield g/plant(C5=fruit yield)

2.4 Discussion

Disease control in organic farming should be based on an integrated (holistic), and ideally preventative approach, that combines a wide range of rotational, variety choice and other agronomic and cultural practices. Crop protection product based approaches (e.g. permitted pesticides, biological control and/or extract based approaches) are not encouraged and are usually restricted to specific circumstances (Agrios, 1997; Sullivan, 2004; Greer and Diver, 2001).

Crop rotations are usually a basic component of organic disease control strategies, but in the case of protected cropping, monocultures or relatively short rotations have remained the norm rather than being an exception (Lampkin, 2000). This is due to (a) higher capital costs (for packing lines and glasshouse machinery and crop arrangements), (b) supply chain limitations and market pressures (e.g. even very large scale production units supplying mainly supermarkets find it difficult to get contracts for more than one or two commodities), but also (c) to a more limited range of crops (many of which share similar pathogen and pest problems) available for the design of rotations (especially in Northern Europe). The contribution that crop rotation can make to crop protection in greenhouse production systems is therefore considered to be minimal.

The reduction in disease (and increase in yield) observed following incorporation of *Brassica* tissues into soils in the studies reported here may indicate that incorporation of *Brassic*as as cash or intercrops into rotations, may contribute to a reduction in disease. However, further research is required to show that *Brassica* crops have the same effect as the application of fresh *Brassica* tissues to soils.

2.4.1 Effect of resistant or tolerant varieties/rootstocks on soil borne disease

In the experiment described above, grafting a variety onto resistant rootstock efficiently controlled corky root rot disease. This confirms that the use of resistant or tolerant varieties, or grafting onto a resistant rootstock, maybe the most reliable tool against soil-borne pathogens in organic farming (Lampkin, 2000).

The results from this study, and subsequent monitoring of the same rootstocks in commercial practice (Rodriguez et al. unpublished), demonstrated that the grafting onto resistant rootstock is the most effective, and currently cheapest way, to control soil-borne diseases in organic systems. With the prohibition of methyl bromide this approach is also thought to become very competitive in conventional soil based production (Morra *et al.*, 1997).

2.4.2 Effect of different organic matter inputs/composts on soil borne disease

The use of organic matter inputs (and especially the use of compost) derived from animal manure, plant material or communal or domestic household wastes, is greatly encouraged in organic farming (Abbasi *et al.*, 2002).

This is because organic matter inputs/composts were shown to improve biological, chemical, and physical properties of amended soils and can induce suppressiveness/biological control of diseases caused by soil-borne plant pathogens (Abbasi *et al.*, 2002; Chellemi *et al.*, 1992; Hardy and Sivasithamparam, 1991; Workeh and van Bruggen, 1994).

The most important mechanisms thought to be involved in the activity of organic matter inputs/composts against soil-borne diseases are (a) increased competition, (b) direct antagonism (c) the presence of antifungal compounds and (d) induction of resistance in plants (Lampkin, 2000).

The results presented here showed that addition of some organic matter inputs (e.g. fresh *Brassica* waste and household waste compost) but not other (e.g. cow manure based compost) reduced root disease.

Previous studies indicate that one likely mechanism for the suppression of disease by fresh *Brassica* waste and household waste compost is increased competition for the pathogen in soil related to the increase of the biological activity in the soil. For example, organic matter amendments were shown to increase the population density of the microflora and microfauna and simultaneously decrease the severity of corky root rot

(Forsberg *et al.*, 1999) and higher microbial activity following organic matter inputs was also shown to correlate with lower corky root disease severity by Workneh *et al.* (1993).

However, the exact mechanism of action was not investigated in the experiments and needs to be investigated in future studies. In subsequent trials (chapter 5), we therefore assessed soil biological activity following addition of different soil amendments.

The increase in yield, and decrease in disease development associated with **house-waste** compost is of particular interest. Great efforts are currently being made to improve the sustainability of (i) waste management systems (minimizing landfill disposal of waste) and (ii) food production (substituting mineral nitrogen fertilizer use), by encouraging the recycling of composted organic household waste back into agricultural soils (Petersen *et al.*, 2003). The added value associated with the “disease suppressive” effect observed in the studies reported here, may make it easier to convince producers to use such products and thereby increase the volumes of organic waste that can be recycled into agriculture.

Although cow manure based compost had no effect on root disease development, the addition of cow manure based compost resulted in a significant increase in the yield compared to the ungrafted plants grown in untreated soils. It can therefore be hypothesized that the cow manure compost, while not suppressing disease, did supply a higher level of nutrients and thereby “compensated” with respect to fruit yield for the loss in root system caused by corky root rot. However, further studies would be required to prove this.

2.4.3 Effect of Chitin soil treatment on soil borne disease

Chitin, a homopolymer of *N*-acetyl-D-glucosamine (Glc-NAc) residues linked by β -1–4 bonds, is the most abundant renewable natural polysaccharide source after cellulose. The main source of chitin is crustacean wastes, but it also occurs in fungi, insects and in some algae (Shahidi *et al.*, 1999). The biological control exercised by several antagonistic microorganisms has been correlated with increases in the population densities of chitin degrading microorganisms and/or chitinase activity in soil (De Boer *et al.*, 1999; Sid Ahmed *et al.*, 2003).

Chitin amendments in soils have also been described to induce crop resistance mechanisms and other authors suggested that the effect of chitin treatments is due to a combination of (i) antagonism of soil-borne diseases by chitinolytic microorganisms, and (ii) elicitation of plant defense mechanisms (De Boer *et al.*, 1999; Manjula and Podile 2001; Sid Ahmet *et al.*, 2003).

Chitin treatment of plants was shown to elicit/induce a range of host responses (e.g. PR- proteins, phytoalexins, proteinase inhibitors, cellulose, lignin) associated with disease resistance in a wide range of plant families (Lyon *et al.*, 1995), and to induce phytoalexin production in fruit of *Capsicum annum* and lignifications in wounded wheat leaves (Watson and Brooks, 1984; Barber *et al.*, 1989).

Furthermore, it was shown that when chitin is being degraded in the soil, ammonia is being produced and it was hypothesized that the ammonia acts as a localized fumigant. However this is only likely to be significant where rates of application are high (Ellis, 2000).

Chitin has been used as a crop protection treatment for a wide range of nematodes and soil and foliar pathogens (both on its own or in combination with different biological control agents). For example, chitin amendments to soil containing potato cyst nematodes reduced egg and larval counts and increased potato yield (Evans, 1993). Davies *et al.*, showed that chitin amendments to soil reduced the severity of black scurf in potato caused by *Rhizoctonia solani* (Davies *et al.*, 2002), Chitin application on tomato canopy controlled grey mould caused by *Botrytis cinerea* (Ploper *et al.*, 1991) and Sid Ahmet *et al.*, (2003) reported that when chitin was applied in combination with *Bacillus spp.* and *Trichoderma harzianum* it increased the efficacy of the BCA against root rot disease of peppers caused by *Phytophthora capsici* and *Rhizoctonia solani*.

Chitin reduced the disease incidence in this experiment as it significantly increased the root fresh weight and yield of plants compared to untreated soil and further investigation of the possible modes of action were therefore carried out in subsequent experiments (see chapter 5).

The source of chitin used in this experiment was a very pure and expensive form of chitin available from Sigma catalogue. Due to its expense, this form of chitin would not be economically viable for use in commercial glasshouse production. Subsequent experiments therefore also investigated whether similar levels of control of soil-borne diseases could also be obtained with cheaper and less purified forms/sources of chitin.

2.4.4. Effect of Brassica soil amendments on soil borne disease

In outside crop production systems incorporation of *Brassicas* into rotations has been shown to be effective against soil-borne pathogens such as *Verticilium dahliae* (Xiao *et al.*, 1998), but the effect on corky root rot has not been investigated in detail previously.

Incorporating un-decomposed *Brassica* residues or green manure crops has been shown to suppress a range of pest and disease organisms including fungi, nematodes and wireworms (Mojtahedi *et al.*, 1991).

The incorporation of intercrops or crop residues of certain *Brassica* spp. into soil was reported to reduce pathogen growth, sporulation and/or survival in soil. This effect was linked to the release of fungicidal and fungistatic compounds during the process of residue breakdown of *Brassica* tissues (Bailey and Lazarovits, 2003), as well as an increased antagonistic potential of the microbial flora in the soil (Cook, 1990).

The antimicrobial compounds found, following incorporation of fresh *Brassica*, belonged mainly to the sulphur containing glucosinolates that are present in all plant parts of species belonging to the family *Brassicaceae* (Kirkegard *et al.*, 1999). Glucosinolates do not have antimicrobial activity by themselves, but are converted to antimicrobial compounds such as isothiocyanates, thiocyanates, nitriles, epithionitriles, and oxazolidine-2-thiones as the plant tissues break down in the soil (Kirkegard *et al.*, 1996).

Of the various antimicrobial breakdown products produced from *Brassica* residues, isothiocyanates (which are also investigated as potential alternative chemical soil disinfectants for the replacement of methyl-bromide) have been studied in most detail. They were shown to suppress fungal growth and have been reported to kill in several fungal root pathogens at very low doses (Angus *et al.*, 1993).

Other compounds released during the decomposition of the *Brassicas* in the soil may indirectly (not via having direct antifungal activity) reduce the persistence of soil-borne pathogens. For example mature broccoli crops which are known to be rich in lignin and the enzymes involved in the lignin biodegradation may degrade fungal melanin, which is known to protect fungi from various biotic and abiotic stresses, and thus reduce the competitiveness of pathogenic fungi in soil (Lampkin, 2000).

Other studies into the mode of action of *Brassica* residues suggested that the suppression of soil-borne pathogens is at least partially caused by an “activation” of biological components that are already in the soil. Increased microbiological activity following *Brassicas* decomposition in the soil was shown to lead to increased competition among root colonizers, and this was hypothesized to contribute to a reduction in root infections by soil-borne pathogens (Xiao *et al.*, 1998).

In the experiments reported here, *Brassica* amendments to soils resulted in the reduction of the disease incidence and increase in yields (compared to ungrafted tomato plants grown in untreated soil), which was similar to that, obtained with plants grown in steam-disinfected soils ($P < 0.05$).

However, most studies into the modes of action of *Brassica* residues on soil-borne diseases were carried out in conventionally managed soils and outdoor vegetable and soft fruit production systems. Further studies are therefore needed to identify the modes of action (and the relative importance of different mechanisms) of *Brassicas* amendments in organically managed soil and under greenhouse conditions.

2.4.5 Effect of steam disinfection of soil on soil borne disease

Steaming has long been known to be a viable alternative to methyl bromide for soil disinfections. Temperatures as low as 50°C were shown to result in significant reductions in soil inoculum levels of many soil-borne pathogens and nematodes (van Loehnen *et al.*, 2003). The main mechanism of thermal kill is thought to be protein coagulation or enzyme inactivation (Langhans, 1990).

Soil can be sterilized by steam either in special containers (soil sterilizers) into which steam is supplied under pressure, or directly on the ground by perforated steam pipes that diffuse steam into the soil (Runia, 2000). In greenhouses, boilers used to heat the greenhouse can often be adapted to supply steam for sterilizing greenhouse benches thus reducing the overall cost of steam sterilization. To aid steam penetration soil should be cultivated as deep as possible and a plastic sheet should cover the soil to delay the rate of cooling of soil after steam treatment.

At about 50⁰ C nematodes, some oomycetous and true fungi, and other water molds are killed whereas most plant pathogenic fungi and bacteria along with some worms, slugs and centipedes are usually killed between 60 and 70⁰C. At about 82⁰C, most weeds the rest of pathogenic bacteria, and most plant viruses in plant debris are killed (Agrios, 1997; van Loehnen *et al.*, 2003). Nearly complete soil sterilization may be obtained when the soil is kept at temperatures >82⁰ C for >30 minutes (Agrios, 1997).

Steam disinfection was shown to be as efficient, and may be less expensive (especially in greenhouse systems) than methyl bromide (Grossman and Liebman, 1995), and leaves no toxic chemical residues. It may also result in a more rapid release of soil nitrogen, and improved N-supply to crops. However, if too high temperature or exposure times are used it may result in excessive release of certain micronutrients (e.g. Mn) from soils, which may result in phytotoxic effects on roots (van Loehnen *et al.*, 2003; van Loehnen, 2003). Also, the use of high temperature steaming (85-100⁰ C) may have significant negative effects on soil aggregate structure.

In the experiment reported here yields over the first 4 weeks, following steam treatment, were lower than those of other treatments that successfully controlled corky root rot. This may indicate the inhibitory effect of the soil steaming treatment used, and should be investigated further.

A major disadvantage of current soil steaming technology is that it cannot be applied to large-scale outdoor field production, due to the slow application speed and its high energy and capital investment costs.

Another disadvantage of soil sterilization is that steaming not only kills the targeted pathogens but also kills non-target organisms and beneficial microbes (e.g. inocula of antagonistic microorganisms and AM-fungi) present in soil. This may create an ecological vacuum, which may result in a more rapid re-colonization of soil by pathogen due to the absence of a competitive micro-flora (Bennett *et al.*, 2003). This problem may at least partially overcome by addition of biological control agents to soil immediately after steam disinfection (Bennett *et al.*, 2003).

2.4.6. Effect of soil application of biological control agents (BCAs) on soil-borne disease

2.4.6.1 Bacillus subtilis

Bacillus species are characterized by their ability to (a) form highly resistant endospores (Foster, 1994) and (b) produce a wide range of antibiotics (Katz and Demain, 1977). Many *Bacillus* species and/or their antibiotics (many of which are peptides) were shown to have antifungal activity against phytopathogenic fungi (Seddon *et al.*, 1996; Leifert *et al.*, 1995). A list of peptide antibiotics produced by this species such as mycobacillin, subtilin, bacilycin, fengumicin, neocidins, subtilin and iturins etc. (Swinburne *et al.*, 1975; Katz and Demain, 1977; Loeffler *et al.*, 1990; Luckner, 1990) has been reported.

B. subtilis strains also produce several other metabolites such as derivatives (precursors) of phytohormones (especially auxins) which have plant growth and health promoting actions (Boshov, 1995).

B. subtilis strains have been extensively investigated for their potential as biocontrol agents against a range of fungal pathogens. For example, (i) Baker *et al.*, (1985) used *B. subtilis* to control *Uromyces phaseoli* (bean rust), (ii) Boshov (1995) reported activity against *Fusarium* spp, *Rhizoctonia solani* and *Pyrenocheta lycopersici* in different greenhouse crops, (iii) Kim *et al.*, (1997) reported biocontrol activity against *Gaeumannomyces graminis* var *tritici*, *Rhizoctonia solani* and *Pythium irregulare* and *P.*

ultimum in wheat, (iv) Tatagiba *et al.*, (1998) showed activity against grey mould of roses, and (v) Sholberg and Gaunce (1995) and Leibinger *et al.*, (1997) showed activity of *B. subtilis* against postharvest infections of apples by *Penicillium expansum*, *B. cinerea* and *Pezicula malicortis*.

In the experiment reported here *B. subtilis* strain MBI500 (which had been developed into a commercial BCA product for seed treatments) did not significantly reduce disease incidence.

This was most likely due to the relatively low soil temperature (for most periods it remained between 15-20⁰C) during the trial period. *P. lycopersici* has a temperature optimum for disease development of between 15 to 20⁰ C (Pohronezny and Volin, 1991), while *Bacillus subtilis* strains were shown to require temperatures > 20⁰ C for significant levels of antagonistic activity (Boscow, 1989; Schmidt *et al.*, 2004).

At higher soil temperatures (22-27⁰C), disease incidence by *P. lycopersici* may have been decreased by *B. subtilis* soil amendments. Therefore, further work comparing the activity of *Bacillus subtilis* at a range of soil temperatures should be carried out identify the “environmental window of opportunity” for *B. subtilis* with respect to control of corky root rot.

2.4.6.2 *Pythium oligandrum*

The ubiquitous oomycete *Pythium oligandrum* was described as a potential biocontrol agent for use against a wide range of pathogenic fungi (Piccard *et al.*, 2000; Bery *et al.*, 1993; Al-Rawahi and Hancock, 1998; Deacon, 1976). The main modes of action described for *P. oligandrum* action against different pathogens were (i) mycoparasitism, (ii) production of antibiotics and (iii) release of fungal cell wall degrading enzymes (Benhamou *et al.*, 1999; Martin and Hancoch, 1987; Lewis *et al.*, 1989).

Furthermore, it has been suggested that, in addition to its direct antimicrobial modes of action, *P. oligandrum* may be able to induce resistance in host plants (Benhamou *et al.*, 1997; Picard *et al.*, 2000). For example, *P. oligandrum* when used against *Fusarium oxysporum* of tomato, displayed the ability to penetrate the roots of tomato plants and to

trigger an array of structural defense-related reactions (Benhamou *et al.*, 1997; Benhamou *et al.*, 1999; Piccard *et al.*, 2000).

In another study it was shown that a low molecular protein, termed oligandrin, is produced by *P. oligandrum*. When oligandrin was applied to decapitated tomato plants, it induced plant defense reactions that contributed to restrict stem cell invasion by *Phytophthora parasitica* (Piccard *et al.*, 2000).

During the experiment reported here *P. oligandrum* did not reduce the disease incidence and, as in the case of *B. subtilis*, the relatively low temperatures during the trial period may have prevented antagonistic activity of the BCA. Therefore, further work comparing the activity of *P. oligandrum* over a range of soil temperatures should be carried out to identify the “environmental window of opportunity” for this BCA with respect to control of corky root rot.

2.4.7 Effect of liquid organic matter fertility inputs (Fish emulsions, Seaweed and Conifer extracts) on soil borne disease

Organic greenhouse crop producers often use seaweed extracts and/or fish emulsion as foliar fertilizers. Conifer extract, on the other hand, is currently not widely used.

2.4.7.1. Sea weed extracts

In addition to supplementing the supply of macro-and micronutrients, seaweed extracts (and to some extent fish emulsions), are known to be sources of certain plant growth regulators including cytokinins, gibberellins, abscisic acid, indole acetic acid, betains and oligosacharides (Verkleif, 1992).

Most work on the effects of seaweed extracts has focused on their effect on yield of different crops. For example Temple (1989) claimed that seaweed extracts resulted in an increased yield in beans of 24%. Passam *et al.* (1995) reported increased yields of cucumbers and Crouch and Van Staden, (1992) reported yield increases in greenhouse grown tomato crops.

However, other studies reported that seaweed foliar sprays failed to increase the yields of tomato (Tourte *et al.*, 2001), onions (Feibert *et al.*, 2003), and wheat crops (Miers and Perry, 1986).

Only a few cases of successful disease control through substrate applications of seaweed extracts have been reported. For example Dixon and Walsh (2004) have shown that when peat-based potting compost was treated with liquid seaweed extract, prior to the inoculation by the pathogen resulted in a reduction of infection of cabbage seedlings by the damping-off pathogen, *Pythium ultimum*. They hypothesized that seaweed extracts increased antagonist populations in soil and thereby reduced soil pathogens, but the exact mode of seaweed extracts was not determined in these studies.

2.4.7.2. Fish emulsions

Fish emulsions have been widely used as a fertilizer in organic greenhouse production (Aung and Flick, 1980; Emino, 1981) but were also shown to contain microbially produced Plant Growth Regulators (PGR). Bacterial and actinomycete isolates capable of producing auxins, gibberellins and cytokinins, were shown to metabolise organic matter in fish waste as a source of nutrients and precursors for PGRs during the fermentation process used to produce fish emulsion fertilizers (El-Tarabilly *et al.* 2003).

Foliar sprays with fish emulsion reduced bacterial spot severity on tomato and pepper, and increased total fruit yield in a two-year study carried out by Abbasi *et al.*, (2003).

2.4.7.3. Conifer extracts

Species within the family Coniferaceae release volatile organic compounds (VOC). VOC may act as plant defense compounds against pathogen and pest attack, but were also shown to enhance the growth development, and reproduction of neighboring plants, animals and microorganisms (Kong, 2001).

Purified conifer VOC were shown to inhibit the invasion, growth and reproduction of a range of airborne fungal pathogens (Kalemba and Kunicka, 2003).

Gao *et al.* (2005) compared the activity of extracts of different conifer species and showed that there is variation between species in the constituents and relative composition of VOCs and identified that the components limonene, β -pinene and aldehydes also had bacteriostatic effects.

In the experiment reported here none of the liquid fertility inputs (extract of seaweed and conifer wastes and the fish emulsion) significantly reduced corky root rot incidence. Also, none of the inputs resulted in increased yields, indicating that there was no significant crop nutrition effect at the levels used in the study. Further research would be needed to investigate whether higher application rates may provide the antifungal or fertiliser effects reported in future studies.

2.5 Conclusions

In the experiments reported here the use of plants grafted onto resistant rootstock was shown to be the most effective method for the control of soil diseases in organic agriculture. This was confirmed when resistant rootstocks were introduced into commercial practice at Cantelo Nurseries (an industrial collaborator in this study). At Cantelo Nurseries five years of monocropping of grafted tomato crops did not result in a significant increase in corky root rot disease (J Rodriguez, personal communication), suggesting that the use of resistant rootstocks is also a very durable method of control.

The use of some organic amendments (e.g. house-waste composts, *Brassica* waste) not only increased the nutrient supplies to the crops, but also reduced the disease severity, possibly due to facilitating an increase of soil microbial activity. Chitin amendments in soil also reduced the disease incidence, but the mechanism of action is unlikely to involve an increase in soil microbial activity.

It is to our knowledge the first report that the use of chitin and brassica amendments reduced diseases incidence of *P. lycopersicii* on tomatoes

Such organic matter inputs may therefore be a useful complementary (to the use of resistant root stocks) tool in integrated crop protection strategies against *P. lycopersici*. All other treatments failed to have a significant effect on root disease incidence and were therefore not included in further studies.

Chapter 3. Effect of treatment concentration and different soil-borne pathogen inocula on root disease development and yield in tomato

3.1. Introduction

Corky root rot caused by *Pyrenochaeta lycopersici* is a soil-borne disease that causes serious problems in both organic and conventional tomato crops. The disease is more severe in countries with cool climate such as countries in North Europe, but it can also cause problems in Southern European countries especially in early open field tomato crops (Shishkoff and Campbell, 1990).

Verticillium is also a major problem in tomato crops and is caused mainly by *V. dahliae* and *V. albo-atrum*. *V. albo-atrum* is more prevalent in Northern countries, whereas *V. dahliae* is more destructive in warmer climates (Pegg and Brady, 2002).

In conventional tomato crops the use of soil disinfection (e.g. with methyl bromide) can give good protection against soil-borne diseases (Duniway, 2002). However, the widespread use of such chemicals has created serious environmental (e.g. the contribution of methyl bromide to atmospheric ozone depletion (Martin, 2003) and residue problems in the fruits and in soils (Gamliel *et al.*, 2000). This has forced many governments to restrict or prohibit the use of methyl bromide. Nevertheless, in many European countries the use of methyl bromide is still common practice.

Other chemicals with systematic action, such as dazomet, fenamiphos, benomyl, thiophanate-methyl, have been used in conventional agriculture against pathogens like *P. lycopersici* and *V. albo-atrum* (Malathrakis *et al.*, 1983; Peg and Brady, 2002). Strains of pathogens that are resistant to some of these chemicals have been developed and, as a result, growers had to either increase the dose or switch to other fungicides (Agrios, 1997).

In organic glasshouse production systems the control of soil borne diseases is being achieved mainly by steam sterilization or solarization of soils and by using

resistant/tolerant cultivars (cultivars with inherent resistance or grafting of susceptible cultivars onto resistant rootstocks), (Lampkin, 2002).

However, both methods have their limitations. Steaming is an expensive method and cannot be used in large areas (e.g. in outdoor production) (Runia, 2000). Moreover, steam sterilization of soils also kills many beneficial soil organisms (including naturally occurring antagonists of pathogens) and creates an ecological vacuum in soil, which may lead to a more rapid re-colonisation of soils with pathogens and more serious epidemics (van Loenen *et al.*, 2003). The repeated use of resistant cultivars (or rootstocks) can also potentially result in the development of resistance in the pathogen population if the same resistant rootstock is used frequently in the same soil (Agrios, 1997).

It is therefore essential to develop new methods for the control of soil borne diseases in particular for organic and low input production systems.

The main objectives of work under chapter 3 were therefore

- (i) to confirm the efficacy of the treatments that successfully controlled *P. lycopersici* in the trials carried out in the previous year (those described in chapter 2)
- (ii) to test the activity of the same treatments against soils infected by *P. lycopersici* in combination with a novel, aggressive strain of *Verticilium albo-atrum* that had emerged in UK organic tomato production systems in the United Kingdom in 2001
- (iii) to investigate the effect of lowering the input levels of selected treatments (those that significantly increased yields and root fresh weights in trials carried out in 2001) (see chapter 2) and
- (iv) to test a range of different (and less expensive) chitin products against *P. lycopersici* and *V. albo-atrum*

3.2 Materials and Methods

In 2002 a glasshouse experiment was conducted at Newcastle University's Close House Experimental station to confirm the efficacy of the previous year's effective treatments. The aim of this year's experiment was also to test lower volumes of these treatments and different types of chitins against soils infected by *P. lycopersici* alone and *P.lycopersici* plus an aggressive strain of *V. albo-atrum*. Soil infected with *P. lycopersici* was collected from the Cantelo nursery, Somerset, UK. The soil was collected from different parts of the glasshouse and then mixed and homogenized with a concrete mixer to minimize differences in substrate texture, structure and inoculum density. It was then placed into 15 litres plastic pots.

3.2.1 Inoculation of soil with *Verticillium albo-atrum*

Inoculum of the aggressive strain of *Verticillium albo-atrum* was provided by the Horticulture Research International (HRI, Wellesbourne, UK) as cultures grown on Potato Dextrose agar PDA (Oxoid). The root-injection method described by Sink and Grey (1999) was used for the infection of the rhizosphere of tomato seedlings.

Immediately before infection, mycelial plugs were taken from the PDA culture and used to prepare a conidial suspension (using sterile distilled water). The suspension was then filtered/passed through a double layer of cheesecloth to remove mycelial fragments. The concentration of conidia was measured using a haemocytometer and the inoculum suspension was then adjusted to 9×10^6 conidia ml^{-1} by dilution of the original suspension with sterile distilled water.

Plants were not watered prior to inoculation to induce a slight drought stress and more rapid absorption of spore inoculum suspension (Sink and Grey, 1999).

Each plant was inoculated with two 5ml injections of inoculum suspension into the rhizosphere.

3.2.2 Soil treatments used

Each treatment was applied to (a) six pots containing soil infected with *P. lycopersicum* only and (b) six pots containing *P. lycopersici* + *V. albo-atrum*. The concrete mixer used to mix soil with the different treatments was cleaned with bleach solution after each treatment had been prepared. The following treatments were applied:

1. Brassica 50%

Chopped up Brussels sprouts (using a food processor) were mixed with soil at a 1:1 (v/v, *Brassica* tissue: soil) ratio using a concrete mixer.

2. Brassica 25%

Chopped up Brussels sprouts were mixed with soil at a 1:3 (v/v *Brassica* tissue : soil) ratio.

3. Cow manure compost 50%

Composted cow manure from an organic farm (Tio Ltd, Inverness, Scotland) was mixed with the soil. Compost was added at a volume of 50% (v/v)

4. Cow manure compost 25%

Composted cow manure was added to the soil to a volume of 25% (v/v), (compost 1 : soil 3).

5. Chitin (S) 1.0%

Chitin (made from crab shells, Sigma catalogue) was added to soil at a level of 1% (v/v).

6. Chitin (S) 0.5%

Chitin (made from crab shells, Sigma catalogue) was added to soil in a volume of 0.5% v/v.

7. Chitin (N) 1.0%

A chitin product from a Newcastle based manufacturer (Travena Organic Products, UK) was also included in the trial, since it was the cheapest product containing chitin available. It was added to soil to a final chitin level of 1% v/v of soil.

8. Chitin (N) 0.5%

The Travena chitin product was mixed with the soil to a final chitin volume of 0.5% v/v of soil.

9. Chitosan 1

A deacetylated chitin product (ChiPro GmbH, Bremen, Germany) was added to the soil every week via the irrigation water (4g of chitosan dissolved in 1000 ml of water was added every week).

10. Chitosan 0.5

A deacetylated chitin product was added via the irrigation water at a rate of 2g of chitosan dissolved in 1000 ml of water every week.

Positive controls

Steam disinfection of soil

Soils infected with either (a) *P. lycopersici* alone or (b) with *P. lycopersici* plus *V. albo-atrum* were disinfected with steam and used as positive controls.

For the steam treatment a commercial soil-steaming machine used for horticultural substrates (Camplex HD5116 Electric Soil Steriliser, THERMOFORCE LIMITED, Cumbria, U.K) was used. Soil was heated to approximately 81° C by steam application for 30 minutes.

Negative controls

- **non grafted plants** grown in soil infected by *P. lycopersici* alone, without application of soil treatments were used as the “**negative control 1**”.
- **non-grafted plants** grown in soil infected with both *P. lycopersici* and *V. albo-atrum*, without application of soil treatments, were used as the “**negative control 2**”.

3.2.3 Experimental design

Six pots/plants of the commercial cultivar “Star Fighter” were used for each treatment/pathogen soil inoculum combination included in the trial and pots placed in a greenhouse in 6 rows (=blocks). A completely randomised block design was used and

one pot of each treatment/pathogen inoculum combination was included (at random) in each row.

Temperature was kept between 15-18° C as low temperatures favour pathogens development.

3.2.4 Assessments

Fruits were collected at a “uniform red” stage and fruit number, weight and size assessments were made as described in chapter 2 (see section 2.2.8).

At the end of the experiment the roots of all plants were rinsed under tap water and root fresh weight was assessed. Finally, roots were dried in an oven (at 80° C for 24h) and root dry weight was measured.

3.3. Results

3.3.1 Effect of different soil-borne pathogen inocula and interactions between pathogen inocula and soil amendments on tomato crop performance (cumulative yield, fruit size, fruit number, root fresh weight and root dry weight)

When the effect of the two different **pathogen inocula** (*P. lycopersici* alone or *P. lycopersici* plus *V. albo-atrum*) and the different **soil amendment treatments** on tomato crop performance (cumulative yield, fruit size, fruit number, root fresh weight and root dry weight) was analysed statistically, results obtained for positive control treatments (= non-grafted plants grown in soil which was steam disinfected) were not included in the initial two-way ANOVA analysis. This was done, because

- (i) steam sterilisation (at >80° C) is known to completely remove pathogen inocula from soil (van Loehnen *et al.*, 2002) and
- (ii) inclusion of the data for positive controls would have “masked” the differences between different pathogen inoculum treatments in the statistical tests.

The initial two-way ANOVA showed that there was

- (i) a highly significant effect of the **pathogen inoculum** (*P. lycopersici* alone or *P. lycopersici* plus *V. albo-atrum*) on (a) cumulative yield, (b) root fresh weight (RFW) and (c) root dry weight (RDW), but not on the number of fruit or on the size of fruit. Cumulative yields, RFW and RDW were significantly lower for tomato plants grown in soils infected with both *P. lycopersici* and *V. albo-atrum*
- (ii) a very highly significant effect of **soil amendment treatments** on all factors assessed, with soil amendments increasing cumulative yield, fruit size, fruit number, root fresh weight and root dry weight and
- (iii) a **significant interaction** between pathogen inoculum type and soil amendment treatments for root fresh and dry weights, but not cumulative yield, fruit size and fruit number (see Table 3.1).

The relative effect of soil amendment treatments on crop performance was assessed in more detail by comparison of (a) crops grown in amended soils with crops grown in either (b) untreated (negative controls) or (c) steam disinfected (positive controls) soils. See below for more detailed separate analysis of the effect of soil amendments on crop performance in either (a) *P. lycopersici* (section 3.3.2) and (b) *P. lycopersici* plus *V. albo-atrum* (section 3.3.3) infected soils.

The interaction between soil pathogen inoculum and soil amendment treatments was then analysed further (by 2 or 3 way ANOVAs) for different (a) soil amendment types (different chitin or soil organic matter inputs) and (b) input levels (see sections 3.3.4 and 3.3.5 below).

Table 3.1 Effect of different soil borne pathogen inocula and interactions between pathogen inocula and soil amendments on tomato crop performance (cumulative yield, number and size of fruit and root fresh and dry weight) (**p-values of two-way ANOVA test**, excluding data for positive controls).

Characteristic assessed	Main Effects		Interaction
			2 way
	P	T	P*T
Cumulative yield	0.004	0.000	0.965
Number of fruit	0.340	0.000	0.181
Size of fruit	0.236	0.001	0.115
Root fresh weight	0.000	0.000	0.006
Root dry weight	0.017	0.000	0.013

P = pathogen inoculum (*P. lycopersici* only; *P. lycopersici* plus *V. albo atrum*)
T = treatment

3.3.2 Effect of soil treatments on tomato crop performance for plants grown in soil infected with *P. lycopersici*

As in the trial reported in chapter 2, the presence of *P. lycopersici* in soil significantly reduced the cumulative yield, fruit size, fruit number and root fresh and dry weight of plants (see results for tomato grown in untreated [negative control] and steam disinfected [positive control] soils in Table 3.2).

One way ANOVA and Tukey’s Honest Significance test also showed that:

1. The **number of fruits** on plants grown in soils treated with fresh *Brassica* tissue amendments (both levels) and with all different types of chitin (at the highest level) was **(a)** significantly higher than the number of fruits on plants grown in untreated soil (negative control), but **(b)** not significantly different to the number of fruits on plants grown in steam sterilised soil (positive control) (Table 3.2).

2. The **size of fruit** on plants grown in soils amended with purified chitin (Sigma) and Chitosan (at the higher input levels) was **(a)** significantly increased compared to fruit of plants grown in untreated soils (negative control), but **(b)** not significantly different when compared to the size of fruits of plants grown in steam sterilised soil (positive control), (Table 3.2).

Table 3.2 Effect of different organic matter amendments to soil and different types of chitin on the performance of tomato plants grown in to soils infected with *P. lycopersici*.

SOIL TREATMENT	Cumulative Yield (kg)/plant	Number of fruit/plant	Mean size of fruit (cm)	Root Fresh Weight (g)/plant	Root Dry Weight (g)/plant
Untreated	0.45c	13b	4.0b	18d	1.7d
<i>Brassica</i> 50%	0.84ab	22a	4.0b	31c	3.4c
<i>Brassica</i> 25%	0.78b	21a	4.3ab	31c	3.1c
Compost 50%	0.73bc	16ab	4.3ab	29c	3.0c
Compost 25%	0.68bc	18ab	4.1b	29c	2.8c
Chitin (S) 1.0%	0.93ab	21a	4.4a	47b	5.0b
Chitin (S) 0.5%	0.9ab	16ab	4.3ab	45b	5.2ab
Chitin (N) 1.0%	0.79ab	20a	4.2ab	31c	3.3c
Chitin (N) 0.5%	0.72bc	16ab	4.4ab	29c	3.4c
Chitosan 1	0.98ab	21a	4.5a	47b	5.1b
Chitosan 0.5	0.88ab	18ab	4.3ab	48b	5.8ab
Steamed	1.1a	22a	4.6a	64a	7.4a

One way ANOVA showed that there were significant differences between treatments (P<0.001). Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05)

3. All soil treatments resulted in significantly higher **root fresh weights** (RFW) and **root dry weights** (RDW) compared to plants grown in untreated soil (negative control) (Table

3.2; figure 3.1). However, the root fresh weight and root dry weight of plants grown in soils amended with purified chitin (Sigma) and Chitosan were higher than those obtained with plants grown in soils amended with all other treatments, but remained significantly lower than those obtained in steam sterilised soil (except for plants grown in soils amended with 0.5% purified chitin or Chitosan, which had similar RDWs to plants grown in steam disinfected soil).

4. Seven of the ten soil treatments resulted in an increase in **cumulative yield** compared to plants grown in untreated soil (negative control) (see Table 3.2 and Fig 3.2). These were (i) fresh *Brassica* tissue (at both input levels), (ii) chitin product produced in Newcastle (higher input level only), (iii) Chitosan (at both input levels) and (iv) purified chitin (Sigma at both input levels).

Application of composted manure (at both levels) and the chitin product produced in Newcastle (at the lower input level) did not have any significant effect on yield, compared to plants grown in untreated soil (negative control) ($P < 0.05$).

The yield of plants grown in soils treated with fresh *Brassica* tissue (at the higher input level), the chitin product produced in Newcastle (at the higher input level), Chitosan (at both input levels) and purified chitin (Sigma at both input levels), was not significantly different to that obtained with plants grown on steam disinfected soil (positive control).

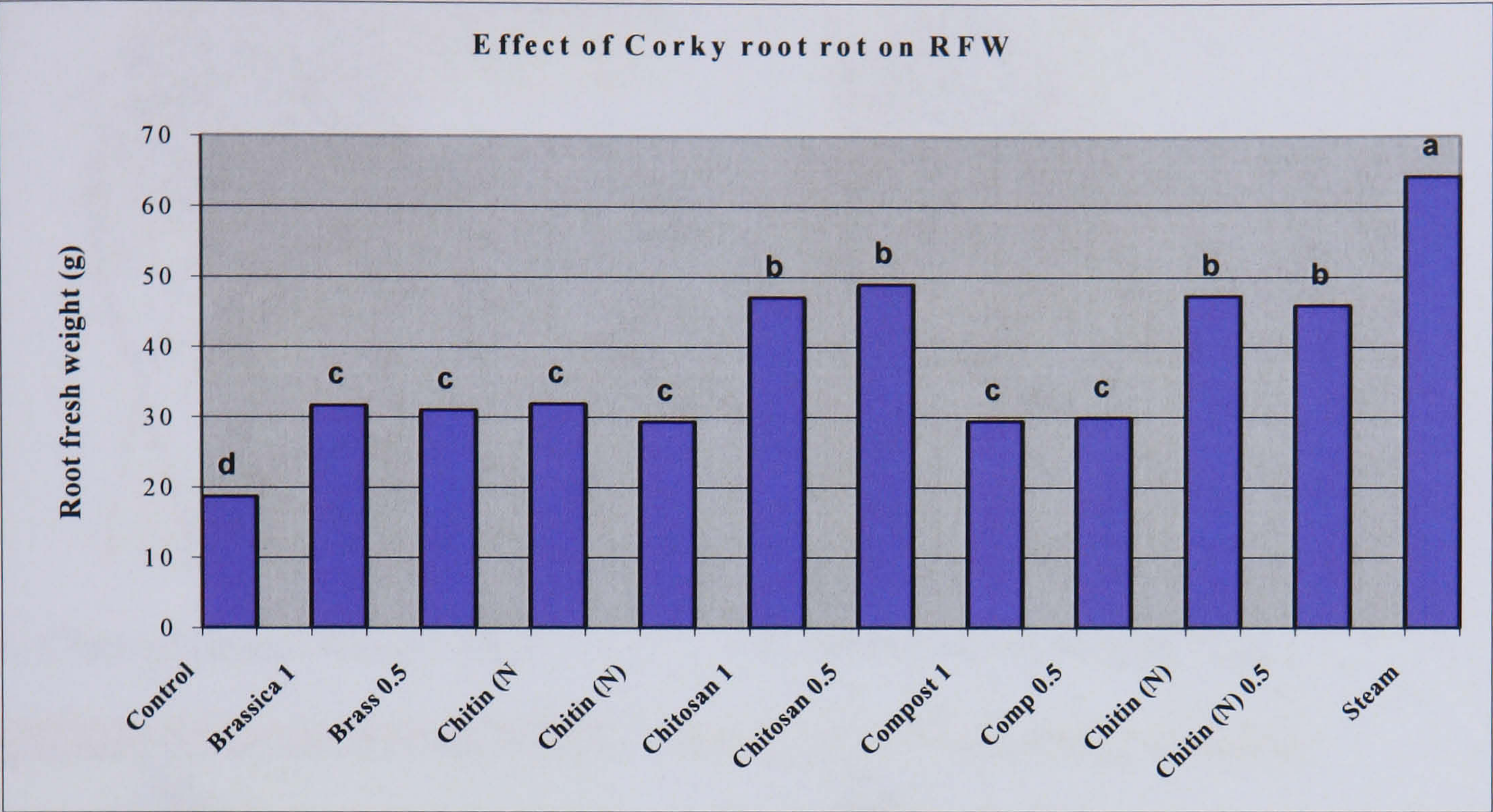


Figure 3.1 Effect of different soil inputs on root fresh weight of tomato plants infected by corky root rot only. Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test ($P<0.05$)

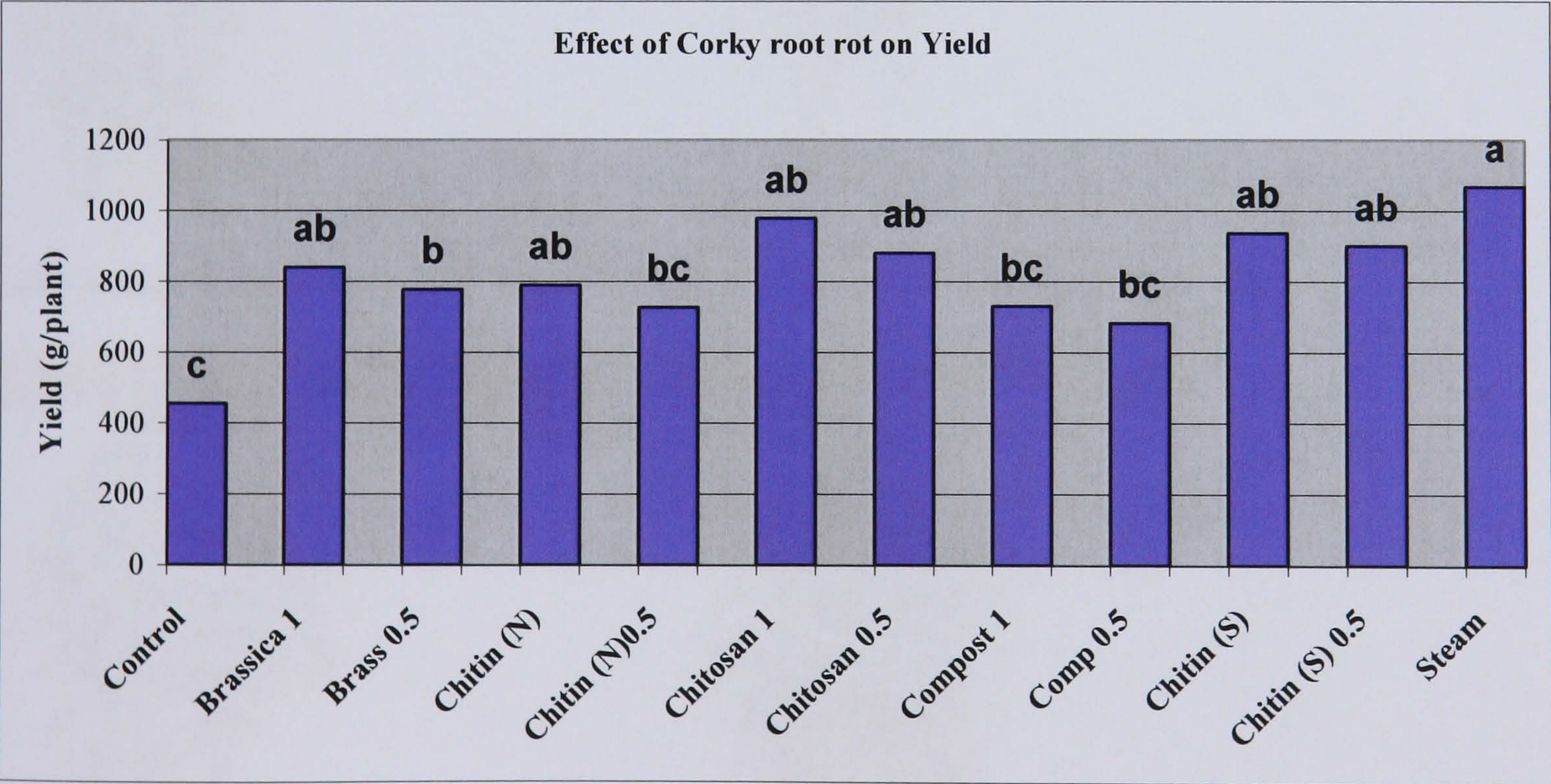


Figure 3.2 Effect of different soil treatments on the yield of tomato plants infected by *P. lycopersici* only. Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test ($P<0.05$)



A. Control (mean weight 13g)



B. Steam (mean weight 77g)



C. Brassica 1 (mean weight 28g)



D. Brassica 0.5 (mean weight 28g)



E. C. man. Comp. 1(mean weight 29g)



F. C. man. Com. 0.5 (mean weight 28g)



G. Chitin (Sigma) 1 (mean weight 47g)



H. Chitin (Sigma) 0.5 (mean weight 30g)



I. Chitosan 1 (mean weight 48g)



L. Chitosan 0.5 (mean weight 47g)



M. Chitin (N) 1 (mean weight 30g)



N. Chitin (N) 0.5 (mean weight 19g)

Figure 3.3 Roots of tomato plants (different treatments) infected by *P. lycopersici* and *V. albo-atrum*.

(Photographs were taken from different distances therefore are not to the same scale)

3.3.3 *Effect of soil treatments on tomato crop performance for plants grown in soil infected with *P. lycopersici* plus *V. albo-atrum**

The combined presence of *P. lycopersici* and *V. albo-atrum* in soil significantly reduced the cumulative yield, fruit size, fruit number and root fresh and dry weight of plants (see results for tomato grown in untreated [negative control] and steam disinfected [positive control] soils in Table 3.3).

One way ANOVA and Tukey's Honest significance tests also showed that:

1. The **number of fruit** of plants grown in soils treated with all soil amendment treatments was **(a)** significantly higher than the number from fruits of plants grown in untreated soil (negative control) (except the lower input level treatments of composted cow manure and the chitin product produced in Newcastle, which were not significantly different to the negative controls), but **(b)** not significantly different to the number of fruits of plants grown in steam sterilised soil (positive control) (except the lower input level treatment of composted cow manure, which gave significantly lower numbers of fruit than the positive controls) (Table 3.3.).
2. None of the soil amendment treatments increased the size of the fruits compared to the **size of the fruit** on plants grown in untreated soil.
3. All soil treatments resulted in significantly higher **root fresh weight** (RFW) and **root dry weight** (RDW) compared to plants grown in untreated soil (negative control) (Table 3.3; figure 3.3) (except for the lower input level of fresh *Brassica* tissue and lower input levels of compost and chitin (N) which showed similar results to negative controls for RDW). The RFWs and RDWs of plants grown in soils amended with purified chitin (Sigma at the higher input level) and Chitosan (at both input levels) were higher than those obtained with plants grown in soils amended with all other treatments.

However, the RFW and RDW for these chitin treatments were significantly lower than those obtained in steam sterilised soil (Table 3.3; Figure 3.3).

Table 3.3 Effect of soil amendments and different types of chitin on tomato plants grown in to soils infected with *P. lycopersici* plus *V. albo-atrum*.

SOIL TREATMENT	Cumulative Yield (kg)/plant	Number of fruit/plant	Mean size of fruit (mm)	Root Fresh Weight (g)/plant	Root Dry Weight (g)/plant
Untreated	0.30d	10b	40.9b	13e	1.9d
<i>Brassica</i> 50%	0.76bc	20a	41.9ab	28c	3.0c
<i>Brassica</i> 25%	0.76bc	19a	43.7ab	28c	2.6cd
Compost 50%	0.66bc	17a	41.4b	29c	3.0c
Compost 25%	0.58cd	15b	41.9ab	28c	2.6cd
Chitin (S) 1.0%	0.86abc	20a	44.0ab	47b	5.7b
Chitin (S) 0.5%	0.76bc	19a	42.0ab	30c	3.4c
Chitin (N) 1.0%	0.76bc	19a	43.4ab	30c	3.3c
Chitin (N) 0.5%	0.56bcd	16ab	40.3b	19d	2.3cd
Chitosan	0.90ab	20a	44.0ab	48b	5.5b
Chitosan	0.87bc	20a	45.3ab	47b	5.3b
Steamed	1.16a	22a	46.9a	77a	9.1a

One way ANOVA showed that there were significant differences between treatments ($P<0.001$). Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P<0.05$)

4. Eight of the ten soil treatments resulted in an increase in **cumulative yield** compared to plants grown in untreated soil (negative control) (see Table 3.3. and Fig 3.4.). Only application of composted manure (at the lower input level) and the chitin product produced in Newcastle (at the lower input level) did not have any significant effect on yield, compared to plants grown in untreated soil (negative control) ($P<0.05$). The yield

of plants grown in soils treated with Chitosan (at the higher input level) and purified chitin (Sigma) (at the higher input level), was not significantly different to that of plants grown on steam disinfected soil (positive control). For all other treatments the yield was lower than that of positive controls (see Table 3.3. and Fig 3.4.).

To identify interactions between the effects of different **(a) soil pathogen inocula (b) soil amendment types** (different organic matter inputs and chitin preparations used) and **(c) input levels**, a series of 2 and 3 way ANOVAs was carried out on selected groups of data. These are described in sections 3.3.4 and 3.3.5 below.

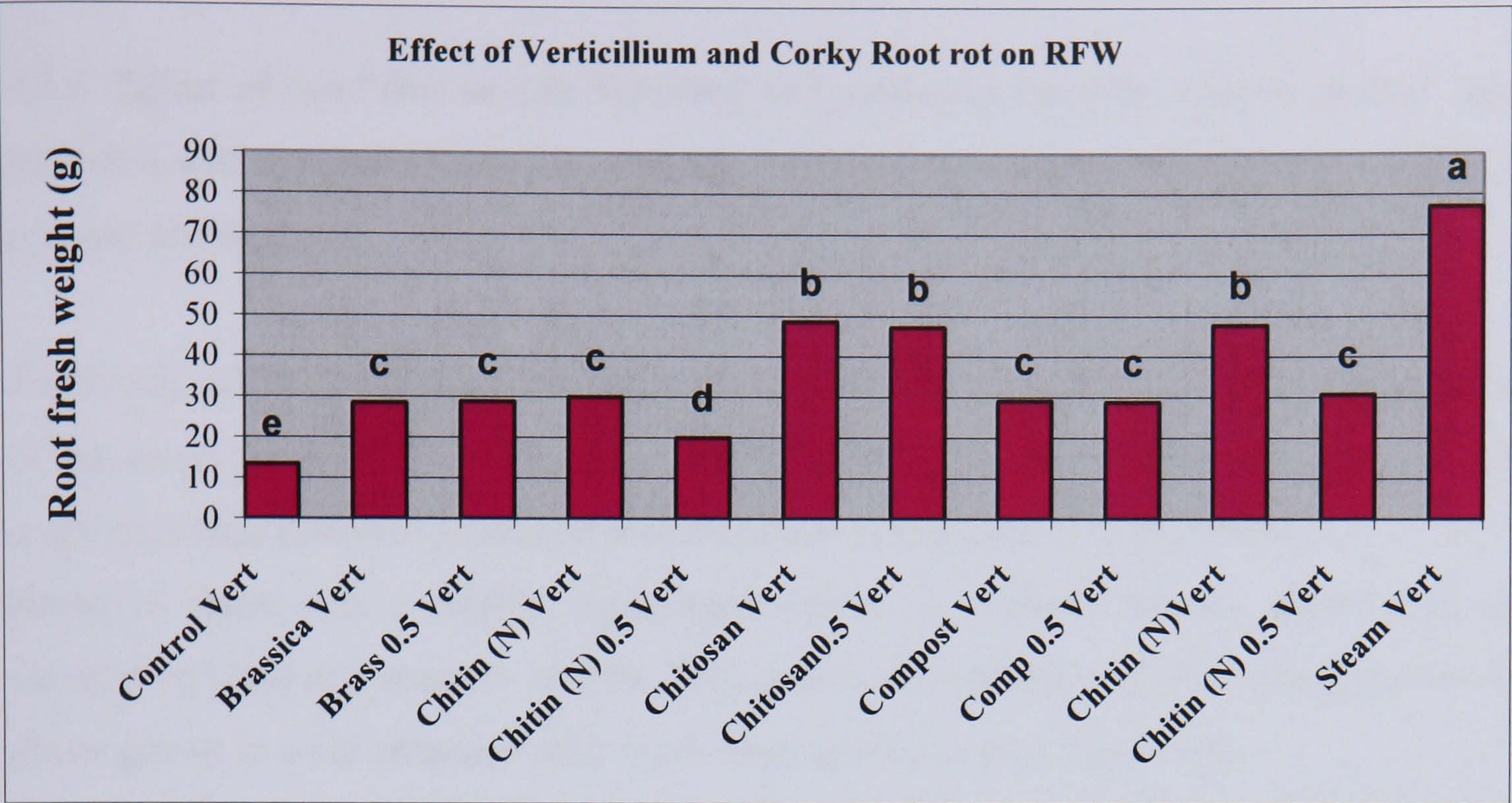


Figure 3.4. Effect of different soil treatments on the root fresh weight of tomato plants infected by *P. lycopersici* + *V. albo-atrum*. Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$)

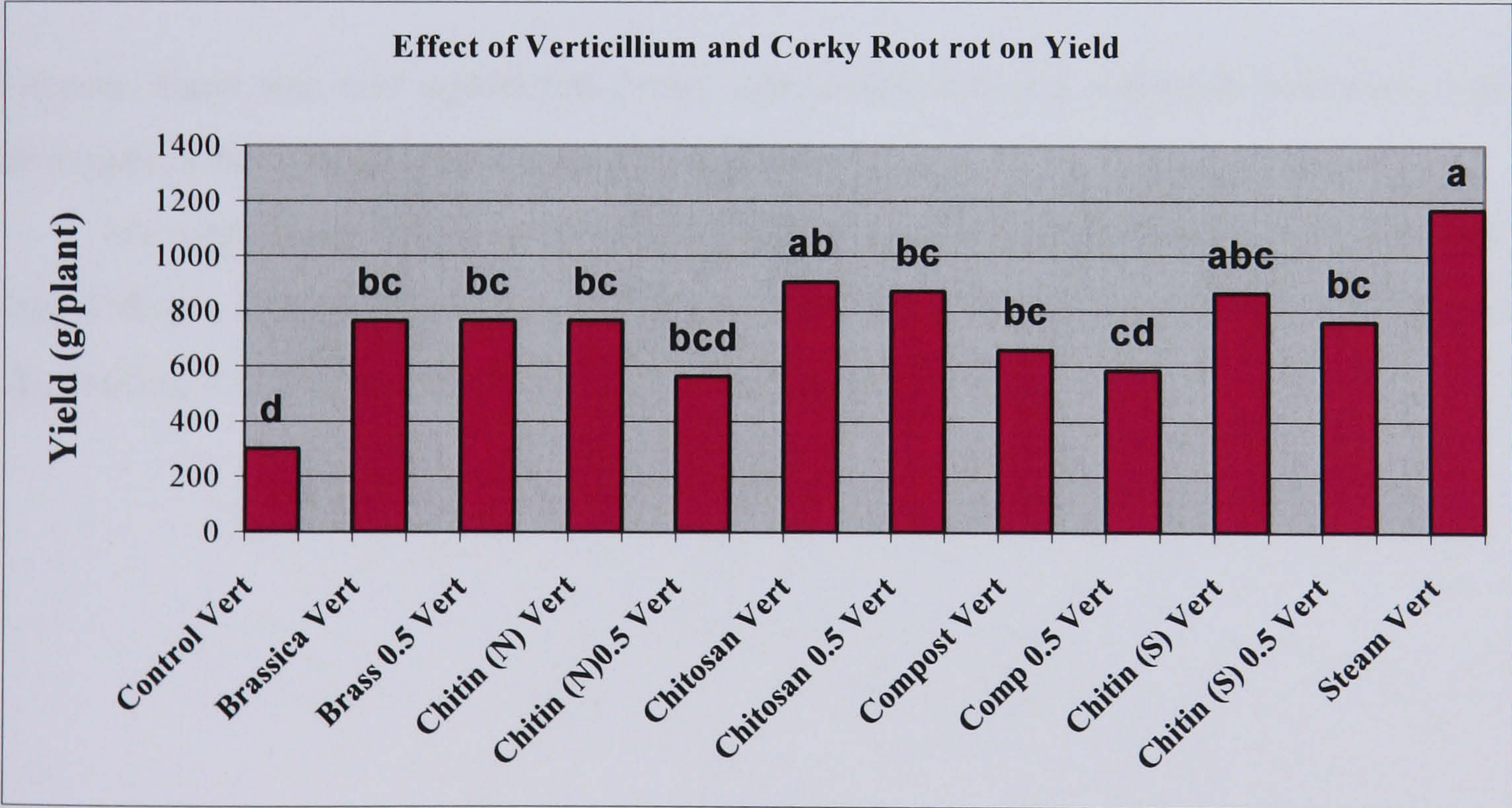


Figure 3.5. Effect of different soil amendments on fruit yield of tomato plants infected by Corky root + *V. albo-atrum*. Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$)

3.3.4. Effect of (and interaction between) soil pathogen inocula, organic matter types (brassica and composted cow manure amendments) and organic matter input levels on crop performance

Three-way ANOVA showed that there was no significant difference in the performance of (a) crops grown in soils inoculated with different pathogen inoculum types and (b) crops receiving different input levels of *Brassica* or composted cow manure.

However, there was a **highly significant effect of organic matter input type** for cumulative yield and number of fruit, with yields and numbers of fruit being greater for plants grown in soils amended with fresh *Brassica* tissue (see Table 3.4).

On the other hand there was no significant difference between soil-input types for root fresh and dry weight and the size of fruit (Table 3.4).

There was no three-way interaction between the pathogen inoculum type, organic matter input type and organic matter input level (see Table 3.4) for any of the crop performance parameters assessed.

However, there was one significant 2-way interaction between pathogen inoculum type and organic matter input type for the size of fruit.

No significant differences between individual treatment means were detected when Tukey's Honest Significant Difference Test ($P < 0.05$) was applied (Table 3.4 and 3.5), making it difficult to explain the nature of the interaction.

Table 3.4 Effect of different organic matter input types (cow manure based compost or fresh *Brassica* tissues) and organic matter input levels on tomato crop performance. (P-values of 3-way ANOVA)

	Main effects			Interactions			
				2 way			3 way
Characteristic assessed	P	OM	L	P*OM	P*L	OM*L	P*OM*L
Cumulative yield	0.121	0.005	0.267	0.635	0.817	0.705	0.579
Number of fruit	0.090	0.000	0.963	0.377	0.074	0.963	0.608
Size of fruit	0.602	0.418	0.667	0.015	0.186	0.830	0.461
Root fresh weight	0.198	0.597	0.816	0.437	0.829	0.855	0.922
Root dry weight	0.093	0.265	0.761	0.452	0.859	0.110	0.719

P = soil pathogen inoculum; OM = organic matter input type; L = Input level

Table 3.5. Effect of (and interaction between) pathogen inoculum and organic matter input type on the size of fruit

	FRUIT SIZE (mm)			
	Input Type		Difference	
Soil inoculum	Compost	<i>Brassica</i>	%	Mean
<i>P. lycopersici</i>	43.8	41.5	5.0	42.6
<i>P. lycopersici</i> and <i>V. albo-atrum</i>	41.7	42.9	2.8	42.3
Mean	42.3	42.2		

There were no significant differences between treatment means according to Tukey's Honest significant difference test

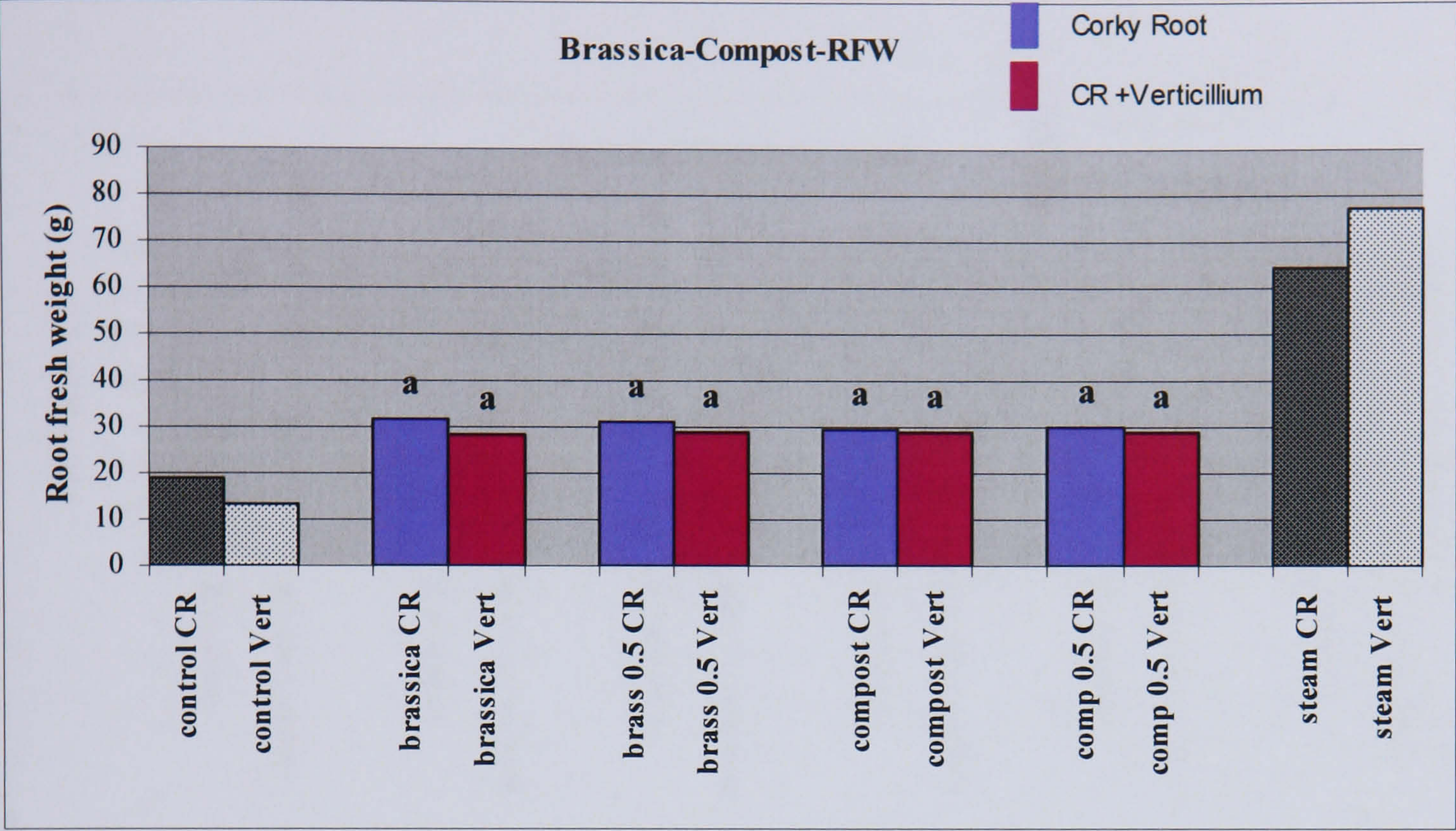


Figure 3.6 Effect of *Brassica* and cow manure compost amendments on the root fresh weight of tomato plants infected by *P. lycopersici* and *P. lycopersici* + *V. albo-atrum*. Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P<0.05$)

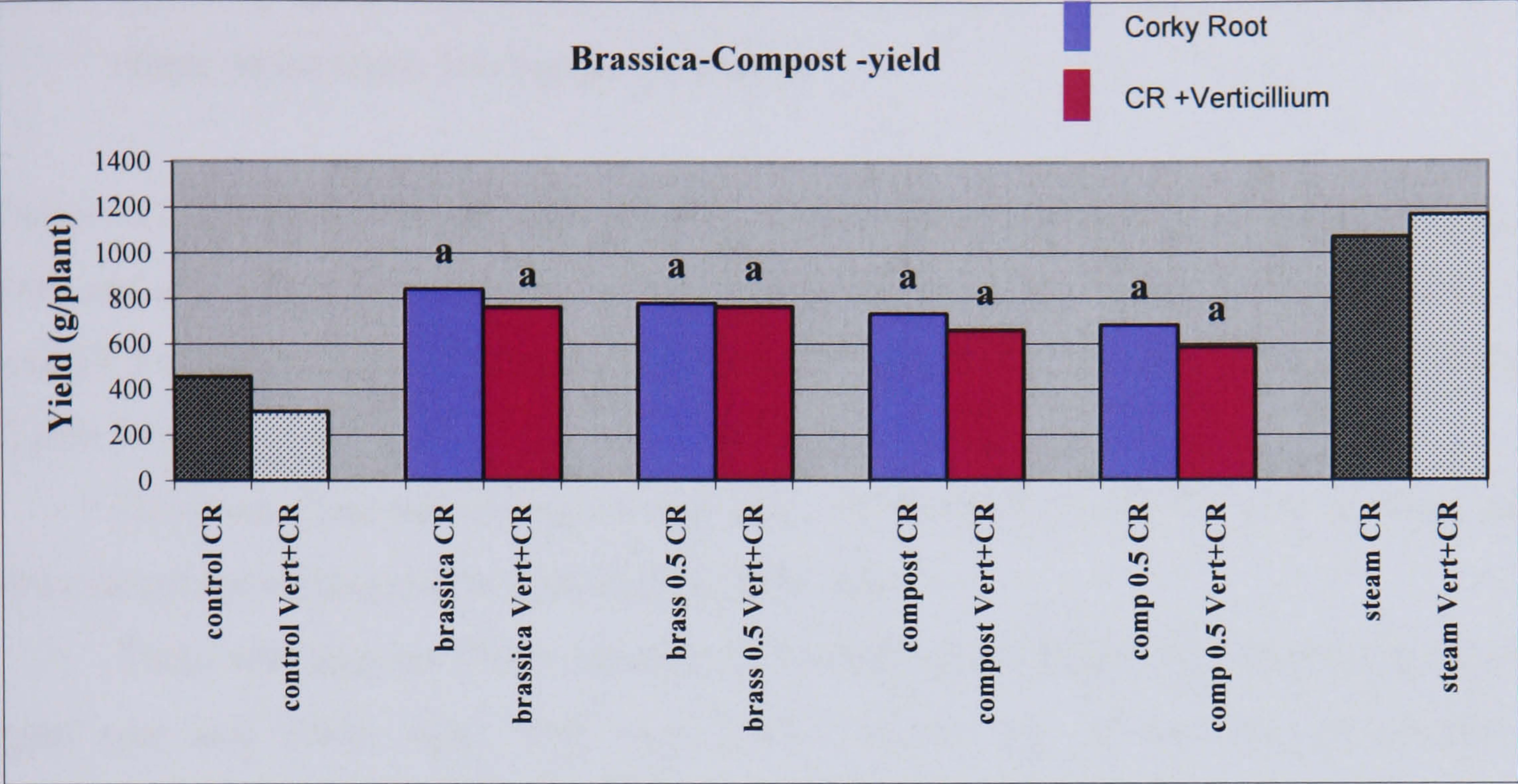


Figure 3.7. Effect of *Brassica* and cow manure compost amendments on the yield of tomato plants infected by *P. lycopersici* and *P. lycopersici* + *V. albo-atrum*. Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$)

3.3.5 *Effect of (and interaction between) soil pathogen inocula, chitin types and chitin input levels on crop performance*

Three-way ANOVA showed that there was a significant difference in yield, and root fresh and dry weight between tomatoes grown in soils with (a) different pathogen inocula (except for yield where $P=0.64$) and (b) chitin input types and (c) chitin input levels (Table 3.6).

However, there was no significant effect of the main factors on fruit number and size (except for chitin type on fruit size), (Table 3.6).

There was also no 3-way interaction between the pathogen inoculum type, chitin input type and chitin input level (see Table 3.6) for any of the crop performance parameters assessed. However, there were 2-way interactions between (a) chitin input type and input level and (b) pathogen inoculum type and chitin input level for the parameters RFW and RDW (Tables 3.7 to 3.10).

While the root fresh weight (RFW) and root dry weight (RDW) remained similar when the chitin input level was reduced in soils infected with *P. lycopersici*, both RFW and RDW decreased significantly when plants were grown in soil inoculated with both *P.lycopersici* plus *V. albo-atrum*.

While the root fresh and dry weights decreased significantly when the levels of purified chitin (Sigma) were decreased, there was (a) no significant decrease in RFW and RDW when the levels of the chitin product produced in Newcastle were reduced and (b) RFW and RDW were very similar for the 2 levels of chitosan used.

The effect of different chitin types and levels on root fresh and cumulative yields are shown in Figures 3.7 and 3.8 respectively.

Table 3.6. Effect of different chitin types and input levels on tomato crop performance. (P-values of 3-way ANOVA)

	Main effects			Interactions			
				2 way			3 way
Characteristic assessed	P	CT	L	P*CT	CT*L	P*L	P*CT*L
Cumulative yield	0.064	0.001	0.037	0.800	0.573	0.767	0.607
Number of fruit	0.682	0.261	0.396	0.800	0.682	0.101	0.074
Size of fruit	0.215	0.014	0.618	0.633	0.132	0.086	0.197
Root fresh weight	0.003	0.000	0.001	0.097	0.003	0.025	0.193
Root dry weight	0.037	0.000	0.037	0.478	0.001	0.041	0.188

P = soil pathogen inoculum; CT = Chitin type; L = Chitin input level

Table 3.7. Effect of (and interaction between) pathogen inoculum and chitin input level on the root fresh weight (g) (RFW). Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05)

	Input level		Difference	
Pathogen	1	2	%	Mean
<i>P. lycopersici</i>	42a	41a	2	42A
<i>P.lycopersici</i> plus <i>V.albo-atrum</i>	42a	33b	21	37B
Mean	42A	37B		

Table 3.8. Effect of (and interaction between) pathogen inoculum and chitin input level on the root dry weight (g) (RDW). Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05)

	Input level		Difference	
Pathogen	1	2	%	Mean
<i>P.lycopersici</i>	4.5a	4.8a	6	4.7A
<i>P.lycopersici</i> plus <i>V.albo-atrum</i>	4.8a	3.7b	22	4.3B
Mean	4.7A	4.3B		

Table 3.9. Effect of (and interaction between) chitin input type and level on the root fresh weight (RFW). Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05)

	Input level		Difference	
Type of Chitin	1	2	%	Mean
Purified Chitin (Sigma)	48a	38b	21	43B
Chitin Product (Newcastle)	31c	25c	20	28C
Chitosan	48a	48a	0	48A

Table 3.10. Effect of (and interaction between) chitin input type and level on the root dry weight (RDW). Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05)

	Input level		Difference	
Type of Chitin	1	2	%	Mean
Purified Chitin (Sigma)	5.4a	4.3b	20	4.9A
Chitin Product (Newcastle)	3.3c	2.9c	9	3.1B
Chitosan	5.3ab	5.6a	9	5.5A

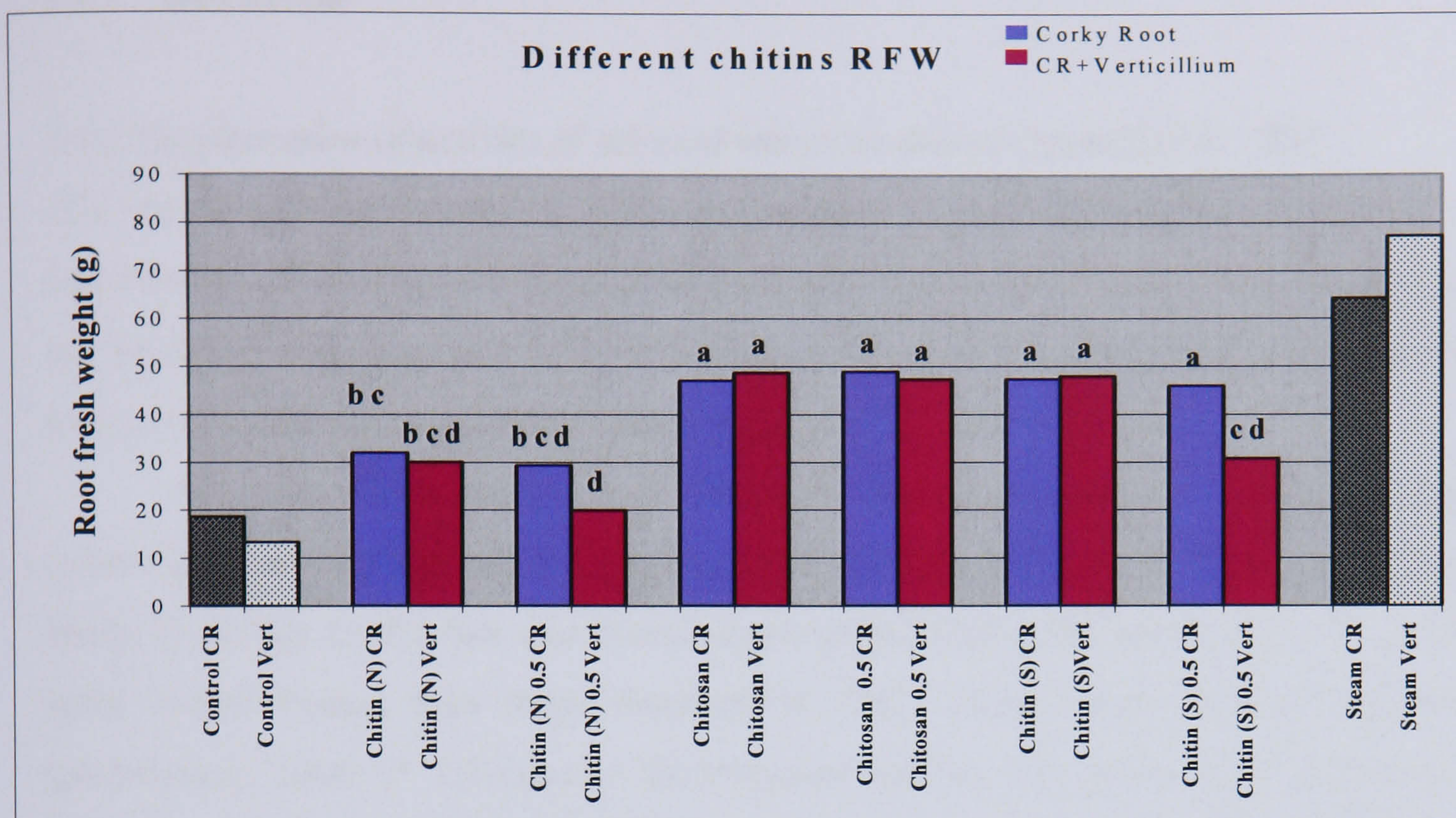


Figure 3.8. Effect of different types of chitin on the root fresh weight of tomato plants infected by *P. lycopersici* and *P. lycopersici* + *V. albo-atrum*. Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$)

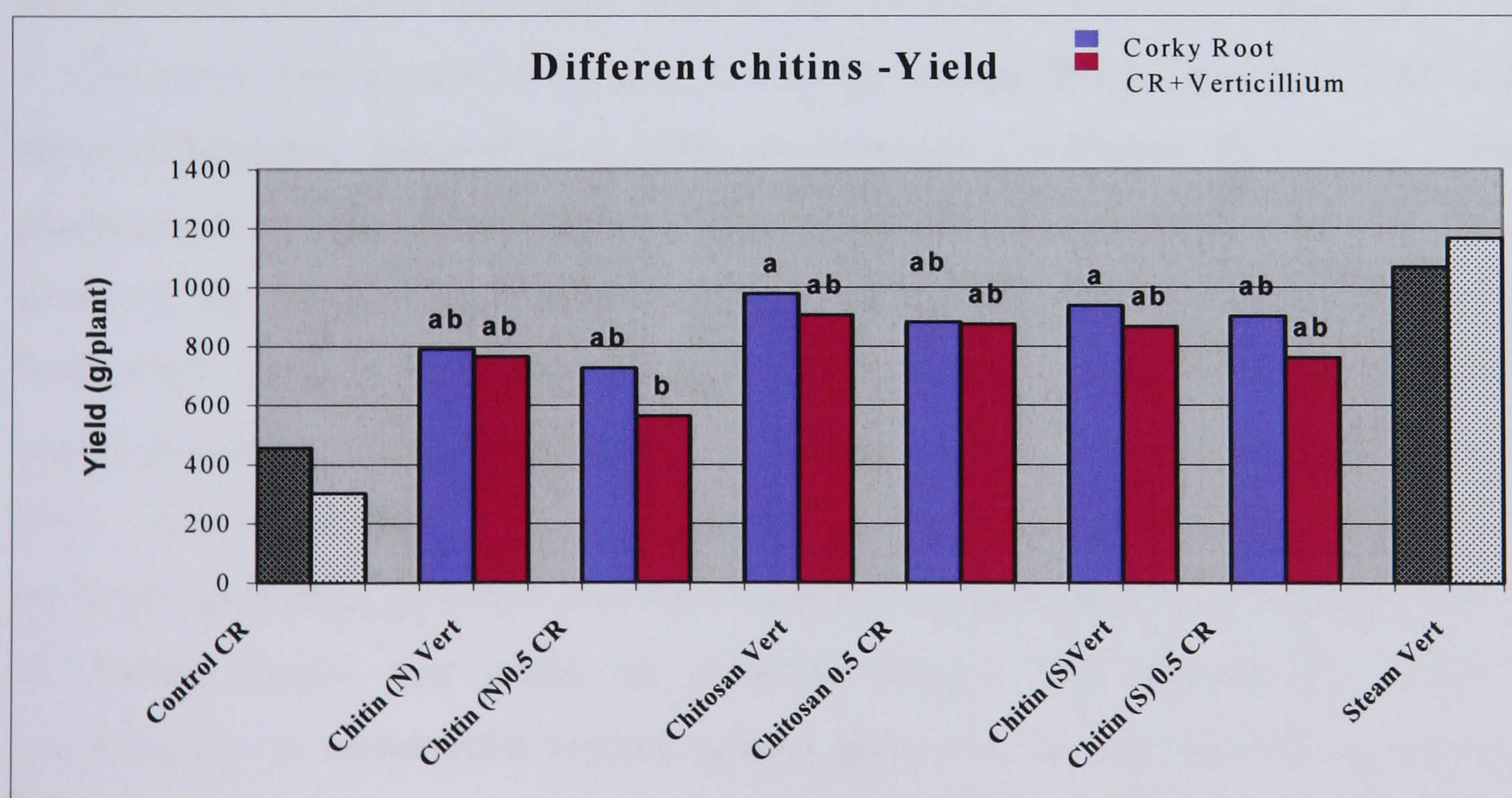


Figure 3.9. Effect of different types of chitin on the yield of tomato plants infected by *P. lycopersici* and *P. lycopersici* + *V. albo-atrum*. Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$)

3.5 Discussion

3.5.1 Confirmation of activity of selected soil amendments identified in 2001

The results of experiments in 2001 (see chapter 2) had shown that a range of soil amendments (fresh *Brassica* tissue, composts and chitin) significantly reduced corky root rot incidence and increased crop performance (especially yield). The activity of these treatments could therefore be confirmed in the 2002 trial reported here.

However, the overall levels of activity (compared to positive control plants: plants grown in steam sterilized soil) were lower in 2002 than 2001 (see Table 3.11). This was likely to be due to the fact that mean temperatures during the growing period in 2002 were overall lower, than those recorded in 2001. It has been reported that lower temperatures favor *P. lycopersici* development and as the temperature increases the severity of disease declines (Malathrakakis, 1983). It was therefore not surprising that yields and RFWs were lower in 2002.

3.5.2 Effect of soil amendment treatments against *Verticillium albo-atrum*

In addition, in 2002, all three treatments (fresh *Brassica* tissue, composts and chitin) were also shown to provide a significant level of disease control when both corky root rot and *V. albo-atrum* were present in the soil. **To our knowledge this is the first report of the effect of brassica, compost and chitin amendments on disease severity on tomato plants grown in soils infected by *P. lycopersici* plus *V. albo-atrum*.**

However, the presence of *V. albo-atrum* did significantly reduce both yields and root fresh weight compared to corky root alone. This confirmed observations in commercial organic nurseries, where *Verticillium* was recognized as a newly introduced disease in 2001 (Juan Rodriguez, Cantelo Nursery, personal communication). Overall, the performance of crops grown in soils infected with both pathogens indicated that the levels of disease control that could be obtained through soil amendments would be unsatisfactory to commercial organic tomato producers. It was decided to investigate further the effect of resistant rootstocks/cultivars in 2003 (see chapter 4) to identify the potential for optimizing root disease control via combined use of soil amendments and resistant cultivars/rootstocks.

Table 3.11. Yield and root fresh weights of plants grown in soils (infected with corky root rot only) amended with fresh *Brassica* tissue, cow manure based compost and Chitin (Sigma) and comparison to yields obtained from plants grown in steam disinfected soil

		Yield (kg/plant)	RFW (g/plant)	Yield (kg/plant)	RFW (g/plant)
Input type	Input level	2001	2001	2002	2002
Steam (positive control)		1.35	81	1.1	64
Fresh <i>Brassica</i> tissue	50%	1.23	80	0.73	33
% difference to steam		8	1	24	48
Compost	50%	1.16	53	0.73	33
% difference to steam		13	34	24	54
Chitin (S)	1%	1.16	81	0.93	47
% difference to steam		13	0	26	26

3.5.3 *Effect of reducing dose rates of soil amendments*

When input levels of soil amendments were reduced by half, yield, root fresh weight and/or other crop performance parameters decreased, but the decrease was not statistically significant for all treatments. It was therefore decided to test the most active treatments identified in the 2002 trials over a range of lower concentrations in 2003 (see chapter 5 for results) to obtain a better estimation of the dose responses for the different soil amendment treatments.

The mode of action of different treatments was not investigated in the trials carried out in 2002. However, since the activity of a range of soil amendment treatments was confirmed, additional assessments were scheduled for the trials planned in 2003, to identify potential mechanisms responsible for the increases in yield and/or reductions in disease incidence observed in 2001 and 2002 (see chapter 5).

3.5.4 Potential for using less expensive chitin products

The different chitin products tested in trials showed very similar levels of activity. It is therefore feasible to replace the very expensive purified (Sigma) product of chitin with more cost effective soluble (chitosan) or insoluble chitin (the product produced in Newcastle).

CHAPTER 4. Effect of variety choice on root disease development in tomato plants grown in soils infected with different pathogen inocula (*Pyrenochaeta lycopersici* alone or *Pyrenochaeta lycopersici* plus *Verticillium albo-atrum*)

4.1 Introduction

The most practical and economically viable method for protecting plants against soil borne pathogens was described to be the use of resistant varieties or rootstocks (Lyon *et. al*, 1995).

4.1.1 Varieties/rootstocks resistant to corky root rot – economic viability of using grafted plants

Resistance to tomato corky root rot, caused by *Pyrenochaeta lycopersici*, has been found in wild tomato plants and in some commercially grown tomato varieties (Cirulli, 1968; Ciccicarese and Cirulli, 1983). However, in most European countries few resistant cultivars are currently commercially available. For example, in the Greek market, only Corno, Piranto, Pyrella, and Sycro are marketed as providing some level of tolerance to corky root rot. However, these varieties were shown to be inferior in plant growth characteristics, fruit yield and fruit quality than more susceptible cultivars (Vakalounakis and Fragiadakis, 2003).

Where corky root rot resistant tomato cultivars are available, but do not provide the desired fruit yield, fruiting pattern and/or fruit quality, grafting of susceptible cultivars (which provide high fruit yields and quality) onto resistant rootstock is therefore often used as an alternative solution for the production of resistant plants (Agrios, 1997). However, grafting onto resistant rootstocks increases the costs of transplants, because twice the number of seed/plants is needed to produce the same planting density and the grafting process causes significant additional labor cost (both technicians with higher skills and a higher labor input per transplant are needed) for the rearing of transplants (Morra *et al.*, 1997). However, the increased yield due to the more vigorous plant root system (and cost savings associated with not having to disinfect soils) usually more than compensate for the increased seedling rearing costs (Mazollier, 1999).

This was confirmed for greenhouse grown tomato crops in the experiment described under chapter 2 and 3, which showed that the level of corky root rot control

provided by use of plants grafted onto resistant rootstocks was similar to that achieved by growing non-grafted plants in steam disinfected soil.

More recently, the so-called “double stem growing technique” has been developed and is now widely used. In this technique, two stems are allowed to develop from one shoot grafted onto a single rootstock. This reduces the number of plants required by half, since plants can be planted at $\frac{1}{2}$ the density without significant loss in yield (Granges and Leger, 1996).

Cultivation of resistant cultivars or grafting on resistant rootstocks not only prevents or significantly reduces, losses caused by soil borne diseases, but also removes the cost associated with chemical (e.g. methyl-bromide) or thermal (e.g. steaming or solarisation) soil-disinfection (Agrios, 1997). It was recently calculated that for organic glasshouse tomato crops, the additional cost associated with the use of resistant rootstocks is less than half the cost of disinfecting soils every 3 years with either steam or methyl bromide disinfection (Juan Rodriguez, personal communication).

4.1.2 Cultivars/rootstocks resistant to *Verticillium* vascular diseases – the need to identify cultivars with resistance to a wider range of diseases

The resistance gene(s) present in rootstocks or cultivars of tomato can be specific for certain diseases (e.g. just provide protection against *P. lycopersici*) or cover a range of diseases (Vacalounakis and Fragiadakis, 2003). There are several commercially available tomato cultivars that are resistant to more than one soil-borne and/or foliar diseases and pests and these are thought to be the most desirable cultivars for use in organic production systems where chemical soil disinfection or pesticide treatments are not permitted and where the use of steam disinfection is increasingly restricted (e.g. recommended not to be used more frequently than every 3-4 years) (Lampkin, 2000).

Corky root rot (*P. lycopersici*) is considered to be one of the most important soil borne disease affecting tomato. However, a range of vascular pathogens (*Fusarium oxysporum* f. sp. *lycopersici* and *Verticillium dahliae* and *V. albo-atrum*) also cause significant losses in yield and quality in commercial production. These soil, seed or

transplant-borne diseases are a problem in many tomato-growing areas in Europe (Scheffer, 1997).

Resistance genes for *Verticillium* diseases have been identified and transferred into commercial tomato cultivars.

For example, a single gene *Ve* from the Peruvian wild species *Lycopersicon pimpinellifolium* was incorporated into commercial cultivars of *L. esculentum* by Blood as early as 1932. This gene confers resistance to race 1 of *V. dahliae* and to certain strains of *V. albo-atrum* (Pegg and Brady, 2002). However, single gene-based resistances often break down rapidly, due to the appearance of new races of the pathogen, which overcomes the resistance (Beckman and Talboys, 1981; Patternotte and Van Kestern, 1993).

As a result, resistance breeding has focused increasingly on identifying polygenic (or horizontal) resistance traits which, although provide partial control of the pathogen (characterized by slower symptom development), are usually more durable (Lampkin, 2000).

For example polygenic, partial resistance to *Verticillim* vascular wilts has been reported for cultivars IRAT L3, Morden Lac, Okitsu Sozai and UC82 and, at present, these resistance traits appear to provide the best defense against race 2 of *V. dahliae* (Gold and Robb, 1995).

However, there is very little information on the level of protection against soil borne diseases provided by the currently available resistant cultivars/rootstocks in soils contaminated with mixed (corky root rot and *V. albo-atrum*) pathogen inocula.

4.1.3 Objectives of work carried out under Chapter 4

The main objectives of chapter 4 were therefore:

- (i) To quantify the level of resistance of three different non-grafted “resistant” tomato cultivars against *P. lycopersici* and a new aggressive strain of *V. albo-atrum*

- (ii) To study the level of resistance provided by resistant rootstocks against *P. lycopersici* and a new aggressive strain of *V. albo-atrum*

4.2. Materials and Methods

4.2.1 Tomato cultivars

Three different tomato cultivars were tested for levels of resistance against both *V. albo-atrum* and *P. lycopersici*. The cultivars used were:

- **Espero**, a “standard fruit size” cultivar that is widely used in UK, was considered to be resistant against *V. albo-atrum* (Agroseed 2001, Seed Catalogue-Vegetables, Athens). However, it was recently reported to become infected by a new, more aggressive strain of *V. albo-atrum* (O’Neil, 2002);
- **72-224** a standard fruit size cultivar that is widely used in Holland, is considered to be resistant to *V. albo-atrum* (Agroseed 2001, Seed Catalogue-Vegetables, Athens) and
- **Piccolo**, a “cherry-type” cultivar that is also considered to be resistant to *V. albo-atrum* (Agroseed 2001, Seed Catalogue-Vegetables, Athens).

4.2.2 Soil used, soil infection and soil steaming methods used

Soils naturally infected with *P. lycopersici* were collected in a commercial glasshouse at Cantelo Nursery Ltd. (Somerset, UK). Soil was collected from different parts of the glasshouse and then mixed and homogenized using a concrete mixer, to minimize differences in soil texture, structure and inoculum density of the soil used in experiments. Soil was then placed into 15 l pots either with or without additional *V. albo-atrum* inoculum having been added.

For steam disinfection the same method as described in chapter 2 was used (see section 2.2.4). For inoculation of soil with *V. albo-atrum* the procedure described in chapter 3 was used (see section 3.2.1).

4.2.3 Treatments applied to cultivars

The following treatments were tested for all three cultivars included in the study:

Cultivars were either (a) grown on their own rootsystem (without grafting) or (b) grafted onto **corky root-rot** resistant rootstocks “**Beaufort**” and then planted into soil that was:

- (i) naturally infected with *P. lycopersici*.
- (ii) naturally infected with *P. lycopersici* and artificially infected with *V. albo-atrum* or
- (iii) steam disinfected soil (that was naturally infected with *P. lycopersici* prior to steam disinfection)

Experimental Design

Five plants/pots were used for each cultivar/soil treatment and grafted cultivar/soil treatment combination and pots/plants were then distributed in the heated greenhouses, at Moorbank experimental station at the University of Newcastle in a completely randomised block design. Rows were used as blocks (= pots were placed in 5 separate rows) in a heated glasshouse and one pot for each each cultivar/soil treatment and grafted cultivar/soil treatment combination was placed randomly in each row.

4.2.4 Assessments

Assessments of fruit weight and size were made at a specific ripening stage (according to colour charts used in commercial glasshouse production = when fruit were uniformly red) (see chapters 2 and 3 for a detailed description of the assessment methods).

After harvesting was finished, in September 2002 root systems were removed from soil and adhering soil removed by gentle washing in a bucket of tap water and root fresh and dry weights determined.

4.3 Results

4.3.1 Effect of (and interactions between), different (a) soil pathogen inocula, (b) cultivars and (c) plant treatments (grafting or non-grafting onto resistant rootstocks) on the performance of organic tomato crops Standard size fruit cultivars

For the two “standard size fruit” cultivars (Espero and 72-224) a three-way ANOVA was carried out to analyze the effect of **(a) soil pathogen inoculum** (none=steam disinfected soil, *P. lycopersici* only and *P. lycopersici* plus *V. albo-atrum*), **(b) cultivar** (Espero and 72-224) and **(c) plant treatment** (grafting or non grafting onto corky root rot resistant rootstocks) and interactions between the three factors with respect to the cumulative yield, number and size of fruit and root fresh and dry weight (see Table 4.1).

The data for the “cherry type” cultivar (Piccolo) included in the experiment were analysed separately for the effect of **(a) soil pathogen inoculum** (none=steam disinfected soil, *P. lycopersici* only and *P. lycopersici* plus *V. albo-atrum*) and **(b) plant treatment** (grafting or non grafting onto corky root rot resistant rootstocks) and **interactions between the 2 factors**, since the “cherry type” varieties are known to have lower yields and fruit sizes and different root systems (see section 4.3.2 below).

When results for the standard fruit size cultivars **Espero and 72-224** were compared:

Soil pathogen inoculum had a significant effect on all characteristics assessed (except for fruit size). Yield, root fresh and dry weight and number of fruits decreased with ‘increasing’ diversity of fungal pathogen inocula present in soil (no inoculum (=steamed soil) < *P. lycopersici* < *P. lycopersici* plus *V. albo-atrum*) (see Tables 4.1 and 4.2).

Cultivar choice had a significant effect on all characteristics assessed except for yield. Cultivar 72-224 had higher root and dry fresh weight and a higher number of fruit, but produced a smaller fruit size than Espero (see Tables 4.1 and 4.2).

Plant treatment (=grafting) also had a significant effect on all characteristics assessed (except for fruit size) with grafted plants having higher fruit number, cumulative yield, root fresh and dry weight (see Tables 4.1 and 4.2).

However, there was also a range of significant 2-way interactions between factors. Interactions were identified between:

- (i) **cultivar and plant treatment (=grafting)** for the number of fruit (see Tables 4.1 and 4.3),
- (ii) **pathogen inoculum and cultivar** for root fresh weight (see Tables 4.1 and 4.4)
- (iii) **pathogen inoculum and plant treatment (=grafting)** for root fresh weight (see Tables 4.1 and 4.5)
- (iv) **cultivar and plant treatment (=grafting)** for root dry weight (see Tables 4.1 and 4.6) and
- (v) **pathogen inoculum and plant treatment (=grafting)** for root dry weight (see Tables 4.1 and 4.7).

However, there was no significant 3-way interaction between soil inoculum, cultivar and plant (=grafting) treatment for any of the characteristics assessed (Table 4.1).

Table 4.1. Effect of (and interactions between), different **(a)** soil pathogen inocula, **(b)** cultivars and **(c)** plant treatments (grafting or non-grafting onto resistant rootstocks) on the cumulative yield, number and size of fruit and root fresh and dry weight data for the two “standard fruit size” tomato cultivars (Espero and 72-224) (**P-values from three-way ANOVA test**)

	Main effects			Interactions			
				2 way			3 way
Characteristic assessed	P	C	G	P*C	C*G	P*G	P*C*G
Cumulative yield	0.000	0.843	0.000	0.661	0.175	0.733	0.516
number of fruit	0.000	0.001	0.05	0.264	0.018	0.88	0.981
Size of fruit	0.594	0.000	0.084	0.278	0.764	0.951	0.993
Root fresh weight	0.000	0.000	0.000	0.002	0.298	0.002	0.344
Root dry weight	0.000	0.000	0.000	0.186	0.022	0.041	0.646

P = soil pathogen inoculum; Cultivar=C; G = Plant treatment (grafting or non grafting)

Table 4.2. Effect of (and interactions between), different **(a)** soil pathogen inocula, **(b)** cultivars and **(c)** plant treatments (grafting or non-grafting onto resistant rootstocks) on the cumulative yield, number and size of fruit and root fresh and dry weight data for the two “standard size” tomato cultivars (Espero and 72-224) (means for individual treatment combinations). Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05).

SOIL INOCULUM	CULTI- VAR	PLANT TREATMENT	CUMU- LATIVE YIELD (kg/plant)	NUMBER OF FRUIT	MEAN SIZE OF FRUIT (cm)	ROOT FRESH WEIGHT (g/plant)	ROOT DRY WEIGHT (g/plant)
Steam	ESPERO	UNGRAFTED	2.1a	62.4ab	3.8a	57b	5.9abc
		GRAFTED	2.2a	52.4abc	4.0a	57b	5.2bcd
	72-224	UNGRAFTED	2.0ab	66a	3.6a	58b	7.0ab
		GRAFTED	2.2a	65a	3.8a	71a	8.7a
<i>P. lycopersici</i>	ESPERO	UNGRAFTED	1.5bcd	43bcde	3.9a	37de	3.8cde
		GRAFTED	2.0ab	50abcde	4.0a	44cd	4.5bcde
	72-224	UNGRAFTED	1.5bcd	43bcde	3.7a	43cd	4.5bcde
		GRAFTED	1.8ab	57abc	3.8a	55b	7.3ab
<i>P. lycopersici</i> + <i>V. albo-atrum</i>	ESPERO	UNGRAFTED	1.1cd	30e	4.1a	20f	1.9e
		GRAFTED	1.5bc	35d	4.1a	28ef	2.9de
	72-224	UNGRAFTED	1.0d	39cd	3.6a	34de	3.1cde
		GRAFTED	1.7bc	53abcd	3.7a	49bc	5.4bcd

Table 4.3 Effect (and interaction between) of cultivar and plant treatment (=grafting) on the number of fruit. Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05).

	Number of fruits		Difference	
Variety	Ungrafted	Grafted	(%)	Mean
Espero	45.20 b	45.87 b	1	45.53 B
72-224	49.80 b	59.00 a	18	54.40 A
Difference (%)	10	29		

There was a significant difference in the number of fruit per plant between grafted and ungrafted plants for 72-224, but not for Espero.

Table 4.4. Effect of (and interaction between) pathogen inoculum and cultivar on the root fresh weight (RFW). Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05).

	RFW (g)		Difference	
Soil inoculum	Espero	72-224	%	Mean
None (Steam sterilized soil)	57 b	65 a	13	61 a
<i>P. lycopersici</i>	41 d	49 c	20	45 b
<i>P. lycopersici</i> and <i>V. albo-atrum</i>	24 e	42 d	71	33 c
Mean	41 B	52 A		

The root fresh weight for 72-224 was higher than that of Espero for plants grown in soil with the two soil inocula and the uninfected control. However, the relative difference in RFW between the two cultivars increased with the number of pathogens present in soil and was 13% in steam disinfected soil, 20% in *P. lycopersici* and 71% in soil infected with both pathogens. The relative reduction in RFW (compared to plants grown in steam disinfected soil) was:

- similar in Espero and 72-224 (40 % and 32 % respectively) when only corky root rot inoculum was present in soil, but
- very different in Espero and 72-224 (135 % and 55% respectively) when both pathogens were present in soil

Table 4.5. Effect of cultivar and plant treatment (=grafting) on root fresh weight. Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05).

	RFW (g)		Difference	
Cultivar	Ungrafted	Grafted	(%)	Mean
Espero	38c	43b	11	40
72-224	45b	58a	22	52
Mean	42 B	51A		

The effect of grafting onto resistant rootstock on the root fresh weight (**RFW**) and the root dry weight (**RDW**) of **cv. Espero** was lower than the effect on the **cv. 72-224**. The difference in **RFW** between the grafted and the ungrafted plants was 11% for **cv. Espero** and 22% of the **cv. 72-224** (Table 4.5) and the difference of **RDW** was 8 and 45% respectively (Table 4.6).

Table 4.6. Effect of (and interaction between) cultivar and plant treatment (grafting) on the root dry weight (RDW). Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05).

	RDW (g/plant)		Difference	
Cultivar	Ungrafted	Grafted	(%)	Mean
Espero	3.9c	4.2b	8	4.0B
72-224	4.9b	7.1 a	45	6.0A
Difference (%)	26	69		

Table 4.7. Effect of pathogen inoculum and plant treatment (=grafting) on root dry weight (RDW). Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05).

Soil inoculum	RDW (g/plant)		Difference	Mean
	Ungrafted	Grafted	%	
None (Steam sterilized soil)	6.5a	7.0a	8	6.7A
<i>P. lycopersici</i>	4.2b	5.9a	40	5.0B
<i>P. lycopersici</i> and <i>V. albo-atrum</i>	2.5c	4.1b	64	3.3C
Mean	4.4b	5.7a		

While the RDW of grafted and ungrafted plants was not significantly different for plants grown in steam disinfected soil, the RDW of plants was significantly higher in grafted than ungrafted plants grown in soils infected with *P. lycopersici* alone and *P. lycopersici*+*V. albo-atrum*. The difference in root dry weight between grafted and ungrafted plants increased with increasing pathogen diversity in soil.

The relative reduction in RDW (compared to plants grown in steam disinfected soil) was lower for both grafted and ungrafted plants (19% and 55% respectively) when only corky root rot inoculum was present in soil, than for grafted and ungrafted plants (71% and 160% respectively) grown in soil in which both pathogens were present.

4.3.2 Effect of (and interactions between), different (a) soil pathogen inocula, and (b) plant treatments (grafting or non-grafting onto resistant rootstocks) on the performance of organic tomato crops Cherry cultivar

For the “cherry type” cultivar (Piccolo) a separate two-way ANOVA was carried out to analyze the effect of **(a) soil pathogen inoculum** (none=steam sterilized soil, *P. lycopersici* only and *P. lycopersici* plus *V. albo-atrum*) and **(b) plant treatment** (grafting or non grafting onto corky root rot resistant rootstocks) and interactions between the two factors with respect to the cumulative yield, the number and size of fruit and root fresh and dry weight.

Similar to the standard size cultivars (see section 4.3.1 above) **soil pathogen inoculum** had a significant effect on all characteristics assessed (except for fruit size).

Yields and number of fruit on plants grown in corky root rot infected soils were not significantly different to those of plants grown in steam sterilised soil. However, the yields were significantly reduced (compared to the plants grown in steam sterilised soil) when plants were grown in soils infected with both pathogens (Tables 4.9 and 4.10).

Root fresh and dry weight decreased with ‘increasing’ diversity of fungal pathogen inocula present in soil (no inoculum (=steamed soil) < *P. lycopersici* < *P. lycopersici* and *V. albo-atrum*) (see Tables 4.9 and 4.10).

Plant treatment (=grafting) had a significant effect on root fresh and dry weight, but not on any of the other characteristics assessed (fruit size, fruit number and yield). Grafted plants had higher root fresh and dry weight than ungrafted plants (see Tables 4.8 and 4.9).

There was no significant interaction between soil inoculum and plant treatment (=grafting) on any of the characteristics assessed (see Table 4.8).

Table 4.8. Effect of (and interactions between) different (a) soil pathogen inocula and, (b) plant treatments (grafting or non-grafting onto resistant rootstocks) on the cumulative yield, number and size of fruit and root fresh and dry weight data for the cherry type tomato cultivar (Piccolo) (**P-values from two-way ANOVA test**)

Characteristic assessed	Main Effects		Interaction
			2 way
	P	G	P*G
Cumulative yield	0.000	0.165	0.865
number of fruit	0.000	0.720	0.920
Size of fruit	0.197	0.631	0.635
Root fresh weight	0.000	0.032	0.676
Root dry weight	0.000	0.003	0.724

P = soil pathogen inoculum; Cultivar=C; G = Plant treatment (grafting or non grafting)

Table 4.9. Effect of (and interactions between) different (a) soil pathogen inocula and, (b) plant treatments (grafting or non-grafting onto resistant rootstocks) on the cumulative yield, number and size of fruit and root fresh and dry weight data for the cherry type tomato cultivar (Piccolo) (means). Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test (P<0.05).

SOIL INOCULUM	PLANT TREAT- MENT	CUMU- LATIVE YIELD (g)	NUMBER OF FRUIT	FRUIT SIZE (mm)	ROOT FRESH WEIGHT (g)	ROOT DRY WEIGHT (g)
Steam	UNGRAFTED	872a	153a	14a	95a	9.9ab
	GRAFTED	911a	152a	14a	100a	12.0a
<i>P. lycopersici</i>	UNGRAFTED	819ab	144a	14a	58bc	6.6cd
	GRAFTED	907a	154a	14a	72b	8.4bc
<i>P. lycopersici</i> + <i>V. albo-atrum</i>	UNGRAFTED	415c	82b	14a	38d	3.7e
	GRAFTED	531bc	87b	14a	47cd	4.8de

Table 4.10. Effect of different soil pathogen inocula on cumulative yield, number of fruits, root fresh and dry weight of the cv. Piccolo. Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P<0.05$).

Soil inoculum	CUMULATIVE YIELD (g)	NUMBER OF FRUIT	FRUIT SIZE (cm)	RFW (g)	RDW (g)
None (Steam sterilized soil)	891a	152a	1.4a	98a	11.0a
<i>P. lycopersici</i>	863a	149a	1.4a	65b	7.5b
<i>P. lycopersici</i> plus <i>V. albo-atrum</i>	473b	84b	1.4a	43c	4.2c

4.3 Discussion

Grafting significantly increased yields of the two standard size cultivars (cv. Espero and cv. 72-224) to levels similar to plants grown in steam sterilized soils when corky root rot was the only pathogen present in soil. However, grafting only had a significant effect on the root fresh weight for one (cv. 72-224) of the two cultivars. This suggests that (i) the expression of resistance to root diseases and/or (ii) root growth and volume, was affected by the variety grafted onto the resistant rootstock. The finding that, even in the absence of soil pathogen inoculum (plants grown in steam disinfected soil), grafted plants of cv. 72-224 had a higher root fresh weight than ungrafted plants of cv. 72-224 suggests that root growth and volume was affected rather than the expression of resistance in the rootstock.

This confirmed earlier reports that grafting onto resistant rootstock can reduce disease and/or the severity of soil borne diseases (Palminha, 1987; Morra *et al.*, 1997; Mazollier, 1999).

While there was no difference in total yield between grafted plants grown in steam disinfected and corky root rot infected soils, the number of fruits was higher for cv. 72-224 (which also showed a higher RFW when grafted) than for cv. Espero (where grafting did not affect RFW). This may have been due to differences in root to shoot signalling (e.g. differences in growth regulator concentrations being produced in roots and transported via the phloem into the shoot), but this will have to be investigated in future studies.

When *V. albo-atrum* inoculum was present in soil in addition to corky root rot inoculum, yields of grafted plants were significantly reduced (compared to control plants grown in steam disinfected soil), but remained significantly higher than those of non-grafted plants.

This is, to our knowledge, the first study which quantified the effect of mixed (corky root rot and *V. albo-atrum*) pathogen inocula on the performance of grafted tomato plants in organically managed soils. It clearly indicated that resistant rootstocks provide only partial protection against disease development in the presence of such mixed pathogen inocula.

These results confirm the aggressiveness of the new *V. albo-atrum* strain found in 2001 in organic tomato nurseries in the UK, and demonstrates that it is able to overcome (at least partially) the *Verticillium* resistance present in cultivars such as **Espero**.

As with plants grown in soils infected with corky root rot only, grafting only had a significant effect on the root fresh weight for one (cv. 72-224) of the two cultivars, confirming that there is an interaction between the rootstock and graft with respect to either **(i)** the expression of resistance to root diseases or **(ii)** the volume of root development is different for the two cultivars.

It should be pointed out that, although not statistically significant, even in steam-disinfected soil, grafted plants of the two standard fruit size cultivars showed a higher yield than ungrafted plants. The percentage of yield increase achieved in pathogen infected soil (but also in steam disinfected soil) would have more than compensated for the higher cost of using grafted plants.

Therefore, for **cv. Espero** and **cv. 72-224**, grafting can be recommended as an economically viable alternative to soil steam sterilization especially if only corky root rot is present as a pathogen.

On the other hand, there was no difference in yield between grafted and ungrafted plants in both steam-disinfected soils and soils infected with the two different pathogen inocula for the cherry cultivar **Piccolo**.

Despite the fact that the roots of the **cv. Piccolo** were infected by the pathogen (root fresh weights were significantly reduced in soil infected with corky root rot) there was no impact on yield. It can therefore be concluded that the **cv. Piccolo** has a high level of tolerance against corky root rot and use of grafting onto resistant rootstock and steaming the soil to protect plants from *P. lycopersici* are not economically viable.

When **cv. Piccolo** was grown in soils infected by both pathogens the overall crop performance was inferior, compared to plants grown in steam disinfected soils, indicating that the **cv. Piccolo** has low levels of tolerance against the simultaneous presence of the two soil pathogens.

Grafting also had no effect on yield, root fresh and dry weights of plants grown in corky root rot and *Verticillium* infected soils.

However, the use of resistant rootstocks may be necessary to maintain yield levels when tomato are grown continuously and/or very frequently in the rotation, since soil inoculum levels of *P. lycopersici* may increase over time and may eventually overcome the tolerance. This should be investigated in future experiments.

Chapter 5. Effect of *Brassica* soil amendments, composted cow manure, chitosan and combination of these treatments on the performance of tomato crops grown in soil infected by *Pyrenochaeta lycopersici* and *Verticillium albo-atrum*

5.1 Introduction

Crop protection strategies, recommended for use in organic farming, focus on creating balanced and buffered micro-ecosystem (especially with respect to nutrient supply and environmental conditions) (Lampkin, 2000), in which plant health is facilitated through a **combination of methods**:

- (i) **measures to increase biodiversity** (in the soil or above ground) and thereby competition for pest and diseases, (Wall *et al.*, 2004). This may involve (a) measures (e.g. rotations, variety mixtures, intercropping), which increase crop diversity and thereby minimise the build-up of crop specific pathogens and pests (Termorshuizen, 2001) and/or (b) strategies which increase soil suppressiveness and/or general soil biological activity and biodiversity (e.g. the addition of specific antagonists and or organic matter inputs to soils) and thereby increase competition for soil-borne pathogens and pests (Bunning and Montanez, 2003), and (c) measures which increase above ground competitions for pests and diseases (e.g. the release of antagonists, predators and parasites) or the planting of non-crop vegetation (intercrops, companion plants and beetle banks) which increases the naturally occurring populations of beneficials (Alabouvette *et al.*, 2004)
- (ii) **measures to optimise crop resistance to pests and diseases**. This may involve the (a) balanced organic matter-based fertility management practices, which avoid “physiological susceptibilities” to pests and diseases resulting from imbalanced supply of essential mineral nutrients to crops (e.g. regimes which avoid both excessive and insufficient supply of N) (Parker *et al.*, 1985) and (b) application of inputs (e.g. plant extract, biological control agents and other elicitors) which induce the expression of plant resistance mechanisms (Lampkin, 2000).

In the experiment in 2002 trials (a) fresh *Brassica* tissue, (b) cow manure compost, and (c) chitosan were identified as providing control against corky root rot and/or to increase crop yields (see chapter 3). In the experiment carried out in 2002 these three soil amendments were tested at two different input levels for their activity against both *Pyrenochaeta lycopersicii* (the only target pathogen used in the 2001 trials) and *Verticilium albo-atrum*.

However, the effects of using combinations of the most successful treatments were not studied in these trials.

Based on the current knowledge/theories about the modes of action of the three types of soil amendments identified in previous trials (see chapters 2 and 3), the use of combinations of chitosan and organic matter inputs was hypothesized to potentially provide additive or synergistic effects with respect to the control of root diseases.

***Brassica* spp.** have been recognized as valuable break crops in cereal rotations, and were shown to increase the yield of subsequent wheat crops by reducing inoculum levels of soil-borne wheat pathogens (Kirkegaard *et al.*, 1996). This break crop effect of *Brassica* spp., does not appear to be due to improved soil fertility, water holding capacity and/or structure (Cresswell and Kirkegaard, 1995). However, it has, at least partially, been linked to the release of break-down products (e.g. glucosinolates) during decomposition of *Brassica* residues in soil. Glucosinolates are the characteristic sulphur-containing constituents found in the tissues of members of the family of *Brassicaceae* (Xiao *et al.* 1998).

Composted animal manures have long been known to improve soil conditions and fertility. They were shown to improve soil functions such as water holding capacity, nutrient content, retention and release characteristics, drainage, aeration and protection of soils from erosion. There is also evidence that composted manures when applied to soil can suppress soil-borne diseases such as *Fusarium* wilts of cucumbers, *Phytophthora* crown rot of peppers and damping-off of vegetable crops caused by *Pythium ultimum* and *Rhizoctonia solani* (Hoitkink and Grebus, 1994; Lumsden *et al.*, 1983; Hoitkin and Boehm, 1999).

In greenhouse experiments, corky root rot of tomatoes was reported to be less severe in soil from organic farms using animal composted manure, than in soils from conventional farms. When soil was sterilized and then re-infected with corky root rot, the increase of disease severity was greater for soils from organic farms, indicating that there was a strong correlation of soil microbial activity and disease severity (Workneh and van Bruggen, 1994). This indicated that the suppressive activity in organically managed soils was due to biological factors in the soil.

Chitosan, (a deacetylated chitin product) is a polycationic β -1,4-linked-D-glucosamine polymer, that is known to induce the expression of plant defense mechanisms. The application of chitin and chitosan was also shown to enable plant tissues to respond more rapidly to pathogen attack by stimulating chitinase and glucanase production (Benhamou, 1996).

In addition, chitosan and its derivatives, such as glycolchitosan and carboxymethylchitosan, are known to form a semi-permeable film around tissues (El Gauth *et al.*, 1994). Recent studies have shown that chitosan induced resistance to *Fusarium oxysporum* in susceptible tomato plants, when applied as a root dressing, foliar spray and seed dressing, by restricting pathogen growth to the outer root tissues and eliciting a number of defense reactions, including structural barriers (Benhamou *et al.*, 1998). Due to its “filmogenic” properties, chitosan may also act as a barrier to the outward flux of nutrients to a level that will not sustain growth of the pathogens (Ait Barka *et al.*, 2003; El Ghaouth *et al.*, 2000).

Chitosan's properties such as biodegradability, antimicrobial potential and elicitor activity, meet the criteria of a promising control agent and has great potential as an

alternative to synthetic fungicides for the control of plant pathogens (El Ghaouth *et al.*, 1994; Ait Barka *et al.*, 2003).

The **main objectives** of the trial reported here (carried out in 2003) were, therefore:

- (i) to identify dose response effects of the different soil treatments; in addition to the lower input level used in the 2002 trials, the effect of further reduced input levels ($= \frac{1}{2}$ and $\frac{1}{4}$ of the lower level used in the 2002 trials) on disease development by *P. lycopersici* and *V. albo-atrum* and crop yield was assessed in 2003 trials
- (ii) to identify potential (antagonistic, additive or synergistic) interactions between different soil amendment types (chitosan, fresh *Brassica* tissue and compost)
- (iii) to identify potential mechanisms of action for disease suppression and yield increases associated with soil amendment with fresh *Brassica* tissues and chitosan

5.2 Materials and Methods

Soil infected with *P. lycopersici* (corky root rot) was collected from Cantelo Nursery Ltd. (Somerset, UK). The soil was collected from different parts of the glasshouse and was mixed and homogenized using a concrete mixer to minimize possible differences in soil texture, structure and inoculum density. Soil was then mixed with different amendments and infected with *V. albo-atrum* and then placed in 15 litre pots.

5.2.1 Soil infection with *Verticillium albo-atrum*

The soil collected from Cantelo Nursery used in experiments was infected by *P. lycopersici* and it was further infected with *V. albo-atrum* using the soil infection method described in chapter 3 (session 3.2.1).

5.2.2 Treatments

Five pots with soil inoculated with *P. lycopersici* and *V. albo atrum* were used per treatment and the following individual (Table 5.2.1) and combination (Table 5.2.2) treatments were applied:

Table 5.1 Individual soil treatments included in trials				
Soil Treatment used	Unit	Amounts used		
Fresh <i>Brassica</i> tissue ¹	% (v/v)	25	12.5	6.25
Composted cattle manure	% (v/v)	25	12.5	6.25
Chitosan ²	g/pot/week	2	1	0.5

¹ chopped up fresh Brussels sprouts were mixed with soil using a concrete mixer

² a soluble chitosan preparation dissolved in 1000ml of water (Chitosan, ChiPro GmbH, Bremen, Germany) was applied with the irrigation water every week

Table 5.2 Combination soil treatments included in trials			
Combination treatment used	Amounts used		
	<i>Brassica</i> tissue % (v/v) ¹	Composted cattle manure % (v/v)	Chitosan mg/pot/week ²
1. Chitosan+ <i>Brassica</i>	12.5		1
2. Chitosan+Compost		12.5	1
3.Chitosan+ <i>Brassica</i> +Compost	6.25	6.25	0.5

¹ chopped up fresh Brussels sprouts were mixed with soil using a concrete mixer

² a soluble chitosan preparation dissolved in 1000ml of water (Chitosan, ChiPro GmbH, Bremen, Germany) was applied with the irrigation water every week

The following control treatments were included in the trials

- **Plants grown in steam-disinfected soil (positive control 1):** a commercial soil-steaming machine for horticultural substrates was used (Camplex HD5116

Electric Soil Steriliser, THERMOFORCE LIMITED, Cumbria, U.K) and soil was heat treated at 81° C for 30 minutes.

- **Plants grafted onto resistant rootstocks (positive control 2):** Grafted plants on the resistant rootstock KVFN (resistant to *V. albo-atrum* and tolerant to *P. lycopersici*) were placed into pots containing untreated (non-steamed) soil.
- **Non-grafted plants planted into untreated soil (negative control):** Plants were planted into soils infected with *P. lycopersici* and *V. albo-atrum*.

5.2.3 Soil Assessments

5.2.3.1 Overall soil biological activity (Dehydrogenase)

One of the most commonly used methods to estimate overall soil biological activity is to measure dehydrogenase activity (Ross, 1971; Trevors *et al.*, 1982; von Mercı and Schinner, 1990). Dehydrogenase is an enzyme fundamental to respiratory metabolism of soil organisms and widely accepted as a good measure of soil biological activity (von Mercı and Schinner, 1990).

For the determination of the dehydrogenase activity a method based on soil treatment with iodonitrotetrazolium chloride described by von Mercı and Schinner (von Mercı and Schinner, 1990) was used. This involved:

Chemicals and Reagents:

- Diluted hydrochloric acid (3M)
100ml of concentrated HCL (37%) was mixed with 300 ml of distilled water.
- Tris buffer (1M, pH 7)
30.28gr of tris(hydroxymethyl) aminomethane was weighed in a 250-ml volumetric flask, it was dissolved in 200ml of distilled water, and adjusted to pH 7 with HCL and was made up to volume with distilled water
- Substrate solution
500mg of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazoliumchloride (INT, Serva 26840) was mixed with 2ml of N,N-dimethylformamide, and was shaken

vigorously. It was then made up to volume with distilled water in a 100-ml volumetric flask and was dissolved in an ultrasonic bath

- Extraction solution
100 ml of N,N-dimethylformamid was mixed with 100ml of ethanol (96% v/v)
- Standard stock solution
10mg of iodonitrotetrazolium formazan (INTF; Sigma I-7375) was weighed in a 100-ml volumetric flask, and was dissolved in 80ml of the extraction solution, and was made up to volume with extraction solution
- Calibration curve
0 (reagent blank), 1, 2 and 5ml of INTF standard stock solution was pipetted into four test tubes and was made up to 13.5 ml with extract solution. Calibration standards correspond to 0, 100, 200 and 500 µg of INTF

Procedure

- 1 g of field-moist soil was put into three test tubes and 1.5 ml of Tris buffer and 2 ml of substrate solution was added.
- Autoclaved soil (autoclaved for 20 min at 121⁰ C and 1.1bar) was used as a “control” treatment.
- The tubes were stoppered, shaken briefly, and incubated for 2h at 40° C in the dark.
- After incubation 10 ml of the extraction solution was added to each of the tubes.
- The tubes were kept for 1 hour in the dark and were shaken vigorously every 20 minutes.
- Immediately after filtering the flask contents, the iodonitrotetrazolium formazan (INFT) content in samples, control and calibration standards were measured photometrically at 464 nm against the reagent blank.
- Dehydrogenase activity is expressed as µg of iodonitrotetrazolium formazan (INTF) per gramme dry matter and incubation time.

$$[(S-C) \times 100] / \%dm = \mu g \text{ INTF} \times g^{-1} dm \times 2h^{-1}$$

S mean value of samples (µg INTF)

C control (µg INTF)

100 x %⁻¹dm factor for soil dry matter.

5.2.3.2 Determination of colony forming units (cfu) of chitosan and cellulose degrading bacteria in soil

For the determination of the population densities of culturable chitosan and cellulose degrading microorganisms in soils treated with different soil amendments, the method developed by Wirth and Wolf (1992) was used. This involved:

1. Preparation of chromogenic substrate containing agar media

Agar-containing basal media (500ml) and soluble chromogenic substrates (500 ml in distilled water) were sterilized separately by autoclaving (120°C, 15 min). Before pouring (45°C) both solutions were mixed. The chromogenic substrates gives the agar medium a red (for chitin) or blue (for cellulose) colour; if the substrate (chitosan or cellulose) is attacked by degrading enzymes, the red or the blue colour becomes lighter or disappears.

2. Preparation of isolation media

A range of media were used to estimate the levels of culturable chitosan and cellulose degrading micro-organisms

1. Xa 1- basal-medium: peptone from soybean (Merck), 0.1 g; KH₂PO₄, 0.5 g; and 20 g agar (bacto-agar, Difco Laboratories, Detroit, Mich, USA) were dissolved in 500 ml distilled water. The pH was adjusted to 7.2 with NaOH before sterilization.

2. Soil Extract Agar: a sandy loam soil collected from the A horizon (top 30 cm) of fertile arable land (Nafferton Farm, Northumberland, UK), was air-dried and sieved (2mm) prior to autoclaving 1 kg in 1.5 litre tap-water (120°C, 15 min).

The suspension was cooled and filtered to yield 1 litre of a clear yellowish extract. Soil Extract Agar was prepared by adding 20g Bacto-agar to 500ml of the fresh extract. The pH of substrate-SEA was adjusted to 7.5 with NaOH or HCL before sterilization.

3. Plating of soil suspensions onto isolation media

Ten fold dilution series of soil suspensions were prepared and samples of the different dilution steps plated onto the isolation media (see above). Colonies with clear halos in

the red and blue substrates of the agar medium were counted and used to estimate the levels of chitosan or cellulose degrading, culturable microorganisms in soil.

5.2.4 Experimental Design

Seventy pots, five per treatment, were arranged in a completely randomized block design (5 replicate blocks = rows of pots) in a heated glasshouse at Newcastle University's Moorbank experimental station.

5.2.5 Temperature and irrigation regime

Temperatures were kept between 20°C and 22°C as relatively low temperatures favour disease development by both pathogens, *P. lycopersici* and *V. albo-atrum*.

Plants were watered regularly (every 2-3 days) to field capacity to provide optimum conditions for disease development (both pathogens are favoured by high soil moisture levels).

5.2.6 Crop assessments

Yield assessments were carried out when the fruits were at full red stage, and fruit number and size were also assessed.

At the end of the experiment the roots of all plants were rinsed under tap water and root fresh weight and dry weights (roots were dried in an oven at 80° C for 24h) were assessed and used as a quantitative measure for the level of overall root disease.

5.3 Results

5.3.1 Effect of soil treatment type, input level and combination treatments on tomato crops (cumulative yield, fruit size, fruit number, root fresh weight and root dry weight)

As in previous trials (see chapters 2 to 4) the presence of pathogen inocula in soil significantly reduced the cumulative yield, fruit size, fruit number and root fresh and dry weight of plants (see Table 5.3). Only the use of (a) resistant rootstocks, (b) application of chitosan (at the highest input level) resulted in levels of disease control similar to those obtained by steam disinfection of soils (see Table 5.3 for data on the different crop characteristics assessed), thus confirming results obtained for grafted plants and chitin-chitosan treatments in experiments under chapters 2, 3 and 4.

For all other treatments except the combination of *Brassica* and chitosan, at least one of the characteristics assessed (cumulative yield, root fresh and dry weight, fruit size or fruit number) was lower than for plants grown in steam-sterilised soil (see Table 5.3).

However, for most treatments, cumulative yield, root fresh and dry weight, fruit size or fruit number were found to be higher than those obtained for non-grafted plants grown in untreated soil (see Table 5.3). The only exceptions were (a) *Brassica* tissue and chitosan at the lowest volumes for the number of fruits, (b) *Brassica* tissue at the two lower volumes, chitin at the lowest volume, the combination of chitin and compost and the combination of all three treatments at the lowest volumes for fruit size, (c) *Brassica* tissue at the lowest volume for RFW and (d) *Brassica* tissue, chitosan and the combination of all three treatments at the lowest volume for RDW volume, which did not result in an increase compared to non-grafted plants grown in untreated soil.

To identify interactions between the effects of different (a) soil input types (chitosan, fresh *Brassica* tissue and compost) and (c) input levels a series of 2-way ANOVAs was carried out on selected groups of data. These are described in separate sections (5.3.2 and 5.3.3) below.

To identify potential synergistic, additive and/or antagonistic effects of combining different soil treatment, a more detailed comparison of combinations of soil treatments with individual treatments is also presented in separate sections (5.3.4 and 5.3.5 and 5.3.6 below).

Table 5.3. Effect of different soil amendments, input levels and combinations of soil amendments on tomato crop characteristics (means and Tukey’s Honest Significant Difference Test results)

SOIL (or plant) TREATMENT	Input Level	Cumulative Yield (kg)/plant	Number of fruit/plant	Mean size of fruit (mm)	Root Fresh Weight (g)/plant	Root Dry Weight (g)/plant
Chitosan	1**	1.8ab	67 ab	39ab	72 abc	8.0bcde
	0.5	1.6bc	54 bcd	36bcd	70abc	8.2bcd
	0.25	1.2 e	38 ef	34de	51de	5.2gh
Brassica tissue	1*	1.5cd	53 bcd	37abcd	65abcd	7.2bcde
	0.5	1.2e	40 e	36cde	60cde	6.2efg
	0.25	1.2e	38 ef	35de	49 ef	4.6gh
Manure Compost	1*	1.4 cde	46 de	37abcd	61 bcde	6.4 cdefg
	0.5	1.2de	40 de	36abcd	58cde	6.2 cdefg
Chitosan + Compost	0.5 each	1.5cd	50 bcde	35cde	59cde	6.4 bcdefg
Chitosan + Brassica	0.5 each	1.8ab	74 a	38abc	71abc	7.6 bcde
Chitosan + Brassica + Compost	0.25 each	1.2 e	46 cde	35cde	52de	5.4 fgh
Untreated (negative control)	-	0.8f	28 f	32e	34f	3.6h
Resistant rootstock (positive control 1)	-	1.7bc	64 abc	39a	76ab	8.2 abc
Steam disinfection (positive control 2)	-	2.0a	79 a	38ab	78a	8.4ab

* Unit for soil amendments (% v/v soil; see section 5.2.2) ** Unit for chitosan (g/pot/week; see methods section 5.2.2 above)

5.3.2. Effect of (and interaction between) three soil amendment types (chitosan, fresh *Brassica* tissue and compost) and two input levels (1 and 0.5) on tomato crop performance

Table 5.4. Effect of (and interaction between) three soil amendment types (chitosan, fresh *Brassica* tissue and compost) and two input levels (1 and 0.5) on tomato crop performance (cumulative yield, number and size of fruit and root fresh and dry weight) (P-values from two-way ANOVA test)

Characteristic assessed	Main Effects		Interaction
			2 way
	IT	L	IT*L
Cumulative yield	0.000	0.000	0.374
Number of fruit	0.000	0.001	0.521
Size of fruit	0.025	0.000	0.112
Root fresh weight	0.005	0.208	0.807
Root dry weight	0.001	0.391	0.273

IT = soil amendment type (chitosan, fresh *Brassica* tissue, compost);
L = soil amendment input level (1 or 0.5)

For **cumulative yield** two-way ANOVA showed a very highly significant effect of soil treatment type (chitosan resulted in a significantly higher yield compared to *Brassica* and compost inputs) (P=0.000) and soil treatment input level (P=0.000) but no significant interaction between soil treatment input type and soil treatment input level (P=0.374) (see Table 5.4 and Figure 5.2).

For the **number of fruit** per plant, there was a very highly significant effect of soil treatment types (chitosan, *Brassica* tissue and compost) and soil input level. Chitosan resulted in a significantly higher number of fruits compared to *Brassica* tissues and composted cow manure soil amendments. As expected the higher level inputs resulted in significantly higher number of fruits, but there was no significant interaction between the volume added and the soil treated types (see Tables 5.3 and 5.4).

For **fruit size** there was a significant effect of soil treatment types (chitosan, *Brassica*, and compost) and a very highly significant effect on input level. Fruit grown in soil

with added chitosan had significantly bigger fruit size than plants grown in soils amended with fresh *Brassica* tissue. As expected, the higher input level resulted in higher fruit size, but there was no significant interaction between the soil treatment type and input level (see Tables 5.3 and 5.4).

For **root fresh weight** two-way ANOVA showed highly significant differences between the soil treatment types (chitosan, fresh *Brassica* tissue and compost) ($P=0.005$). Chitosan soil amendments resulted in a higher root fresh weight compared to soil amendment with fresh *Brassica* tissue or composted cow manure. However, there was no significant difference between input levels ($P=0.208$) and there was no significant interaction between soil treatment type and soil treatment input level ($P=0.807$) (see Table 5.4 and Figure 5.1).

For **root dry weight** two-way ANOVA showed that there was a very highly significant effect of soil treatment type, as plants grown on chitosan had significantly higher root dry weight, compared to soil with added *Brassica* tissue or composted cow manure (see Table 5.3). However, there was no significant effect of different input levels and there was no significant interaction between the soil treatment type and soil input level (see Table 5.4).



A. Control (mean weight 34g)



B. Steam (mean weight 78g)



C. Brassica 1 (mean weight 65g)



D. Compost 1 (mean weight 61g)



E. Chitosan 1 (mean weight 72g)



F. Grafted (mean weight 76g)



G. Brassica 2 (mean weight 60g)



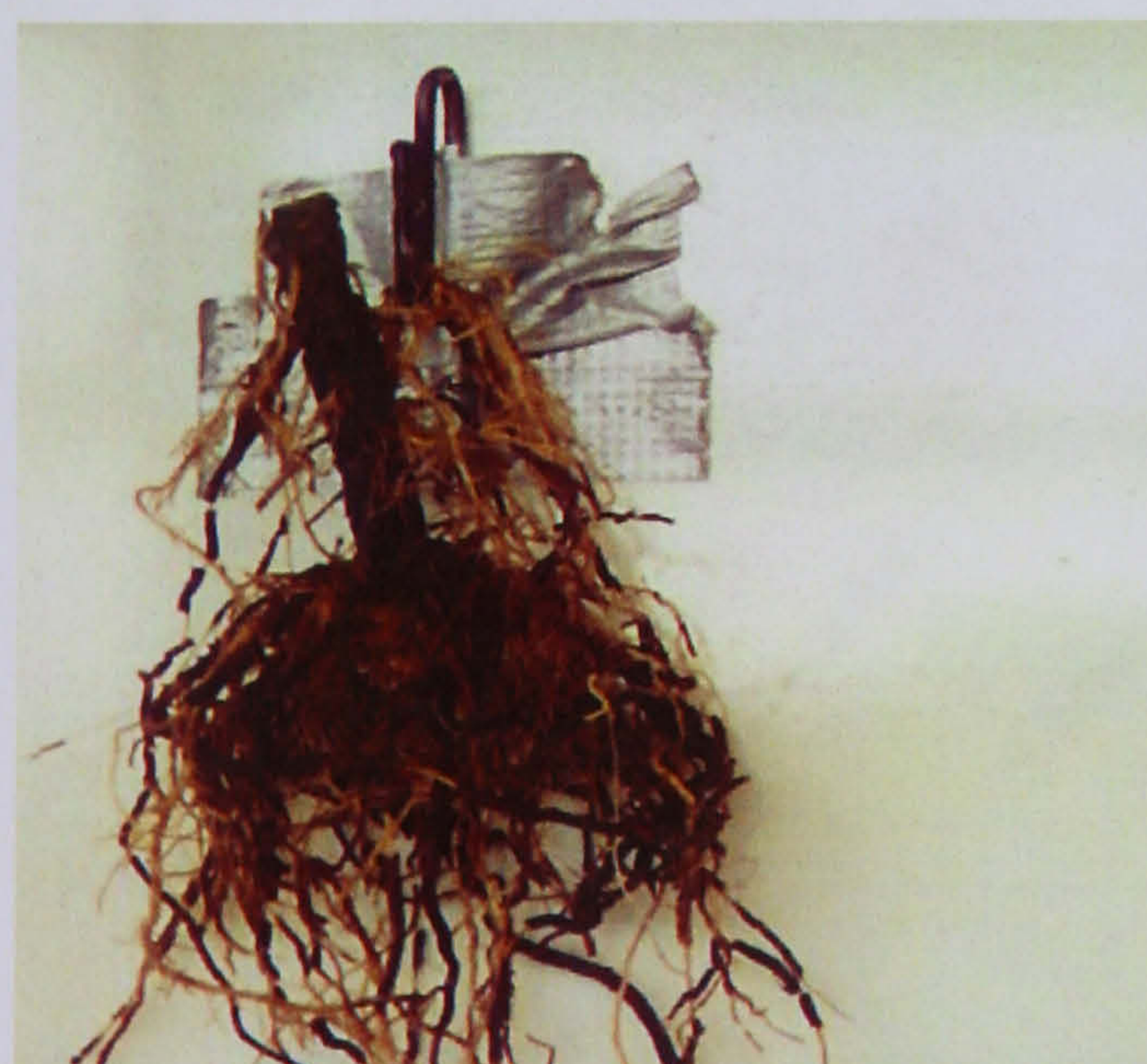
H. Brassica 3 (mean weight 49g)



J. Chitosan 2 (mean weight 70g)



K. Chitosan 3 (mean weight 51g)



L. C. man. Comp. 2(mean weight 58g)



M. Chit.+Brassica (mean weight 71g)

Figure 5.1 Tomato roots (with different treatments) infected by *P. Lycopersici* and *V. albo-atrum*.

(Photographs were taken from different distances and therefore not to the same scale)

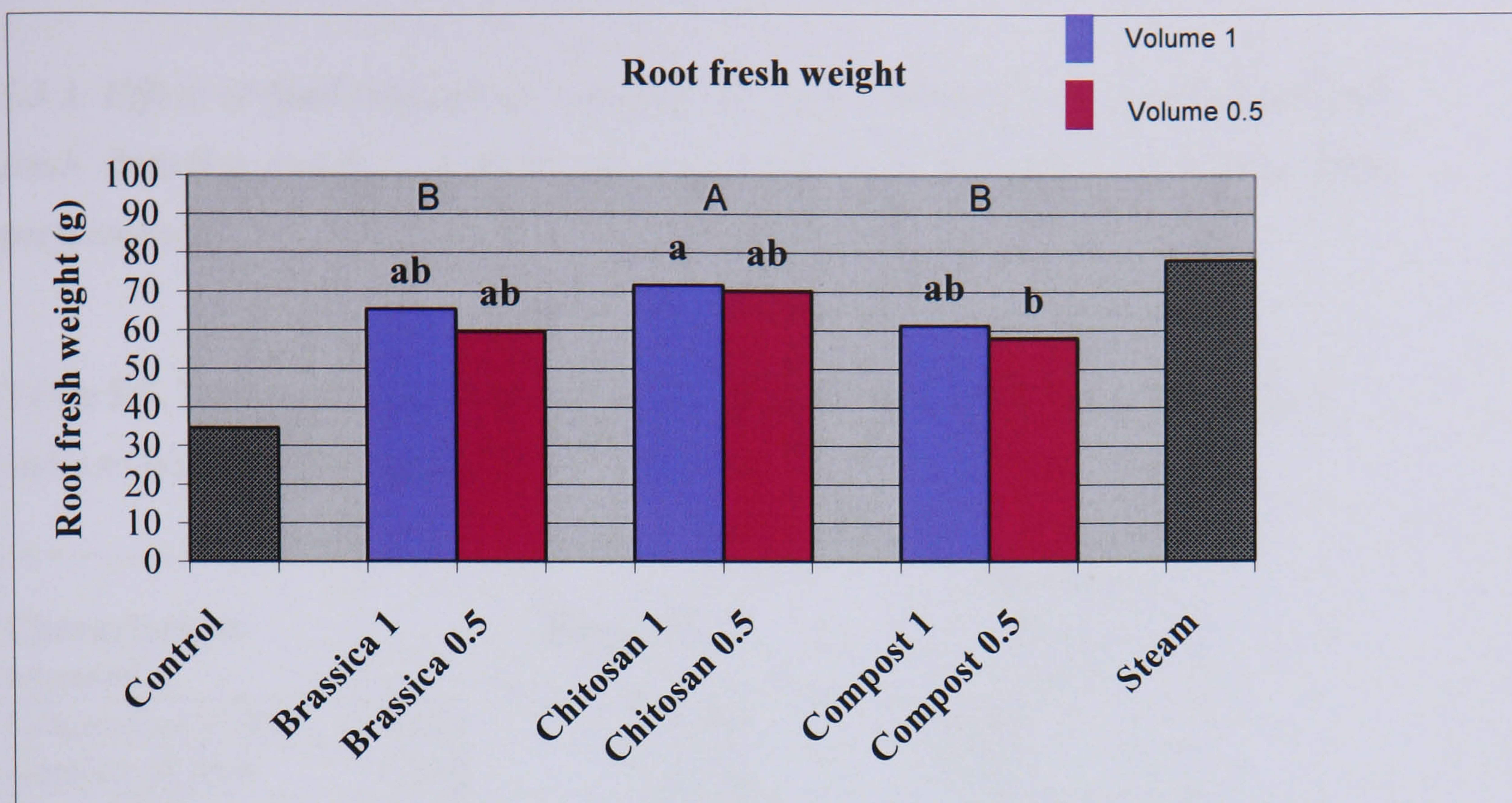


Figure 5.2. Effect of two different volumes of *Brassica*, chitosan, and compost on root fresh weight. Means with the same letters (capital: overall amendment comparison; small: individual treatment) are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$).

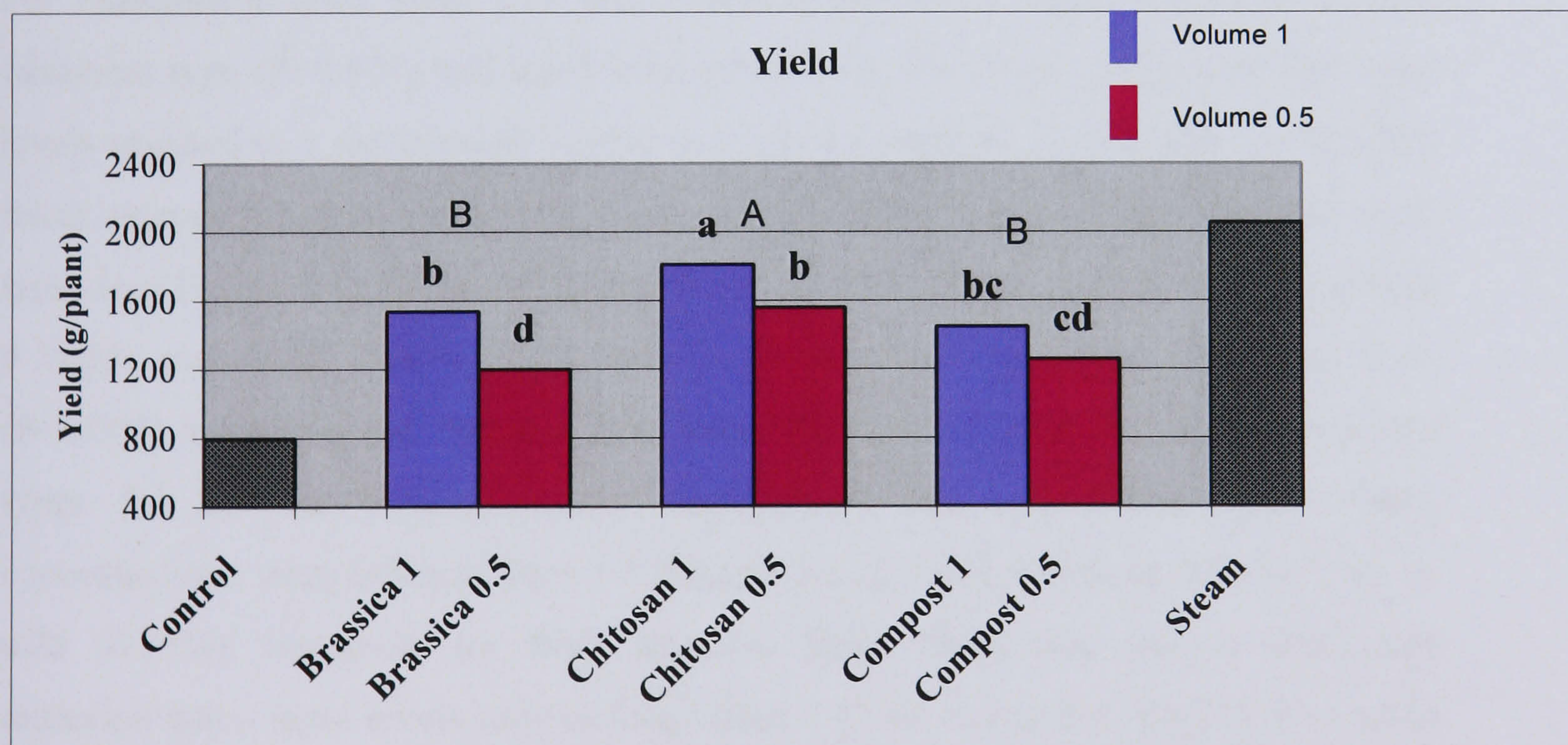


Figure 5.3. Effect of two different volumes of *Brassica*, chitosan, and compost on yield. Means with the same letters (capital: overall amendment comparison; small: individual treatment) are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$).

5.3.3. Effect of (and interaction between) two soil amendment types (chitosan and fresh Brassica tissue) and three input levels (1, 0.5 and 0.25) on tomato crop performance

Table 5.5. Two-way ANOVA results for cumulative yield, number and size of fruit and root fresh and dry weight data

Characteristic assessed	Main Effects		Interaction
			2 way
	IT	L	IT*L
Cumulative yield	0.001	0.031	0.004
Number of fruit	0.012	0.000	0.063
Size of fruit	0.235	0.000	0.085
Root fresh weight	0.016	0.001	0.375
Root dry weight	0.002	0.000	0.152

IT = soil amendment type (chitosan, fresh *Brassica* tissue, compost);
L = soil amendment input level (1 or 0.5)

For **cumulative fruit yield** two way ANOVA showed a significant effect of soil treatment type (P=0.001) and input level (P=0.031). The range of chitosan soil input levels resulted in a significantly higher root yield compared to the range of *Brassica* tissue amendment levels, and yield increased with increasing input level for both input types (see Figure 5.4). However, different to root fresh weight assessments, there was a highly significant interaction between soil treatment input type and input level (P=0.004) indicating that the dose response differs between the two soil amendment types. For chitosan yields decreased significantly and to a similar extent when concentrations were lowered from 1 (25%) to 0.5 (12.5%) and from 0.5 (12.5%) to 0.25 (6.25%). However, for fresh *Brassica* tissue there was only a significant reduction when input levels were reduced from 1 (25% v/v) to 0.5 (12.5 % v/v) while there was no difference in yield between level 0.5 (12.5% v/v) and 0.25 (6.25% v/v). (see Table 5.5 and Fig. 5.4).

Two-way ANOVA showed that, for the **number of fruits**, there was a significant effect of the soil treatment type, with the plants that were grown in soil with chitosan

amendments having significantly higher numbers of fruit than those plants grown in soils with *Brassica* tissues (see Tables 5.3 and 5.5). Soil input level also had a significant effect on the number of fruits, with the highest input level resulting in significantly higher number of fruits, compared to both reduced levels (see Tables 5.3 and 5.5). However, there was no interaction between soil input type and soil input level, indicating that this trend was similar for both soil treatment types (see Table 5.5).

For the **size of fruits** two-way ANOVA showed a significant effect of soil input level (see Table 5.5). Plants grown in soils with the highest input level had significantly bigger fruit compared to those grown in both lower input levels (see Table 5.3).

However, there was no significant effect of soil input type and there was no significant interaction between soil treatment type and soil input level (see Table 5.5).

For **root fresh weight** two-way ANOVA showed a significant effect of soil treatment type ($P=0.016$) and input level ($P=0.001$) (see Table 5.5). The range of chitosan soil input levels resulted in a significantly higher root fresh weight compared to the range of *Brassica* tissue amendment levels and root fresh weight increased with increasing input level for both input types. However, there was no significant interaction between soil treatment input type and input level ($P=0.375$) indicating that there was a similar dose response for both soil amendments (see Table 5.5 and Fig. 5.3).

Similarly to RFW, for **root dry weight** two-way ANOVA showed that there was a significant effect of soil treatment type and soil input level, as plants grown in soil with chitosan amendments had significantly higher root dry weight compared to plants grown in soil with fresh *Brassica* tissue amendments (see Tables 5.3 and 5.4).

The root dry weight of plants grown in soils with the two higher input levels of chitosan, but with only the highest *Brassica* tissue input level, were significantly higher than the root dry weight of plants grown in soils with the lowest soil treatment input level.



Figure 5.4. Effect of three different volumes of *Brassica* and chitosan on root fresh weight. Means with the same letters (capital: overall amendment comparison; small: individual treatment) are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$)

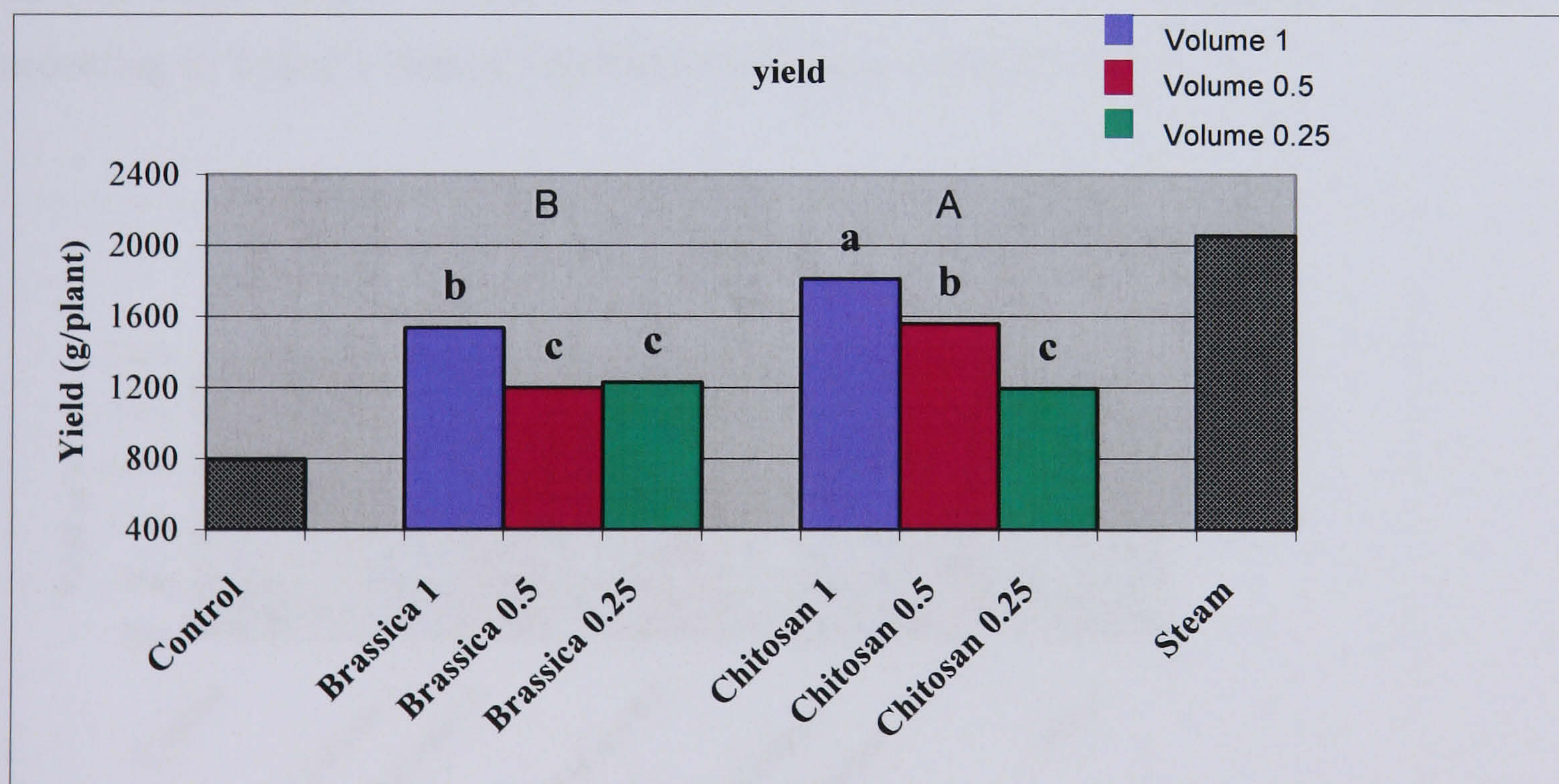


Figure 5.5. Effect of three different volumes of *Brassica* and chitosan on yield. Means with the same letters (capital: overall amendment comparison; small: individual treatment) are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$)

5.3.4 Effect of combined chitosan and compost amendment on crop performance

Using a combination of chitosan and compost (both applied at the 0.5 level) did not result in a significant difference in root fresh weight or yield, compared to plants grown in substrate treated with individual chitosan or compost treatments at levels 1 or 0.5 (Table 5.3 and Figures 5.5 and 5.6). The same was true for fruit size, fruit number and root dry weight assessments (see Table 5.3).

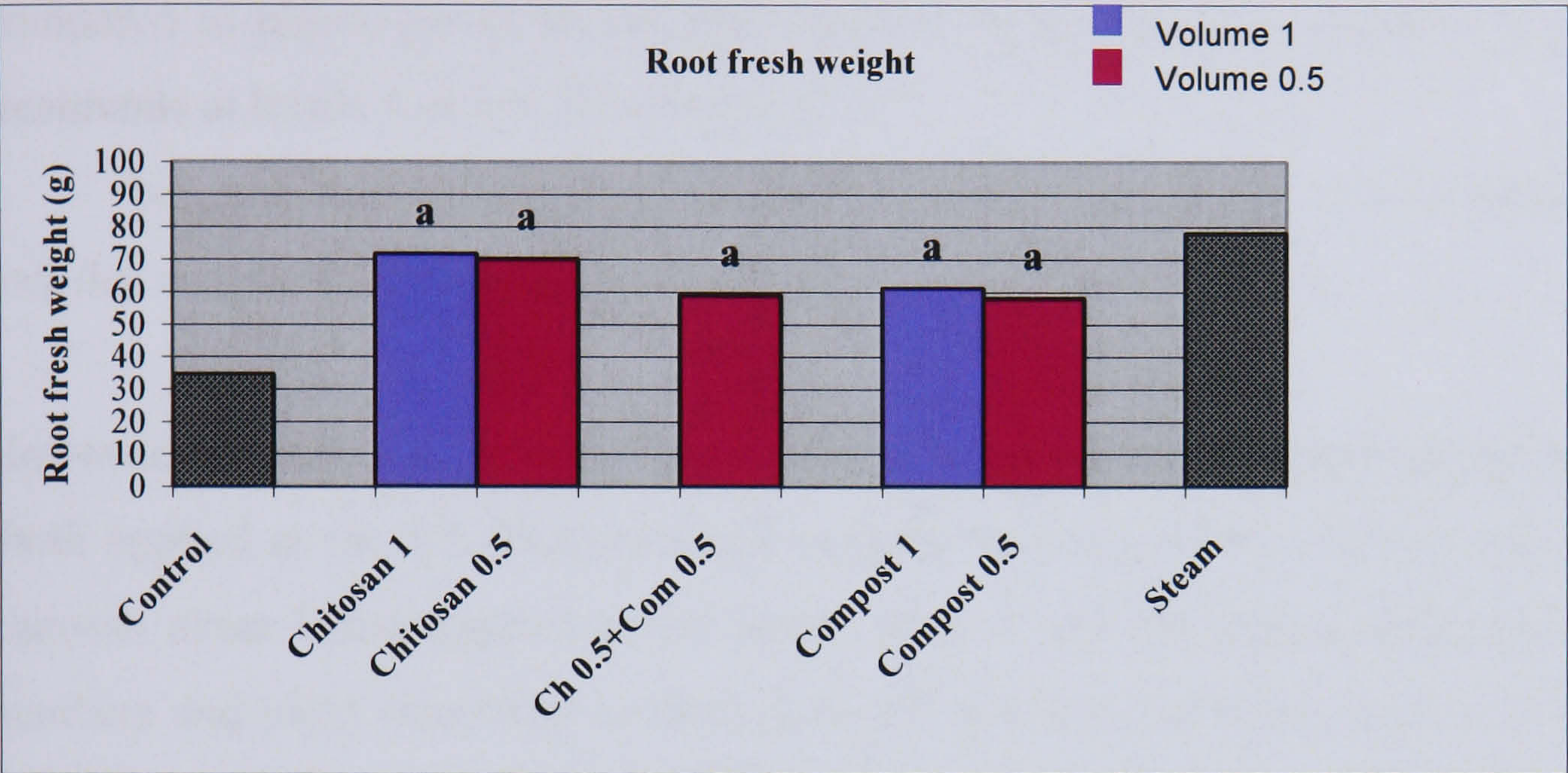


Figure 5.6. Effect of combining cattle manure compost and chitosan soil amendments on root fresh weight. Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test ($P<0.05$)

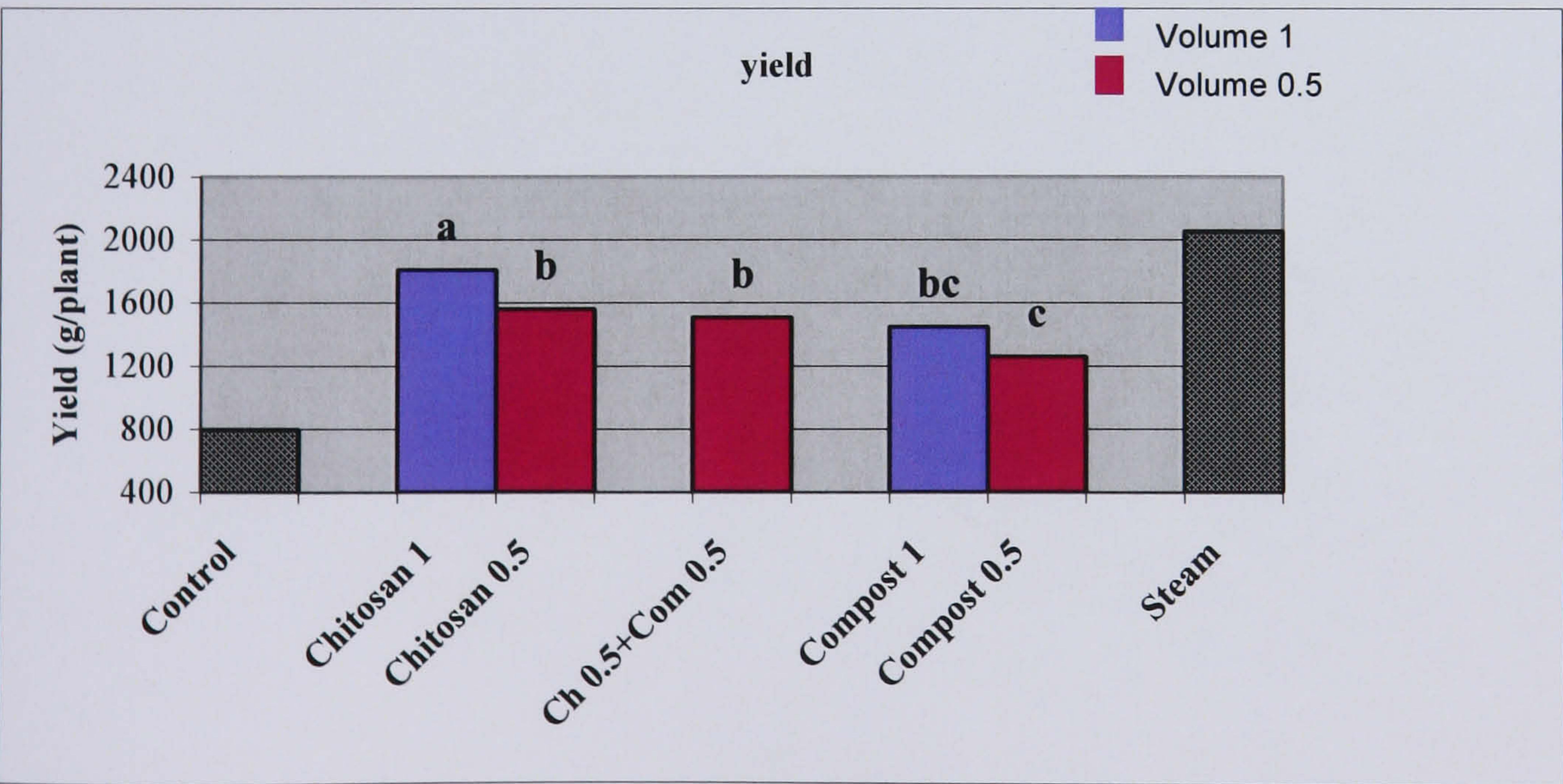


Figure 5.7. Effect of combining cattle manure compost and chitosan soil amendments on yield. Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test ($P<0.05$)

5.3.5 Effect of combining chitosan and fresh Brassica treatments on crop performance

Using a combination of **chitosan and fresh *Brassica* tissue** as a soil treatment (both applied at the 0.5 level) did not result in a significant difference in root fresh weight compared to plants grown in substrates treated with individual chitosan or compost treatments at levels 1 or 0.5 (see Figure 5.7).

No additive or synergistic effects of this combination could be detected for root dry weight, fruit size (see Table 5.3).

However, amending soils with a combination of **chitosan and fresh *Brassica* tissue** (both applied at the 0.5 level) resulted in **(a)** a similar number of fruits and yield to chitosan alone being applied at the higher level 1 and **(b)** significantly higher fruit numbers and yield compared to fresh *Brassica* tissue alone being applied at levels 1 and 0.5 and chitosan being applied alone at level 0.5. This clearly indicates an additive effect of combined treatment of chitosan and fresh *Brassica* tissue (see Table 5.3 and Figure 5.8).



Figure 5.8. Effect of combining *Brassica* tissue and chitosan soil amendments on root fresh weight of tomato plants grown in soil infected by *P. lycopersici* and *V. albo-atrum*. Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test ($P<0.05$).

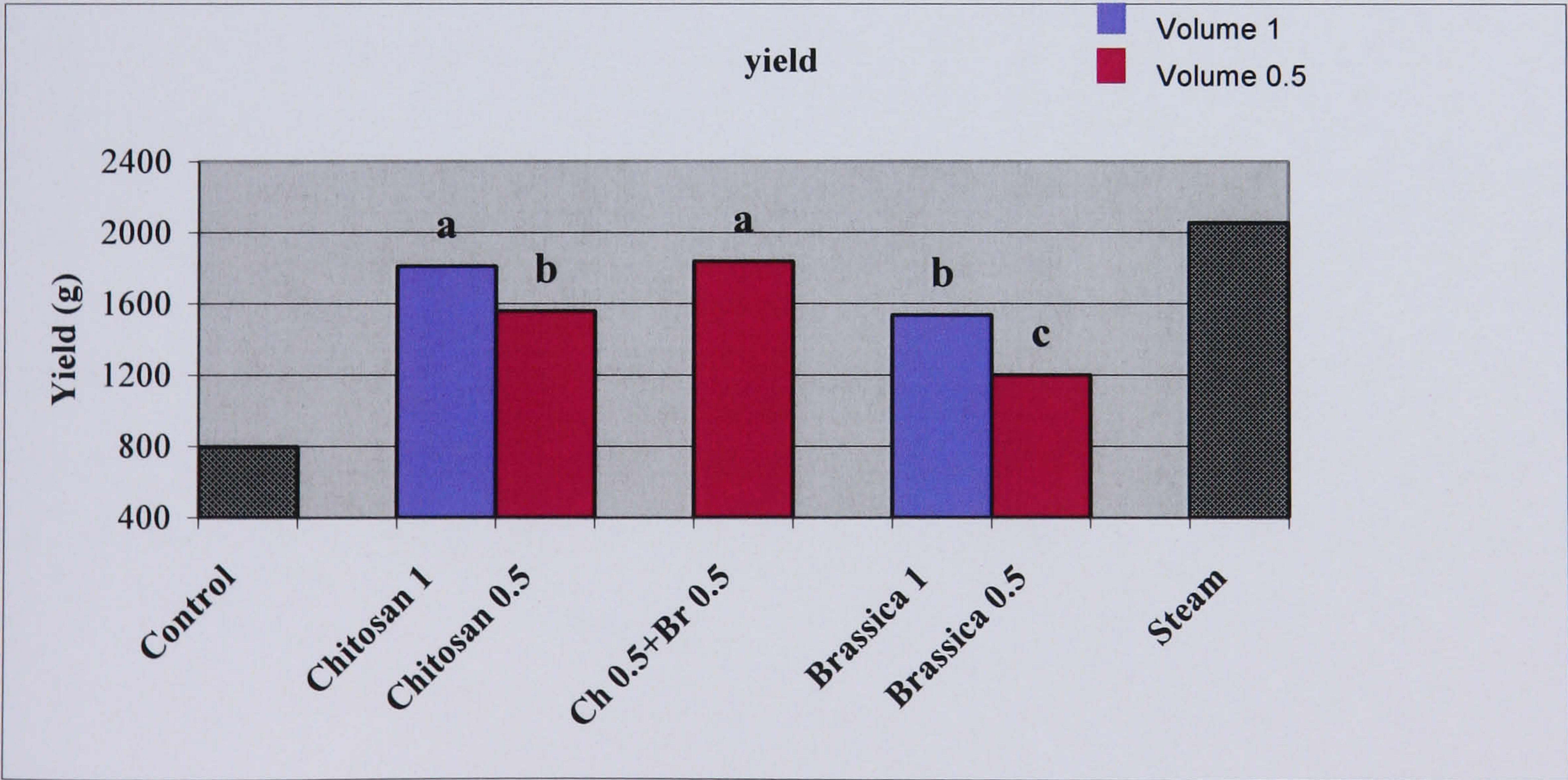


Figure 5.9. Effect of combining *Brassica* tissue and chitosan soil amendments on yield of tomato plants grown in soil infected by *P. lycopersici* and *V. albo-atrum*. Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test ($P<0.05$)

5.3.6. Effect of combining chitosan, compost and fresh Brassica treatment on crop performance

The combination of chitosan, *Brassica* and compost (added at the level of 0.25), did not significantly increase the root fresh weight or crop yield, when compared to the individual treatments applied at the same level (0.25) (see Figure 5.9), indicating that there was no additive effect between the three treatments.

No additive or synergistic effects of this combination could be detected for root dry weight, fruit size (see Table 5.3).

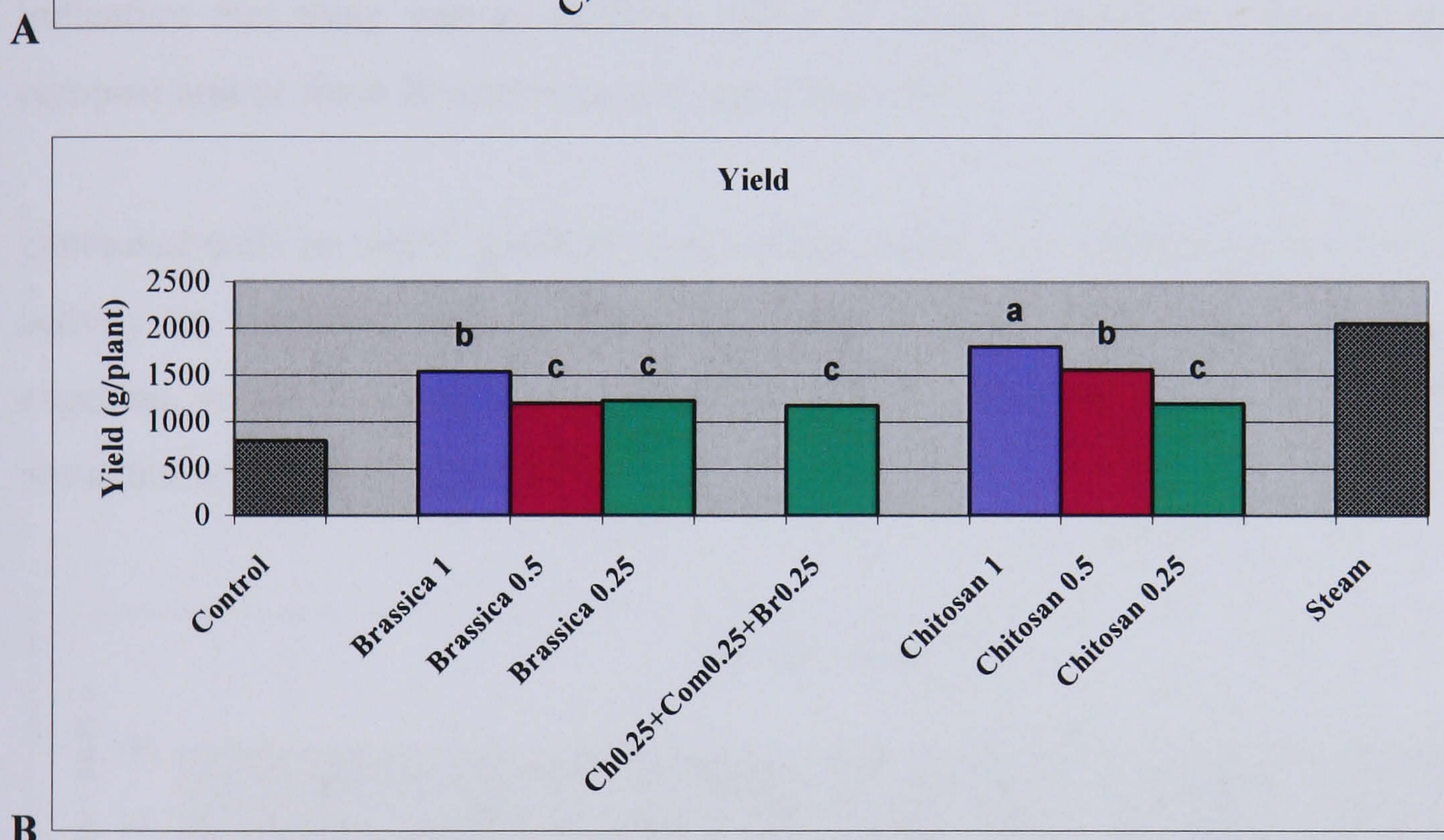
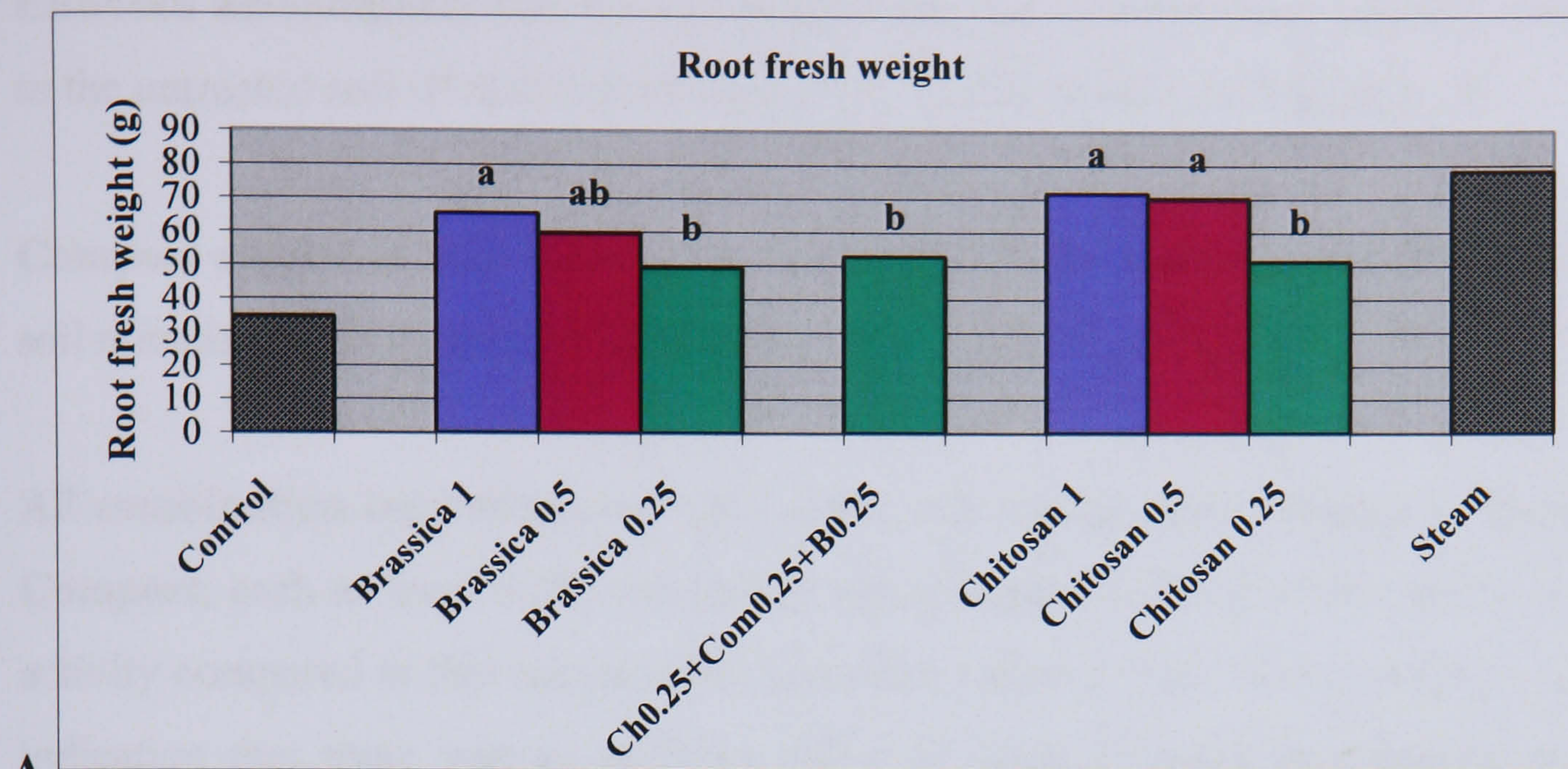


Figure 5.10. Effect of combining cattle manure compost, *Brassica* tissue and chitosan soil amendments on root fresh weight (A), and yield (B). Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$)

5.3.7 Effect of soil treatments on microbial activity

Fresh *Brassica* tissue amendments increased soil biological activity, but this was only significant ($P < 0.05$) for the highest input level used. Microbial activity decreased with decreasing levels of *Brassica* tissue added and was very similar to that of untreated control soils at the lowest *Brassica* tissue input level (see Figure 5.10).

Chitosan amendments had no significant effect on soil microbial activity compared to the untreated soil ($P<0.05$) at all three input levels tested (see Figure 5.10).

Compost applied at both input levels (1 and 0.5) significantly increased ($P<0.05$) the soil microbial activity compared to untreated soil (see Figure 5.10).

All **combination treatments** included in the trial (except for **Chitosan + Brassica + Compost**; each at level 0.25) resulted in significantly higher ($P<0.05$) soil microbial activity compared to that measured in untreated control soils. However, there was no indication that there was an additive effect of using chitosan in combination with compost and/or fresh *Brassica* tissues (see Figure 5.11).

Untreated soils in which **grafted plants** were grown had similar levels of microbial activity as untreated soils in which non-grafted plants were grown. However, as expected, **steam treatment** significantly reduced the biological activity compared to untreated soils (see Figure 5.11).

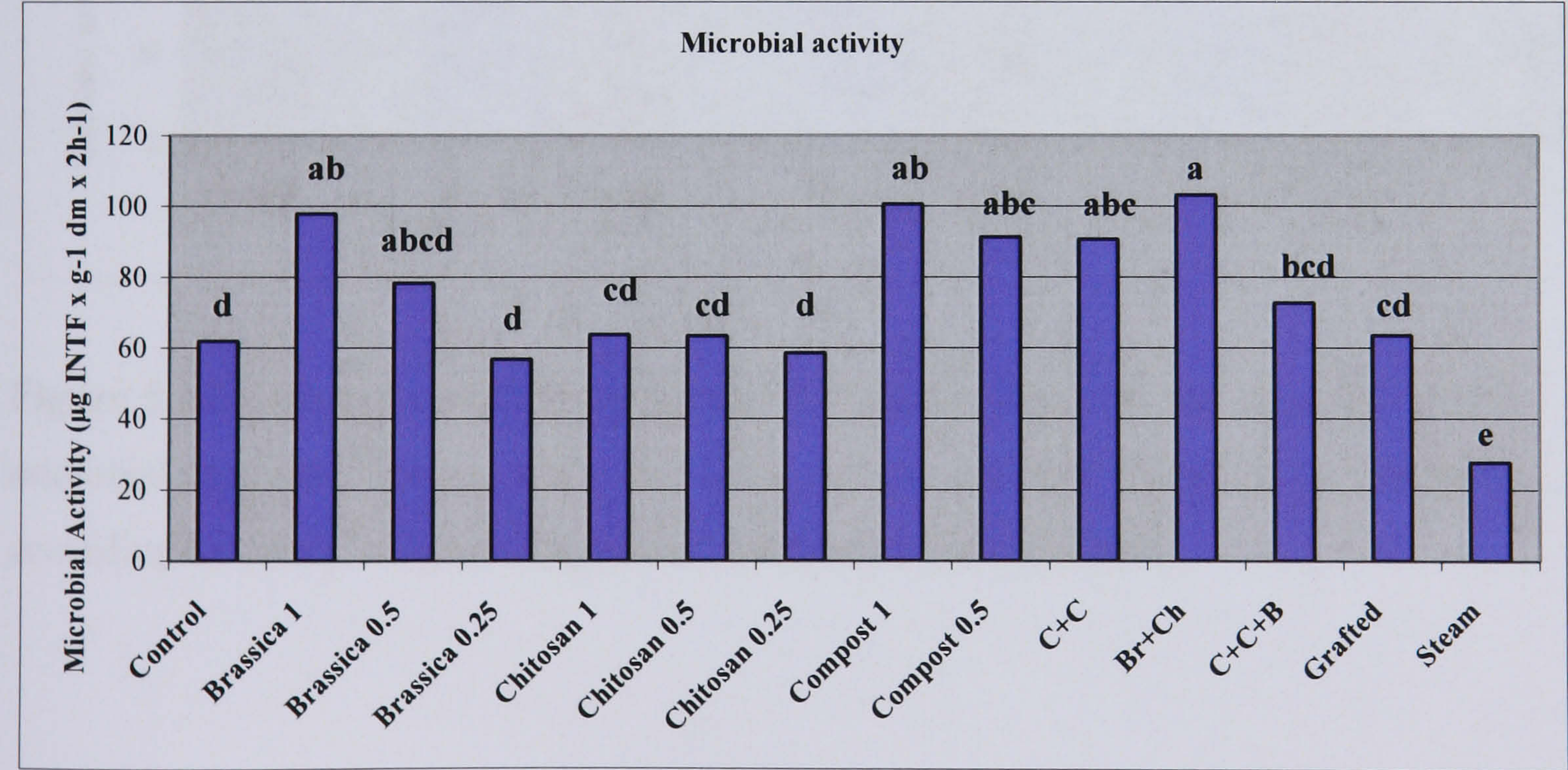


Figure 5.11. Effect of different soil amendment type and application level on soil microbial activity. Means with the same letters are not significantly different according to Tukey’s Honest Significant Difference Test ($P<0.05$).

To test for potential interactions between treatment types and input levels two-way ANOVA were carried out on selected groups of treatments.

Two way ANOVA showed a significant effect of soil treatment type ($P<0.001$) on soil microbial activity when all **three treatment types and two input levels (1 and 0.5)** were included in the analyses (see Figure 5.11). There was also a trend for differences between soil treatment input levels, but this was not significant ($P=0.054$). There was no significant interaction between soil treatment type and soil treatment input level ($P=0.280$) (see Figure 5.11).

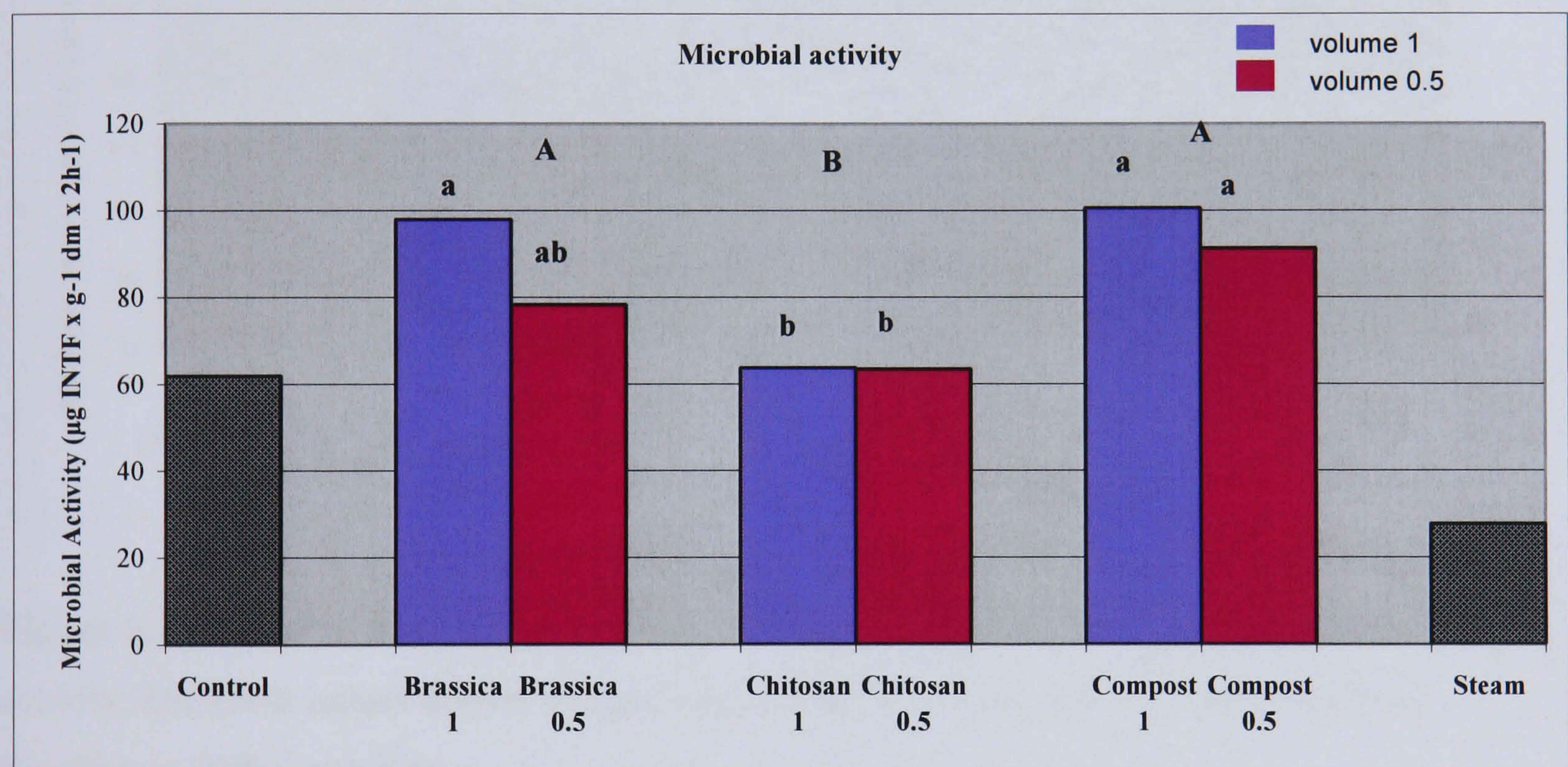


Figure 5.12. Effect of two different levels of *Brassica*, chitosan, and compost on soil microbial activity. Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P<0.05$)

When **two input types** (chitosan and fresh *Brassica* tissue) and **all three input levels** (1, 0.5 and 0.25) were included in the analyses, two-way ANOVA showed a significant effect of soil treatment type ($P=0.004$) and soil treatment input level ($P=0.002$) and a significant interaction between soil treatment type and input level ($P=0.023$). For fresh *Brassica* tissue microbial activity decreased with decreasing input level, while for chitosan soil microbial activity remained similar at all three input levels.

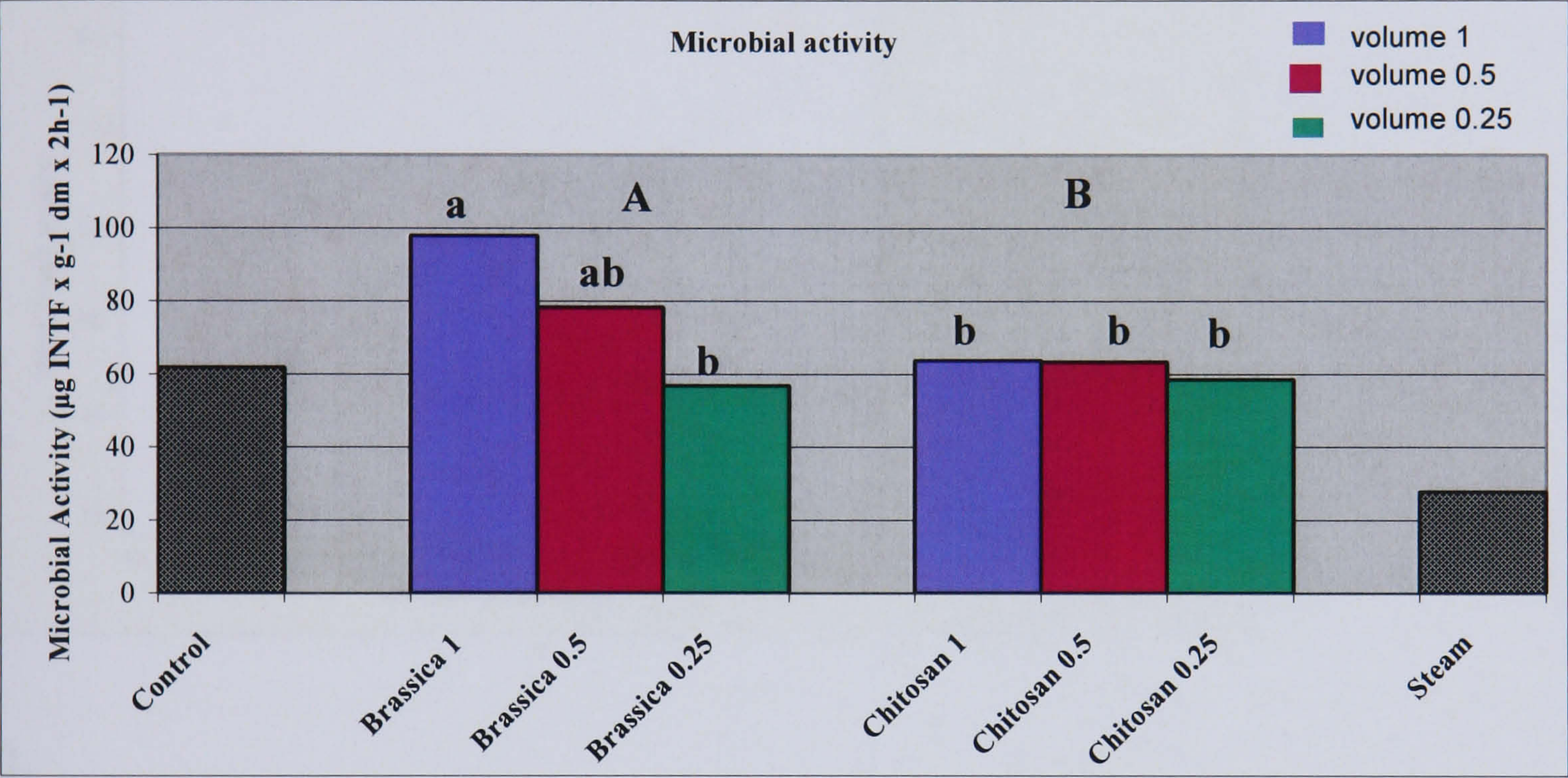
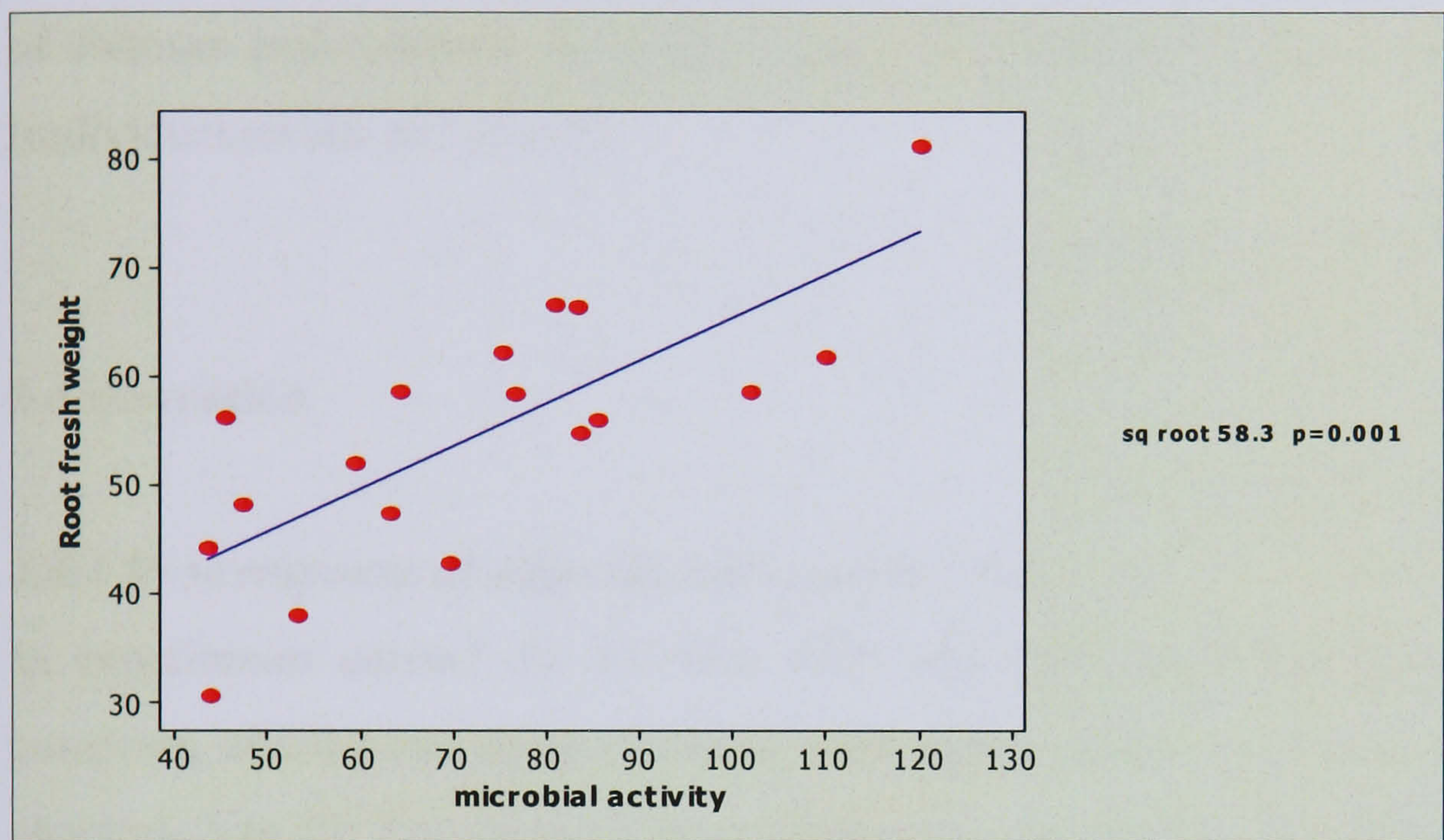


Figure 5.13 Effect of three different levels of *Brassica* and chitosan on soil microbial activity. Different letters denote a significant difference according to Tukey’s Honest Significant Difference Test.

5.3.8 Correlation between soil microbial activity and RFW and yield of plants grown in soils with Brassica amendments

There was a clear correlation between soil microbial activity and RFW and yield of plants grown in to soils with *Brassica* amendments (see fig. 5.13)

A



B

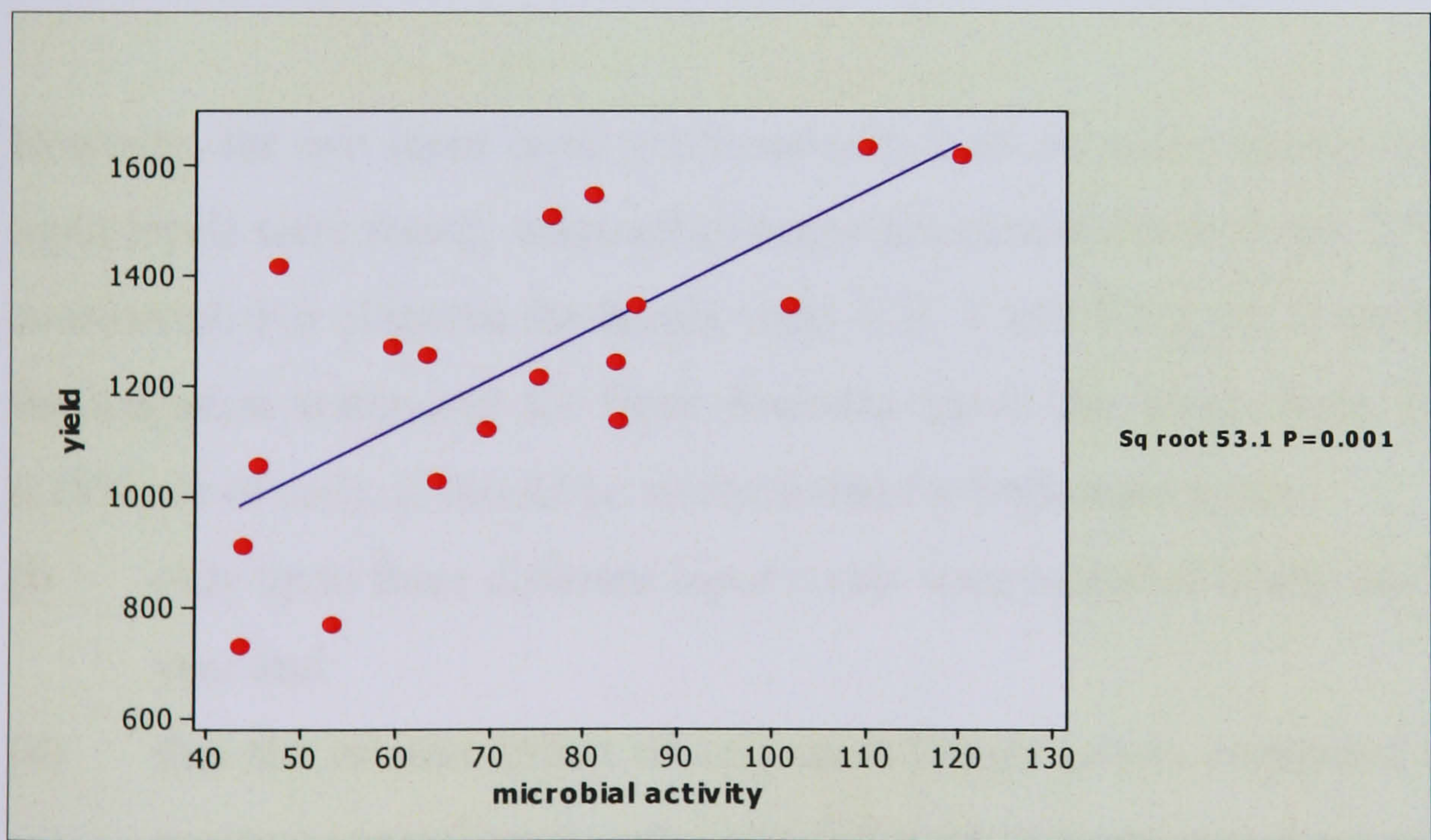


Figure 5.14 Correlation between soil microbial activity ($\mu\text{g INTF} \times \text{g}^{-1} \text{ dm} \times 2\text{h}^{-1}$) and (A) RFW (g) and (B) yield (g/plant) of plants grown in to soils with *Brassica* amendments

5.3.9 Effect of soil steaming and addition of organic matter inputs on chitosan and cellulose degrading fungi and bacteria

When the populations of chitosan and cellulose degrading bacteria and fungi were estimated, by plate counts on soil extract agar, no significant differences in the density of chitosan and cellulose degrading organisms could be detected between treatments (individual results not shown).

5.4 Discussion

5.4.1 Dose response of different soil amendments

In experiments carried out between 2001 and 2003 most soil treatments (the two composts, and the two chitin products) were only tested at one or two input levels (see chapters 2 to 5). For most of these treatments the higher concentration resulted in higher yield and/or root fresh weights, but it was not possible to establish dose-response curves.

However, for two input types (chitosan and fresh *Brassica* tissue) four different soil input levels were tested, when all experiments carried out between 2001 and 2003 are considered. For chitosan the levels were 4, 2, 1 and 0.5 g per 2 weeks, applied with the irrigation water and for fresh *Brassica* tissue the levels were 50, 25, 12.5 and 6.25% v/v of soil). It should be stressed that for both input types

- (i) only up to three different input levels were included in any one experiment and year and
- (ii) that the relative effect of soil amendments (when compared to negative and positive control treatments) was different between experiments.

However, when data from all experiments, and the two types of soil pathogen inocula are combined and best fit curves are plotted through the means of results obtained for different input levels, a first tentative dose response curve can be constructed (see Figures 5.15 and 5.16).

The **dose response curves for yield** indicate that the maximum level of efficacy that can be obtained with chitosan is higher than that for fresh *Brassica* tissue (when the yield and root fresh weight are compared) (see Figures 5.15 and 5.16). This difference between the two input types was confirmed by the statistical analyses in individual experiments (see chapters 2 to 5). The dose response curve for yield also indicates that yields increase with increasing input levels up to 1g/week of chitosan and 12.5% (v/v) fresh *Brassica* tissue, but remain at the same level if soil input levels are increased further (saturation curve).

The **dose response curve for root fresh weight (RFW)**, on the other hand, shows increasing RFW up to 1g/week of chitosan and 12.5% (v/v) fresh *Brassica* tissue, but then decreases again if higher input levels are used (saturation curve).

This reduction in root fresh weight could have been due to the two inputs supplying significant levels of plant nutrients to the crop at higher input levels. Chitosan is known to contain significant levels of nitrogen, while *Brassica* tissues contain a range of different nutrients. The density and total volume of roots is known to decrease with increasing availability of nutrients, and this could explain why yields remained constant, while roots fresh weight decreased at the higher input levels (see Figs 5.15 and 5.16). However, the reduction in root fresh weight at higher levels could have also been due to toxic effects on root growth caused by the inputs or their breakdown products.

However, it should be stressed that these interpretations/conclusions will have to be confirmed in future experiments in which a wider range of concentrations of the different inputs are used in the same experiment.

Crop Yield (% of positive control*)

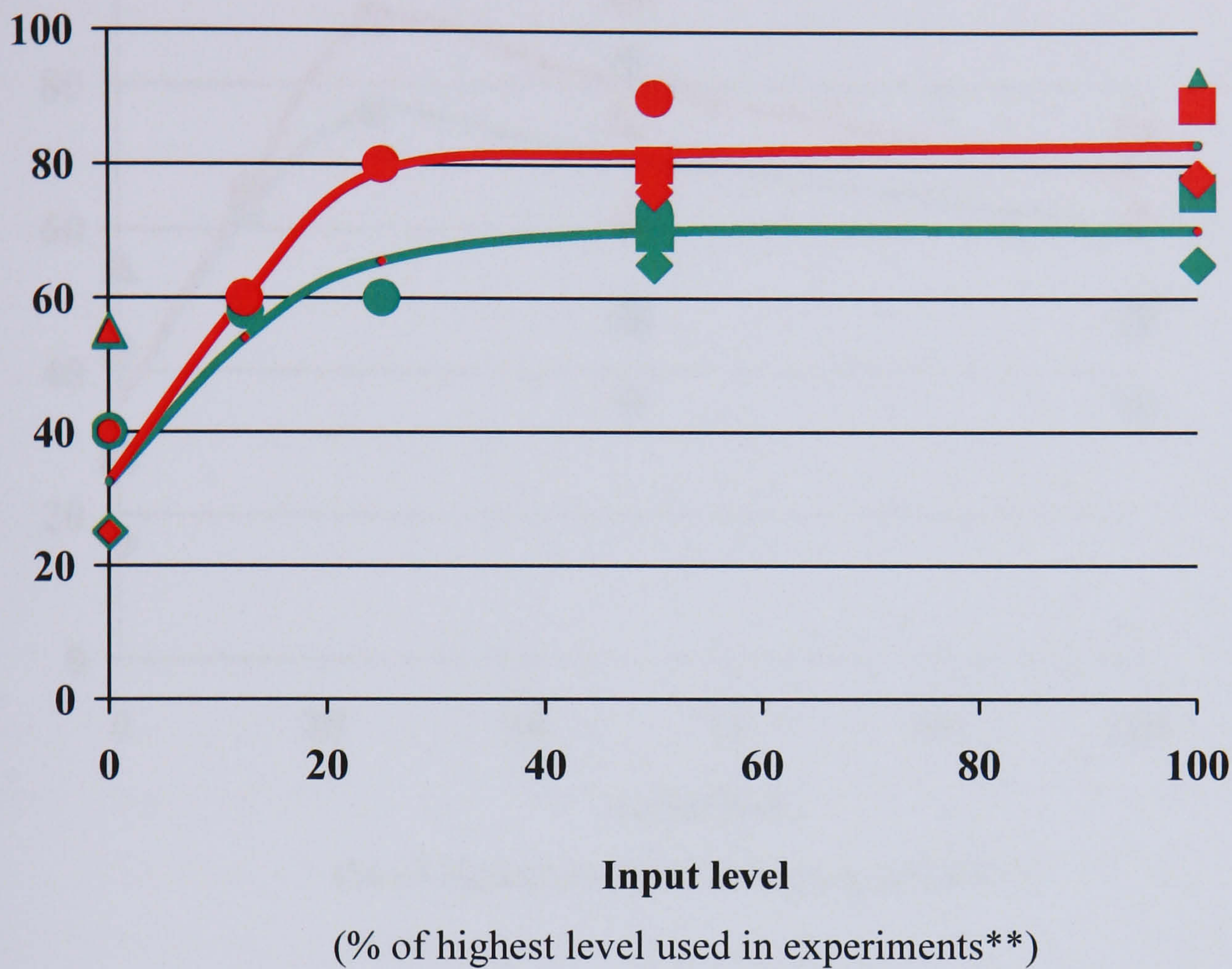


Figure 5.15. Dose response curve for **yield** for (a) fresh *Brassica* tissue (green line and symbols) and (b) chitosan (red line and symbols); * positive controls were plants grown in steam sterilised soil; ** the highest input level used for fresh *Brassica* tissue was 50% v/v of substrate and for chitosan 4g applied with the irrigation water per plant and week. Different symbols represent data from different experiments:

- ▲ = means from the 2001 experiment (soils infected with corky root rot only)
(see chapter 2)
- = means from the 2002 experiment (soils infected with corky root rot only)
(see chapter 3)
- ◆ = means from the 2002 experiment (soils infected with both corky root rot and *Verticillium*) (see chapter 3)
- = means from the 2003 experiment (soils infected with both corky root rot and *Verticillium*) (see chapter 5).

Means of untreated control plants grown in non-steam treated soils were used as 0 input level.

Root fresh weight (% of positive control*)

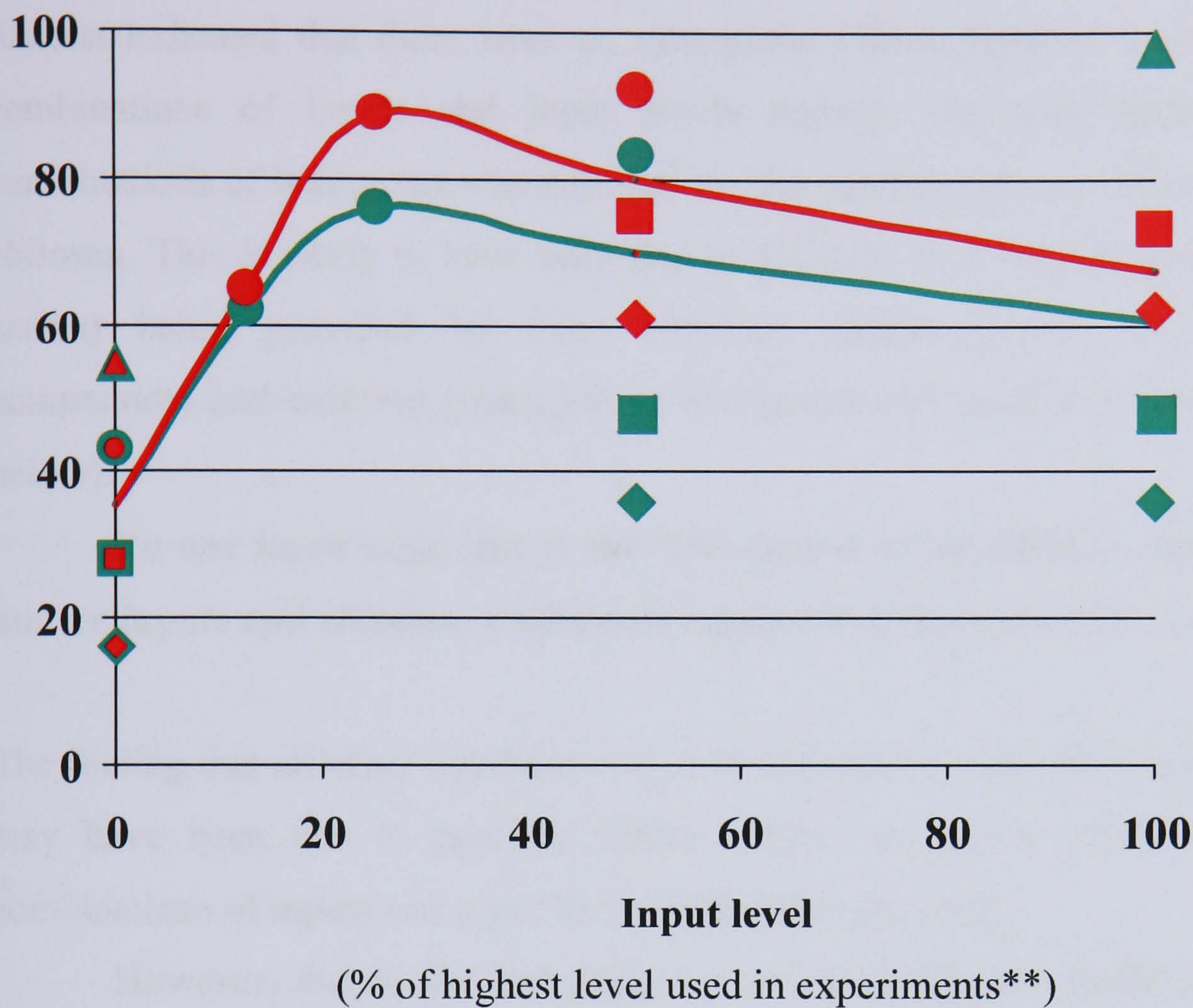


Figure 5.16. Dose response curve for **root fresh weight** for (a) fresh *Brassica* tissue (green line and symbols) and (b) chitosan (red line and symbols); * positive controls were plants grown in steam sterilised soil; ** the highest input level used for fresh *Brassica* tissue was 50% v/v of substrate and for chitosan 4g applied with the irrigation water per plant and week. Different symbols represent data from different experiments:

- ▲ = means from the 2001 experiment (soils infected with corky root rot only)
(see chapter 2)
- = means from the 2002 experiment (soils infected with corky root rot only)
(see chapter 3)
- ◆ = means from the 2002 experiment (soils infected with both corky root rot and *Verticillium*) (see chapter 3)
- = means from the 2003 experiment (soils infected with both corky root rot and *Verticillium*) (see chapter 5).

Means of untreated control plants grown in non-steam treated soils were used as 0 input level.

5.4.2 Potential additive or synergistic effects of combining different soil amendments

Results indicated that there were no synergistic effects between treatments (for the combinations of inputs and input levels tested). The only additive effect of combinations of treatments was detected for the combined use of *Brassica* tissue and chitosan. This is likely to have been due to different (but not antagonistic modes of action) being provided by fresh *Brassica* tissues (release of anti-microbial compounds) and chitosan (induction of resistance) (see section 5.1 above and 5.4.3 below).

To our knowledge this is the first report of an additive effect of organic matter inputs and chitosan treatments against *P. lycopersici* and *V. albo-atrum*.

The finding that all other combinations of treatments did not result in additive effects may have been due to possibly similar modes of action and/or to the limited combinations of inputs and input levels included in the study.

However, due to the lack of space in the glasshouse facilities available for trials at Newcastle University, only two volumes of composted cow manure were used, so it is not clear if a further reduction of the compost input would still provide beneficial effect against the pathogens. Future work should therefore assess a wider range of combinations of inputs at different input levels.

5.4.3 Potential mechanisms of actions of different soil amendments

5.4.3.1 Increased competition through overall stimulation of soil biological activity

***Brassica* amendments** at the two higher input levels significantly increased the root fresh weight and the yield of plants compared to plants grown in untreated soil. The suppression of soil-borne pests and pathogens associated with the inclusion of *Brassica* crops in the rotation was previously attributed mainly to the release of biocidal compounds, principally isothiocyanates, when the glucosinolates in the *Brassica* tissues are hydrolysed during the decomposition of crop residues in soil (Kirkegaard *et al.*, 1999).

However, the experiment described here (see correlation between soil biological activity and, root fresh and yield in Figure 5.13) indicates that increased competition (due to increased soil biological activity) may have also been involved in the “suppressive” effect observed for fresh *Brassica* tissues, since the microbial activity in soils treated with the two higher input levels of fresh *Brassica* tissue volumes was also increased.

If isothiocyanate production would have been the main mode of action of the *Brassica* treatment, one would have expected a negative impact of *Brassica* tissues on soil microbial activity, since isothiocyanate is known to inhibit/kill a wide range of micro-organisms (Bailey and Lazarovits, 2003). Since only one soil microbial assessment was made at 60-70 days after the incorporation of fresh *Brassica* tissue, the release and inhibitory effect isothiocyanate may have occurred earlier and was not detected by the assessment. Future studies should therefore assess microbial activity and different times after incorporation of *Brassica* tissues.

In a previous study (Xiao *et al.* 1998) it was reported that the incorporation of *Brassic*as in soils resulted in an increase in the population of actinomycetes, spore forming bacteria and fungi, indicating a possible role of these components of the soil micro-flora in the disease suppression effect observed.

Composted cow manure also increased soil microbial activity at the two higher input levels compared to the untreated soil ($P < 0.05$). As with the *Brassica* tissue this could indicate a general effect of increased competition by the soil micro-flora. However, whether the exact mechanism of competition was antibiotic release, antagonism for space and nutrients and/or induced resistance will have to be identified in future studies. The excess of nutrients resulting from the decomposition/mineralisation of organic matter may also have had a direct “nutritional” effect on plant yield..

Chitosan amendments to soil, especially 1 and 0.5, increased root fresh weight (decreased disease incidence) and yields were significantly higher compared to those obtained in untreated soils. However, the microbial activity in soil treated with chitosan was not significantly different to that of untreated soil, and increased competition resulting from increased soil biological activity levels could therefore not be confirmed as mode of action for the observed disease suppression for chitosan.

Chitosan has been reported to induce plant resistance mechanisms and thereby improve the plants ability to protect themselves from pathogens such as fungi (Suzuki and Shinshi, 1998; Reddy *et al.*, 1999) and promotes plant and root growth (Hirano, 1998; Tsugita *et al.*, 1993; Harada *et al.*, 1995).

However, it has also been suggested that the growth promotion could at least, be partially due to increased nitrogen supply, because chitosan contains 8.7% N (Ohta *et al.*, 1999). More recently “filmogenic” properties of chitosan were described to result in a chitin layer/barrier around plant roots, thus preventing **(a)** the invasion of root tissues by different soil pathogens and **(b)** the exudation of nutrients from roots to a level, that removes chemotactic responses and growth of pathogens in the rhizosphere (Ait Barka *et al.*, 2003; El Ghaouth *et al.*, 2000).

5.4.3.2 Increased competition from chitin and cellulose degrading fungi and bacteria

There were no significant differences in chitin and cellulose degrading soil microorganisms between treatments. Most surprisingly, even steam-disinfected soil (which did show a lower level in overall biological activity, based on respiration rate assessments) had similar numbers of chitin and cellulose degrading fungi and bacteria.

Since assessments were only carried out at the end of the experiment, and because assessments were not repeated in other experiments, no conclusions should be drawn at this stage. However, in future experiments testing the effect of chitin, chitosan and organic matter amendments, assessments of chitin and cellulose degrading activity in soils should be repeated and, if necessary improved procedures/assays should be used to ensure that the lack of differences between populations was not due to the timing of sampling and deficiencies in the experimental methods used.

CHAPTER 6. Effect of cultivar resistance and alternative foliar treatments on powdery mildew in glasshouse grown cucumber crops

6.1 Introduction

Powdery mildews (*Erysiphaceae*) are one of the most commercially important groups of plant pathogens, and most of the vegetable and ornamental plants grown in greenhouses suffer from powdery mildews (Elad *et al.*, 1996).

Agrios (1997) concludes: “*Powdery mildews are so common, widespread and ever present among crop plants and ornamentals that the total losses, in plant growth and yield, they cause every year on all crops probably surpass the losses caused by any other single type of plant disease*”.

Podosphaera xanthii (syn. *Sphaerotheca fuliginea*) is the most important powdery mildew species in greenhouse grown cucurbit crops (Molot and Lecoc, 1986) and is currently the only disease that prevents long “English”-type cucumbers from being produced without the use of synthetic pesticides (Daayf *et al.*, 1995).

Due to its commercial importance, and the ability to rely on high natural infection levels, the *P. xanthii* – cucumber system was therefore chosen as the model system for the development of improved control methods for mildew diseases in glasshouse production systems.

The development of alternatives to fungicides is not only important for organic production. In conventional protected cucumber production systems agriculture, the widespread use of chemosynthetic pesticides has resulted in the development of pathogen strains resistant to the main mildew fungicides used such as triadimefon and benomyl and resistant strains are now widespread (Dekker and Gielink, 1979; Schepers, 1983; Elad *et al.*, 1996).

Public concerns about the environmental impact and potential consumer health risks, associated with pesticide residues in foods, have resulted in public pressure to reduce the use of chemosynthetic pesticides.

In both conventional and organic greenhouse production systems the use of sulphur fungicides is permitted. They provide effective control against powdery mildew, but are toxic for plants at temperatures above 27⁰ C. This limits their use to the cooler

parts of the season, especially in Southern European countries (Belanger and Labbe, 2002; Bourbos and Skoudridakis, 1993). Sulphur fungicides were also shown to have negative effects on natural enemies and commercial preparations of beneficial invertebrates, used to control greenhouse pests.

Cultivars resistant to powdery mildews have been developed for a number of cucurbit crops. For example, there are several cultivars of melon with resistance against races 1, 2, and 3 of *P. xanthii*, but, for cucumber, only a few **tolerant** cultivars are available, which provide moderate protection against powdery mildew (Elad *et al.*, 1998).

An alternative, to both the use of pesticides and resistant/tolerant cultivars, for the control of powdery mildew of cucumbers is the use (i) elicitors of plant resistance (such as plant extracts and chitin) and (ii) biological control agent (BCAs) (see chapter 1).

Several elicitors were previously reported to provide significant levels of control of powdery mildew (Konstantinidou-Doltsini and Tzempelekou, 1998; Petsikos-Panayotarou *et al.*, 2002; Singh and Prithivira, 1997).

However, for both fungal and bacterial BCAs a **mismatch between environmental conditions** (especially temperature and humidity) **required by (a) antagonists** for germination, infection, growth and/or biological control activity of antagonists and **(b) powdery mildew fungi**, for infection and disease development in host plants, was described as the main reason for the relatively poor biological control activity of most antagonists under glasshouse conditions (see chapter 1 and Elad *et al.*, 1996).

The **main objectives** of the experiment carried out under chapter 6 were therefore to:

- (i) to compare powdery mildew disease development pattern and yields of 2 cultivars (Gloria and Palmera) with different levels of genetic resistance to powdery mildew
- (ii) to quantify the effect of different alternative, non-chemical foliar treatments on powdery mildew disease development and fruit yield

- (iii) to identify interactions between cultivar resistance and foliar treatments
(= compare the relative effect of alternative treatments in different cultivars)

6. 2 Material and Methods

6.2.1 *Field trial sites*

The trial was conducted in 2003 at the experimental glasshouse facilities at the Technological Educational Institution of Ionian Islands, Department of Organic Agriculture, Argostoli, Greece.

The minimum and maximum temperatures measured in the glasshouse during the trials reported in this chapter and chapter 7 were 18 and 32⁰C respectively.

6.2.2 *Cultivars used*

Cucumber seeds of the cultivars Palmera (Rijk Zwaan, Holland; **susceptible to powdery mildew**) and Gloria (Tezier, France; **moderately tolerant to powdery mildew**), were sown in a peat-based substrate, and later transplanted into soil in the glasshouse, (after approximately 2-3 weeks). No artificial inoculum was used to infect plants, and plants became naturally infected with powdery mildew.

At the end of the experiment cleistothecia of the powdery mildew were examined and it was confirmed that the fungus was *Podosphaera xanthii* (contained only one ascus)

6.2.3 *Experimental Design*

Treatment plots of four cucumber plants were arranged as a completely randomized block design with four replicate blocks in the glasshouse (see Figure 6.2.1 for the arrangement of plots in the glasshouse)

Six treatments were applied as foliar sprays for both cultivars:

- **Control (water spray)**
- **Fungicide control Mixture of two fungicides: myclobutanil (Systhane 12 E, EC, 12.5% a.i. w/v, ALFA) at the rate of 0.4 ml l⁻¹, penconazole (Topas 10 EC, 10% a.i. w/v., SYGENTA), at 0.15 ml l⁻¹.**
- **Chitosan as soluble chitin 1kg/ha**
- **Milsana VP 2002 (plant extract of the perennial weed *Reynoutria sachalinensis*) 50kg/ha**
- ***Ampelomyces quisqualis* (AQ10) 70g/ha**
- ***Pythium oligandrum* (Polyversum) oospore preparation (10⁶ oospores/g), 0.1kg/ha.**

BLOCK 1		BLOCK 2		BLOCK 3		BLOCK 4	
P2	G6	P1	G6	P3	G5	P1	G4
G4	P6	G2	P3	G1	P5	G3	P2
P5	G3	P4	G5	P1	G2	P6	G1
G1	P4	G1	P6	G3	P2	G6	P3
P3	G2	P2	G4	P6	G6	P5	G2
G5	P1	G3	P5	G4	P4	G5	P4

Table 6.1. Experimental design

P= 4 plants of cv. **PALMERA**
G= 4 plants of cv. **GLORIA**

1. CONTROL (water sprays)
2. MILSANA
3. CHITOSAN
4. AQ 10
5. POLYVERSUM
6. FUNGICIDES

Distance between plants on a plot = 0.5m
Distance between treatment plots = 2m
Distance between rows in a block = 1.5m
Distance between different blocks = 2m

The first treatment spays were applied five days after transplanting, before any disease symptoms appeared and were then repeated weekly. All treatments were applied at medium volume spray (500-1000 l ha⁻¹) with a 20l knapsack sprayer (Berthoud Vermorel 2000 HP, 3 bars).

For chemical control usually a mixture of fungicides with different modes of action are used to avoid infection of resistant strains of the targeted pathogen. Since the glasshouse used for these trials belongs to the department of Organic Agriculture and no chemosynthetic fungicides were used previously, no resistant strains of *P.xanthii* were expected to be present. That is why two chemical fungicides that belong to the same group (EBI) were used.

6.2.4 Crop assessments

Marketable cucumbers were harvested from all plants and weighed (g/plant) at three to five day intervals. The number of fruits per plant was also assessed.

Disease severity (% infected leaf area) was evaluated/scored at regular intervals after symptoms were first detected on secondary leaves, using a scale were of 1=0.1-25%, 2=25-50%, 3=50-75%, 4=75-100% of leaf area infected.

Five disease assessments were carried out in total. The mean disease severity per plant was calculated using the formula of Townsend Heuberg. These AUDPC values were subjected to statistical analysis.

AUDPC

For some plant disease epidemics, where the purpose is to summarize a disease progress curve for comparative or analytical purposes, the area under the disease progress curve (AUDPC) can be used as a descriptor for the epidemic (Shaner and Finney, 1977). Variation in time of disease onset, r^* , and final y (y_f) are all incorporated into AUDPC. Area Under Disease Progress Curve is simply y (disease intensity) integrated between two times.

The AUDPC can be estimated as:

$$\text{AUDPC} = \sum_{i=1}^{n-1} (y_i + y_{i+1})/2 (t_{i+1} - t_i)$$

Where i : is the time dimension (in assessment), y : is the disease incidence, t : is time in days (between two successive assessments) and n : is the number of total observations. Units of AUDPC were % days or proportion-days.

6.2.5 Statistical analysis

Data on disease severity obtained from both greenhouse experiments (reported in this Chapter and Chapter 7) were plotted and day 0 in the plots was set to be the day of

transplanting in the greenhouse.

The effectiveness of the treatments in the greenhouse was analyzed using the area under disease progress curve (AUDPC) approach (Campbel and Madden, 1990) as explained above. The AUDPC ($\sum[(y_i+y_{i+1})/2] \times d_{ti}$) was calculated for disease severity (in terms of percentage infected leaf area).

Data obtained on: (a) percentage infected leaf area (as values of AUDPC - % days) and (b) fresh weight per plant (square root transformed values) were subjected to Analysis of Variance (ANOVA) and differences between means were tested by Duncan's multiple range test.

In the first case, two-way ANOVA was performed where treatments and cultivars were the two independent variables. The effect of their interaction was also estimated.

In addition, disease progress in the controls of both cultivars was studied using epidemiological models that aimed to estimate the impact of cultivar on powdery mildew progress on leaves. Linearized logistic models were used with the assumption that the maximum level of infection was 100% or 1 (K asymptote). The logit transformation of y [$\ln(y/1-y)$] was carried out for the mean values (per plot) of disease severity per cultivar, and linear regression analysis was performed on the transformed data. Disease records for each cultivar were summarized as the slope (r_L) and the intercept (y_o) of the regression of the logit of disease on time during the periods when the records were changing. Residuals were reasonably distributed and no pattern was systemically present (Campbel and Madden, 1990).

In order to estimate the relationship between yield and disease severity in controls, as a first step yield was plotted against disease (AUDPC values) in order to visualize any relationship. Hence, yield was regressed on disease.

6.3 Results

6.3.1. *Effect of cultivar resistance (disease progress in untreated control plants)*

Disease progress (linearized logistic models) on the untreated controls for both cultivars is shown in Fig 6.1. Disease parameters in terms of r_L and y_o are presented in

Table 6.2. Disease onset was delayed in the less susceptible cultivar Gloria as indicated by the y_o value (Table 6.2). The rate of infection in the initial phase of disease development appears to be similar to that of the susceptible cv. Palmera (see r_L values), while final disease severity was higher in Palmera (78 %) compared to Gloria (56%) (see Figs. 6.1 and 6.2). In addition, disease severity was statistically lower in untreated Gloria control plants (Fig. 6.2) (this was confirmed by a t-test comparing values of AUDPC for Palmera and Gloria divided by the total number of days of the experiment) (data not shown).

Table 6.2. Slope r_L and intercept y_o for regression of logit transformation of the percentage infected leaf area over time

Model		Coefficients	SE Coef.	Sig.	Adj R ²
PALMERA	y_o	-3.0676	0.1419	.000	0.949
	r_L	0.1355	0.0072	.000	
GLORIA	y_o	-4.4088	0.1969	.000	0.911
	r_L	0.1402	0.0100	.000	

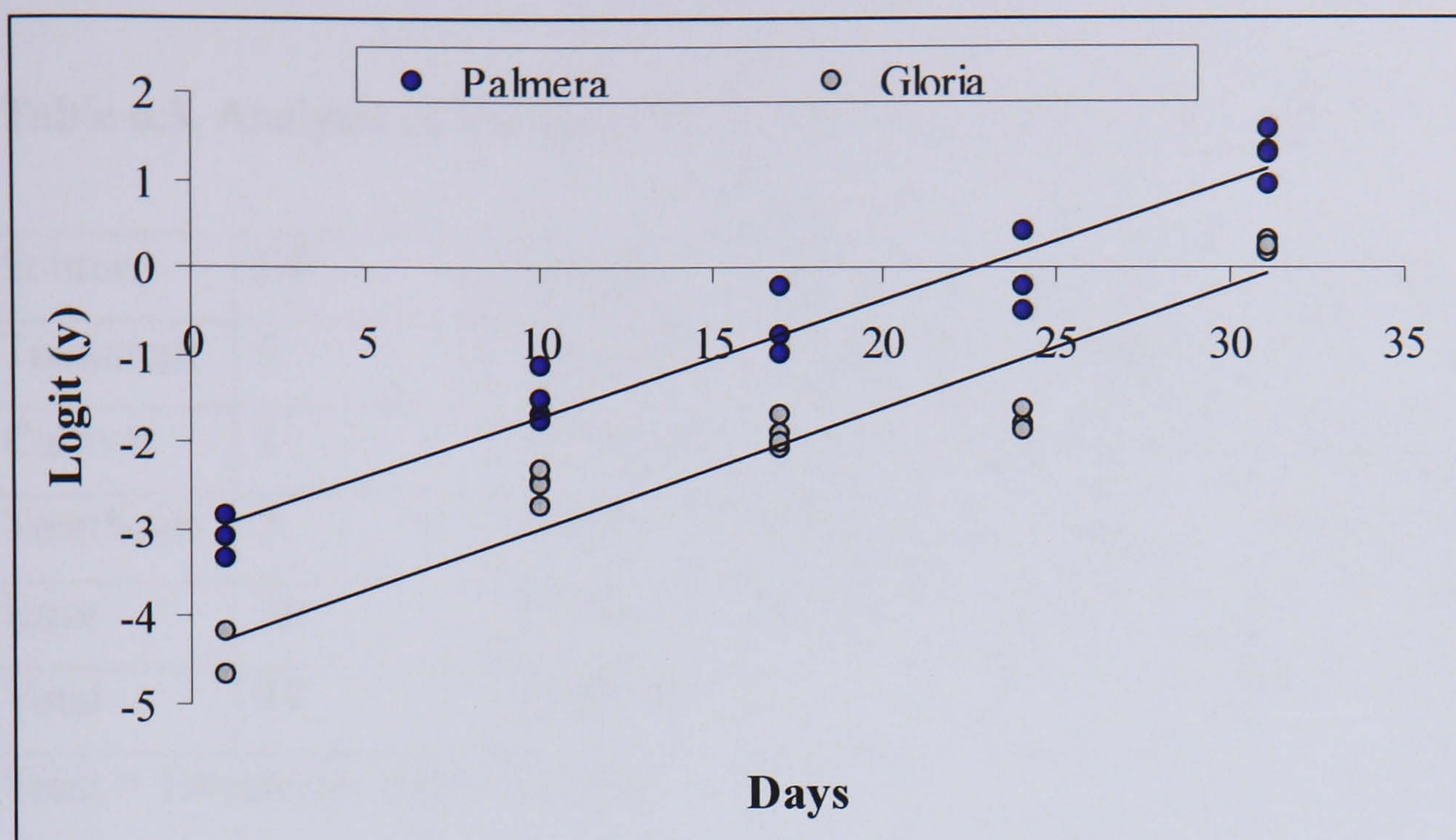


Figure 6.1. Linear logistic models of disease progress on two cultivars with different levels of susceptibility to powdery mildew

6.3.2 *Effect of foliar treatments on foliar disease development in tolerant and susceptible cultivars*

Results of a two-way ANOVA, where AUDPC values were the dependent variable, are presented in Table 6.3. The analysis showed that there was a very highly significant effect of both foliar treatment and cultivar on disease severity. There was also a very highly significant interaction between cultivar and foliar treatment (see P-value in Table 6.3.). This means that the relative effect of foliar treatments depends on the level of cultivar resistance. As expected, foliar treatments had less of an effect (in terms of the difference in disease severity between treated and untreated control plants) in the tolerant (Gloria) compared to the susceptible cultivar (Palmera).

Table 6.3. Analysis of Variance for AUDPC values, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	5	3766018	3766018	753204	4277	0.000
Cultivar	1	1271976	1271976	1271976	297.41	0.000
Treat*cult	5	563287	563287	112657	26.34	0.000
Error	36	153964	153964	4277		
Total	47	5755245				

Treat = Treatment; cult = cultivar

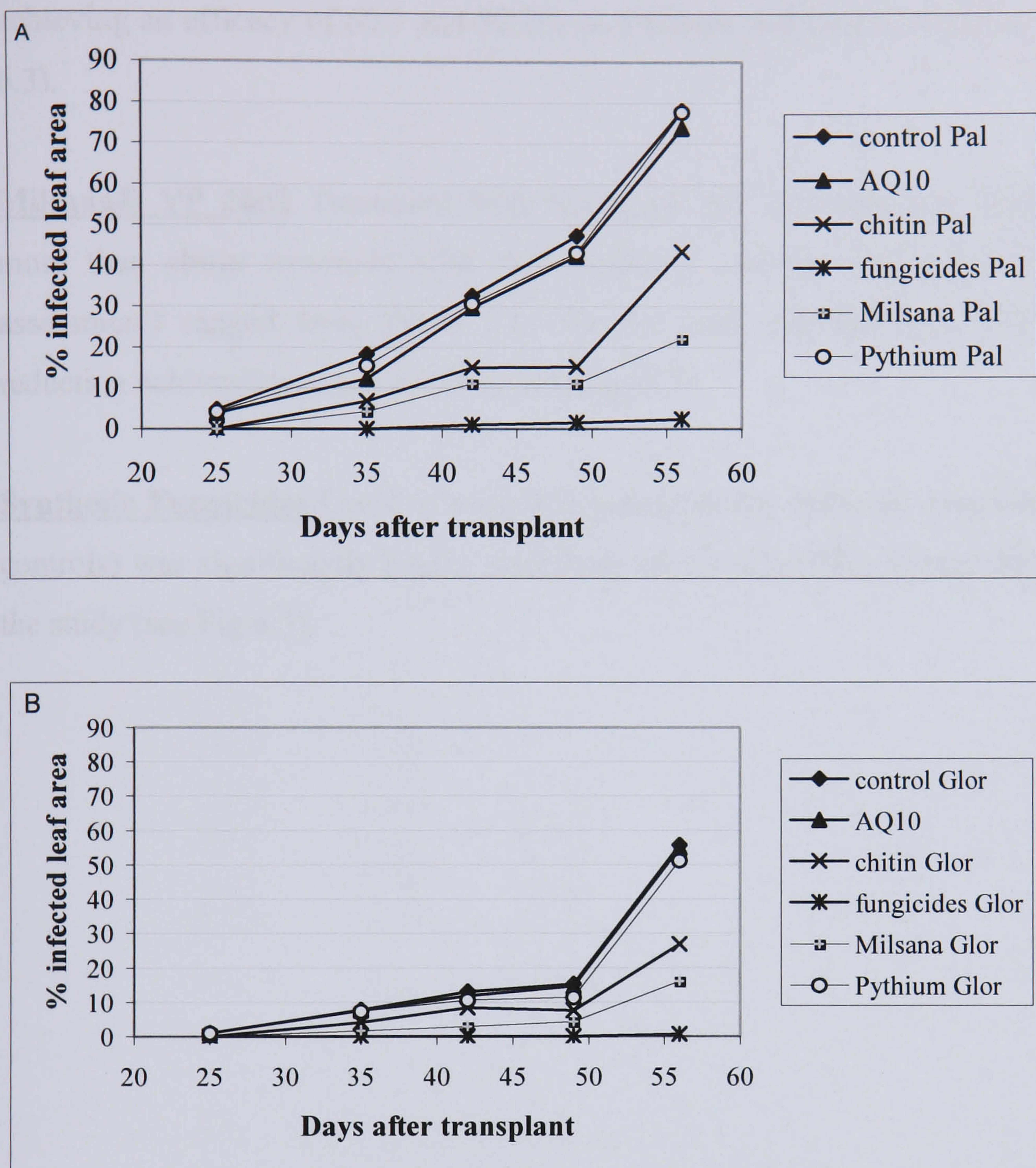


Figure 6.2. Disease progress on Palmera (A) and Gloria (B) treated with different foliar treatments

Ampelomyces quisqualis Treatment with the fungal biological control agent AQ10 did not significantly reduce disease severity compared to the control for both cultivars when the AUDPC values were compared (Fig. 6.3 A & B).

Pythium oligandrum Applications of the oomycete fungus based biological control product (Polyversum) did also not significantly ($P=0.05$) reduce powdery mildew levels in Palmera, but for Gloria (the more tolerant cultivar) there was a small but significant effect (14.2% reduction based on AUDPC values) (see Fig 6.3).

Chitosan Foliar treatment with chitosan was more effective than Polyversum with both cultivars and significantly reduced disease severity (compared to the control) achieving an efficacy of 60.3 and 50.1% on Palmera and Gloria, respectively (see Fig 6.3).

Milsana® VP 2002 Treatment with Milsana® VP 2002 reduced disease severity more than chitin treatment with both cultivars and the infected leaf area (final assessment) ranged from 16 to 22.3 %. On both cultivars the level of disease reduction achieved was around 75% (see Fig. 6.3).

Synthetic Fungicides Level of control obtained by the synthetic fungicides (positive controls) was significantly higher than those of all alternative treatments included in the study (see Fig 6.3).

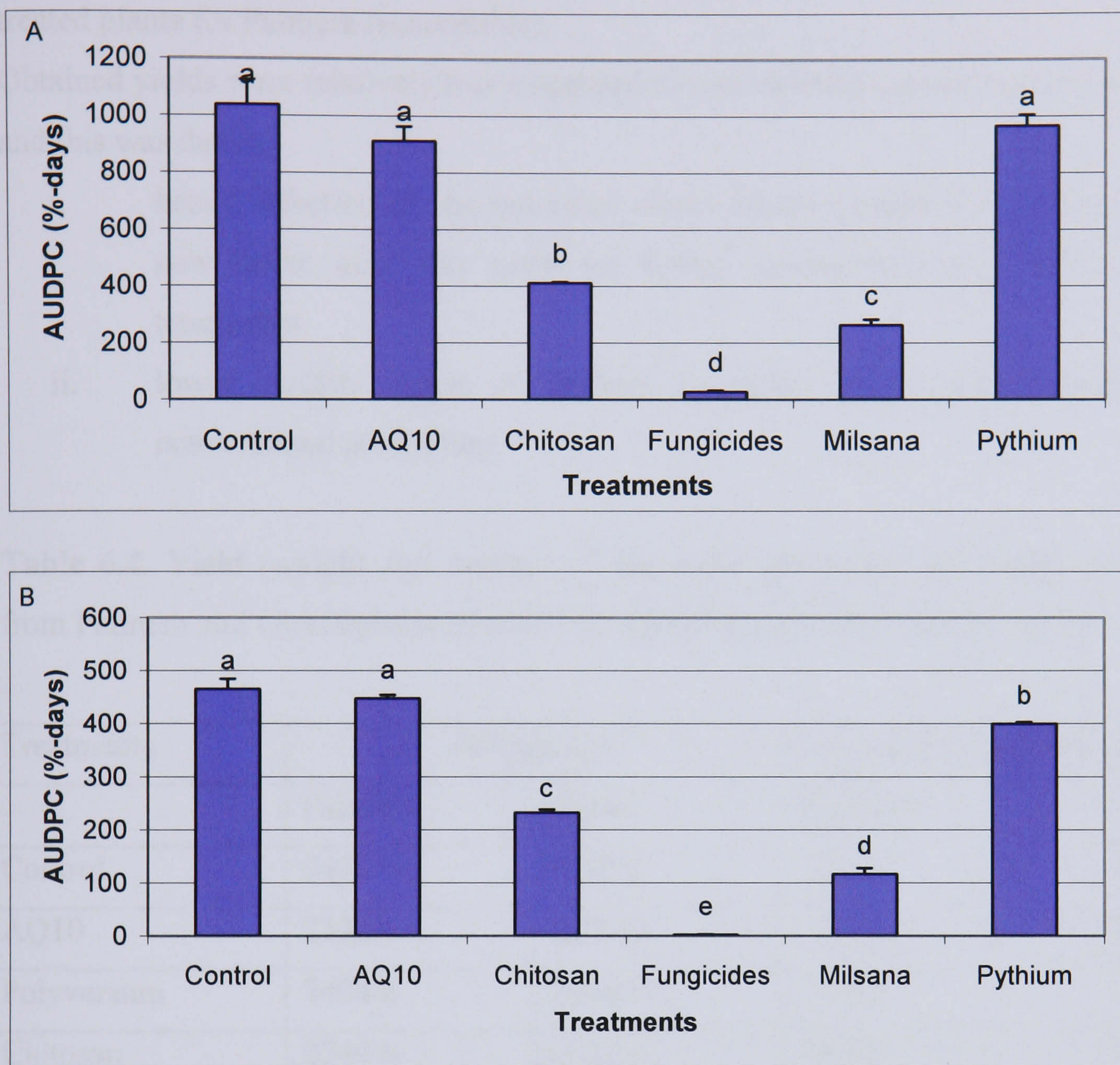


Figure 6.3. Values of the Area Under Disease Progress Curve (AUDPC %-Days) of powdery mildew (± 1 SE) on the upper surface of Palmera (A) and Gloria (B) with different treatments. Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$)

6.3.3 Effect of foliar treatments on yields of tolerant and susceptible cultivars

The weight of harvested fruits per plant was significantly increased ($P = 0.05$) only in the Milsana® VP 2002, chitosan and fungicides treated plots (Table 6.4). Statistically significant differences in yield were also detected between Milsana VP 2002 and chitosan treated plants of both cultivars (with Milsana VP 2002 resulting in higher yields), and the weight of harvested fruits was significantly higher in the fungicide

treated plots compared to Milsana® VP 2002 treated plots for Gloria (tolerant), while there was no significant difference in yield between Milsana® VP 2002 and fungicide treated plants for Palmera (susceptible).

Obtained yields were relatively low compared to conventional-commercial production and this was due to:

- i. heavy infection of the untreated plants which stopped the production of new fruits; after this point no further assessments were taken in all treatments
- ii. lower fertility inputs in organic cucumber production compared to conventional production

Table 6.4. Yield (weight and number of harvested cucumbers per plant) obtained from Palmera and Gloria plants treated with different alternative control means

Treatments	Weight (g)		Cumulative number of cucumbers	
	Palmera	Gloria	Palmera	Gloria
Control	2415 c	2242 d	9.8 c	8.6 d
AQ10	2333 c	2271 d	9.5 c	8.7 d
Polyversum	2404 c	2248 d	9.6 c	8.6 d
Chitosan	2744 b	2512 c	10.8 b	9.5 c
Milsana VP 2002	3289 a	2797 b	12.6 a	10.5 b
Fungicides	3252 a	3012 a	12.6 a	11.2 a

Different letters in a column denote statistically significant differences at P=0.05

6.3.4 Correlation between disease severity and yield

The result of the linear regression analysis between disease on yield is shown in the Table accompanying Figure 6.4. There was a strong negative correlation between disease severity on leaves and cumulative yield (Fig. 6.4). From the regression tables, only slopes were used for the estimation of yield loss. In this study, results obtained were :**(a)** Palmera –yield loss=-0.951(±0.078)*AUDPC **(b)**Gloria-yieldloss = -1.675(±0.1)*AUDPC.

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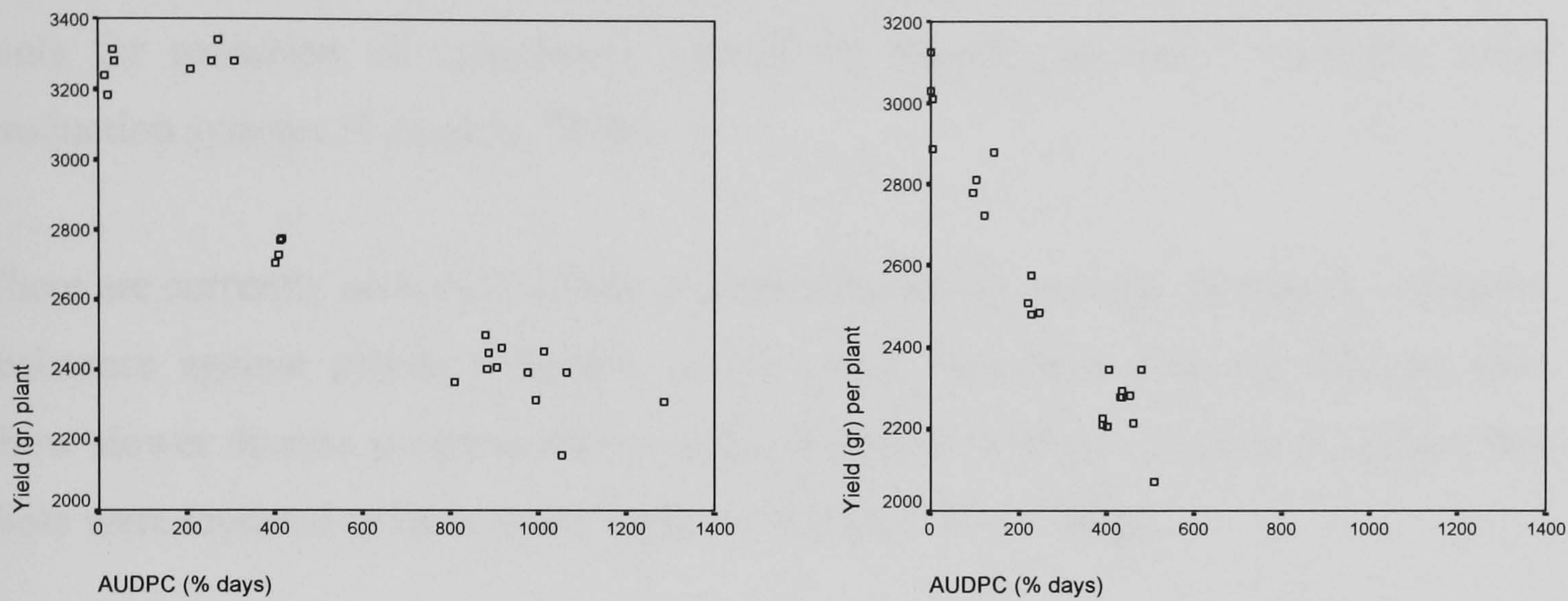


Figure 6.4. Regression plots showing the correlation between disease (AUDPCs) and yield (g harvested fruits per plant) for the two different cultivars: Palmera (left) and Gloria (right)

Model		Coefficients	SE. Coef.	Sig.	Adj R ²
PALMERA	<i>slope</i>	-0.951	0.078	0.00	0.871
GLORIA	<i>slope</i>	-1.675	0.100	0.00	0.925

6.4 Discussion

6.4.1 Potential for improving powdery mildew control and yields of cucumber through the use of tolerant cucumber cultivars

The use of resistant and/or tolerant cultivars is thought to be one of the most effective tools for reduction of crop losses caused by fungal pathogens in organic crop production systems (Lampkin, 2000).

There are currently no commercially available cucumber cultivars that show complete resistance against powdery mildew of cucumber. However, tolerant cultivars that show slower disease progress and/or reduced disease severity are now available, but these were reported to have lower yield levels (Elad *et al.*, 1998).

For example, of the two cultivars used in experiments reported here, the cv. Palmera is known as a high yielding cultivar, but is also highly susceptible to powdery mildew. In conventional farming the maximum yield potential of Palmera can therefore only be achieved through use of mildew fungicides.

In organic farming systems (where chemo-synthetic fungicides are not permitted), it was hypothesized that the choice of less susceptible cultivars with a lower yield potential, such as Gloria may achieve higher yields than the more susceptible cultivars. However, with and without additional protection by alternative treatments (that are permitted in organic systems) the yields of the tolerant cultivar (Gloria) remained lower than that of the susceptible cultivar (Palmera).

As a result, organic farmers may still choose the more susceptible cultivars, and increase the yield through use of alternative treatments, if the additional yield and/or fruit quality justifies the additional cost (alternative treatments are significantly more expensive than fungicides).

However, several cucumber cultivars are resistant to more than one soil and/or foliar disease such as *Alternaria alternata*, *Cladosporium cucumerinum*, *Fusarium oxysporum* and, since alternative treatments may not cover the same range of diseases, the use of the resistant cultivars may still be commercially viable and become the

main strategy for crop protection in organic systems (Vakalounakis and Fragiadakis, 2000)

Use of the more tolerant cultivar (Gloria) reduced mildew severity, but did not result in higher yields. In fact, yields of untreated control plants of the susceptible cultivar (Palmera) were higher than untreated plants of the tolerant cultivar (Gloria), despite higher levels of mildew disease.

Based on the data from these trials the use of the **particular** tolerant cultivar (Gloria) would provide little commercial benefit to farmers. However, if the increased mildew disease levels were shown to have a negative impact on fruit quality and/or shelf-life, the use of more tolerant cultivars could still be beneficial.

Whether or not there are differences in fruit quality and shelf life between cultivars relating to the levels of mildew disease, was not assessed in this study. This will therefore have to be investigated in future studies. Moreover, other tolerant cultivars, that could be more productive than Gloria, should be included in future trials, if they become available.

6.4.2 Potential for improving powdery mildew control and yields of cucumber through the use of the mycoparasite *Ampelomyces quisqualis*

Mycoparasitism of powdery mildews by *Ampelomyces* spp is one of the best-known systems of fungal antagonism. However, the performance of *Ampylomyces* in field trials was variable, and use of the commercial product (AQ10) was not adopted widely by glasshouse producers (Kiss, 2003).

In some experiments conidial suspensions of isolates of *Ampelomyces* or the formulated product AQ10 (marketed as a Biofungicide) achieved satisfactory levels of control of the disease, while in some others they showed low or no significant control (Kiss, 2003).

In the experiments reported here AQ10 did not reduce disease incidence and had no effect of crop yield compared to untreated control plants of both cultivars. The poor performance of AQ10 was most likely due to the dry conditions in the greenhouse where the experiment took place (although no relative humidity measurements were

taken), which allowed fast development of powdery mildew, but did not fulfill the known high humidity requirements of the hyperparasite (Hajlaoui and Belanger, 1991; Phillip and Helstern, 1986). Since similar conditions would be expected in most growing seasons in Greece, the use of this product can therefore not be recommended, unless improved formulations or isolates with higher activity at lower humidity become available.

In some cases, the use of oil formulations of *A. quisqualis* was shown to increase the capacity of the hyperparasite to control powdery mildew (Urquhart & Punja, 1997; Belanger *et al.*, 1994; Verhaar *et al.*, 1999).

However, this was not investigated as part of the study reported here, but could be investigated in future studies.

6.4.3. Potential for improving powdery mildew control and yields of cucumber through the use of the oomycete antagonist *Pythium oligandrum*

Pythium oligandrum is a ubiquitous oomycete and was first described as a potential biocontrol agent against a wide range of soil-borne pathogens (Al-Rawahi and Hancock, 1998; Berry *et al.*, 1993; Deacon, 1976; Picard *et al.*, 2000).

The mode of action of antagonism of *P. oligandrum* was reported to involve the production of cell wall hydrolytic enzymes and/or antibiotics (Benhamou *et al.*, 1999; Picard *et al.*, 2000). However, the exact contribution of antibiotics/enzymes to the observed plant protective activity is not clearly understood, and other authors suggested that, in addition to its antimicrobial properties, *P. oligandrum* may be able to induce resistance in host plants (Benhamou *et al.*, 1997; Picard *et al.*, 2000).

During the experiments reported here, application of *Pythium oligandrum* significantly reduced the values of areas under disease progress curve (AUDPC -% days) compared to untreated control plants, but only for Gloria, the tolerant cultivar. However, the level of disease control obtained did not have an effect on cucumber yield, which was similar to untreated control plants.

As with AQ10 the low level of biocontrol activity obtained was most likely due to the low levels of relative humidity in the greenhouse, since proliferation of

oomycete fungi is known to require high humidity and/or free water films on plant surfaces (Piccard *et al.*, 2000).

6.4.4. Potential for improving powdery mildew control and yields of cucumber through foliar applications of chitosan

Chitin is a high molecular mass polymer of β -1,4-N-acetylglucosamine and is the second most abundant natural polymer on earth, after cellulose. Chitin is a major structural component of the cells of crustaceans, exoskeletons of insects, and cell walls of fungi and some algae (Shahidi *et al.*, 1999).

Chitin was initially studied as a potential control treatment against potato cyst nematodes. For example, chitin amendments to soil containing potato cyst nematode (PCN) increased yield and reduced PCN egg and larval counts (Evans, 1993).

Some of these early studies suggested that the addition of chitin to soil stimulates the population of bacteria, actinomycetes and a limited number of fungi with chitinolytic properties. It was hypothesized that this increased chitinolytic activity may have a negative effect on the growth and survival of fungal plant pathogens, which have chitin as a structural compound in their cell wall (Muzzarelli, 1977).

More recently, chitin has been tested for its ability to control plant pathogens and it was found to be able to elicit host resistance responses (e.g. phytoalexins, protein inhibitors, callose, lignin production), which are known to be associated with inducible disease resistance responses in plants (Lyon *et al.*, 1995; Watson & Brooks, 1984; Barber *et al.*, 1982; Hahn *et al.*, 1993). It was also reported that the application of chitin to tomato canopies provided significant control against grey mould caused by *Botrytis cinerea* (Ploper *et al.*, 1991). However, to our knowledge, chitin has not previously been tested for activity against cucumber powdery mildew in glasshouse trials.

In the experiments reported here, foliar sprays of chitin significantly reduced powdery mildew severity compared to untreated control plants and led to an increase in both the number and total weight of fruits per plant for both cultivars included in trials,

Palmera and Gloria. **It is to our knowledge the first report of a reduction of powdery mildew incidence in cucumbers by foliar treatment of chitosan.**

Although experiments were not focused on determining the mode of action of chitosan, the development of significant chitinolytic activity by the soil surface microflora is unlikely to have played a role, since the extremely dry conditions during trials are likely to have prevented significant microbial activity in the phyllosphere. This should be confirmed in future experiments, by measurement of microbial densities and activity in the phyllosphere.

Induced resistance could be a possible way of action of chitin, since **(a)** this mode of action is thought to be less dependent on relative humidity and **(b)** chitin has been shown to elicit plant resistance responses in other crop-fungal pathogen systems. This should be confirmed for the mildew-cucumber systems.

Following mineralization in soil, chitin may also release significant levels of nitrogen. The proportion of foliar sprays that reached the soil surface may, therefore, have contributed to the plants' nitrogen supply, which may have contributed to the positive effect of chitin on crop yield. Furthermore the release of N on the leaf surface could have increased microbial population and therefore microbial activity against the thallus of *P. xanthii*. However, such a mechanism would also have to be investigated via monitoring of N-supply pattern (through soil and plant mineral analyses) in future studies.

6.4.5. Potential for improving powdery mildew control and yields of cucumber through foliar applications of Milsana VP 2002

A range of plant extracts was shown to induce resistance to fungal pathogens including powdery mildews (Belanger and Benyagoub, 1997; Dik *et al.*, 1995; Seddon and Schmitt, 1999; Konstantinidou-Doltsinis and Tzempelikou, 2000).

For example, it was reported that treatment of cucumber plants with Milsana resulted in a build up of phenolic-type phytoalexins in infected cucumber leaves. This led to the conclusion that induced resistance is the major mode of action of this plant extract against powdery mildew (Dayyf *et al.*, 1995 & 1997; Petsikos-Panayotarou *et al.*, 2002).

In these experiments Milsana VP 2002 significantly reduced powdery mildew development and was the most effective alternative treatment against mildew. As with chitin, it significantly increased the number and total weight of fruits per plant and yields were significantly higher than those of chitin treated plants.

Most importantly, yields of Milsana VP 2002 treated plants of the more susceptible, but higher yielding cultivar Palmera, were not significantly different from those of fungicide treated plants. In the Milsana VP 2002 treated plants of the more mildew tolerant, but lower yielding cultivar Gloria, the total weight of fruits was lower than that of fungicide treated plants.

The fact that Milsana VP 2002 treatment has a greater yield effect in susceptible cultivars, makes it likely that the introduction of Milsana VP 2002 treatments into organic cucumber production protocols, will favour the use of more susceptible, higher yielding cultivars. Similar to conventional production systems, where the availability of fungicides facilitates the use of high yielding, susceptible cultivars, the widespread use of Milsana VP 2002 is therefore likely to increase the dependence of organic cucumber production on crop protection products.

CHAPTER 7. Effect of combinations of alternative foliar treatments on powdery mildew in glasshouse grown cucumber crops

7.1 Introduction

Powdery mildew fungi (Erysiphaceae) are one of the most conspicuous groups of plant pathogens and, despite extensive research on their pathogenesis, epidemiology and control, powdery mildew infections remain among the most commercially important plant pathological problems worldwide (Kiss, 2003).

In organic agriculture the use of sulphur fungicides, the use of tolerant varieties/cultivars and the use of biological control and plant extracts are the main strategies against cucumber powdery mildew, but all of them have limitations.

The use of sulphur fungicides provides plants good control against powdery mildews but it can be toxic in temperatures over 27⁰ C (Bourbos and Skoudridakis, 1993).

The use of resistant cultivars is the most efficient and cheap mode of controlling the disease and, for a range of crops, powdery mildew resistant cultivars have been developed. However, for crops such as tomatoes and cucumbers, only hybrids that are susceptible or partially resistant (tolerant) to powdery mildew are available (Vakalounakis and Fragiadakis, 2003). The yield of many of the more tolerant cucumber hybrids is known to be lower, a fact that was confirmed under chapter 6.

As a result, even organic growers often prefer to grow cultivars susceptible to powdery mildew (personal communication, cooperative of greenhouse vegetable producers-Preveza, Greece).

Biological control agents (BCAs) and a range of plant extracts and other elicitors have been studied extensively as alternatives to the use of mildew fungicides, and are now available as commercial products in several countries. However, they are so far not widely used by farmers, because of their relatively low levels of activity (Lampkin, 2000; Shtienberg and Elad, 2002) and often variable efficacy between seasons (Elad, 1990). This was confirmed under chapter 6, where both BCAs tested had no

significant effect and only one showed some low level of control against powdery mildew.

High temperatures and dry conditions favour the pathogen whereas the fungal, and especially oomycete and bacterial antagonists, require more humid conditions for activity (Elad *et al.*, 1996). This incompatibility is thought to be the main reason for the low activity of BCAs under Greek greenhouse conditions (Bourbos and Skoudridakis, 1993)

Plant extracts such *Azadirachta indica* and *Reynoutria sachalinensis* have been reported that can induce resistance in peas and cucumber respectively against powdery mildew, but they are efficient when they are applied preventively and when the disease pressure is not high (Daayf *et al.*, 1997; Singh and Prithivira, 1997). The potential of controlling powdery mildew in cucumbers by application of elicitors was clearly demonstrated under chapter 6, with both chitin and Milsana showing significant activity againsts powdery mildew.

One approach to overcome the variability of biocontrol efficacy of currently available BCAs may, therefore, be to combine the use of different BCAs with different modes of actions or to combine BCAs with elicitor treatments (Shtienberg and Elad, 2002).

The combination of tolerant cultivars, biological control agents and elicitors may provide more consistent control levels against powdery mildew (Malathrakakis, 1997).

The main **objectives** of the experiment carried out under chapter 7 was therefore to:

- (i) to **compare** disease development pattern and yields of **two cultivars** with different levels of genetic resistance to powdery mildew
- (ii) to **quantify the effect of combinations** of different alternative, non-chemical foliar treatments on powdery mildew disease development and fruit yield.
- (iii) to **identify interactions** between cultivar resistance and combinations of foliar treatments (=compare the relative effect of alternative treatments in different varieties)

7.2 Materials and methods

7.2.1 Field trial sites

The trials were conducted in 2004 at the glasshouse experimental facility at the Technological Educational Institution of Ionian Islands, Department of Organic Agriculture, Argostoli, Greece.

The minimum and maximum temperatures were 16 and 34°C respectively.

7.2.2 Cultivars used

Cucumber seeds of the cultivars Palmera (Rijk Zwaan, Holland; **susceptible to powdery mildew**) and Gloria (Tezier, France; **less susceptible to powdery mildew**), were sown in a peat-based substrate, and later transferred to the glasshouse, (approximately after 2-3 weeks). No artificial inoculum was used to infect plants and plants became naturally infected with powdery mildew.

7.2.3 Treatments

Eleven treatments were applied as foliar sprays for both cultivars:

1. **Control** (water spray)
2. **Fungicide control.** Mixture of two fungicides: myclobutanil (Systhane 12 E, EC, 12.5% a.i. w/v, ALFA) at the rate of 0.4 ml l⁻¹, penconazole (Topas 10 EC, 10% a.i. w/v., SYGENTA), at 0.15 ml l⁻¹
3. **Chitosan** as soluble chitin 1kg/ha
4. **Milsana VP 200** 50kg/ha
5. *Ampelomyces quisqualis* (AQ10) 70g/ha
6. *Pythium oligandrum* (Polyversum) Oosporic preperation 10⁶ spores/g, 0.1kg/ha
7. **AQ 10 + *P. oligandrum***
8. **Milsana VP 2002 + Chitosan**
9. **Milsana VP 2002 + Chitosan + AQ10**
10. **Milsana VP 2002 + Chitosan + Polyversum**
11. **Milsana VP 2002 + Chitosan + AQ10 +Polyversum**

Treatment applications started five days after transplanting, before any disease symptoms were visible and were repeated weekly. All treatments were applied at medium volume spray (500-1000 l ha⁻¹) with a 20 L knapsack sprayer (Berthoud Vermorel 2000 HP, 3 bars).

7.2.4 Experimental Design

Treatment plots of four cucumber plants were arranged as a completely randomized block design with four replicate blocks in the glasshouse (see Table 7.1 for the arrangement of plots in the glasshouse).

7.2.5 Crop assessments

Crop assessments were carried out as described in section 6.2.4.

7.2.6 Statistical analysis

Statistical analyses were carried out as described in section 6.2.5.

BLOCK 1		BLOCK 2		BLOCK 3		BLOCK 4	
P1		P8		P4		P4	
	G5		G5		G10		G8
G9		G3		G2		G7	
	P11		P11		P9		P2
P8		P1		P8		P6	
	G10		G8		G1		G5
G2		G11		G3		G3	
	P3		P2		P11		P3
P5		P6		P5		P5	
	G1		G4		G4		G10
G11		G9		G9		G11	
	P7		P3		P2		P9
P6		P5		P1		P8	
	G4		G6		G6		G4
G7		G2		G11		G2	
	P9		P9		P7		P7
P10		P4		P6		P10	
	G8		G10		G5		G6
G3		G7		G7		G9	
	P2		P7		P3		P11
P4		P10		P10		P1	
	G6		G1		G8		G1

Table 7.1. Plots arrangement in the glasshouse

P= 4 plants of cv. **PALMERA**

G= 4 plants of cv. **GLORIA**

- CONTROL (water sprays)
- MILSANA
- CHITOSAN
- AQ 10
- POLYVERSUM
- MILSANA + CHITOSAN
- MILSANA +CHITOSAN+ AQ 10
- MILSANA + CHITOSAN + POLYVERSUM
- AQ 10 + POLYVERSUM
- MILSANA + CHITOSAN + AQ 10 + POLYVERSUM
- FUNGICIDES

Distance between plants on a plot = 0.5m
Distance between treatment plots = 2m
Distance between rows in a block = 1.5m
Distance between different blocks = 2m

7.3 Results

7.3.1 Disease progress in untreated control plants of both cultivars

The disease progress (linearised logistic models) on the untreated control plants of both cultivars is shown in Fig 7.1. Disease parameters in terms of r_L and y_o are presented in Table 7.2. There was a delay in the onset of the disease in the less susceptible cultivar Gloria, as indicated by the y_o value (Figure 7.1). While the final disease incidence on the control plants of susceptible cultivar Palmera was higher compared to the control plants of the less susceptible cultivar Gloria, the rate of infection appears to be similar.

Table 7.2. Slope r_L and intercept y_o for regression of logit transformation of the percentage infected leaf area over time

Model		Coefficients	SE Coef.	Sig.	Adj R ²
PALMERA	y_o	-3.075	0.0532	.000	0.993
	r_L	0.1361	0.0027	.000	
GLORIA	y_o	-4.398	0.1817	.000	0.924
	r_L	0.1413	0.0093	.000	

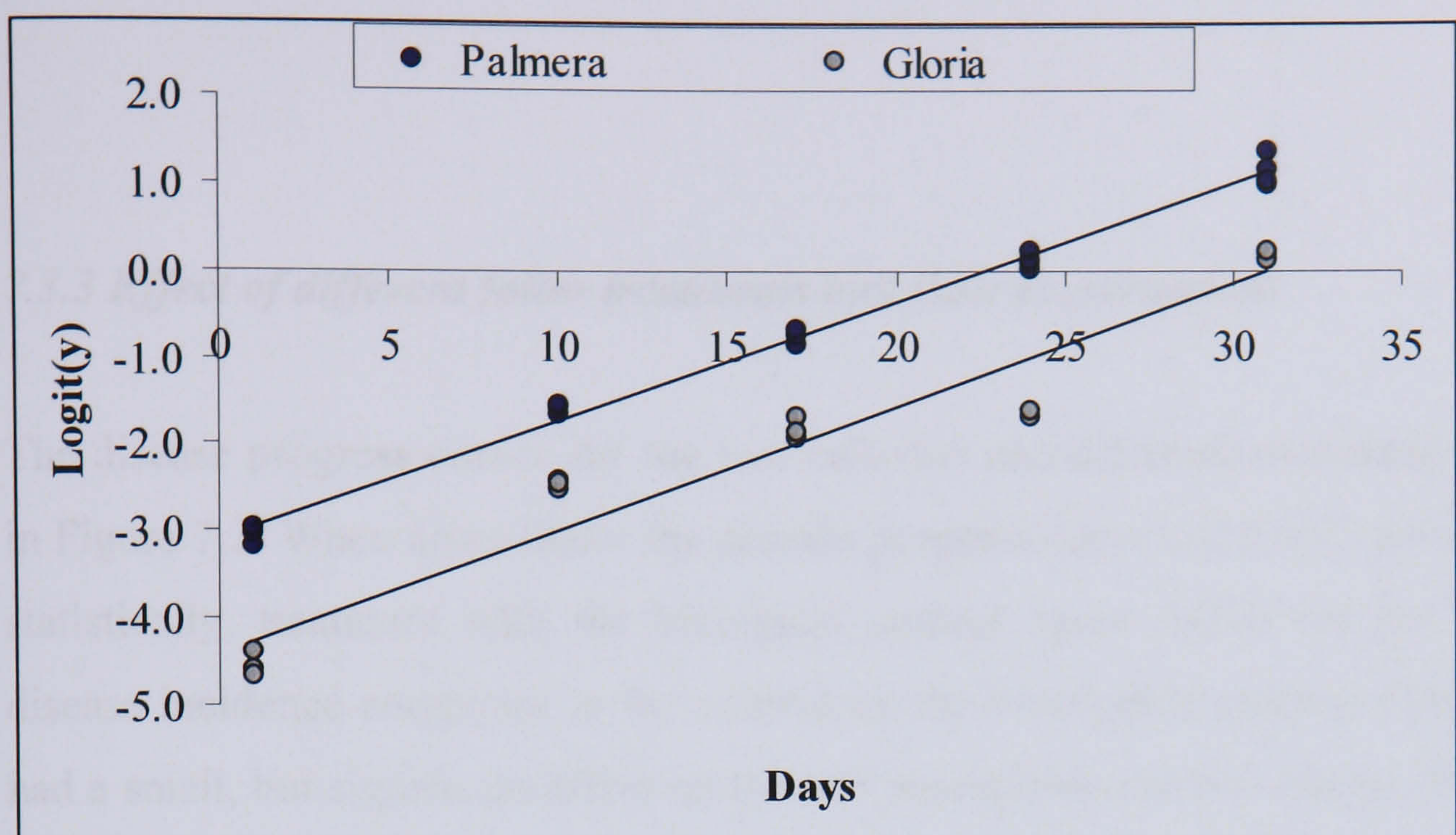


Figure 7.1. Linear logistic models of disease progress on two cultivars with different levels of susceptibility to powdery mildew

7.3.2 Effect of foliar treatments and their combination on tolerant and susceptible cultivars

Results of a two-way ANOVA, where AUDPC values were the dependent variable are presented in Table 7.3. The analysis showed that there was a highly significant effect of treatment type and cultivar on disease severity ($P=0.002$). There was also a very highly significant interaction ($P=0.000$) between treatment type and cultivar (Table 7.3).

Table 7.3. Analysis of Variance for AUDPC values, using Sequential SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Treatment	10	5335658	5335658	533566	7.12	0.002
Cultivar	1	1391170	1391170	1391170	18.56	0.002
Treat*cult	10	749416	749416	74942	46.93	0.000
Error	66	105399	105399	1597		
Total	87	7581642				

7.3.3 Effect of different foliar treatments and their combinations

The disease progress curves for the two cultivars and different treatments are shown in Figure 7.2. When areas under the disease progress curve (AUDPC) were compared statistically, treatment with the biological control agent AQ10 did not reduce the disease incidence compared to the control on the susceptible cultivar Palmera, but it had a small, but significant effect on the less susceptible cultivar Gloria (P=0.05; Fig. 7.3).

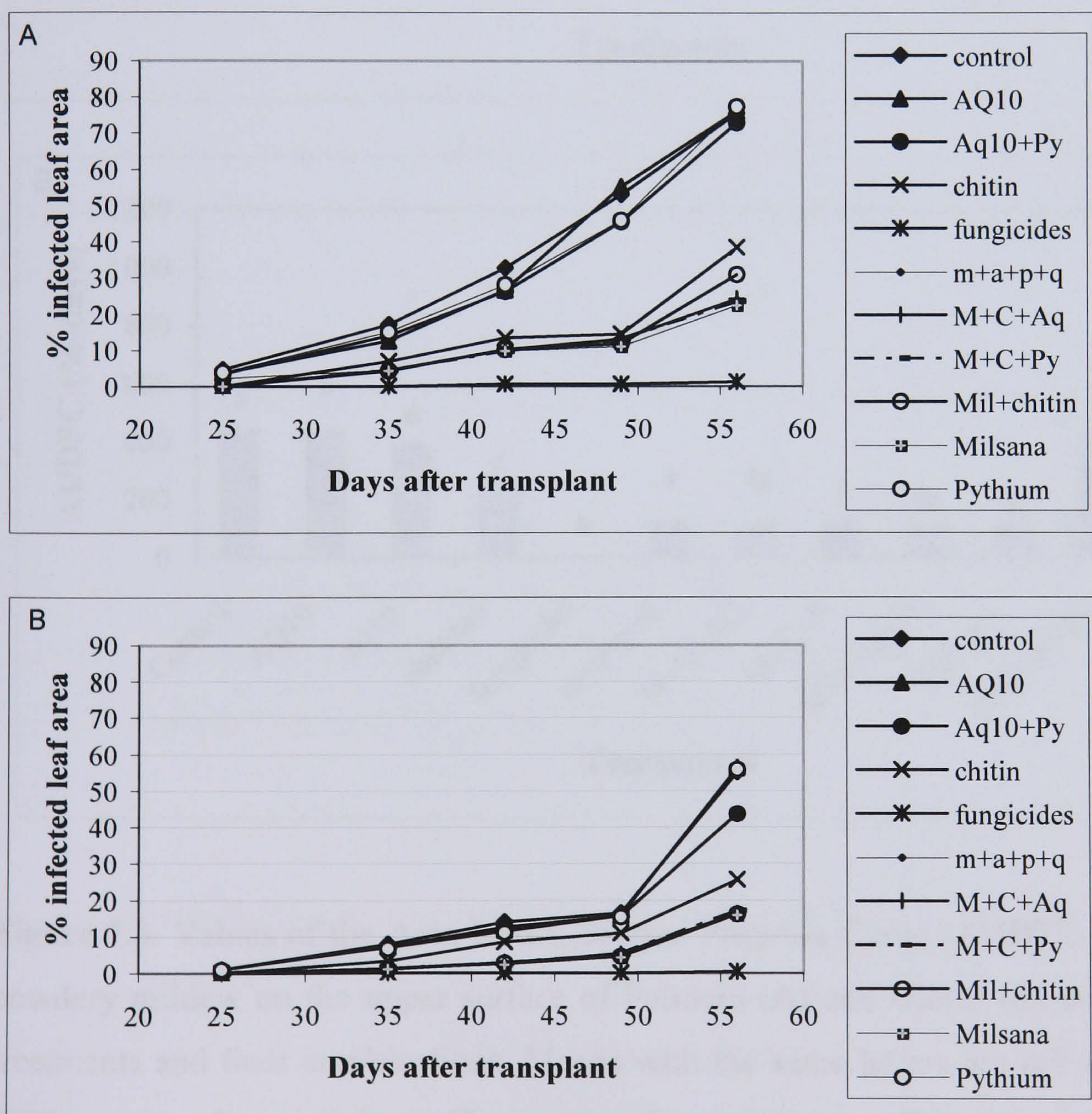


Figure 7.2. Disease progress on Palmera (A) and Gloria (B) treated with different control means and combinations of them.

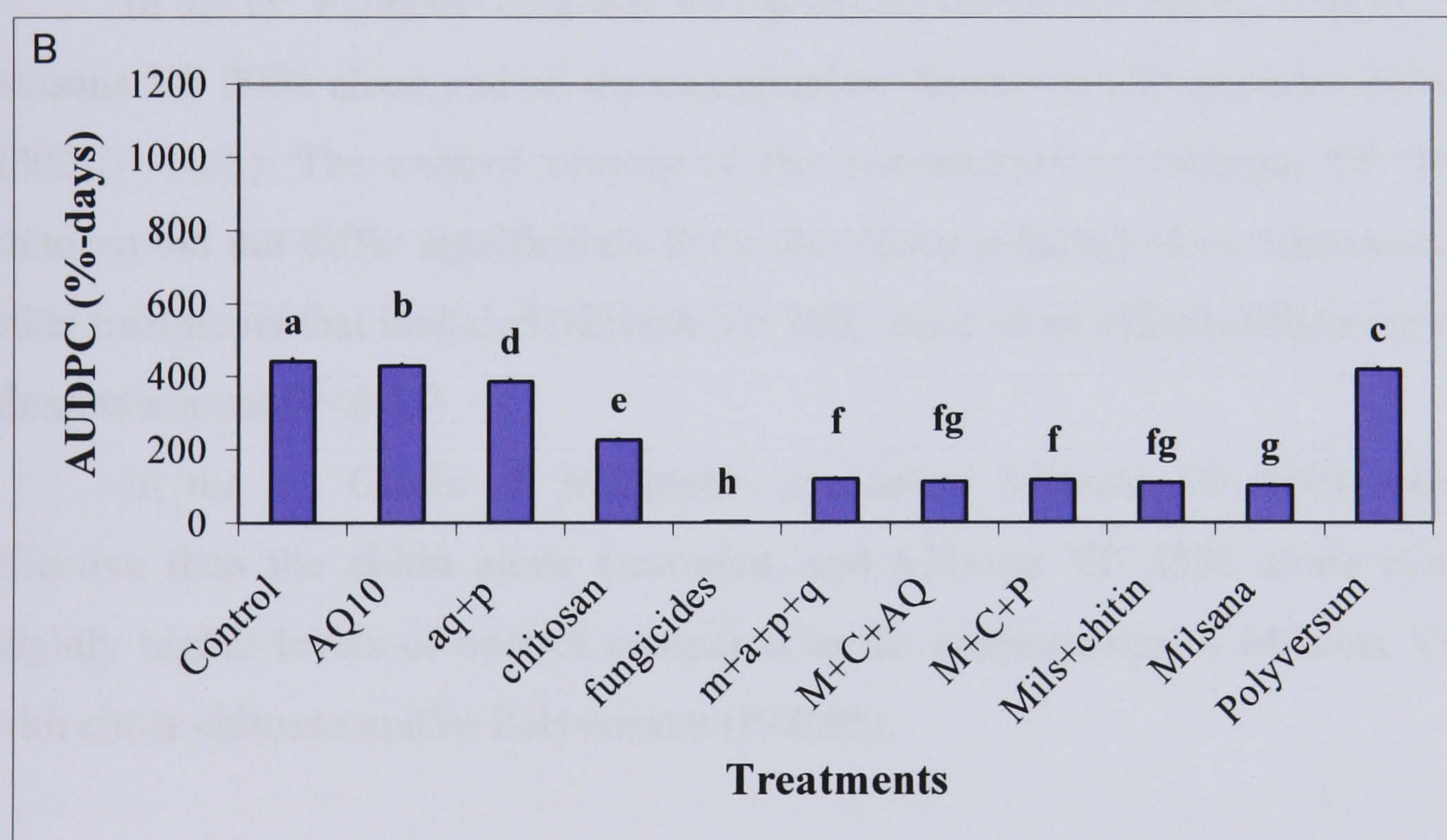
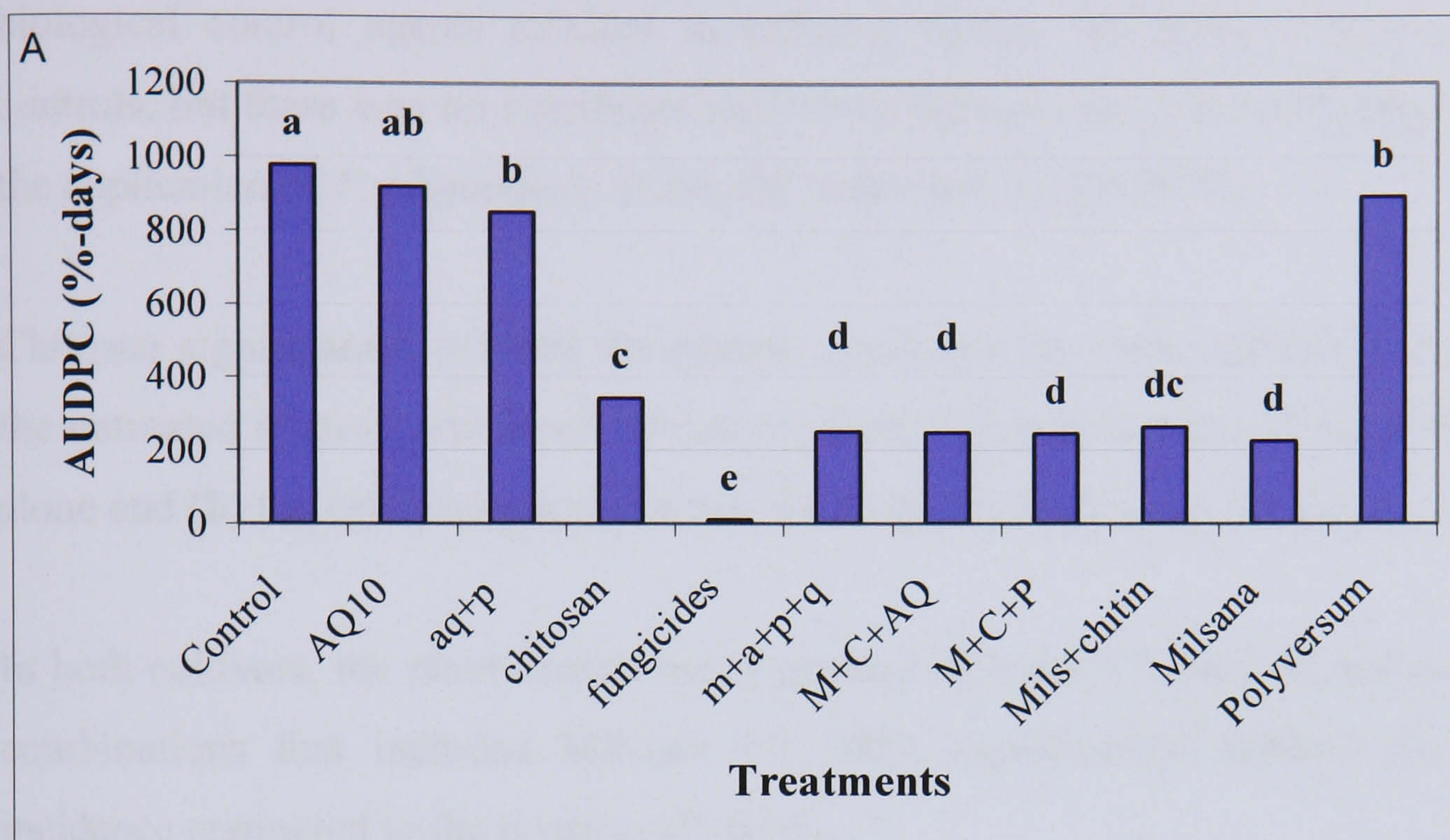


Figure 7.3. Values of the Area Under Disease Progress Curve (AUDPC- %Days) of powdery mildew on the upper surface of Palmera (A) and Gloria (B) with different treatments and their combinations. Means with the same letters are not significantly different according to Tukey's Honest Significant Difference Test ($P < 0.05$).

Applications of biological control product (Polyversum), based on the oomycetes fungus *Pythium oligandrum*, significantly reduced the disease incidence on both cultivars ($P<0.05$) but by a relatively small amount. The combination of the two biological control agents resulted in reduced disease incidence compared to the controls, but there was no significant difference between the combined treatment and the application of *P. oligandrum* alone, for both cultivars ($P<0.05$).

Chitosan significantly reduced the disease incidence for both cultivars compared to the untreated controls and levels of control were higher than those of (a) Polyversum alone and (b) the combination of the two BCAs for both cultivars ($P<0.05$).

In both cultivars, the plant extract based product Milsana VP 2002 alone, and all the combinations that included Milsana VP 2002, significantly reduced the disease incidence compared to the controls ($P<0.05$).

In the cv. Palmera there was no significant difference between applications of Milsana VP 2002 alone and all the combination treatments that included Milsana VP 2002 ($P<0.05$). The control activity of the combination of Milsana VP 2002 and chitosan did not differ significantly from that of the chitosan alone treatment, but all other treatments that included Milsana VP 2002 were more effective than the chitosan alone treatment ($P<0.05$).

In the cv. Gloria all treatments containing Milsana VP 2002 were more effective than the chitin alone treatment, and Milsana VP 2002 alone resulted in slightly higher levels of control compared to the combination of Milsana VP 2002, with either chitosan and/or Polyversum ($P<0.05$).

7.3.4 Effect of treatments and their combination on yield

Milsana VP 2002 alone, and all combination treatments including Milsana VP 2002, significantly increased the total weight of fruits per plant compared to the control for both cultivars, and the cumulative weight of harvested fruits was not significantly different to that of plants from fungicide treated plots (Table 7.4).

Chitosan increased the total weight of fruits per plant (for both cultivars) compared to the untreated control, but the total yield per plant was lower than that obtained in plots treated with Milsana VP 2002, and combinations that included Milsana VP 2002.

AQ10, Polyversum and a combination of the two treatments did not increase the total yields per plant compared to the untreated control for both the susceptible and the less susceptible cultivar.

Table 7.4. Yield (weight and number of harvested cucumbers per plant) obtained from Palmera and Gloria plants treated with different alternative control treatments

Treatments	Weight (g)		Cumulative number of cucumbers	
	Palmera	Gloria	Palmera	Gloria
Control	2212 c	2188 c	9.0 c	8.1 c
AQ10	2215 c	2168 c	9.1 c	8.3 c
Polyversum	2283 c	2169 c	9.1 c	8.3 c
AQ10+Polyversum	2237 c	2205 c	9.3 c	8.4 c
Chitosan	2628 b	2441 b	10.3 b	9.1 b
Milsana VP 2002	3246 a	2775 a	12.2 a	10.3 a
Milsana VP 2002+Chit.	3160 a	2774 a	12.0 a	10.4 a
Milsana VP 2002 +Chitosan+AQ10	3171 a	2779 a	12.0 a	10.3 a
Milsana VP 2002 + Chitosan+Polyv.	3206 a	2798 a	12.1 a	10.4 a
Mil+Chit+AQ10+Pol	3201 a	2749 a	12.2 a	10.2 a
Fungicides	3266 a	2882 a	12.4 a	10.6 a

Different letters in a column denote statistically significant differences at P=0.05

7.4 Discussion

7.4.1 Potential for improving powdery mildew control and yields of cucumber through use of resistant cultivars and/or foliar application of alternative treatments

With respect to the effect of resistant cultivars and individual alternative treatments on yield experiments under chapter 7, this confirmed the results described in chapter 6. Most importantly:

- **Application of BCAs** resulted in a small but significant reduction in disease levels but this had no impact on yields and can therefore not be recommended for use in organic greenhouse production
- **Application of chitosan and Milsana VP 2002** resulted in both (a) a significant reduction in disease and (b) a significant increase in fruit yield and therefore has potential as an alternative treatment for powdery mildew
- The tolerant cultivar (**Gloria**) produced lower yields even when untreated control plants were compared, and its use can therefore not be recommended, unless a significant effect of fruit quality and/or shelf life can be identified in future studies

7.4.2. Potential for improving powdery mildew control and yields of cucumber through foliar applications of combinations of alternative treatments

Some of the treatments used at this experiment have been previously described as BCAs (Milsana VP 2002, AQ10) that employ different modes of action in the control of powdery mildew. The combination of BCAs with different modes of action, and/or BCAs, which target different stages in the infection process, was suggested as one approach to increase the reliability and level of activity of biological control treatments (Schmitt *et al.*, 2001). Such an approach was described to be essential in situations where a single biological method does not give sufficient protection (Malathrakakis, 1997).

However, in the experiments reported here no additive or synergistic effect was detected in any of the combinations of treatments tested. In particular

- (i) combinations of the two biological control agents with Milsana VP 2002 or chitosan did not result in an increase in activity over and above that achieved by Milsana VP 2002 and/or chitosan alone. This indicates that the BCAs remain inactive when applied in combination with chitosan and Milsana VP 2002
- (ii) combining Milsana VP 2002 and chitosan did not result in an increase of activity over and above that achieved by Milsana VP 2002 alone. This may be due to the similar mode of action (induced resistance) of chitosan and Milsana VP 2002, and the fact that the level of resistance that can potentially be induced is already achieved by either of the compounds on their own

Use of the type of combinations included in trials carried out under chapter 7 can therefore not currently be recommended. However, a wider range of combinations and concentrations of individual compounds in mixtures should be examined before conclusions on potential synergistic effects can be drawn.

Chapter 8 General discussion

8.1 Progress - Control of soil borne diseases without soil steam disinfection

To develop strategies for the control of soil borne diseases, tomato (the most widely grown glasshouse crop in Greece and the UK) was chosen as the model crop.

A range of methods was identified which effectively controlled the main soil borne disease currently reported to cause significant reductions in crop yield and quality. These are discussed in separate sections (8.1.1 to 8.1.3) below.

8.1.1. *Grafting onto resistant rootstocks*

The use of **corky root rot resistant rootstock (Beaufort)** provided effective control in soils infected with corky root rot (*P. lycopersici*) only. However, the same rootstock was only moderately effective when tomato plants were grown in soils infected by both *P. lycopersici* and a new aggressive *V. albo-atrum* strain.

The use of another resistant rootstock KVFN, which is resistant to *Verticillium* and tolerant to corky root rot, was as effective as soil steam sterilization for the prevention of root disease development in soils infected by both *P. lycopersici* and a new aggressive *V. albo-atrum* strain.

The **most important component of an integrated strategy of the control of soil borne disease** in greenhouse crops should therefore be the **use of grafted plants**, using rootstocks that provide protection against the local complement of soil borne diseases. Given that some rootstocks only provide resistance against specific pathogens, farmers should be advised to carry out soil tests to determine the pathogen spectrum present in their greenhouse soils, before selecting rootstocks.

However, the **continuous use of resistant rootstocks or cultivars** could lead to the development of strains of the pathogens able to overcome this resistance (Agrios, 1997). In these experiments it was confirmed that the new aggressive strain of *V. albo-atrum* that emerged in England in 2001 can overcome the resistance of cultivars such as Espero, 72-774 and Piccolo. It is therefore important to identify additional methods for the control of soil borne diseases, which provide a second “layer” of control and/or delay the development of resistant pathogenic populations. The methods evaluated included the:

- (i) use of **organic matter soil amendments** (manure and household waste based compost and fresh *Brassica* tissues) which may suppress soil borne diseases via (a) release of antimicrobial compounds and/or (b) by increasing soil microbial activity (see 8.2.2 below) and
- (ii) use of **alternative soil treatments** (chitin, extracts and biological control agents) which were reported to (a) induce resistance in the plant, (b) antagonize/compete with soil borne disease inocula and/or (c) increase overall biological activity and/or specific populations of soil microbes with antagonistic activities to soil borne diseases (see section 8.2.3 below)

8.1.2. Organic matter amendments

The **addition of composts** to soils is recommended in organic agriculture since it not only provides crops with the complete set of essential macro and micro nutrients, but also improves the biological, physical and chemical properties of soil and can significantly reduce the severity of certain soil-borne pathogens (Abbasi *et al.*, 2002; Chellemi *et al.*, 1992; Hardy and Sivasithamparam, 1991; Workeh and van Bruggen, 1994).

The studies reported here showed that addition of both household waste and cow manure-based composts increased yields and root fresh weight in the presence of corky root rot alone and mixed inocula of both *P. lycopersici* and *V. albo-atrum*. Compost addition was associated with a significant increase in soil respiration rates, indicating that increased soil microbial activity, and associated competition for soil-borne diseases, was a major factor of the reduction in soil borne diseases (see chapter 5). Apart from confirming the beneficial impact of compost use it was also indicated that the choice of compost can have a significant effect on the efficacy of compost amendments on soil borne diseases. Large scale trials carried out in a commercial nursery (Cantelo Nursery Ltd) confirmed these results and also showed that, apart for household waste based compost, pine bark based composts also provided high levels of control of *P. lycopersici* and *V. albo-atrum* (Juan Rodriguez, personal communication).

Amendment of soils with fresh *Brassica* tissues also gave significant levels of control against soil borne diseases in tomato. This “suppressive effect” of incorporating *Brassica* break crops on soil borne diseases has long been known (Xiao

et al., 1998; Kirkegaard, *et al.*, 1996) and is one of the reasons why wide **crop rotation** is considered as an important component in disease management systems in organic farming systems (Lampkin, 2000).

The use of *Brassica* residues as soil amendments has been shown to achieve similar results to the inclusion of *Brassica* break crops in the rotation (Mojtahedi *et al.*, 1991). In greenhouse crop systems rotations are usually short (1-3 years) and dictated by market demands (high value crops, such as tomatoes are grown very frequently, and apart from a few specialist crops such as kohlrabi, very few *Brassica* crops are grown in greenhouses) (Greer and Diver, 2000).

Using *Brassica* wastes (in our experiments fresh chopped-up Brussels sprouts tissue) is therefore the only commercially feasible approach to utilize the disease suppressive effect of *Brassica* tissues for greenhouse crops. *Brassica* amendments effectively reduced the disease incidence when tomato plants were infected by *P. lycopersici* alone and when both *P. lycopersici* and *V. albo-atrum* were present. Previous studies have identified the production of isothiocyanates, a compound with high antimicrobial activity, especially under acidic conditions as the main mode of action of *Brassica* tissues (Kirkegaard *et al.*, 1996).

In the experiments reported here the amendment of *Brassica* tissues in soils increased the microbial activity two months after incorporation (chapter 5). It can therefore also be hypothesized that increased competition by the soil microflora has contributed to the “suppressive effect” of *Brassica* wastes on soil borne diseases.

The beneficial effect of adding fresh *Brassica* tissue was observed in all three trials carried out over a three-year period. The **use of *Brassica* wastes** (where available) as a component of the overall management system for soil borne diseases **can therefore be recommended** for use in organic farming systems.

8.1.3. Alternative Soil Treatments

The use of **plant (e.g. sea-weed and conifer extracts) or other organic liquid extracts (e.g. fish emulsion)** has been reported in scientific literature (and by companies marketing extract based products and composts extraction machines) to provide control against different soil and foliar pathogens. The proposed mode of action of different plant extracts is increased competition, antibiosis and induced resistance (Dixon and Walsh, 2004; Kong, 2001). However, in the experiments

conducted in this project (chapter 2) **none of the extracts** (seaweed, conifer or fish emulsion) **resulted in a reduction in corky root rot disease** incidence and/or an increase in yield. Therefore those treatments **cannot be recommended** for the control of soil borne diseases in tomato production.

On the other hand, the **use of chitin** soil amendments significantly reduced soil borne diseases in both corky root rot and *Verticillium* infected soils (see chapters 2 to 5). This confirms previous studies which reported that the use of chitin and its **deacetylated** form (chitosan) can reduce the disease incidence of soil, foliar and post harvest diseases (Davies *et al.*, 2002; Ploper *et al.*, 1991; Sid-Ahmet *et al.*, 2003; El Ghaouth *et al.*, 2000). However, this is, to our knowledge, the first time that the use of chitin was tested against *P. lycopersici* and against both *P. lycopersici* and *V. albo-atrum* in organic tomato production systems.

The main possible modes of action described in the literature for chitin and chitosan are (i) induction of plant resistance and (ii) increase in populations of chitinolytic microorganisms in soil (Benhamou, 1996; Sid Ahmed *et al.*, 2003). Chitinolytic microorganisms can degrade the body of different fungal pathogens that consists of, in many cases, chitin (De Boer *et al.*, 1999). In the experiments reported here chitin amendment did not increase the total microbial activity (see chapter 5) and no increase in chitinolytic activity in soil could be detected based on plate counts. However, the assessment for chitinolytic activity in soil should be repeated in future experiments, using more sensitive methods to confirm these results.

However, from the results obtained, it is more likely that induced resistance is the main mode of action for observed activity of chitin against soil borne diseases.

An alternative third possible mode of action of chitin was recently discussed by Benhamou *et al.* (1998); El Ghaouth *et al.* (1994) who described results indicating that chitosan will form a film around root tissues and may act as a physical/chemical barrier to pathogen invasion and/or to nutrients leaking from roots into soils (thus preventing a chemotaxic stimulus for invading pathogen hyphae).

Overall, the experiments showed that chitin and chitosan have great potential as a crop protection tool (see also foliar diseases below) in organic farming. Costs of **the product containing chitin that was obtained locally in Newcastle in the UK** and the **chitosan** used in the experiments described in this research work were similar to those for common fertilizers-plant strengtheners used in organic farming.

Therefore, the use of chitin amendments as a component of the overall management system for soil borne diseases **can therefore be recommended** for cost-effective use in organic farming systems.

8.2 Progress - Control of foliar diseases without sulphur fungicide use

To develop and evaluate strategies for the control of powdery mildew, cucumber (the glasshouse crop most affected by powdery mildew in Greece and the UK) was chosen. A range of methods was identified which effectively control the powdery mildew, the main foliar disease currently causing significant reductions in crop yield and quality. These are discussed in separate sections (8.2.1 to 8.2.2) below.

8.2.1. Use of resistant cultivars

To identify the level of control of mildew that can be achieved through choice of cultivars with different levels of susceptibility to powdery mildew, two commercial cucumber cultivars Palmera (susceptible to powdery mildew) and Gloria (which is a tolerant cultivar) were compared with respect to disease incidence and yield.

It is well known that there is a negative interaction between powdery mildew resistance and yield potential (and sometimes eating quality) in tomato. This lower yield potential has prohibited the widespread use of less susceptible cultivars in commercial practice (Vakalounakis and Fragiadakis, 2003).

As expected, results showed that the incidence of powdery mildew in the tolerant cv. Gloria was lower compared to the susceptible cv. Palmera. Nevertheless, even untreated plants of cv. Palmera were more productive compared to untreated plants of Gloria. Therefore, despite tolerance to powdery mildew it is difficult to recommend the use of less susceptible cultivars such as cv. Gloria because of its low yield potential. Clearly, there is a need for the introduction new varieies that combine tolerance with high yield potential.

Sensory analyses were not carried out as part of the experiments reported here against cucumber powdery mildew. Other cultivars that combine tolerance to powdery mildew and high productivity should be tested in future trials.

8.2.2 The use of Plant extract and alternative foliar treatments

The use of plant extracts of the perennial weed *Reynoutria sachalinensis* (Milsana® VP 2002) against cucumber powdery mildew reduced disease incidence and it was as effective as the chemical fungicides used. This confirms previous reports for the efficacy of Milsana (other types, see chapter 1) against cucumber powdery mildew. This product is on registration in Germany as a plant strengthener and its price is very similar to other common products used in organic farming, therefore **it can be recommended** for use against powdery mildew of cucumber in organic greenhouse production.

Chitosan reduced the disease incidence and it can be recommended against powdery mildew of cucumbers, though it was less effective than Milsana® VP 2002.

However, this is to our knowledge the first time that the use of chitosan was tested against *P. xanthii* in organic cucumber production systems.

8.3 Biological control against soil and foliar pathogens of organic greenhouse crops

The use of **biological control** against soil and foliar pathogens should be seen as a last resort once all other steps to create an optimal environment for plant growth have been taken (Lampkin, 2000).

There are a lot of fungi and bacteria that have been tested for their potential as BCAs against different pathogens but only a few of them have been formulated and are commercially available to greenhouse growers (Paulitz and Belanger, 2001).

During the experiments reported here, the use of *Pythium oligandrum* and *Bacillus subtilis*, against tomato corky root rot, and the use of *Ampelomyces quisqualis* and *Pythium oligandrum*, against cucumber powdery mildew, did not reduce the disease incidence of both soil and foliar diseases. One of the main disadvantages of the use of BCAs is that, due to the fact that they are living organisms, their efficacy is very much dependent on environmental conditions (Elad *et al.*, 1996).

For example, *P. lycopersici* is favored by cool soil temperature and can severely harm tomatoes when temperatures are between 15-20°C (Shishkoff and Campbell, 1990) On the other hand the BCAs used against the pathogen have an optimal temperature for development, which is between 23-25°C, and subsequently their action against the pathogen was very low (Boscow, 1989; Piccard *et al.*, 2000)

Similarly *Pythium oligandrum* and *Ampelomyces quisqualis* need high relative humidity for their development, while *P. xanthii* can thrive under dry conditions (Elad *et al.*, 1996; Bery *et al.*, 1993).

One approach to overcome this weak point of biological control is to use one BCA, with different modes of action, or to use a **combination of BCAs**, with different modes of action, with no negative effects of each to the other (Shtienberg and Elad, 2002). Therefore, different treatment combinations of BCAs that were successful against *P. lycopersici*, *V. albo-atrum* and *P. xanthii* when applied alone were tested to examine possible additive or synergistic effects against the pathogens. From these experiments no additive or synergistic effect was detected for any pathogen, but due to the lack of space not all the possible volumes were tested.

Conclusions

According to the results of the work presented in this thesis the following conclusions can be drawn:

The use of **resistant** cultivars-rootstocks is the most efficient and cheap mode for the control of corky root and Verticillium wilt of tomato crops.

The use of **composts** not only improves the soil chemical, physical and biological characteristics but also can provide control to different pathogens. The amendment of compost in soils increased the soil microbial activity and it is assumed that it increased the antagonism between the pathogen and non-pathogen microorganisms in soils, and possibly induced resistance to crops.

The use of **Brassica** tissues can provide effective control of tomato plants grown in soils infected by *P. lycopersici* alone or by both *P. lycopersici* and *V. albo-atrum*.

Since rotations are not so easy to be adopted in greenhouse cropping systems, the incorporation of fresh tissues can be an effective alternative.

The use of **chitin** and **chitosan** reduced the disease incidence of *P. lycopersici* alone and *P. lycopersici* plus *V. albo-atrum*. Those components, that have specific characteristics such as biodegradability, antimicrobial potential and elicitor activity, meet the criteria of a promising biocontrol agent and have great potential as an alternative to synthetic fungicides for the control of plant pathogens.

The use of **plant extracts** of *Reynoutria sachalinensis* (Milsana® VP 2002) have been previously reported to provide effective control against powdery mildew. The experiments conducted here confirm its efficacy.

Topic areas identified as targets for future research

Breeding and selection for resistance for all the three pathogens but mainly for cucumber powdery mildew

Investigate more combinations of the successful treatments against all three pathogens

Further studies to confirm possible modes of action for all successful treatments

Apply the successful treatments to other pathogens of greenhouse crops

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